SUSTAINABLE MANAGEMENT OF BOTRYTIS BLOSSOM BLIGHT IN WILD

BLUEBERRY (*VACCINIUM ANGUSTIFOLIUM* AITON)

by

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ABSTRACT

Botrytis blight is an important disease of wild blueberries with control dependent on fungicide usage. Given concerns over human health, environment, and fungicide resistance, field and laboratory experiments were conducted during 2015 and 2016. Laboratory radial growth expansion assessments of *Botrytis cinerea* isolates on media amended with pyraclostrobin, boscalid, penthiopyrad, cyprodinil, and fludioxonil were conducted. Field trials investigating the susceptibility of four phenotypes at different flower stages as well as burning, lime sulfur and the use of biofungicides and their rotation with a botryticide were undertaken. Resistance towards cyprodinil, pyraclostrobin and boscalid in *B. cinerea* isolates was detected. No shifts toward resistance to penthiopyrad or fludioxonil were detected. *Vaccinium angustifolium* was susceptible while *V. myrtilloides* was less susceptible. Floral stages F6 and F7 were susceptible while F5 and F8 were less susceptible. Burning and lime sulfur demonstrated disease control, and biofungicides used in rotation with Switch® provided Botrytis blight control.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ANOVA – Analysis of Variance
a.i. - Active ingredient
AP - Anilinopyrimidine
cm - Centimeter
EC_{50} - Effective concentration that inhibits mycelial growth by 50%
FB - Floral bud
g - Gram
ha - Hectare
kg - Kilogram
kPa - Kilopascal
L - Liter
m - Meter
mg - Milligram
mL - Milliliter
mm - Millimeter
N - North
PDA - Potato dextrose Agar
PSI - Pound-force per square inch
QoI - Quinone outside inhibitor
r - Coefficient of correlation
RH - Relative humidity
SDH - Succinate dehydrogenase
SDHI - Succinate dehydrogenase inhibitor
spp. - Species
VB - Vegetative bud
W - West
µg - Microgram
ºC - Degrees Celsius
® - Registered trademark
% - Percent
± - Margin of error of a quantity
< - Less than
> - More than

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CHAPTER 1

INTRODUCTION

1.0 Introduction

Wild blueberry or lowbush blueberry (*Vaccinium angustifolium* Aiton, *Vaccinium mytilloides* Machx, and *Vaccinium boreal*) is a low-growing perennial shrub belonging to the Ericaceae or Heath family (Eck, 1966). It is a native and economically important crop in North America, specifically eastern Canada and the state of Maine in the United States (Drummond *et al*., 2010; Hall *et al*., 1979; Vander Kloet, 1978). Wild blueberry is a leading horticultural commodity in Nova Scotia, Quebec, New Brunswick, Prince Edward Island and Newfoundland with about 17,700, 28,000, 13,600, 5,000 and 700 ha, respectively, representing approximately 50% of Canada's land area in fruit and nut production (Statistics Canada, 2015).

Wild blueberry is affected by several diseases including Monilinia blight (mummy berry), Botrytis blight, Septoria leaf spot, Valdensinia leaf spot and leaf rust (Percival, 2013; Delbridge *et al*., 2011). Botrytis blight, caused by *Botrytis cinerea* Pers.:Fr., is found in most wild blueberry fields and can be destructive, especially during bloom (Lambert, 1995). Botrytis blight has been a problem in coastal areas with extended periods of rainfall and fog providing a suitable environment for infection (Lambert, 1990). The disease has become widespread across the wild blueberry industry and is directly responsible for over 20% reduction in yield annually in coastal areas such as Parrsboro shore (NS, Canada) (WBPANS, 2013 unpublished; Delbridge and Hildebrand, 1997).

The fungus typically infects individual flowers or entire inflorescences at the mid to late bloom stage (Lambart, 1995). *B. cinerea* is a necrotrophic pathogen that can survive as a saprophyte (Lambert 1995; Sutton, 1991; Bisiach *et al*., 1984) and overwinters as

sclerotia or dormant mycelium on shoots, woody stems and dormant buds (Lambert, 1995; Nair *et al*., 1995). In the spring, sclerotia and mycelia germinate to produce conidia. These conidia serve as a source of primary inoculum. Conidial dispersion is usually by rain and wind, and the conidia germinate at temperatures between 5 and 30°C (20°C being the optimal) in free surface water or high relative humidity (Latorre and Rioja, 2002; Williamson *et al.,* 1995).

The most susceptible tissues to *B. cinerea* are senescent floral tissues (Keller *et al*., 2003) and profuse sporulation is observed on these tissues during spring when conditions are favorable for pathogen development. There has been an increase in Botrytis blossom blight disease recently, especially across coastal wild blueberry fields. This can be attributed to the significant increase in flower densities due to improved management practices such as fertilization and weed management (Percival, 2013). Combined with cool and wet conditions during bloom, an increase in leaf and berry debris and burning not being employed as a pruning and sanitizing tool, presents a suitable condition for the rapid escalation of this disease.

The use of chemical fungicides such as boscalid, cyprodinil, penthiopyrad and pyraclostrobin have been employed over the years for the control of Botrytis blossom blight (Percival, 2013). However, *B. cinerea* is a typical high-risk pathogen which readily develops resistance to these fungicides (Percival, 2013; Brent and Hollomon, 1998). This has resulted in loss of efficacy of fungicide. This has caused the number of conventional fungicide applications in a season to escalate resulting in increase in the cost of production (Percival, 2013).

Given the widespread resistance of Botrytis to fungicides on various crops in different countries (Leroux et al., 2010; Baroffio et al., 2003; Forster and Staub, 1996), it is important to assess the status of Botrytis resistance to the common active ingredients used in wild blueberry production in Nova Scotia. In the face of resistance development and search for more sustainable disease management practices, it is key to identify and obtain knowledge about the relative susceptibility of wild blueberry floral stages and phenotypes. Assessment of resistance and identification of susceptible phenotypes is an important tool that can be integrated into Botrytis blight management programs.

Recent studies in other berry crops have shown that Botrytis blight can be significantly reduced with the use of lime sulfur and biofungicides applied both while the plant is dormant and during bloom (Schilder *et al.*, 2002; 2006). Therefore, there is a need to evaluate selected biofungicides and reduced risk fungicides as well as inoculum reduction strategies as an alternative Botrytis blossom blight management.

1.1. Hypothesis and Objectives
1.1.1. Hypothesis

Botrytis cinerea control is mainly dependent on conventional fungicide applications. The control of Botrytis and other phytopathogens with biofungicides demonstrated by researchers in other crops presents an alternative for *B. cinerea* control and support fungicide resistance management strategies. Coupled with reports of widespread *B. cinerea* resistance to fungicides, and varying susceptibility among wild blueberry phenotypes to *Monilinia* blight, it is anticipated that the application of inoculum eradication techniques to reduce mass of leaf litter and Botrytis inoculum, and application of biofungicides have the potential to reduce Botrytis blight. Also, knowledge of *B.*

cinerea sensitivity/resistance status to fungicides has the potential of contributing to Botrytis blight management through the development of an effective and resistance management strategy. The specific hypotheses for this research work are:

1. There is no loss of sensitivity of *B. cinerea* to the fungicides fludioxinil, cyprodinil, pyraclostrobin, boscalid and penthiopyrad.

2. Phenotypic and phenological variation as well as floral developmental stages associated with wild blueberries will affect the susceptibility of Botrytis blossom blight in the field.

3. Application of inoculum eradication techniques and biofungicides can reduce Botrytis blossom blight

4. Biofungicides used alone and in rotation with chemical fungicides can reduce Botrytis blossom blight

1.1.2. Objectives

1. Examine the *in vitro* sensitivity of *Botrytis cinerea* to active ingredients fludioxinil, cyprodinil, pyraclostrobin, boscalid and penthiopyrad in fungicides used for the management of Botrytis blight.

2. Determine the relative susceptibility of four wild blueberry phenotypes at different floral stages to Botrytis blight, and verify the specific location of infection (floral part).

3. Examine the main and interaction effects of thermal pruning (burning), lime sulfur and *Trichoderma* spray applications on Botrytis blight.

4. Evaluate the efficacy of biofungicide applications used alone and in rotation with conventional fungicides in the management of Botrytis blight.

1.2. Literature review

1.2.1. Taxonomy and botany of wild blueberry

Lowbush blueberry is a member of the Ericaceae (Heath) family (Vander Kloet, 1978; Eck, 1966) and subfamily *Vaccinioideae* (Gray and Fernald, 1950). *Vaccinium* (e.g. blueberry) and *Gaylussacia* (e.g. huckleberry) are two important genera of *Vaccinioideae*. The wild blueberry crop falls under the genus *Vaccinium* and subgenus *Cyanococcus* (Eck, 1966). Four kinds of lowbush blueberry are found in Canada, these include *V. angustifolium, V. angustifolium* f. *nigrum, V. myrtilloides* Michx. and *V. boreale* (Kinsman, 1993; Eck, 1996).

Vaccinium myrtilloides is mostly found in woodlands and is most abundant in fields recently developed from woods. However, it tends to be eliminated through repeated burning. *V. boreale* is the smallest in number and is mostly found in the northern regions of Cape Breton, Nova Scotia and the highlands of Newfoundland (Kinsman, 1993). Commercial wild blueberry fields consist of native clones of *V. angustifolium* and/or *V. myrtilloides* (Strik and Yarbourough, 2005). However, *V. angustifolium* (common lowbush blueberry) is the most abundant species in eastern North America representing about 80% of species on commercial fields (Eck, 1996).

Wild blueberry plants are low growing, 10 to 60 cm in height, with new shoots of maturing plants emerging from dormant buds on underground stems called rhizomes which originate from seedlings (Kinsman, 1993). Wild blueberry plants are spread by rhizomes. The roots are shallow and fibrous. Though the root is thickened, deep tap roots can sometimes be found. A piece of rhizome with well-developed roots, if separated from the parent plant, can grow to be a separate plant. (Kinsman, 1993). In addition to spread through rhizomes, wild blueberry can also be propagated by seed. Plants which originate

from a seed are known as mother plants. The mother plant develops rhizomes which enables the plant to spread (Agriculture, Aquaculture and Fisheries, 2010).

Wild blueberry is a deciduous shrub with broad to elliptic shaped leaves that are glossy blue-green in summer and turn red to purple in the fall (Hall *et al*., 1979). The leaves possess a thick epicuticular wax layer and are hypostomatous. These characteristics help to support crop survival during drought periods (Glass, 2000). The cylindrical to urceolate or bell-shaped floral tube is white or pinkish-white with four or five lobes, 5 mm long and inverted with the opening of the corolla at the bottom (Kinsman, 1993; Eck, 1966) and 5 mm long (Hall *et al*., 1979)

1.2.2. Production and characteristics

Production of the wild blueberry is unique because the fields are populated by volunteer plants (Kender and Eggert, 1966) which are primarily spread by rhizomes or underground runners. All shoots arising from the same rhizome possess similar genetic characteristics and are therefore referred to as a blueberry clone (Kinsman, 1993). Wild blueberries can be found on a wide range of soil types, but they thrive best on well-drained, infertile podzols with low pH of glacial or alluvial origin (Sanderson *et al.,* 2008; Jensen and Yarborough, 2004). The optimum pH of soils for wild blueberries is between 4.0 and 5.5 (Kinsman, 1993). Soils with these levels of acidity are normally inappropriate for other types of agricultural crops (Howatt, 2008; McIsaac, 1997).

The majority of wild blueberry production follows a two-year production cycle: the sprout or pruning year and the crop or bearing year. In year one, the plants are pruned in either the spring or fall by burning or flail mowing to near ground level (Kinsman, 1993). Following pruning, plants regenerate naturally from rhizomes at the start of each

production cycle (Hall et al., 1979). The plants are pruned every other year to make the most of floral bud initiation, fruit set, yield and easy harvest (Percival and Sanderson, 2004). Though cultivated on a two-year cycle, a small portion of blueberry fields in Nova Scotia is managed on a three-year cycle where an additional cropping year is incorporated before pruning (Eaton and Nams, 2006).

Pruning is accomplished by removing as much of the above-ground portion of the plant as possible. Basically, two methods of pruning exist: thermal pruning and the mower pruning (DeGomez, 1988). Pruning in late fall after harvest encourages the growth of new shoots, maximizes floral bud formation, and increases berry yield and easy harvest (Mclsaac and Reid, 2000). Growth and development of wild blueberry in the sprout year consists of shoot emergence and vegetative growth until termination of the apical meristem (tip dieback) initiates floral bud development on the upper portion of the shoot in late summer and fall (Aalders and Hall, 1964; Barker and Collins, 1963).

In the second or cropping year, growth and development comprise leaf expansion and flowering in spring (Hall et al., 1979). During early spring of the crop year, fields are managed with appropriate herbicides, pesticides, fungicides, and fertilizers as needed (Santiago and Smagula, 2012). Due to the predominantly self-incompatibility of blueberry plants, cross-pollination is vital for fruit set (Wood, 1968). This is normally attained using native (bumblebee) and honeybees in May and June when the blueberry plants bloom (Drummond *et al*., 2010; Yarborough, 2009; Mclsaac and Reid, 2000) and the berries are then harvested in August (Drummond *et al*., 2010)

1.2.3. Botrytis spp. and *Botrytis cinerea* Pers.:Fr.

Botrytis, is a genus of anamorphic fungi which belongs to the Ascomycota with about 28 identified species that are pathogenic (Dewey & Grant-Downton, 2016). *Botrytis* species are reported to infect over 595 genera of vascular plants representing more than 1400 plant species (Elad, et al., 2016). Among the *Botrytis* species, *B. cinerea* Pers.:Fr.*,* is reported to infect over 230 host species (Choquer et al., 2007; Walker et al., 2015) belonging to about 170 families of plants of agricultural importance (Elad et al., 2016). The next species identified to also have a wide host range is *B. pseudocinerea* (Plesken et al., 2015; Walker et al., 2011). Apart from *B. cinerea* and *B. pseudocinerea,* all other species of *Botrytis* are host specific or usually infect limited number of hosts (Elad et al., 2007). Although numerous *Botrytis* species have been identified, *B. cinerea* is the most studied. This species has received much attention and is a model pathogen due to its wide host range and ubiquitousness.

Botrytis cinerea is the asexual stage (anamorph) of the fungus *Botryotinia fuckeliana* (De Bary) Whetzel (teleomorph) and is believed to have obtained its name from the Ancient Greek word 'botrys' which means grape, because the conidia of the fungus look like bunches of grapes (**Figure 1**) (Agrios, 2005).

1.2.3.1. Morphology

Botrytis cinerea colonies are identifiable on host tissues or culture media by gross morphological features. The pathogen produces abundant colonies: initially the colony is white to grayish, followed by dark brown colour. The mycelium of *B. cinerea* is branched, olive brown in colour, cylindrical, and has septate hyphae (Mirzaei *et al*., 2007). The fungus produces conidia in clusters from enlarged apical cells at the end of

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branched, slender conidiophores (1-3 mm long) (Pearson and Goheen, 1988), which originate from enlarged foot cells (Jarvis, 1980, 1977). The conidia are smooth, single-celled, faintly ash-coloured structures, fairly large (8-14 × 6-9 µm) and oval in shape (Horst, 2008; Willetts, 1997). Under unfavourable (dry/cold) environmental conditions, *B. cinerea* produces sclerotia that can be considered the most important structures for the survival of the fungus as it can survive for years (Erental *et al*., 2008; Holz *et al.*, 2007). Sclerotia serve as survival structures for *B. cinerea* and contain viable hyphae which serve as primary inoculum for disease development. They are flat or convex, hard, and adherent to the substrate. They measure (0.36) 1- 11 (20) × (0.36) 1-8 (11) mm; when young, they are whitish then becomes black at maturity (Mirzaei *et al*., 2007). Sclerotia may germinate producing mycelium, conidiophores and conidia or apothecia and ascospores. Apothecial production in *B. cinerea* is rarely found in nature (Lorbeer, 1980), hence, it is worth stating that Anton de Bary described *Peziza* (*Botryotinia*) *fuckeliana* and *B. cinerea* from grapevine in Switzerland well over a century ago (Gregory, 1949)

1.2.3.2. Life cycle

Botrytis cinerea is present in fruit production systems including vineyards as part of the environmental microflora (Keller *et al*., 2003). Various forms of overwintering inoculum have been identified within the field, these include mycelium, sclerotia (Nair *et al*., 1995), and chlamydospores (Coley-Smith, 1980). In the spring, when the environmental conditions are favourable, the formation of fresh conidia from these sources provides an abundance of inoculum **(Fig. 1)**. This becomes the source of inoculum for primary infection of flowers and leaves. These conidia are produced throughout the growing season (Pearson and Goheen, 1988). Most susceptible tissues to *B. cinerea* are senescing

floral tissues, and profuse sporulation is usually observed on these tissues during spring when conditions are favourable for pathogen development (Keller *et al*., 2003) **(Fig. 1)**. As infected flowers die, the conidia germinate and colonize dead floral parts followed by the infection of other living tissues such as young and succulent shoots, particularly in wounded or cracked plant parts (Ellis, 2008). Usually, blossom infection is followed by a period of latency in berries without causing disease symptoms, until the berries begin to soften and ripen and the sugar level increases (Pezet *et al*., 2003; Nair *et al*., 1995; McClellan and Hewitt, 1973). After latency, the fungus starts to colonize mature berries and senescent plant tissues. This phase is believed to be important in the epidemiology of *B. cinerea* (Nair *et al*., 1995).

The sexual life cycle of *B. cinerea* occurs through the spermatization of sclerotia leading to the production of apothecia and asci with eight binucleate ascospores (Williamson *et al*., 2007). Ascospores released from these apothecia can infect leaves of plants in the production fields and thereby serve as a source of primary inoculum of the fungus.
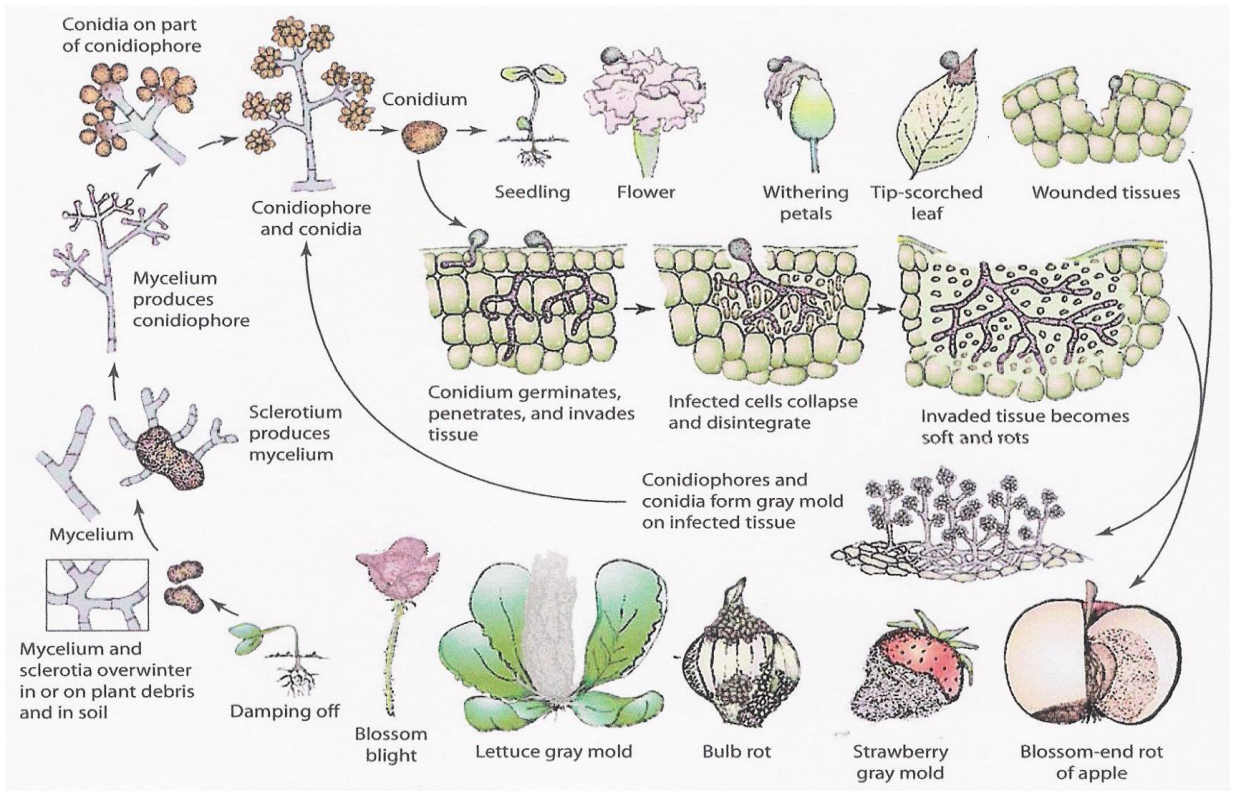
Figure 1.0. Life cycle of *Botrytis cinerea* and disease cycle. (Adopted from Agrios, 2005)

1.2.3.4. Pathogenicity

Botrytis cinerea is an opportunistic fungus that can cause infection at wound sites or previously infected sites. Nevertheless, it is able to directly penetrate intact host surfaces through the cuticle (Cole *et al*., 1996; Williamson *et al*., 1995). The fungus can also enter the host through stomata and other natural openings (Hsieh *et al.,* 2001; Fourie and Holz, 1995; Clark and Lorbeer, 1976). The pathogen requires wet or damp conditions for germination of conidia and infection to occur (Coertze and Holz, 2002). Wet periods and moderate temperature are considered to be important factors that influence the germination and infection severity by *B. cinerea*. The risk of *B. cinerea* infection is dependent on surface wetness and temperature. For instance, wet periods are needed for

infection, and the length of time of the wet period essential for infection increases as the temperature decreases (Table 1.0) (Delbridge and Hildebrand, 2007).

Following the deposition, attachment and germination of conidia, the first barrier to overcome is the plant cuticle. Normally, overcoming the cuticle does not involve physical or mechanical damage (Cole *et al*., 1996; Williamson *et al*., 1995) indicating the involvement of enzymatic activity in penetrating the cuticle (Salinas and Verhoeff, 1995). Enzymes such as cutinase, lipase and cellulase produced by *B. cinerea* have been observed to contribute to *B. cinerea* infection (Commenil *et al.,* 1995; Jarvis, 1977).

Plants produce defense compounds (phytoalexins) and accumulate pathogenesis-related (PR) proteins during interaction with the pathogen. Significant evidence supports the observation that build-up of phytoalexins and PR proteins during infection is one mechanism by which plants resist disease (Diaz et al., 2002; Benito et al., 1998; Darvill and Albersheim, 1984). Nevertheless, *B. cinerea* can withstand toxic effects of plant defense compounds with varying structures and mechanisms.

Prior to invasion of host cells by hyphae, *B. cinerea* kills underlying host cells after penetration of the cuticle (Clark and Lorbeer, 1976). As a necrotrophic pathogen, *B. cinerea* induces host cell collapse possibly by secretion of toxic metabolites producing necrosis of host tissues (Van Kan, 2006). *B. cinerea* also secrets oxalic acid, which may have direct toxic effects by lowering the pH of the environment (Germeier *et al*., 1994). The activities of oxalic acid and pectinolytic and non-pectinolytic enzymes cause the breakdown of plant cell walls, thereby making nutrients available to the pathogen (ten Have *et al*., 2002).

Table 1.0. Rating of *B. cinerea* infection periods based on environmental conditions (Delbridge and Hildebrand, 2007)

Wetness Duration (Hrs)	Mean Temperature (°C) during Infection Period				
	4°	8°	12°	16°	20°
4	Low	Low	Low	Low	Low
6	Low	Low	Low	Low	Low
8	Low	Low	Low	Low	Med
10	Low	Low	Low	Low	High
13	Low	Low	Low	High	High
24	Low	Med	High	High	High
36	Low	High	High	High	High
48	Med	High	High	High	High

1.2.4 *Botrytis* management strategies

Management of *Botrytis cinerea* is difficult especially when inoculum levels in the fields are high and conditions are favourable for the pathogen. The fungus is very difficult to eradicate once established in the field due to the production of long-lived sclerotia in the field. Therefore, integrated management of the pathogen and disease using chemical methods, cultural activities and biocontrol agents is the best practice.

1.2.4.1. Cultural methods

Several cultural practices are useful for the management of Botrytis diseases. There are various practices that can help ease the effect of *B. cinerea* during production. However, some of these practices are usually host specific and depend on the cropping system in place. The removal of infected and infested debris from the field and storage rooms and the provision of conditions for proper aeration to facilitate quick drying of plant surfaces

is key to reducing infection (Williamson *et al*., 2007; Agrios, 2005). At high relative humidity (RH), conidial generation is high and also germination and penetration of the host is promoted (Williamson *et al*., 2007).

In wild blueberries, it was observed that *B. cinerea* colonized fruits from sites pruned by mowing more frequently than by biennial burning (Lambert, 1990). This is due to the fact that thermal pruning reduces the amount of overwintering *B. cinerea* propagules in the field which will serve as source of primary inoculum under favourable conditions.

Conventionally, wild blueberry fields were pruned by free-burning of blueberry plants with the hope that the whole field would burn. Free-burning offers a very inexpensive method of pruning, however, it is very hard to control and often does not burn the field completely (DeGomez, 1988). In an attempt to reduce the cost of pruning with straw or fuel oil, flail mower which is inexpensive yet acceptable pruning method was introduced (Kinsman, 1993; DeGomez, 1988). Thermal pruning offers many advantages over flail mowing. The heat produced by the fire will not only kill the stem, but may reduce the incidence of insects, diseases, and weeds. The heat produced during thermal pruning may also destroy overwintering fungal propagules such as mummy berry, dormant mycelia and sclerotia in the field (DeGomez, 1988). Similarly, apple orchards were also observed to produce apples free from scab caused by *Venturia inaequalis* the following year after the leaf litter was burned (Gomez *et al.,* 2004; Hardison, 1976).

There are many weed species in agricultural crops that may significantly influence disease incidence and spread (Wisler and Norris 2005). Weed management is key to the management of *B. cinerea* as some of these weeds may serve as hosts for the pathogen. In wild blueberry fields, weeds that have been observed to be sources of disease include

bunchberry (*Cornus Canadensis* L), sheep sorrel (*Rumex acetosella* L.), goldenrod (*Solidago Canadensis L.*) and Pearly everlasting (*Anaphalis margaritacea*) (Delbridge and Hildebrand, 1997).

1.2.4.2. Biological methods

Extensive knowledge has been obtained on biological control of various plant diseases. The control of *Botrytis* with fungi, bacteria and yeasts has been intensively investigated over the last two decades (Blakeman, 1993; Blakeman and Fokkema, 1982; Dubos, 1992; Tronsmo, 1992; Elad and Freeman, 2002). Biocontrol presents an alternative or addition to the use of conventional means for disease control since microbial biocontrol agents are considered to be less damaging to the environment and their generally complex mode of action reduces the risk of resistance development (Elad *et al*., 2007).

The search for and use of biological control agents for disease control begun over 6 decades ago and several researchers over the years have used biological agents to control Botrytis diseases. Newhook (1957) managed to control gray mould in glasshouse tomato by spraying a spore suspension of *Cladosporium herbarum* and *Penicillium* sp. on floral remains on the fruits. Also Bhatt and Vaughan (1962) controlled gray mould of strawberry with *Cladosporium herbarum* by protecting flowers of strawberry under field conditions. The antagonistic effect of bacteria on *B. cinerea* on chrysanthemum and beetroot leaves has also been reported (Blakeman and Fraser, 1971). The inhibition of *B. cinerea* and other pathogens by epiphytic bacteria has been described as a general phenomenon (Blakeman and Brodie, 1976).

Trichoderma spp. are the best known and extensively investigated mycoparasites (Elad, 1995). *Trichoderma* proliferation was observed on the boundaries between healthy and

necrotic zones on rotting grape berries. Microscopic observations revealed the coiling and penetration of the mycelium of *B. cinerea* by the antagonist *Trichoderma* (Dubos, 1987). Spraying of the fungal antagonist *Trichoderma viride* spores on strawberry blossoms and young fruits reduced significantly the disease during pre- and postharvest (Agrios, 2005). A number of antagonistic yeasts have also been identified to protect grapes and tomatoes from *B. cinerea* infection (Agrios, 2005). Yeast species such as *Aureobasidium pullulans* (de Bary), *Metschnikowia pulcherrima* and *Pichia guilliermondii* were observed to provide good and effective biocontrol of *B. cinerea* on grapes (Raspor *et al*., 2010). *Aureobasidium pullulans* has been widely used in the control of *B. cinerea* in apple (Mari et al., 2012; Zhang et al., 2010; Vero et al., 2009) and strawberry (Adikaram et al., 2002). A number of experiments have pointed out competition for nutrients and the secretion of enzymes such as chitinase and glucanase as the main mode of action (Zhang et al., 2010; Castoria et al., 2001). A few commercial products have been developed *A. pullulans* for *B. cinerea* control. These include Boni Protect[®], Blossom Protect[®] and Botector[®].

Several researchers have investigated the use of bacteria as a potential biocontrol agent. The antifungal activity of various strains in the genus *Bacillus* against *B. cinerea* have been reported (Lee et al., 2006; Walker et al., 1998). For instance, in both *in vitro* and field tests, *Bacillus subtilis* S1-0210 was observed to significantly reduce gray mould infection in strawberries by over 85% (Hang et al., 2005). *Bacillus* spp. have been described to be effective in the management of several plant diseases due to the production of broad-spectrum antibiotics and extended shelf lives through endospore formation (Emmert and Handelsman, 1999). From research into *Bacillus* as a biological control agent, various commercial products have been developed from *Bacillus* spp. and

tested for their gray mould control abilities. These include Kodiak HB, *B. subtilis* GB03 (Mahaffee and Backman, 1993) and Serenade, *B. subtilis* QST-713 (Marrone, 2002). Nonpathogenic microorganisms mostly inhibit pathogen growth through the production of inhibitory metabolites and parasitism, induced resistance, competition for nutrients, or modification of plant surfaces. Through these events, they naturally limit plant disease in the environment (Elad *et al*., 2007).

Plant-based compounds such as proteins and peptides, essential oils, and plant-based crude extracts have been extensively studied for control of pathogens including *B. cinerea.* For example, Vitoratos et al., (2013) demonstrated the antifungal activity of essential oils from oregano (*Origanum vulgare* L.) and lemon (*Citrus limon* L.) against *B. cinerea* both *in vitro* and *in vivo* where oregano and lemon oils significantly reduced gray mould severity of infection in tomatoes, strawberries and cucumbers. Similar to essential oils, polypeptides from plants such as sweet lupine known as Banda de Lupinus albus doce (BLAD) have been extracted, tested and patented for Botrytis control (Taghavi et al., 2015). BLAD binds to chitin of the fungal cell wall and inhibits fungal growth. It also degrades chitin by catalyzing and removing N-acetyl-D-glucosamine terminal chitin monomers, leading to the destruction of the fungal cells (APVMA, 2017).

1.2.4.3. Chemical control

The use of synthetic fungicides was introduced in the 1950s and some of the early fungicides used to control Botrytis include pthalimides (captan, folpet), sulphamides (dichlofluanid) and dithiocarbamates (thiram) (Elad *et al*., 1995). All the early fungicides used were multi-site inhibitors, which affected many target sites in fungal cells and therefore, acted as general enzyme inhibitors. The multi-site fungicides act as a protectant

only, hence require repeated applications at high doses (Leroux, 2004). The risk of resistance to these multi-site fungicides is low, and control failure due to resistance development is infrequent (Williamson *et al.,* 2007; Leroux, 2004). Currently, several families of site-specific Botryticides are available (Rosslenbroich and Stuebler, 2000) and they can be classified according to their biochemical modes of action. Five categories have been distinguished: 1) anti-microtubule toxicants (benzimidazoles); 2) fungicides affecting fungal respiration (fluazinam, boscalid and multi-site inhibitors; 3) compounds affecting osmoregulation (dicarboximides, fludioxonil); 4) inhibitors of methionine biosynthesis (anilinopyrimidines) and 5) sterol biosynthesis inhibitors (fenhexamid) (Leroux, 2007). Although the low toxicity and high effectiveness of these fungicides is advantageous, nevertheless, they come with the cost of high susceptibility to resistance build up because of their site-specific mode of action. Resistance was reported soon after the release of some of these chemical groups (Thind, 2012). For instance, anilinopyirimidines were highly effective against *B. cinerea* but a high risk of resistance development was obvious at the preregistration stage in the laboratory (Birchmore and Forster, 1996). A few years after registration, cyprodinil (anilinopyirimidine) resistance were reported by Latorre et al. (2002) in table grapes (*Vitis vinifera* L.). Also other recent studies have reported anilinopyirimidine resistance among *B. cinerea* isolates (Fernández-Ortuño et al., 2012).

Chemical control remains the primary approach to decreasing the occurrence of gray mould and other *Botrytis* diseases on major crops. In chemical control of Botrytis diseases, timing is critical for effective control. Synthetic fungicides provide good control of *B. cinerea*: however, *B. cinerea* is a typical high-risk pathogen which readily develops

resistance to these chemicals (Percival, 2013; Brent and Hollomon, 1998). Therefore, fungicides with different modes of action are recommended for Botrytis disease control (Agrios, 2005).

1.2.5. Predictive modelling

Given the complexity of *B. cinerea* epidemics, a number of predictive models have been developed that allow users to understand historical weather data and make predictions about disease outbreaks. Predictive models help allocate resources (control products and cultural methods) efficiently for a successful and cost effective disease management in the field (Mukamel et al., 1997). Different models have been developed and validated for some Botrytis management system especially in grapes production. Some of these models are weather based (Marcuzzo and Haveroth, 2016; Broome et al., 1995; Nair and Allen, 1993) while others incorporate plant development stage and Botrytis infection pathways (González-Domínguez et al., 2015) and *B. cinerea* biology, economic thresholds, and fungicides (Ellison et al., 1998). Though a number of models exist, some of them lack robustness and failed under certain field conditions. Due to this, predictive models are not widely used in disease management systems (González-Domínguez et al., 2015).

1.2.6. Fungicide resistance

The use of chemical fungicides in agricultural production has a very long history. Starting with the use of inorganic compounds, such as salt water which was used in the seventeenth century for treatment of grain followed by liming to control rot. These were followed by the discovery of sulfur dust and then the popular Bordeaux mixture (a

mixture of copper sulphate and hydrated lime) (Hahn, 2014). Other antifungal organic compounds with broad-spectrum activity were developed, most of these fungicides are multisite inhibitors. These include captan and folpet, which are active on thiol groups of glutathione and proteins (Bernard and Gordon, 2000). A novel group of fungicides was developed in the 1960s, starting with the benzimidazoles. Their mode of action directed towards a specific target protein in fungal pathogens. They are very active and show low phytotoxicity with most of them being systemic fungicides (Hahn, 2014).

Few years after the introduction of site-specific fungicides, resistance development in the pathogen populations were observed. *B. cinerea* was one of the early fungi to be described to have developed fungicide resistance (Hahn, 2014). The term fungicide resistance, as used by the fungicide resistance action committee (FRAC), refers to a developed, heritable reduction in sensitivity of a fungus to a specific anti-fungal agent/fungicide. Cross-resistance is also a phenomenon that occurs when resistance arises to one fungicide that also results in resistance to another fungicide (http://www.frac.info/resistance-overview).

Botrytis cinerea is a high risk pathogen for fungicide resistance acquisition due to its high genetic variability, the abundant sporulation, the short life cycle, wide host range, and the high number of fungicide sprays needed for its effective control (Leroux et al., 2002; Yourman *et al.,* 2001). Specific resistance may be observed a few years after a release of a new fungicide group for Botrytis control. Resistance to fungicides usually results from an alteration at the site of fungicidal action in the target pathogen. Thus knowledge of the mode of action can indicate risk (Brent and Hollomon, 2007). For instance, resistance to boscalid and carboxin have been linked to single mutations in one of the subunits (B, C

or D) of the succinate dehydrogenase (SDH) complex (Miles et al., 2014; Avenot et al., 2008; Broomfield and Hargreaves, 1992).

Specific resistance has been described for the various Botryticide groups available on the market. These include benzimidazoles (e.g., benomyl and carbendazim) (Yourman and Jeffers, 1999), anilinopyrimidines (e.g., cyprodinil and pyrimethanil) (Myresiotis et al., 2007), carboxamides/SDHIs (Leroux et al., 2010), dicarboximides (Myresiotis et al., 2007; Leroux, 2004) and quinone outside inhibitor (QoI) fungicides (Bardas et al., 2010; Ishii et al., 2009). Cross resistance has also been described for several fungicides and pathogens. These include cross-resistance between SDHIs (boscalid and penthiopyrad) in *Botrytis cinerea* (Amiri et al., 2014), *Alternaria alternata* (Avenot et al., 2009), and *Didymella bryoniae* (Thomas et al., 2012). Cross-resistance between boscalid (SDHI) and pyraclostrobin (QoI) has also been documented for *B. cinerea* (Amiri et al., 2013) and *Alternaria solani* (Gudmestad et al., 2013).

In crop production, proper selection and usage of partner fungicides is key in any resistance management strategy. Generally, multi-site inhibitors are good compliments in resistance management because they have low resistance risk compared to site-specific fungicides. The use of unrelated fungicides is also important in resistance management programs as it helps reduce the possibility of cross-resistance.

CHAPTER 2

PRELIMINARY ASSESSMENT OF FUNGICIDE RESISTANCE AMONG *BOTRYTIS CINEREA* ISOLATES FROM WILD BLUEBERRY FIELDS IN NOVA SCOTIA

2.0. Abstract

Botrytis cinerea is a pathogen with a high risk of developing resistance to various groups of fungicides. Fifteen single-spore isolates of *Botrytis cinerea* were collected from commercial wild blueberry fields in Nova Scotia. Eight baseline isolates were also collected and used to evaluate resistance development. The isolates were evaluated for their sensitivity to the fungicides cyprodinil, fludioxonil, boscalid, penthiopyrad, and pyraclostrobin using a mycelium growth assay. The EC_{50} values for the 15 isolates ranged from 0.04 - 10.03, 0.0047 - 0.0073, 0.47 - 9.25, 0.41 - 12.40 and 0.15 -1.88 for cyprodinil, fludioxonil, boscalid, pyraclostrobin and penthiopyrad, respectively. Results from this study also revealed the existence of cyprodinil, pyraclostrobin and boscalid-resistant strains at frequencies of 100, 100 and 73.3%, respectively. Two (2) isolates were found to be resistant to penthiopyrad whereas no isolate resistant to fludioxonil was detected. Only two (2) isolates were found to be resistant to all fungicides except fludixonil. Eleven (11) isolates were resistant to cyprodinil, boscalid and pyraclostrobin. A significant cross-resistance relationship existed between the two SDHI fungicides boscalid and penthiopyrad when their EC_{50} values were subjected to a linear correlation analysis (r = 0.671, p = 0.006). A strong cross-resistance association was observed between boscalid and pyraclostrobin (r = 0.737, p = 0.002). Similarly, cross-resistance was detected between pyraclostrobin and penthiopyrad (r = 0.655, p = 008). A negative correlation was detected between fludioxonil and boscalid (r = -0.583, p = 0.023).

Though some isolates were simultaneously resistant to more than one fungicide, no cross-resistance relationships were detected in the remaining fungicide pairs. This study reveals a significant shift of *B. cinerea* isolates towards resistance development to cyprodinil, pyraclostrobin and boscalid. It also suggests a high risk for prompt widespread occurrence of *B. cinerea* populations resistant to penthiopyrad unless suitable resistant management strategies are employed to curb any future resistance challenges.

Keywords: cyprodinil, fludioxonil, boscalid, pyraclostrobin, penthiopyrad, fungicide resistance, Botrytis blight

2.1. Introduction

Botrytis cinerea Pers.: Fr. is an important fungal pathogen that infects over 200 plant species of agricultural and horticultural importance, both in the field and in storage (Agrios, 2005; Jarvis, 1977). It is also an important pathogen of wild blueberry (*Vaccinium angustifolium*), with greatest impact along coastal areas with persistent wet and cool weather conditions causing over 20% yield loss annually (WBPANS, 2013, unpublished data; Delbridge and Hildebrand 1997). The pathogen mainly infects flowers and inflorescence of the blueberry plant (Lambert, 1995). The fungus is important in field blueberry production but of little importance in post-harvest handling of wild blueberry fruit.

Currently, the general recommendations for Botrytis disease control involve a combined approach using environmental modifications to reduce field inoculum, canopy humidity, and leaf wetness together with the use of various fungicides. Some of the first fungicides developed for Botrytis control were site-specific and included benzimidazoles and dicarboximides; and the multi-site group such as chlorothalonil and dichlofluanid

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(Myresiotis et al., 2007; Elad et al., 1995). Due to reports of development of resistance among *B. cinerea* populations to some of these fungicides in the mid-1990s (Pappas, 1997; Elad et al., 1992), new fungicides belonging to different groups with excellent activity against the pathogen were developed. These new groups include anilinopyrimidines (APs), phenylpyrroles and hydroxyanilides (Rosslenbroich and Stuebler, 2000).

Anilinopyrimidines derivatives such as pyrimethanil and cyprodinil do not affect spore germination, but prevent germ-tube elongation and initial mycelial growth of *B. cinerea* (Rosslenbroich and Stuebler, 2000). Biochemical studies indicated that these compounds inhibit the biosynthesis of methionine (Fritz et al., 1997). Anilinopyrimidines have also been found to inhibit the fungal secretion of extracellular proteins such as hydrolase which is associated with plant pathogenesis (Milling and Richardson, 1995; Miura et al., 1994).

The phenylpyrroles fungicide fludioxonil is a non-systemic and preventive fungicide which inhibits spore germination, germ-tube elongation and mycelial growth of *B. cinerea*. Biochemical work has shown that the protein kinase PK-III, which is potentially associated with the osmoregulation signal transmission pathway, is the target of phenylpyrrole fungicides (Pillonel and Meyer, 1997). A course of events has been proposed that originates in the inhibition of the protein kinase PK-III and leads to the significant accumulation of fungal polyols such as glycerol in the mycelium and cell death (Pillonel et al., 2003).

Recently developed groups of site-specific fungicides for Botrytis and other disease control are the succinate dehydrogenase inhibitors (SDHIs) such as boscalid and

penthiopyrad; and the quinone outside inhibitors (QoIs) such as pyraclostrobin. Both groups are respiration-inhibiting fungicides. SDHIs have a distinctive mode of action which acts by blocking the fungal respiration process by binding to the ubiquinone reduction site of complex II of the respiratory chain known as succinate dehydrogenase. SDHIs aim at the succinate dehydrogenase complex which blocks the citric acid cycle and ATP production necessary for respiration of the fungus (Avenot and Michailides, 2010; Stammler et al., 2008). The mode of action of QoI fungicides depends on their ability to hinder mitochondrial respiration by binding to the quinone outside site. This blocks the transfer of electrons between cytochrome *b* and cytochrome *c1*, which leads to an energy deficit in the fungal cells preventing ATP production which leads to the death of fungal cells (Fernández-Ortuño et al., 2010).

In Canada, the main active ingredients of fungicides registered for Botrytis blight control in wild blueberries are cyprodinil, fludioxonil, boscalid, pyraclostrobin, fenhexamid and penthiopyrad (Agriculture, Aquaculture and Fisheries, 2016; Percival, 2013). For resistance management purposes, some of these active ingredients are premix on the market as Switch® (cyprodinil + fludioxonil), and Pristine® (boscalid + pyraclostrobin). Switch® and Pristine® were registered for use in wild blueberries in 2006, and Fontelis® (penthiopyrad) was registered in 2012 for the suppression of Monilinia blight. Following registration of these products, they have been used extensively on wild blueberry fields for Botrytis control with three fungicide applications per cropping season.

The site-specific nature of these fungicides allows the fungus to develop resistance based on very few genetic alterations. The most important mechanism of resistance in *B. cinerea* is mutation in the genes coding for the target site protein which leads to reduced

fungicide binding activity. These alterations usually determine the specific resistance to a single or a fungicide class (Leroux et al., 2010; Fillinger et al., 2008). These site-specific fungicides have been classified into low, medium and high-risk fungicides according to their intrinsic risk for resistance evolution. QoIs (FRAC group 11) are classified as high-risk fungicides, anilinopyrimidines (FRAC group 9) are classified as medium-risk fungicides, SDHIs (FRAC group 7) are classified as medium- to high-risk fungicides whiles phenylpyrroles (FRAC group 12) and hydroxyanilides (FRAC group 17) are classified in the low- to medium-risk group (FRAC, 2016).

The development of fungicide resistance in pathogen populations from continual exposure is of concern because it can negatively affect the efficacy of fungicides (Brent and Hollomon, 2007). The use of site-specific fungicides to control high-risk pathogens, such as *B. cinerea* contributes to an increase in the development of field resistance among the pathogen population. Hence, *B. cinerea* isolates resistant to most of the fungicide groups have been reported in different studies and from different countries (Leroux et al., 2010; Baroffio et al., 2003; Forster and Staub, 1996). For these reasons and lack of information on resistance/sensitivity of *B. cinerea* to these fungicides in wild blueberry fields, this research was conducted to provide data on sensitivity to APs, SDHIs, QoIs and phenylpyrroles among *B. cinerea* isolates obtained from wild blueberry fields in Nova Scotia. Specifically, the objective of this study was (i) to determine in vitro sensitivity to boscalid, cyprodinil, penthiopyrad, fludioxonil, and pyraclostrobin.

2.2. Materials and Methods

2.2.1. PDA medium preparation and incubation conditions

Potato dextrose agar (PDA) medium was used in most of the experiments. Autoclaved medium was cooled to 45°C before antibiotics or fungicides were added. Amended or unamended medium was poured into Petri dishes at 20-25 mL per dish. All Petri dishes were carefully marked and sealed with parafilm (Bemis North America, USA) before incubation at 22-24°C in the dark.

2.2.2. Isolation of *Botrytis cinerea*

Fifteen isolates of *B. cinerea* were isolated from diseased floral tissues from different commercial wild blueberry fields in Debert, Rawdon, Kemptown, Webb Mountain, Dean and Parrsboro in Nova Scotia, Canada. Eight wild isolates (baseline isolates) were also isolated from diseased blossoms collected from a field in Masstown, NS that had never been treated with any fungicides. Conidiophores were transferred onto PDA and incubated for 5-7 days to obtain abundant conidia. Standard microscopic observation and literature descriptions of the conidia, conidium-bearing structures and mycelium by Williamson *et al.* (2007) were used to confirm the fungus as *Botrytis cinerea*. Afterwards, all isolates were purified by single spore isolation. Small pieces of mycelium were placed in a falcon tube containing 10 ml of sterile distilled water and agitated. Ten microliters (10 μl) of the spore suspension were spread onto water agar (WA) plates and incubated in the dark for 24 hours. Pieces of agar containing only one spore were removed from the water agar plates and placed on PDA amended with 100 mg/L streptomycin sulfate and 100 mg/L chloramphenicol to inhibit bacterial growth. The isolates were incubated and stored on PDA at 4 °C.

2.2.3. Fungicide preparation and sensitivity test

The sensitivity test was carried out following the protocol described by Kim and Xiao (2010) and Zhang *et al*. (2007). Five technical-grade fungicides were used in this study: fludioxinil, cyprodinil (Syngenta Crop Protection, Canada), pyraclostrobin and boscalid (BASF Cooperation NC, USA), and penthiopyrad (Dupont Crop Protection, Canada). Stock solutions of the fungicides were prepared in acetone. All the fungicides were added to PDA medium. The final acetone concentration was 1 ml/L (0.1% vol/vol) in all fungicide concentrations and control media. Autoclaved agar media were cooled to 45°C and amended with appropriate volumes of the fungicide stock solutions to obtain the following test concentrations: 0.0, 0.01, 0.05, 0.5, 1 µg/ml of fludioxinil; 0.0, 0.1, 1, 10, 100 µg/mL of cyprodinil; 0.0, 0.01, 0.1, 1, 10 µg/mL of pyraclostrobin; 0.0, 0.05, 0.5, 1, 5 µg/mL ofr boscalid and 0.0. 0.1, 0.5, 1, 5 µg/mL of penthiopyrad.

The grouping of sensitivity of isolates to the fungicides was based on previousely published discriminatory doses (DD). A single discriminatory dose for each fungicide was defined. The discriminatory dose was defined as the concentration at which *B. cinerea* isolates could be separated in two groups: those inhibited in the presence of the fungicide and those not inhibited (Panebianco, 2013). Discriminatory dose (DD) used were 0.03 µg•mL^{-1} for cyprodinil (Myresiotis et al., 2007), 0.1 µg•mL^{-1} for fludioxonil and pyraclostrobin (Baroffio et al., 2003; Fernandez-Ortuno et al., 2016), 1.0 µg•mL^{-1} for boscalid, and penthiopyrad (Fernandez-Ortuno et al., 2016).

2.2.4. Sensitivity of mycelial growth

Two perpendicular lines were marked on the bottom of the Petri dishes (Figure 2.1). Individual 4.0-mm-diameter mycelial plugs from a 3-day-old culture were removed from

the margin of the colony with a sterile cork borer and placed upside down onto the center

(intersection of the two lines) of the Petri dish (90 × 15 mm) containing amended PDA at

each fungicide concentration. The cultures were incubated for 3 days in the dark (Figure

2.2). The colony diameter (minus the diameter of the inoculation plug) was measured in

two perpendicular directions. The experiment was repeated four times for each isolate.
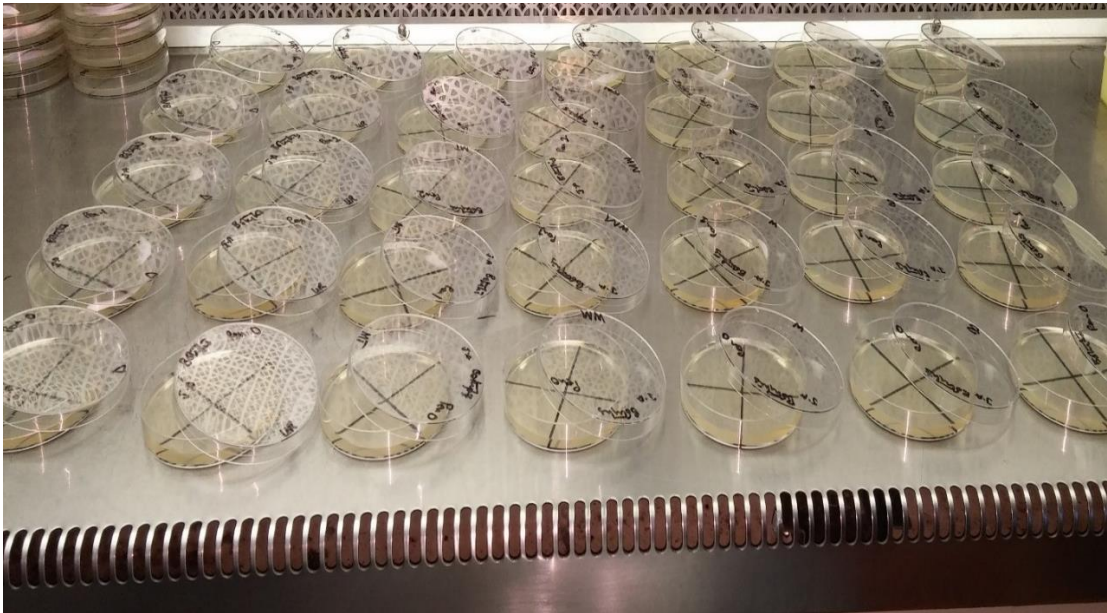
Figure 2.1. Experimental set-up of Potato dextrose agar (PDA) amended with different concentrations of fungicides for the assessment of mycelial growth of *B. cinerea* isolates from wild blueberry fields in Nova Scotia.
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Figure 2.2. Incubation of *Botrytis cinerea* isolates from wild blueberry fields in Nova Scotia on Potato dextrose agar (PDA) amended with different concentrations of fungicides at 22-24°C.

2.2.5. Statistical analysis

The results were expressed: percent inhibition of mycelial growth relative to growth on the control or unamended medium multiplied by 100. EC_{50} values (μL/mL) were calculated by nonlinear regression of the relative growth against the log of the fungicide concentrations using SigmaPlot (version 12.0, software Inc., San Jose, California, US) (Leroux, 2007). EC_{50} is the effective concentration of fungicide causing a 50% reduction in the mycelial growth, compared to the mycelial growth on the unamended plate (control). Subsequently, the EC_{50} range and the relative mean were determined for each chemical compound. Correlation analysis was performed using Minitab statistical software (Version 17) to determine whether there was cross-resistance among the fungicides.

2.3. RESULTS

2.2.1. Sensitivity tests

The sensitivity of 15 *B. cinerea* isolates to fungicides and the EC_{50} values for each active ingredient are shown in Table 2.1. Also, the EC_{50} values of the eight baseline isolates are presented (Table 2.3).

Cyprodinil

The sensitivity test performed on the 15 isolates revealed that all the isolates had reduced sensitivity to cyprodinil based on a discriminatory dose of 0.03 µg·mL^{-1}. EC_{50} values ranged from 0.04 to 10.03 µg·mL^{-1}, with a mean EC_{50} value of 4.38 (± 1.01) µg·mL^{-1}. These isolates exhibited good mycelial growth on PDA amended with concentrations >1.0 µg.ml^{-1}. Thus, these isolates were all classified as having decreased sensitivity (resistance) to cyprodinil (Table 2.1). The sensitivity of the eight baseline isolates revealed EC_{50} values of 0.03 to 8.91 µg.ml^{-1} with a mean of 2.32 (± 1.32) µg.ml^{-1} (Table 2.3).

Fludioxonil

The fludioxonil sensitivity test conducted on the 15 *B. cinerea* isolates revealed that all the isolates were sensitive to fludioxonil with EC_{50} values of 0.0047 to 0.0073 µg·mL^{-1} (mean EC_{50} = 0.006 ± 0.0002 µg·mL^{-1}). Using a discriminatory dose of 0.1 µg·mL^{-1}, all the 15 isolates were classified as sensitive to fludioxonil (Table 2.1, 2.2). None of the isolates grew on the media amended with fludioxonil at concentration >0.05 µg·mL^{-1}. The eight baseline isolates revealed EC_{50} values of 0.006 to 0.010 µg.ml^{-1} with a mean of 0.008 (± 0.001) µg.ml^{-1} (Table 2.3).

Boscalid

The boscalid sensitivity test carried out on the 15 *B. cinerea* isolates revealed EC_{50} values which ranged from 0.47 to 9.25 µg·mL^{-1} with a mean of 2.79 ($\pm$ 0.71) µg·mL^{-1} (Table 2.1). Among the 15 isolates tested, the majority (11) had EC_{50} values higher than the discriminatory concentration, 1.0 µg·mL^{-1}, and were boscalid resistant (Table 2.2). Four of the isolates were classified as boscalid sensitive. The EC_{50} values of the baseline isolates were observed to be between 0.70 and 2.55 µg·mL^{-1} with a mean of 1.35 ($\pm$ 0.26) µg·mL^{-1} (Table 2.3).

Penthiopyrad

The EC_{50} values for the 15 isolates on penthiopyrad ranged from 0.15 to 1.88 µg·mL^{-1} with a mean of 0.46 ($\pm$ 0.13) µg·mL^{-1} (Table 2.1). Most of the isolates (13) had EC_{50} values between 0.15 and 0.92 µg·mL^{-1}, and hence, were considered as sensitive. Only two isolates had EC_{50} values greater than 1.0 µg·mL^{-1} (Table 2.2). The EC_{50} values of the baseline isolates were observed to be between 0.11 and 0.32 µg·mL^{-1} with a mean of 0.18 ($\pm$ 0.03) µg·mL^{-1} (Table 2.3)

Pyraclostrobin

The sensitivity test performed on the 15 isolates revealed that all the isolates had reduced sensitivity to pyraclostrobin using a discriminatory dose of 0.1 µg·mL^{-1}. EC_{50} values ranged from 0.41 to 12.40 µg·mL^{-1}, with a mean EC_{50} value of 2.79 ($\pm$ 1.01) µg·mL^{-1} (Table 2.1). All the 15 isolates exhibited good mycelial growth on PDA amended with concentrations > 0.1 µg.ml^{-1}. Thus, these isolates were all classified as having decreased sensitivity (or resistance) to pyraclostrobin (Table 2.2). The sensitivity of the eight

baseline isolates revealed EC_{50} values of $0.41 - 2.98$ µg.ml^{-1} with a mean of 0.86 (± 0.30)

µg.ml^{-1} (Table 2.3).

Table 2.1. Sensitivity of *Botrytis cinerea* isolates obtained from wild blueberry fields in Nova Scotia to cyprodinil, fludioxonil, boscalid, pyraclostrobin and penthiopyrad.

Fungicide	Number of isolates	EC_{50} values (µg·mL^{-1})	
		Range	Mean ($\pm$ SEM)
Cyprodinil	15	$0.04 - 10.03$	4.38 (± 1.01)
Fludioxonil	15	$0.0047 - 0.0073$	0.006 (± 0.0002)
Boscalid	15	$0.47 - 9.25$	2.79 (± 0.71)
Pyraclostrobin	15	$0.41 - 12.40$	3.33 (± 1.01)
Penthiopyrad	15	$0.15 - 1.88$	0.46 (± 0.13)

Table 2.2. Characterization of *Botrytis cinerea* isolates from wild blueberry fields in Nova Scotia based on discriminatory doses (DD[x]) of different fungicides.

Isolate	Cyprodinil	Fludioxonil	Boscalid	Pyraclostrobin	Penthiopyrad
Debert 1	+[y]	-[z]	+	+	+
Debert 2	+	-	-	+	-
Debert 3	+	-	+	+	+
Debert 4	+	-	-	+	-
Kemptown 1	+	-	+	+	-
Kemptown 2	+	-	-	+	-
Kemptown 3	+	-	+	+	-
Dean 1	+	-	+	+	-
Dean 2	+	-	+	+	-
Parrsboro 1	+	-	+	+	-
Parrsboro 2	+	-	+	+	-
Webb Mt 1	+	-	+	+	-
Webb Mt 1	+	-	+	+	-
Rawdon 1	+	-	-	+	-
Rawdon 2	+	-	+	+	-

[x]The discriminatory dose (DD) used were 0.03 µg•mL^{-1} for cyprodinil (Myresiotis et al., 2007), 0.1 µg•mL^{-1} for fludioxonil and pyraclostrobin (Baroffio et al., 2003; Fernandez-Ortuno et al., 2016), 1.0 µg•mL^{-1} for boscalid, and penthiopyrad (Fernandez-Ortuno et al., 2016; Kim and Xiao, 2010).
[y]+ Indicates resistant isolate.
[z]– Indicates sensitive isolate.

Table 2.3. Sensitivity of baseline *Botrytis cinerea* isolate collected from a field that had never been sprayed with fungicides in Nova Scotia to cyprodinil, fludioxonil, boscalid, pyraclostrobin and penthiopyrad.

Fungicide	Number of isolates	EC$_{50}$ values ($\mu g \cdot mL^{-1}$)	
		Range	Mean ($\pm$ SEM)
Cyprodinil	8	0.03 – 8.91	2.32 ($\pm$ 1.32)
Fludioxonil	8	0.006 – 0.010	0.008 ($\pm$ 0.001)
Boscalid	8	0.70 – 2.55	1.35 ($\pm$ 0.26)
Pyraclostrobin	8	0.41 – 2.98	0.86 ($\pm$ 1.01)
Penthiopyrad	8	0.11 – 0.32	0.18 ($\pm$ 0.03)

Cross-resistance patterns

Sensitivity of isolates to each fungicide was correlated against the sensitivity to the other fungicides. Significant correlations of the EC$_{50}$ values, indicating cross-resistance, were seen among some of the fungicides tested for these isolates (Table 2.4). The highest correlation of sensitivity was seen between boscalid and pyraclostrobin (r = 0.737, p = 0.002). A significant correlation of sensitivity was observed between the two SDHI fungicides, boscalid and penthiopyrad (r = 0.671, p = 0.006). Also, there was a significant correlation between pyraclostrobin and penthiopyrad sensitivity (r = 0.655, p = 0.008). Fludioxonil and boscalid sensitivities were negatively correlated with a negative correlation coefficient of -0.583 (p = 0.023). The correlations indicate cross-resistance patterns between these fungicides. All the other combinations tested showed very low correlation coefficients and none of them were significant (P > 0.05) (Table 2.4).

Table 2.4. Patterns of cross-resistance between cyprodinil, fludioxonil, boscalid, pyraclostrobin and penthiopyrad in *Botrytis cinerea* isolates obtained from commercial wild blueberry fields in Nova Scotia.

	Fungicide							
	Fludioxonil		Boscalid		Pyraclostrobin		Penthiopyrad	
Fungicide	r^y	p^z	r	p	r	p	r	p
Cyprodinil	-0.269	0.332	0.123	0.661	0.213	0.445	0.064	0.821
Fludioxonil	-	-	-0.583	0.023	-0.154	0.583	-0.343	0.210
Boscalid	-	-	-	-	0.737	0.002	0.671	0.006
Pyraclostrobin	-	-	-	-	-	-	0.655	0.008

[y]Pearson correlation coefficient (r) values of EC_{50}
[z]Correlation coefficients are significant at $P < 0.05$

2.4. Discussion

The development of fungicide resistance among *B. cinerea* populations is of great concern to commercial wild blueberry, strawberry, grape and vegetable growers. In the past years, the most severe challenges of fungicide resistance development have occurred within *B. cinerea* (Brent and Hollomon, 2007). This study demonstrates that these concerns are justified and that resistance has emerged towards some of the fungicides used for Botrytis blight control in wild blueberry fields. Our study reports preliminary information on *B. cinerea* isolates resistance to anilinopyrimidine fungicide cyprodinil, phenylpyrrole fungicide fludioxonil, QoI fungicide pyraclostrobin, and the SDHI fungicides boscalid and penthiopyrad in *B. cinerea* isolates from wild blueberry fields in Nova Scotia. Furthermore, this study also determined the sensitivity of a few *B. cinerea* isolates from a baseline location that had never been sprayed with fungicides. Baseline isolates used in this study refer to *B. cinerea* isolates from field that has never been

sprayed with fungicides and completely isolated from any commercial blueberry or other farming operation. It is believed that these isolates if not native to the area, were disseminated some years ago and have been separated from the commercial field isolates for many years before the introduction of the tested active ingredients in Nova Scotia. Gene flow (migration) is a key evolutionary element in the determination of genetic variation and hence, differentiation between populations (Isenegger et al., 2008). Given the difference between the conditions of the two fields (commercial field and wild), and the less likelihood of isolates from a commercial field being dispersed to the field from which the baseline isolates where collected, the possibility of gene flow was likely restricted to only isolates within the baseline population and not between the baseline population and the commercial population.

This study revealed a high incidence of *B. cinerea* resistance to cyprodinil, pyraclostrobin and boscalid but less resistance to fludioxonil and penthiopyrad. *B. cinerea* isolates with increased tolerance to cyprodinil were observed in every field sampled, indicating that resistance is widespread in the wild blueberry fields. This is supported by the fact that Switch® which contains cyprodinil has been extensively used on wild blueberry field due to its effective disease control ability. Though some previous reports indicated less than 1% resistance frequencies of *B. cinerea* to a cyprodinil (Hilber and Hilber-Bodmer, 1998; Forster and Staub, 1996), our study revealed high frequency of isolates with reduced sensitivity. The results from this study confirms reports of widespread occurrence of *B. cinerea* strains resistant to cyprodinil in other studies (Fernández-Ortuño et al., 2012; Latorre et al., 2002). Cyprodinil resistant isolates have been reported in table grapes in Chile where about 38.5% of *B. cinerea* isolates were observed to be resistant to

cyprodinil after four applications of cyprodinil within two years (Latorre et al., 2002). Also, Fernández-Ortuño et al. (2012) reported 52.7% of 216 *B. cinerea* isolates recovered from strawberry fields in the Carolinas. In comparing the mean EC_{50} value (4.38 µg·mL^{-1}) from the 15 isolates to the mean EC_{50} (2.32 µg·mL^{-1}) value of the baseline isolates in our study, there is a clear indication of resistance development to cyprodinil.

A similar trend towards reduced sensitivity was observed in pyraclostrobin. Like Switch®, Pristine® which contains pyraclostrobin as one of its active ingredients has been used extensively in the region over the past decade. Pristine® application is usually made during the later stages of bloom to also provide Septoria leaf spot control on wild blueberry fields. Thus, the product has been used successively during every cropping phase of production in the region. This result is not surprising because in the literature, pyraclostrobin resistance in *B. cinerea* has been reported from many countries and on different crops. In an experiment with *B. cinerea* isolates from strawberries in Florida, Amiri et al. (2013) reported that 86.5% of 392 isolates were resistant to pyraclostrobin. In a similar experiment, Fernández-Ortuño et al. (2012) reported that 66.7% of 216 isolates were resistant to pyraclostrobin. Some researchers have also reported resistance to pyraclostrobin but with lower frequencies (Yin et al., 2011; Leroux et al., 2010). In comparing the mean EC_{50} value (3.33 µg·mL^{-1}) from the 15 isolates to the mean EC_{50} (0.86 µg·mL^{-1}) value of the baseline isolates, there is a clear indication of resistance development to pyraclostrobin.

Boscalid which is one of the active ingredients in Pristine® also had a high resistance frequency. This is not surprising because boscalid and pyraclostrobin are both respiration-inhibiting fungicides and found in the same product. The high resistance

frequency observed in this study is may be explained by the extensive use of Pristine® every year on wild blueberry fields in the region. Similar outcomes have been reported in the literature which corroborate our results. For instance, 85.4% of 392 *B. cinerea* were found to be resistant to boscalid in strawberry (Amiri et al., 2013). Also, Fernández-Ortuño et al. (2012) found that 61.6% of 216 isolates from strawberry fields in the Carolinas were resistant to boscalid. Comparing the mean EC_{50} value of the fifteen isolates (2.79 µg·mL^{-1}) to the mean baseline EC_{50} value (1.35 µg·mL^{-1}) in our study suggests a shift of *B. cinerea* isolates toward resistance to boscalid. Also, baseline EC_{50} values reported in literature; 2.09 µg·mL^{-1} (Myresiotis et al., 2008), and 1·07 µg·mL^{-1} in tomato, cucumber, aubergine and pepper (Zhang et al., 2007) also suggest a shift of *B. cinerea* isolates in the region towards resistance to boscalid.

Given the extensive use of Switch® for disease control, and fludioxonil premixed with cyprodinil as active ingredients in Switch®, it would be expected that these isolates would develop resistance to fludioxonil. However, none of the isolates were found to be resistant to fludioxonil in this study. This could be attributed to the polygenic (at least two different genes) control of resistance in *B. cinerea* as described by Vignutelli et al. (2002). Also, Ziogas et al. (2005) reported that fludioxonil resistance was coded by three unlinked chromosomal loci in *B. cinerea*. Hence, the inability of the pathogen to easily develop resistance to fludioxonil. In the literature, resistance in *B. cinerea* to fludioxonil is rarely reported (Fernandez-Ortuno et al., 2013a; Vignutelli et al. 2002). In some cases, just a single isolate is found to be resistant to fludioxonil (Fernandez-Ortuno et al., 2013b; Weber and Wichura, 2013). Our results confirm the outcome of previous studies that reported no resistance of *B. cinerea* isolates to fludioxonil (Panebianco et al., 2015;

Yin et al., 2015; Latorre and Torres, 2012; Myresiotis et al., 2007; Baroffio et al., 2003). Comparing the mean EC_{50} value (0.006 μg·mL^{-1}) of fludioxonil to the baseline EC_{50} (0.008 μg·mL^{-1}) in this study as well as the baseline sensitivity of fludioxonil in other crops, such as 0.005 μg·mL^{-1} in apple and pear in Washington by Zhao et al., (2010), there is no indication of a shift in sensitivity towards resistance to fluidioxonil hence, the EC_{50} value from this study can be used as a baseline for monitoring shifts in sensitivity to fludioxonil in the future.

Among all the fungicides used disease control in wild blueberries, penthiopyrad (Fontelis®) is one of the recent product registered. Therefore, it has not been extensively used compared to those registered earlier. This may explain the low level of resistant isolates observed in this study. Although there are not many resistance studies on penthiopyrad in the literature because it is one of the recently developed SDHIs, the low frequency of resistance observed in this study supports the outcome of previous work with penthiopyrad and other SDHI fungicides (Hu et al., 2016; Amiri et al., 2014). For instance, only 7.4% of 2570 isolates collected from strawberry fields in the eastern United States, showed resistance to penthiopyrad after over a decade of usage of SDHI fungicides for *B. cinerea* control (Hu et al., 2016). Although the study reveals that *B. cinerea* isolates are sensitive to penthiopyrad, a comparison of the mean EC_{50} value (0.46 μg·mL^{-1}) from the 15 isolates to the mean EC_{50} (0.18 μg·mL^{-1}) value of the baseline isolates, suggests a shift towards resistance development. A significant shift towards resistance to penthiopyrad is likely to occur in the near future as penthiopyrad is beginning to lose its efficacy. This could possibly be due to the fact that chemically, the structures of SDHI's are very diverse, however, they have an essential feature which is

the amide bond (Sierotzki and Scalliet, 2013). The extensive resistance to boscalid could have caused or increased the likelihood of *B. cinerea* resistance emerging.

From the results of boscalid (SDHI) and pyraclostrobin (QoI) obtained in this study, it is surprising that a strong positive correlation was observed between the two fungicides indicating possible cross-resistance. This is may be because the two fungicides come together in one product which has been extensively used for Botrytis blight control on wild blueberries in Nova Scotia. Cross-resistance between fungicides with a different mode of action is less common. Although they have different modes of action, they are both respiration-inhibiting fungicides, and both boscalid and pyraclostrobin resistance has been reported in Botrytis (Amiri et al., 2013) and other fungi such as *Alternaria solani* (Gudmestad et al., 2013). Nevertheless, some previous work on *B. cinerea* reported no cross-resistance between them because of the existence of isolates resistant only to either pyraclostrobin or boscalid (Kim and Xiao, 2010; Myresiotis et al., 2008).

Similarly, cross-resistance was detected between the two SDHI fungicides, boscalid and penthiopyrad. It has mostly been postulated that fungicides belonging to the same family or a similar mode of action are likely to develop cross-resistance. Our results confirm the cross-resistance reported with *B. cinerea* (Amiri et al., 2014) and other pathogens such as *Didymella bryoniae* (Thomas et al., 2012) and *Alternaria alternata* (Avenot et al., 2009). Cross-resistance in SDHI fungicides are conferred, in most fungi, by mutations in the SdhB subunits of these fungi (Amiri et al., 2014; Veloukas et al., 2013). It is therefore possible that the extensive usage of boscalid (Pristine®) in wild blueberry fields would have favoured *B. cinerea* isolates with a mutation in their SdhB subunits, hence, the positive correlation observed between penthiopyrad and boscalid although penthiopyrad

is a relatively new product. Interestingly, cross-resistance was also detected between penthiopyrad and pyraclostrobin. With boscalid and penthiopyrad belonging to the SDHI group, the likelihood of penthiopyrad developing cross-resistance with pyraclostrobin was high since boscalid showed cross-resistance with pyraclostrobin.

Sensitivity to fludioxonil was negatively correlated with the sensitivities to all the other fungicides tested. With the exception of boscalid, the sensitivity to fludioxonil was not significantly correlated with the sensitivities to the other fungicides tested. In a similar study, fludioxonil was negatively correlated with boscalid although the correlation was not statistically significant (Amiri et al., 2013). The result from this study also confirms the outcomes of previous work that observed no cross resistance between fluidioxonil and cyprodinil (Amiri et al., 2013; Myresiotis et al., 2007; Forster and Staub, 1996).

The information obtained in this study indicates that *B. cinerea* isolates from commercial wild blueberry field in Nova Scotia have shifted toward decreased sensitivity to cyprodinil, pyraclostrobin and boscalid. Nevertheless, the *B. cinerea* populations are stillsensitive fludioxonil and penthiopyrad. Given the significant cross-resistance relationship between penthiopyrad and boscalid as well as pyraclostrobin suggests there could be a high risk of widespread occurrence of *B. cinerea* populations resistant to penthiopyrad unless suitable resistant management strategies are employed to curb any future resistance challenges. Even though no resistance or cross-resistance was observed with fludioxonil, resistance management implementation is important to avoid a shift toward resistance.

This study, due to the limited number of isolates examined, is a preliminary work. Fifteen commercial and eight baseline isolates were used in this study. In sensitivity/resistance

analysis of fungi to fungicides, a minimum of 45 isolates are recommended to capture accurate data (Russell, 2004; Latorre, 2002; LaMondia and Douglas, 1997). Future work expanding on the present study would be useful to obtain the complete picture of the resistance status of *Botrytis cinerea* in Nova Scotia, Canada.

2.5. Conclusion

The present study revealed several cases of fungicide resistance in *B. cinerea* isolates from some Nova Scotian wild blueberry fields, showing different levels of reduced sensitivity (resistance) to single fungicides and/or multiple resistance to different combinations of fungicides. Preliminary data obtained in the current study indicate that *B. cinerea* populations from the selected wild blueberry fields in Nova Scotia have shifted toward decreased sensitivity to cyprodinil, boscalid and pyraclostrobin. Despite the implementation of resistance management strategies such as the combination of different actives ingredient in a product, selection of resistant strains to anilinopyrimidines, quinone outside inhibitors (QoIs) and some succinate dehydrogenase inhibitors (SDHIs) fungicides have occurred. Nevertheless, *B. cinerea* isolates from Nova Scotian wild blueberry fields had not shifted towards resistance development to fludioxonil and penthiopyrad. The appearance and selection of resistant isolates, mostly exhibiting resistance to multiple fungicides, highlight the importance of suitable resistance management measures through the adoption of IPM schemes and restriction of the number of fungicide sprays with the same mode of action in a season.

CHAPTER 3

BOTRYTIS BLOSSOM BLIGHT OF WILD BLUEBERRIES: PHENOTYPE AND FLORAL BUD SUSCEPTIBILITY

3.0. Abstract

Botrytis blossom blight, caused by *Botrytis cinerea*, is an important disease of wild blueberries (*Vaccinium angustifolium*). Two field experiments were designed in 2016 and 2017 to determine the susceptibility of four phenotypes (*V. angustifolium*, *V. angustifolium* f. *nigrum*, *V. myrtilloides* and *V. angustifolium* var. Fundy) and wild blueberry floral stages [(Bud break (F5, Pink or white bud prebloom (F6), corolla fully open (F7), and senescent corolla (F8)] to Botrytis infection. Specific flower clusters on tagged stems from different phenotypes were inoculated with a *B. cinerea* conidial suspension (10^6 conidia/ml). The differences in the infection among the flower stages and phenotypes were visible 8 days after inoculation. Disease incidence and severity in phenotype ranged from 14.1 to 22.6% and 37.4 to 42.3% in 2016, and 39.8 to 44.1% and 9.70 to 19.1% in 2017, respectively. Results indicated that *V. angustifolium* was more susceptible followed by *angustifolium* f. *nigrum* and *V. angustifolium* var. Fundy. *V. myrtilloides* was found to be least susceptible among all the phenotype tested. Disease incidence and severity on floral developmental stages ranged from 2.95 to 36.4% and 7.81 to 75.5% in 2016, and 7.28 to 66.9% and 11.1 to 27.1% in 2017, respectively. Floral stage F7 was the most susceptible with incidence up to 66.9% and severity up to 75.5%. This was followed by stages F6, F5 and F8. Floral stages F8 and F5 were the least susceptible with incidences of less than 3% and 7.5% and severity of 7.81% and 11.1%, respectively. The outcome of this study indicates that *V. myrtilloides* is less susceptible to *B. cinerea* than *V. angustifolium* phenotypes. Also F6 and F7 floral stages are more

susceptible to Botrytis blight. The outcome of this study has the potential of helping growers make informed decisions on the timely application of disease control measures based on plant developmental stage. This finding could also be useful in blueberry breeding programs.

Keywords: *Vaccinium angustifolium,* phenotype, *Botrytis cinerea,*

3.1 Introduction

Wild blueberry (*Vaccinium angustifolium*) production represents a valuable component of the agricultural industry in Atlantic Canada. It is a high-value export crop with approximately 65,000 ha under production representing about 50% of Canada's land area in fruit and nut production (Statistics Canada, 2015). The blueberry plant is affected by several fungal diseases such as Monilinia blight, Septoria leaf spot and Botrytis blossom blight (Percival, 2013; Delbridge et al., 2011). Botrytis blight caused by *Botrytis cinerea* Pers.:Fr is one of the most important and destructive diseases especially in the coastal regions with prolonged periods of cool and wet conditions (Lambert, 1990).

Botrytis blossom blight has been found to cause over 20% crop loss annually on the field (WBPANS 2013, unpublished data; Delbridge and Hildebrand, 1997) but is usually not important in storage. On blueberries, the pathogen attacks individual flowers or entire inflorescences at the mid to late bloom stage (F6 and F7 stages), though it can also attack leaves and tender green twigs. It is an opportunistic fungus that usually infects wound sites or senescing tissues but it can also penetrate intact host surfaces (Cole et al., 1996; Williamson et al., 1995), and enter its host through natural openings (Hsieh et al., 2001; Fourie and Holz, 1995), causing necrosis. Infected tissues show symptoms similar to and are sometimes mistaken for freeze injury especially at the early stages. However,

Botrytis-infected tissues turn brown or black and then die. Infected tissues show the typical gray mould sign with abundant masses of conidia (Delbridge and Hildebrand, 1997). Infection and outbreak of the disease occurs when there are several hours to days of wet conditions during bloom or when wetness and moderate temperatures (14 to 28 °C) occur at bloom (Sapkota et al., 2015; Rivera et al., 2013). In addition to abundant sporulation during favorable weather conditions, conidial germination has been found to be enhanced by the presence of sugar such as fructose and sucrose (Nassr and Barakat, 2013).

The current strategy for control of Botrytis diseases mainly relies on the combination of proper cultural management techniques such as canopy management and fungicide applications. However, the unique nature of wild blueberries does not allow for canopy management. Nonetheless, cultural management techniques including thermal treatments (burning) used for Botrytis management have been phased out due to high cost and deleterious environmental effects. Therefore, Botrytis control in wild blueberry fields is basically through the application of fungicides. In others crops such as strawberries, resistant cultivars are used to help reduce the impact of the disease (Chandler et al., 2004) though Botrytis-resistant cultivars are presently not available for most crops.

Wild blueberry fields are extremely heterogeneous with about four phenotypes of blueberry plants. These include *V. angustifolium*, Ait, *V. angustifolium* f. *nigrum*, *V. myrtilloides* Michx. and *V. boreale* (Kinsman, 1993; Eck, 1996). Despite the importance of Botrytis blossom blight on wild blueberry fields, little is known about the susceptibility of the various phenotypes to Botrytis blight. Not much is known about the susceptibility of the various developmental stages of the blueberry flower to Botrytis

infection (Hildebrand et al., 2001). There is substantial information on the environmental conditions necessary for Botrytis disease development both in blueberries and other crops (Sapkota et al., 2015; Rivera et al., 2013; Hildebrand et al., 2001). Therefore, the information on the host developmental stage and host susceptibility is critical to developing disease management strategies and breeding programs. In view of this, the objectives of this study were to determine (i) the susceptibility of wild blueberry flowers at specific developmental stage to Botrytis blossom blight (ii) the relative susceptibility of various phenotypes to Botrytis blossom blight.

3.2. Materials and Methods
3.2.1. Site selection and experimental design
Two trials were conducted during the 2016 and 2017 cropping years in a commercial wild blueberry field at Debert, Nova Scotia (NS) (coordinates = 45°26'35.65 N, 63°27'5.69 W) in June 2016 and May-June 2017. The trials were set up in a split plot experimental design with four replications. Four phenotypes consisting of *V. angustifolium*, *V. angustifolium* f. *nigrum*, *V. myrtilloides* and *V. angustifolium* var. Fundy (Figure 3.1) were used as whole plot factor. Four flower growth and developmental stages, were used as subplot factors: corolla half developed (F5), Pink or white bud prebloom (F6), corolla fully open (F7), and senescent corolla petal (fall) (F8) (Figure 3.2) (Hildebrand et al., 2001). The experiment was conducted in Debert, NS because patches of all the wild blueberry phenotypes were present in that field. In the 2016 trial, *V. angustifolium* var. Fundy was excluded because the patch for that phenotype was in the vegetative phase, therefore it was not possible to use it. Due to the difference in the growth and development among the blueberry plants, obtaining all the

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four floral development stages was challenging. As the flowers developed and the growth stages advanced, number of flowers in early stages decreased, thus, as flowers approached the F8 stage, the number of flowers in the F5 stage decreased. This posed a challenge in obtaining all the four stages in 2016. Due to this observation, the experiment in 2017 was conducted earlier, in May-June, hence, the F8 stage was excluded. The exclusion of the F8 stage was also informed by the outcome of a pilot experiment in 2015 and that of 2016.

The field for the experiment was equipped with a Watchdog® model 2700 weather station (Aurora, IL, USA) to monitor air temperature, relative humidity, wind speed and direction, and leaf wetness and logged data every 15 min for the duration of the trial.

Figure 3.1. *V. angustifolium* (A), *V. angustifolium* f. *nigrum* (B), *V. myrtilloides* (C) and *V. angustifolium* var. Fundy (D).
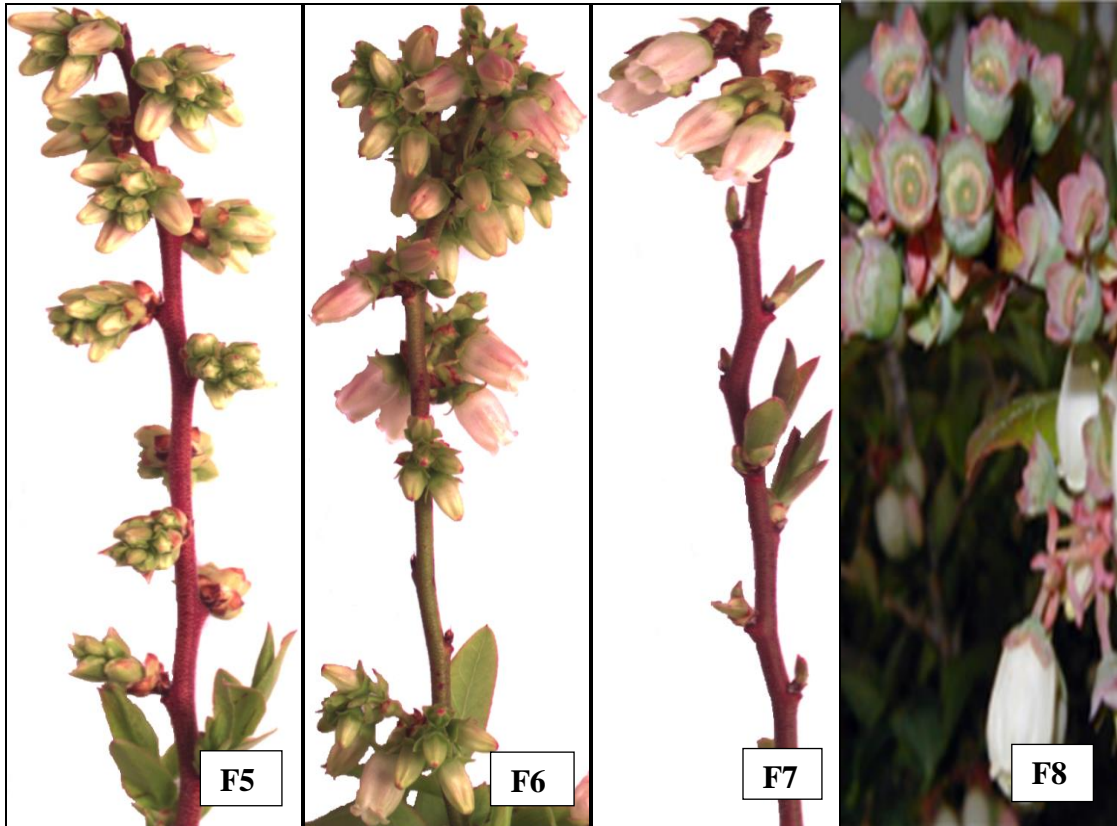
Figure 3.2. Lowbush blueberry floral bud stages. F5. Buds break; F6. Pink or white bud; F7. Anthesis or corolla fully open; F8. Senescent corolla.

3.2.2. Inoculum production and preparation

B. cinerea was isolated from a diseased blueberry flower from the field by collecting conidia with a sterile scalpel and spreading them on the surface of potato dextrose agar (PDA) in a Petri dish. The cultures were sub-cultured on PDA and incubated at 22-24°C in the dark to obtain abundant conidia.

Fungal inoculum was prepared from 10- to 14-day-old cultures. A conidial suspension was prepared by flooding plates with about 5ml of sterile distilled water and dislodging conidia with a glass rod. The conidial suspension was filtered through a double layer of cheesecloth and conidia were counted with a hemacytometer (BLAUBRAND® Neubauer improved). The suspension was diluted and adjusted to a concentration of 10^6 conidia/ml

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using a hemacytometer. Tween 20 (0.04%) was added to the suspension prior to inoculation. The germination percentage of the conidial suspension used was between 65 and 70%.
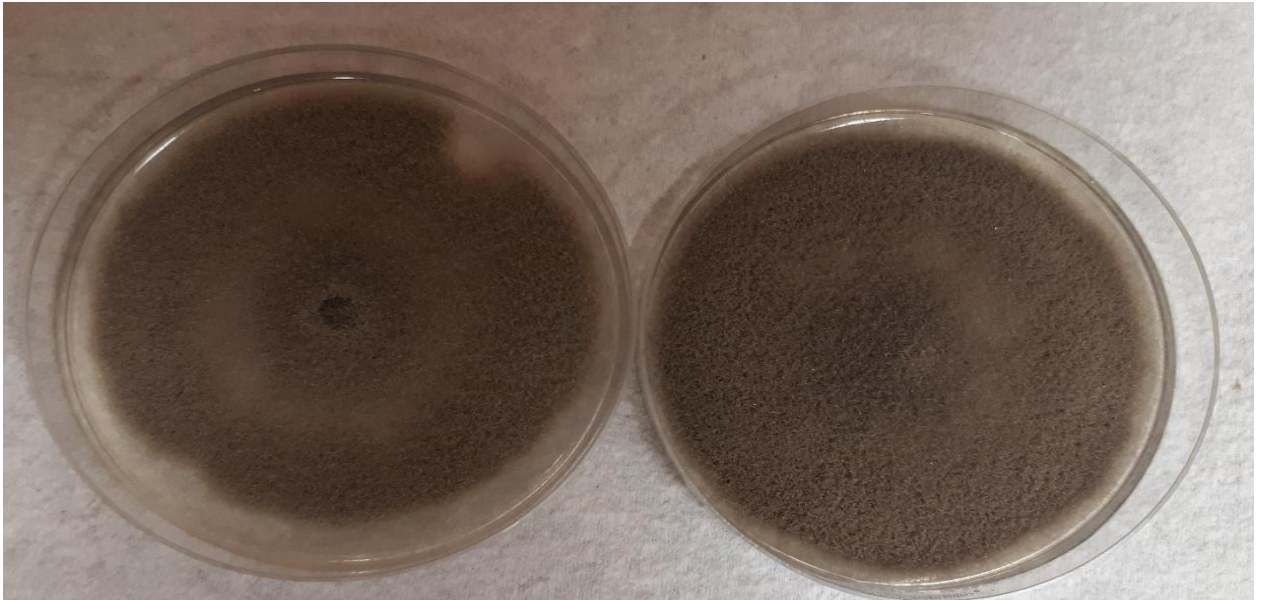
Figure 3.3. A three-week-old culture of *Botrytis cinerea* used for flower inoculation.

3.2.3. Preparation of plants and inoculation

The blueberry plants were not sprayed with any *Botrytis* control fungicide. Ten stems of each split plot with flowers of the specified phenotype at a specific growth stage were tagged with flagging tape and all other flowers were removed. Since the stage of individual flower development within clusters varied, only clusters showing a uniform flower development were tagged. All other flowers were removed from the plants. Clusters were scored based on the most advanced flower (Hildebrand et al., 2001). *Botrytis cinerea* conidial suspension was applied to the flowers using a hand pump sprayer to produce very fine evenly distributed droplets on each plant to the point of runoff. Immediately after inoculation, the plots were covered with a hoop structure with

DeWitt Plant & Seed Guard row cover and a 2-mm plastic film for 24 hours to provide conducive conditions for infection after which the plastic film was removed. In order to assess the presence of pre-infection or inoculum deposition prior to the experiment, random plots near the experiments also were covered with a hoop structure and row cover during the same period of the experiment. Assessment of these random plots for disease infection were done after 10 days and it could be concluded that the fields was sufficiently clean from background inoculum.

Figure 3.4. Experimental set up of wild blueberry stems within a phenotype with specific flower stages tagged with a flagging tape before inoculation with *B. cinerea* conidial suspension at Debert, NS in June 2017.

Figure 3.5. Whole plots covered with a row cover and plastic film immediately after inoculation to create humid conditions for *Botrytis* infection in wild blueberry.

3.2.4. Disease assessment

Assessment of Botrytis blossom blight was carried out 8 days after inoculation. Disease incidence and severity were recorded and attention was given to the specific site of infection (corolla, stigmatic surface or ovary). Disease incidence was determined by the percentage of floral buds per stem with visual symptoms of Botrytis blight. Severity was estimated by proportion tissue area per flower with visual symptoms of Botrytis blight within a stem. All the data collected from the experiments above were analyzed using the PROC GLIMMIX procedure of SAS (α=0.05).

3.3. Results

The inoculation of the blueberry flowers with a *B. cinerea* suspension resulted in substantial disease development. Significant differences were observed among the various phenotypes in this experiment. Similarly, significant differences were also

observed among the various floral developmental stages. The specific floral part of infection observed was the corolla with 98.4% and 98.9% of the total flower cluster infected in 2016 and 2017, respectively.

In 2016, disease incidence and severity ranged from 14.1 to 22.6% and 37.4 to 42.3%, respectively, among phenotypes. Disease incidence was significantly higher in *V. angustifolium* (22.6%) compared to *V. angustifolium* f. *nigrum* (16.6%) and *V. myrtilloides* (14.1%). No significant difference was observed among the phenotypes with respect to disease severity (Table 3.1).

Among the flower developmental stages, disease incidence and severity ranged from 2.95 to 36.4% and 7.81 to 75.5%. Floral stage F7 had the highest disease incidence (36.4%) followed by floral stage F6 with incidence of 28.5%. Floral stages F8 and F5 had the least disease incidences of 2.95% and 3.32%, respectively. There was no significant difference between the incidences in floral stages F8 and F5. Similar trend was observed with the severity of the floral stages but the values were higher than those of the incidence. Floral stage F7 remained the highest with severity of 75.5% followed by floral stages F6, F5, and F8 with 66.1%, 8.20% and 7.81%, respectively. No significant difference was observed between the severities of F8 and F5 as observed in incidence (Table 3.2).

There was a significant treatment effect on interaction between phenotype and floral bud stage with respect to disease incidence. Although, significant interaction was observed, there was no difference among F5 and F8 interaction with all the phenotypes. Floral stages F5 and F8 interaction with the phenotypes had the least incidence whereas *V. angustifolium* * F7 had the highest incidence (50.5%) followed by *V. angustifolium* f.

Nigrum * F7 and *V. angustifolium* * F6. There was however, no significant treatment effect on the severity of phenotype and floral bud stage interaction.

In 2017, observations among phenotypes were a reciprocal of that observed in 2016. Incidence was not significant but it was significant in 2016. Nevertheless, severity was significant in 2017 whereas it was insignificant in 2016. Disease incidence ranged from 39.8 to 44.1% whereas severity ranged from 9.70 to 19.1 %. Disease severity was significantly higher in *V. angustifolium* var. Fundy (19.1%) followed by *V. angustifolium* f. *nigrum*, *V. angustifolium* and *V. myrtilloides* (Table 3.3).

Among the flower developmental stages, similar trend was observed compared to 2016 outcome. Disease incidence and severity ranged from 7.28 to 66.9% and 11.1 to 27.1%. F7 had the highest incidence (66.9%) followed by F6 (51.6%) and F5 (7.28%). With respect to severity, F7 remained the highest with 27.1% followed by F6 and F5, with 12.4% and 11.1% respectively (Table 3.4)

There was significant interaction effect on both incidence and severity. Similar trend was observed in both incidence and severity interactions with the interaction of *V. angustifolium* var. Fundy, *V. angustifolium* f. *nigrum* and *V. angustifolium* with F7 being the most susceptible. Interaction of all the phenotypes with F5 was the least susceptible. Generally, the interaction between phenotypes and floral stages were low with F5 but increased with increasing flower stage with the exception of *V. mytilloides* * F7 (Table 3.3).

Table 3.1. Incidence of Botrytis infections observed on wild blueberry stems 8 days after inoculation with *B. cinerea* conidial suspension in 2016.

| Phenotypes | Flower Developmental Stage | | | | Main effect (Phenotypes) |
	F5	F6	F7	F8	
V. angustifolium	1.85d	35.9b	50.5a	2.48d	22.6a
V. angustifolium f. *nigrum*	0.63d	24.8c	38.4b	2.50d	16.6b
V. myrtilloides	7.51d	24.9c	20.4c	3.87d	14.1b
Main effect (Flower stages)	3.33c	28.5b	36.4a	2.95c	

% Incidence = 0 to 100% where 0 = no blossoms infected and 100 = all blooms are infected with at least one lesion. ANOVA: Phenotype * floral bud stage   Sig. (p=0.0001), Phenotype   Sig. (p=0.0008), Floral bud stage   Sig. (p=0.0001)**.** Means followed by the same letters in a column/row are not significantly different from each other.

Table 3.2. Severity of Botrytis infections observed on wild blueberry stems after 8 days of inoculation with *B. cinerea* conidial suspension in 2016.

| Phenotypes | Flower Developmental Stage | | | | Main effect (Phenotypes) |
	F5	F6	F7	F8	
V. angustifolium	2.36	63.5	80.1	3.75	37.4
V. angustifolium f. *Nigrum*	2.50	66.3	77.1	5.00	37.7
V. myrtilloides	19.7	68.3	66.4	14.6	42.3
Main effect (Flower stages)	8.20b	66.0a	75.5a	7.81b	

Severity = 0 to 9 rating scale where 0 = no disease and 9 >= 90% of each blossom/leaf tissue is infected. ANOVA: Phenotype * floral bud stage = NS, Phenotype = NS, Floral bud stage   Sig. (p=0.0001). Means followed by the same letters in a column/row are not significantly different from each other.

Table 3.3. Incidence of Botrytis infections observed on wild blueberry stems 8 days after inoculation with *B. cinerea* conidial suspension in 2017.

Phenotypes	Flower developmental stage			Main effect (Phenotypes)
	F5	F6	F7	
V. angustifolium	5.55fg	57.01bcd	69.80ab	44.12
V. angustifolium f. *Nigrum*	0g	61.98abc	67.38abc	43.12
V. angustifolium var. Fundy	5.83fg	41.97e	74.30a	40.70
V. myrtilloides	17.7417f	45.7464de	56.1764dc	39.88
Main effect (Flower stages)	7.28c	51.68b	66.92a	

% Incidence = 0 to 100% where 0 = no blossoms infected and 100 = all blooms are infected with at least one lesion. ANOVA: Phenotype * floral bud stage   Sig. (p=0.0003), Phenotype = NS, Floral bud stage   Sig. (p=0.0001). Means followed by the same letters in a column/row are not significantly different from each other.

Table 3.4. Severity of Botrytis infections observed on wild blueberry stems 8 days after inoculation with *B. cinerea* conidial suspension in 2017.

Phenotypes	Flower developmental stage			Main effect (Phenotypes)
	F5	F6	F7	
V. angustifolium	0.40f	9.05de	23.95b	11.13bc
V. angustifolium f. *Nigrum*	0f	15.67cd	26.98b	14.18b
V. angustifolium var. Fundy	1.93fe	14.55cd	40.90a	19.12a
V. myrtilloides	2.11ef	10.56cd	16.67c	9.78c
Main effect (Flower stages)	11.11c	12.43b	27.13c	

Severity = 0 to 9 rating scale where 0 = no disease and 9 >= 90% of each blossom/leaf tissue is infected ANOVA: Phenotype * floral bud stage = Sig. (p=0.0001), Phenotype = Sig. (p=0.0001), Floral bud stage   Sig. (p=0.0001). Means followed by the same letters in a column/row are not significantly different from each other. Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ɑ́=0.05).

3.4. Discussion

Although Botrytis blossom blight is important and widespread in wild blueberry fields along coastal areas, to our knowledge, this is the first study that compares the susceptibility of lowbush blueberry phenotypes to Botrytis blossom blight. Nonetheless, there are a few reports that compared susceptibility of floral developmental in lowbush blueberry and highbush blueberry (Hildebrand et al., 2001; Smith, 1998).

Traditionally, interaction between environment, host and pathogen has been diagrammed into a triangle to illustrate the individual components of disease development (Agrios, 2005). In the absence of any of these three components, disease is less likely to occur. Given the conditions under which this experiment was conducted, thus, similar weather conditions and inoculum, it is highly possible that any difference in the susceptibility of the various phenotypes is could be due to host factors which could be genetically or physically influenced.

In our study, it was observed that *V. myrtilloides* was relatively less susceptible to Botrytis blight than *V. angustifolium* species. In a pilot study, *V. myrtilloides,* was also observed to be less susceptible compared to the *V. angustifolium* (Harris, 2015, unpublished data). Botrytis blight infection is usually variable in the field due to a number of factors such as difference in bud development among clones, genotypic make up of plants and varying environmental conditions. The phenotypic and genetic diversity of *B. cinerea* found in wild blueberry fields is unknown, therefore it is likely that plants in some fields or within some clones may be infected by more virulent *B. cinerea* strains than others leading to variable levels of infections. The difference among the phenotypes observed in this study could be due to genotypic difference among the phenotypes since

the conditions and the isolates employed in this study were similar. Generally, it can be said that the *V. myrtilloides* group are less susceptible to Botrytis infection than the *V. angustifolium* groups. This is interesting because, similar outcome was observed with Monilinia blight where *V. myrtilloides* was found to be less susceptible (Stretch et al., 2001; Ehlenfeldt and Stretch, 2001). The resistance in *V. myrtilloides* may be due to the difference in ploidy among the two species: *V. myrtilloides* is a diploid whereas *V. angustifolium* is a tetraploid (Kinsman, 1993). This could partly account for the resistance observed with *V. myrtilloides*. In addition to genetic factors, morphological features could be attributed to the difference in susceptibility. For example, *V. myrtilloides* possess pubescence/ hair-like structures on the on their stems and peduncles. This structure has the potential of preventing conidia from landing on plant surfaces.

Another important factor that may be attributed to the susceptibility is the phenological difference among the phenotypes. Although there exist appreciable variation in the phenology of wild blueberry species, *V. myrtilloides* is generally the late clone and this could contribute to their less susceptibility to infections. For plant reproduction, timing is important and it has been pointed out that early flowering plants might not had time to accumulate sufficient material resources for seed production and vigorous whereas the late flowering species might gain higher capacity for seed production (Elzinga et al., 2007). This phenomenon could also influence disease susceptibility on the field and would be a valuable source of information if investigated.

The outcome of this study may partially account for the high levels of Botrytis infections observed within commercial wild blueberry fields. This is because in commercial fields, about 80% of the plants are susceptible phenotype (*V. angustifolium* groups). The

combined effect of flower susceptibility as floral bud stage progressed and the presence of conducive weather conditions explains the observation of severe Botrytis infections.

A few studies have reveal that, the susceptibility of flowers (not limited to only blueberries) is dependent on the environmental conditions and flower developmental stage (Del Ponte et al., 2007; Mertely et al., 2002). In the present study, disease incidence was observed to be very low at the F5 stage but increased to over 85% and 89% on F6 and F7, respectively. A sharp decrease in incidence and severity was observed with F8. This observations are consistent with the reports of Hildebrand et al., (2001) on lowbush blueberry and Smith, (1998) on highbush blueberry. The susceptibility of floral buds begins to increase from floral stage F5 and peaks at stage F7 as was also demonstrated by Hildebrand et al., (2001) who reported no infection on flower buds at the F4 floral stage. After floral stage F7, susceptibility begins to decrease due to the formation of immature berries which are resistant to infection. A number factors have been identified to affect flower infection by pathogen in other crops. These include the role, quantities and importance of phenols (resveratrol) (Keller and Cole, 2003). Also, physiological changes, such as increased membrane permeability and increased pollen and pollen exudates are known to ensue in flower tissues and in plants as they age, hence increasing their susceptibility to infections. (Fourie and Holz, 1998). Some of these factors could account for the susceptibility of wild blueberry flowers as they advanced.

Similar trend as observed with the interaction effect on incidence was seen with floral bud infection progression. This shows little/no influence of phenotype on the floral stage infections. Although significant variation was observed among phenotype infections, F6 and F7 are the most important developmental stages in Botrytis disease management.

In the literature, Botrytis infections in a number of crops have mostly been associated with corolla (Rheinländer et al., 2013; Hartill and Campbell, 1974). Majority of the infections were observed on the corolla. Many of the flowers showed signs and symptoms on the entire flower tissues (the pistil, the stamen, ovary and nectiferous surfaces), such flowers had the entire corolla showing signs and symptoms of the disease. Visual observation of floral part infected in this study coroborates the report of Hildebrand et al., (2001) who observed that lesions spread from the corolla to the peduncle. Botrytis destroys entire tissue once infection begins from any part of the flower. In this study, corolla was found to be infected mostly and this could probably be due to the fact that corolla have large surface area compared to the other parts of the flower and also shield the androecium and gynoecium, hence, it is the first line of contact for inoculum deposition.

3.5. Conclusion

This study indicates that the variability among plants and the different floral bud developmental stages influence the extent of Botrytis infection on the field. Outcome of this study have illustrated that *V. angustifolium* and *V. angustifolium* var. Fundy is the most susceptible phenotype on wild blueberry fields compared to *V. angustifolium* f. *nigrum* and *V. myrtilloides* which were relatively less susceptible. Also it was found that floral buds are most susceptible at F7 stage (corolla fully open) for all the phenotypes whiles F5 and F8 were less susceptible to Botrytis infection.

CHAPTER 4

MANAGING BOTRYTIS BLOSSOM BLIGHT THROUGH FIELD SANITATION, LIME SULFUR AND *TRICHODERMA* APPLICATION

4.0. Abstract

Botrytis blossom blight is an important disease of wild blueberries which causes substantial yield loss annually. To determine the main and interaction effects of burning, lime sulfur and *Trichoderma* application on Botrytis disease development, and yield, field trial that involved primary inoculum reduction and disease reduction techniques was conducted from May, 2015 to August, 2016. Fields were burnt, in May, 2015 after pruning, lime sulfur (Green earth lime sulfur) and *Trichoderma* (Trianum P) were applied in November, 2015 and May, 2016, respectively as single and combined treatments. Burning-sulfur-*Trichoderma* and burning-lime sulfur combinations reduced Botrytis blossom blight incidence and severity by over 74% and 61% respectively compared to the untreated control. Stand-alone treatments of burning, lime sulfur and *Trichoderma* as well as burning-*Trichoderma* combination also reduced Botrytis blight infection compared to the untreated control. However, none of the treatments had significant effect on Botrytis blight on vegetative bud, Monilinia blight, and Septoria leaf spot. Burning, lime sulfur and *Trichoderma* treatments did not increase harvestable berry yield compared to the untreated control. Outcome from this study points out that Botrytis blossom blights can be managed adequately, and fungicide usage reduced when lime sulfur is integrated and used in tandem with burning and biofungicides.

Key words: Burn-pruning, Lime sulfur, *Botrytis cinerea*, Biofungicide, *Vaccinium angustifolium*

4.1. Introduction

Wild blueberry (*Vaccinium angustifolium)* fields are developed from native stands (AAFC, 2005) and consist of variable and distinctive clones that spread by rhizomes (Glass and Percival, 2000). The crop is managed on a two-year production cycle. Plants are pruned close to the ground to promote vegetative growth in the first or sprout year and flowering, fruit development, and harvest in the second or crop year.

Botrytis blossom blight is one of the most destructive diseases of both wild and cultivated blueberries. In wild blueberries, the disease has been reported to cause 30-35% yield loss annually (Delbridge and Hildebrand, 1997). It is of importance to fields along coastal areas with prolonged periods of cold and wet conditions (Delbridge and Hildebrand, 1997). Botrytis blight occurs during the bloom period and may be a recurrent problem in some fields or in seasons when extended wet periods occur during bloom or shortly after petal fall.

The disease is caused by the fungus *Botrytis cinerea* Pers.; Fr. It is one of the world's most important and damaging fungal pathogens, and has been reported to infect over 200 host plants of agricultural and horticultural important (Agrios, 2005; Jarvis, 1977; Hennebert, 1973). The pathogen has unique characteristics that enable it to survive for many years once they are established in the field. *B. cinerea* is a necrotrophic pathogen capable of growing and reproducing on dead and senescent plant tissues and plant debris as saprophyte. It is an opportunistic pathogen that initiates infection at wound sites or at sites previously infected by other pathogens. Nonetheless, it can also invade healthy or intact plant tissue when conditions are favourable (van Kan, 2003). Research findings have indicated that the fungus overwinters on weeds within and outside the blueberry

field. During wet weather conditions especially in the spring the fungus produces conidia on the overwintering diseased tissue, which are wind-blown to developing blueberry blossoms (Delbridge and Hildebrand, 1997). The principal plant part infected in blueberry is floral tissues but can also infect other parts such as leaves and stems (Delbridge and Hildebrand, 1997). The pathogen causes initial disease symptoms that are sometimes mistaken for frost injury. Infected flowers turn brown or black and die. *B. cinerea* produces abundant gray masses of conidia that can rapidly spread throughout the field. High humidity (>95%) and moderately cool temperatures (15 to 20°C) are ideal for Botrytis infection (Smith, 1998).

Wild blueberry production continues to expand due to improved management practices such as fertilization and weed management. The improvement in management practices has led to an increase in number of flowers from 34 million flower/acre to aver 150 million flowers/acre (Percival, 2013). Coupled with continuous periods of cold and wet conditions as well as increased in wild blueberry debris on fields has resulted in escalated *B. cinerea* infections. Though *B. cinerea* infections are on the rise, control of the disease is presently achieved using chemical fungicides. In Canada, the registered fungicides for Botrytis blight control include Switch® (a.i. cyprodinil + fludioxonil), Pristine® (a.i. boscalid + pyraclostrobin), Cantus® (a.i. boscalid) and Elevate® (a.i. fenhexamid) (Agriculture, Aquaculture and Fisheries, 2016).

Fungicidal suppression of Botrytis blossom blight often has been unsatisfactory as the fungus is a high-risk pathogen that easily acquires resistance to these chemical fungicides (FRAC, 2014; Percival, 2013; Brent and Hollomon, 1998). The development of resistance to these chemical fungicides has led to an increase in the number of fungicide

applications from no fungicide to three fungicide applications per season in wild blueberry fields. This has also resulted in increased cost of production associated with Botrytis blight control and increased concern about fungicide residue on berries. Due to the growing concern about the possibly harmful effects of chemical pesticides on the environment and pathogen resistance, there is increasing interest in adopting alternative approaches for disease management (Bélanger, 2006; Paulitz and Bélanger, 2001).

Field sanitation including the removal, and/or the destruction of infected plant parts and crop debris is encouraged for Botrytis disease management especially in greenhouse-grown flowers (Hausbeck and Moorman, 1996). In wild blueberries, *B. cinerea* colonized fruits from fields pruned by mowing were often observed than fields pruned by biennial burning (Lambert, 1990). Burn-pruning every second or third crop cycle has been found to reduce overwintering *Botrytis* (Delbridge and Hildebrand, 1997) and other pests and diseases (Jensen and Yarborough, 2004; Yarborough, 2004; Smith and Hilton, 1971).

Dormant sprays of vines with lime sulfur have been found to help in the reduction of Botrytis bunch rot in grapes. Lime sulfur (calcium polysulfide) has long been used as the product of choice for dormant applications on grapevines. In blueberries, lime sulfur can be sprayed at the dormant stage of the crop for Botrytis blight management (Douglas, 2003). It has been found to be a good cleanup product (Bettiga, 2013). Research has shown that the application of lime sulfur during dormant or delayed dormant will reduce overwintering sclerotia of *Botrytis cinerea* by 70-75% (Bettiga, 2013). The product kills the sclerotia and hence significantly reduces inoculum (Gubler and Bettiga, 2012).

Biological control of diseases is an alternate means of controlling foliar pathogens. One of the most studied commercial biocontrol agents are *Trichoderma harzianum* strains.

Several strains including T-22 and T39 have been found to suppress foliar pathogens such as *Botrytis cinerea, Sclerotinia sclerotiorum* and *Sphaerotheca fusca* on several crops (Elad, 2000a; Harman, 2000).

The importance and disease control capabilities of field sanitation (burning), lime sulfur and *Trichoderma harzianum* have been studied individually on several crops and very little if any in wild blueberries. Nonetheless, their combined or interaction effect in managing Botrytis blight in blueberries have not been exploited. Hence, this study was conducted to investigate the main and interaction effects of burning, dormant lime sulfur and *Trichoderma* applications on Botrytis blossom blight development and yield in commercial wild blueberry fields.

4.2. Materials and Methods
4.2.1. Site selection and experimental design

Field trial was conducted between 2015 and 2016 in a commercial wild blueberry field over the two-year production cycle of the crop. The field trial was set up at Debert, Nova Scotia (NS) (coordinates = 45°26'35.65 N, 63°27'5.69 W) in May, 2015. The field for the experiment was equipped with Watchdog® model 2700 weather station (Aurora, IL, USA) to monitor air temperature, relative humidity, wind speed and direction and leaf wetness and logged every 15 min for the duration of the trial. The trial was set up in a 2^3 factorial experiment in a randomized complete block design (RCBD) with 5 replications, a plot size of 4×4 m with 2 m buffers between plots. The plots were staked out and treatment tags were installed.

4.2.2. Treatment combination and application

The factors were (+/-) Propane burner (burning) (Weed Dragon® Model VT2-23SVC 100,000 BTU, LaCrosse, KS, USA) (Figure 4.1), (+/-) Green earth lime sulfur (Premier Tech Home & Garden Inc., QC, Canada) (Calcium polysulfide) and (+/-) Trianum P® (Koppert Biological Systems, ON, Canada) (*Trichoderma harzianum* T-22). Treatment combinations used in the trial were: 1. Untreated control, 2. Burning & Lime sulfur & Trianum P®, 3. Burning & Lime sulfur, 4. Burning & Trianum P®, 5. Burning, 6. Lime sulfur & Trianum P®, 7. Lime sulfur and 8. Trianum P®. Burning was done in May, 2015 after pruning using a Weed Dragon® Model VT2-23SVC 100,000 BTU propane burner. The lime sulfur was applied at 11.25 L· ha^{-1} in November, 2015 when 2/3 of the leaves had dropped and *Trichoderma* was be applied at 3.0 g ·m^{-2} in May, 2016. The lime sulfur and *Trichoderma* were applied using a Bell spray Inc.® hand-held research sprayer with 2m carbon dioxide propelled boom with 4 Tee Jet Visiflow 8003VS nozzles at a pressure of 220 kPa (32 PSI). The nozzle discharge rate was 12.5 mL·s^{-1} and application ground speed was approximately 1.2 m·s^{-1}

Figure 4.1. Application of treatments (burning) on the field with propane (arrowed).

4.2.3. Disease assessment, yield components and berry yield

Before bloom, ten blueberry stems were randomly selected from each plot for the assessment of Botrytis and Septoria canker. Twenty stems were also randomly selected at mid-bloom (between 40-50% blooms) and full bloom. The stems were cut diagonally at 30 cm interval along a 4.5-m line transect in each plot. Stems were cut as close to the base as possible to avoid vegetative stems. The stem samples were placed in plastic bags and brought to the laboratory for examination of Botrytis disease development (incidence and severity) and other diseases including Monilinia blight (First sample collection) and Septoria leaf spot.

Disease incidence was determined as the proportion of floral/leaf buds with visual symptoms of Botrytis blight within a stem (Nutter et al., 2006; Kranz, 1988). Disease severity was quantified as the proportion of flowers or leaf tissue area infected with visual symptoms of Botrytis blight using a 0-9 disease severity rating scale (Table 4.0).

Disease development on plants from treated plots were compared to the disease development on plants in the untreated control plots to determine disease suppression efficacy of the treatments. Phytotoxicity of each treatment was examined visually observing whether there was physical damage on the floral and/or vegetative buds of each stem.

Other physical development parameters that were recorded included stem length, number of vegetative buds per stem, floral node numbers per stem, and floral bud and vegetative bud stages. Fifteen stems were randomly selected in mid-July to assess yield components. The parameters recorded on each stem were length, number of vegetative and floral buds, side branches, fruit set and pinheads (small, unmarketable berries).

To determine harvestable berry yield, blueberries were harvested in August, 2016 with a forty-tine, commercial wild blueberry hand rake from four randomly selected 1 m^2 quadrants in each plot. Harvested berries from each plot were weighed with an Avery Mettler PE 6000 digital balance, and the data recorded. Composite 500 mL berry sample were taken from each plot and brought to the lab for further analysis (berry incubation) (Figure 4.2).

Figure 4.2. Berry samples (50 berries from each plot) placed in sterile Petri plates for incubation at 22-24 ºC and 100% RH.

Table 4.0. A 0-9 scale disease severity rating scale for Botrytis assessment in wild blueberries.

Scale	Rating (%)
0	No sign of disease infection observed on flowers or leaves
1	10-19
2	20-29
3	30-39
4	40-49
5	50-59
6	60-69
7	70-79
8	80-89
9	90-100

4.2.4. Statistical analysis

Data collected on Botrytis blight disease development, parameters of physical development of wild blueberries (stem length, number of floral and vegetative buds,

development stages of floral and vegetative buds), yield components (stem length, number of vegetative buds, floral buds, set fruits, pinheads, and side branches), and harvested berries were analyzed using the PROC GLIMMIX procedure of SAS (version 9.4, SAS institute, Inc., Cary, NC). LSD was used for multiple means comparison at α=0.05. Prior to the analysis, the data set was subjected to normality test. Data were not normally distributed. However, all transformation attempted could not normalize the data. Therefore, the central limit theorem was applied in the analysis due to large sample size, n > 30 (Hogg et al., 2015).

4.3. Results

Natural epidemics of *Botrytis* infection developed in the field during the experimental season. After burning and lime sulfur applications, incidence of Botrytis and Septoria canker before bloom ranged from 0 to 44.5 % and 10.0 to 83.3 %, respectively (Table 4.1). Burning and all treatment combinations with burning had the least incidence of Botrytis and Septoria canker with over 90 % less Botrytis canker and over 58 % less Septoria canker than the untreated control. There was however, no significant treatment effect of stand-alone application of lime sulfur, *Trichoderma* and the lime sulfur-*Trichoderma* combination on Botrytis and Septoria incidence compared to the untreated control. Generally, plots that were burnt had lower canker infections compared to those that were not burnt (Table 4.1).

Infections by *Botrytis* were significant for both mid and full bloom disease assessment, whereas infections by Monilinia did not reveal any significant difference. Across all treatments, Botrytis blight incidence and severity at mid bloom ranged from 0 to 3.35%

and 0 to 3.37%, respectively. At full bloom, Botrytis blight incidence and severity ranged from 2.27 to 13.18% and 2.87 to 9.30%, respectively. Disease levels were lowest in the Burning-lime sulfur as well as lime sulfur-*Trichoderma* treatment and highest in the untreated control (Tables 4.2a and 4.3a). Unlike floral infections, very low *Botrytis* infections of less than 0.5 % was recorded on vegetative buds for both mid and full bloom disease assessment (Tables 4.2a and 4.3a).

At mid bloom, Botrytis blossom blight incidence and severity varied among the treatments significantly. Botrytis floral incidence and severity were significantly reduced with burning, lime sulfur and *Trichoderma* combinations as well as stand-alone applications of burning, lime sulfur and *Trichoderma* (Table 4.2a). Compared to the untreated control, burning–lime sulfur–*Trichoderma*, burning–lime sulfur, burning–*Trichoderma*, burning, lime sulfur–*Trichoderma*, lime sulfur and *Trichoderma* reduced Botrytis blossom blight with over 50% and 43% less incidence and severity, respectively. All treatments but untreated control did not vary significantly from each other. There was no significant treatment effects on the development of Botrytis disease on vegetative bud. Similarly, there were no significant treatment effects on the development of Monilinia vegetative and floral bud blights (Table 4.2a). However, Septoria leaf infection was significant. Burning–lime sulfur, burning, lime sulfur–*Trichoderma*, lime sulfur, *Trichoderma* reduce Septoria leaf spot with over 60% and 32% less incidence and severity, respectively compared to the untreated control. Interestingly, burning–lime sulfur–*Trichoderma* combination was not able to suppress Septoria disease development (Table 4.2b).

Botrytis blight development was low at mid bloom but increased substantially compared to disease development at full bloom. Burning–lime sulfur–*Trichoderma*, burning–lime sulfur, and Lime sulfur –*Trichoderma* provided the most effective Botrytis blight suppression with 74.9, 82.8 and 81.8% less incidence and 61.1, 69.1 and 68.3 % less severity, respectively compared to the untreated control. Burning–*Trichoderma*, burning, lime sulfur and *Trichoderma* were able to reduce Botrytis blight compared to the untreated control with 43.0, 52.2, 38.6 and 54.6% less disease incidence, respectively. All treatments had over 40% less severity compared to the untreated control. Unlike floral bud infections, there was no significant treatments effect on the development of Botrytis disease on vegetative buds as observed during mid bloom. Contrary to that of mid bloom, there was no significant treatment effects on Septoria leaf spot infections (Table 4.3a). The difference in the blossom blight infection between mid-bloom and full bloom is influenced by floral growth stage.

There were significant treatment effects on some physical development parameters whereas other parameters did not reveal significant difference (Tables 4.1, 4.2b, 4.3b, and 4.4). Generally, stems from burnt plots and its combination with lime sulfur and *Trichoderma* were taller than stems from other treatments plots. Plots treated with burning–lime sulfur had relatively more number of floral buds (Tables 4.2b and 4.3b) compared to the rest of the treatments that were not significantly different from each other. Burning and all treatment combinations with burning had the highest number of vegetative buds compared to the other treatments without burning (Table 4.3b, and 4.4). No significant treatment effect was observed with yield component parameters (set fruit and side branches per stem). However, there were significant treatment effects on number

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of pinheads. Although pinheads were significant, most of the treatments did not vary significantly from each other and also the untreated control (Table 4.4).

Harvestable berry yields in the burning–lime sulfur, burning–*Trichoderma,* as well as stand-alone burning and *Trichoderma* treatments were relatively higher (over 710g$.$m^{-2}) than the other treatments. However, yields among burning–lime sulfur, burning–*Trichoderma,* burning and *Trichoderma* treatments were not significantly different from each other. Lime sulfur treated plots had the least berry yield (590.95 g$.$m^{-2}) which was not significantly different from the untreated control and burning–lime sulfur–*Trichoderma* and lime sulfur–*Trichoderma* (Table 4.4)*.* There was no indication of significant treatment effect on harvested berry samples incubated to assess the Botrytis fruit rot incidence (Table 4.5).

Table 4.1. Effect of burning, Lime sulfur and *Trichoderma* treatment on Botrytis and Septoria canker incidence before bloom from a commercial wild blueberry field at Debert, Nova Scotia.

Treatment	Stem length (cm)	Floral bud number	Botrytis Canker (%)[1]	Septoria Canker (%)[1]
Untreated control	17.7c	3.95	39.5a	76.5a
Burning - Lime sulfur - *Trichoderma*	20.5ab	4.26	0b	32.0b
Burning - Lime sulfur	21.9a	5.58	0b	18.0b
Burning - *Trichoderma*	18.9bc	4.52	0b	10.0b
Burning	18.6bc	3.72	2.00b	22.0b
Lime sulfur -*Trichoderma*	18.3bc	4.01	34.2a	74.7a
Lime sulfur	19.4bc	4.10	38.0a	82.0a
Trichoderma	18.1bc	4.62	44.5a	83.4a
ANOVA Results[3]	P=0.0152	NS	P<0.0001	P<0.0001

[1] % Disease incidence: % of number of stems showing visual signs and symptom of *Botrytis* infection.
[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.

Table 4.2a. Effect of burning, Lime sulfur and *Trichoderma* treatment on incidence and severity of Botrytis and Monilinia blight observed at mid bloom from a commercial wild blueberry field at Debert, Nova Scotia.

	Monilinia blight				Botrytis blight			
	Disease incidence (%)[1]		Disease severity (%)[2]		Disease incidence (%)[1]		Disease severity (%)[2]	
Treatment	FB	VB	FB	VB	FB	VB	FB	VB
Untreated control	0.40	0	1.20	0	3.35a	0	3.37a	0
Burning - Lime sulfur -*Trichoderma*	0	0	0	0	1.66b	0	1.89ab	0
Burning - Lime sulfur	0	0	0	0	0b	0	0b	0
Burning - *Trichoderma*	0	0	0	0	0.59b	0	0.62b	0
Burning	0	0	0	0	0.56b	0	0.87b	0
Lime sulfur - *Trichoderma*	0.44	0.08	1.33	0.67	0b	0.33	0b	0.04
Lime sulfur	0	0	0	0	0.56b	0	0.72b	0
Trichoderma	0.47	0	2.33	0	1.00b	0	1.89ab	0
ANOVA Results[3]	NS	NS	NS	NS	P=0.0014	NS	P=0.010	NS

[1] % Disease incidence: % of number of floral/vegetative buds showing visual signs and symptom of *Botrytis* infection.
[2] Disease severity = 0 to 9 rating scale where 0 = no disease and 9 = 90-100% where infected whole blossom/leaf tissue area is affected. [3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ὰ=0.05). Means in a column with the same letters are not significantly different from each other.
VB: Vegetative bud, FB: Floral bud

Table 4.2b. Effect of burning, Lime sulfur and *Trichoderma* treatment on physical development of wild blueberries and Septoria leaf spot observed at mid bloom from a commercial wild blueberry field at Debert, Nova Scotia.

Treatment	Stem length (cm)	FB number	VB number	FB stage	VB stage	Septoria incidence (%)[1]	Septoria severity[2]
Untreated control	19.6c	4.62c	9.13	5.88ab	4.89a	6.67b	0.56bc
Burning - Lime sulfur - *Trichoderma*	20.8ab	5.58b	10.06	5.68cd	4.87a	11.3a	1.49a
Burning - Lime sulfur	20.9a	7.21a	9.71	5.62cd	4.18c	0c	0d
Burning - *Trichoderma*	20.3abc	5.03cb	9.59	5.80bc	5.00a	6.23b	0.78b
Burning	19.4c	4.47c	10.35	6.01a	5.05a	0c	0d
Lime sulfur -*Trichoderma*	19.5c	4.49c	9.08	5.63cd	4.63b	2.53c	0.38cd
Lime sulfur	19.9bc	5.11cb	9.15	5.91ab	4.62b	0.10c	0.03d
Trichoderma	20.0abc	5.62b	9.43	5.51d	4.36c	0.13c	0.04d
ANOVA Results[3]	P=0.022	P<0.0001	NS	P<0.0001	P<0.0001	P<0.0001	P<0.0001

[1] % Disease incidence: % of number of no blossoms/leaves with visual signs and symptoms of *Botrytis* infection.
[2] Disease severity = 0 to 9 rating scale where 0 = no disease and 9 = 90-100% where infected whole blossom/leaf tissue area is affected. [3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.
VB: Vegetative bud, FB: Floral bud

Table 4.3a. Effect of burning, Lime sulfur and *Trichoderma* treatment on incidence and severity of Botrytis blight observed at full bloom from a commercial wild blueberry field at Debert, Nova Scotia.

Treatment	Botrytis incidence of floral buds (%)[1]	Botrytis incidence of vegetative buds (%)[2]	Floral bud severity of Botrytis (%)[1]	Vegetative bud severity of Botrytis (%)[2]	Septoria incidence (%)[1]	Septoria severity (%)[2]
Untreated control	13.1a	0	9.30a	0	0b	0b
Burning - Lime sulfur - *Trichoderma*	3.31c	0	3.62b	0	2.09a	0.69a
Burning - Lime sulfur	2.27c	0	2.87b	0	0b	0b
Burning - *Trichoderma*	7.51b	0.30	4.45b	0.30	0.14b	0.07b
Burning	6.30bc	0	4.10b	0	0b	0b
Lime sulfur - *Trichoderma*	2.40c	0	2.95b	0	0.47b	0.21b
Lime sulfur	8.09b	0	5.65b	0	0b	0b
Trichoderma	5.98bc	0	5.32b	0	0.33b	0.21b
ANOVA Results[3]	P<0.0001	NS	P=0.0033	NS	P=0.0008	P=0.0023

[1] % Disease incidence: % of number of floral/vegetative buds showing visual signs and symptom of *Botrytis* infection.
[2] Disease severity = 0 to 9 rating scale where 0 = no disease and 9 = 90-100% where infected whole blossom/leaf tissue area is affected. [3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.

Table 4.3b.3 Effect of burning, Lime sulfur and *Trichoderma* treatment on physical development of wild blueberries and Septoria leaf spot observed at full bloom from a commercial wild blueberry field at Debert, Nova Scotia.

Treatment	Stem length (cm)	FB number	VB number	FB stage	VB stage
Untreated control	19.5abc	5.10b	10.1bc	7.14	5.95ab
Burning - Lime sulfur - *Trichoderma*	20.2a	4.80b	11.3a	7.11	5.93ab
Burning - Lime sulfur	19.8ab	5.72a	10.7abc	7.03	5.92ab
Burning - *Trichoderma*	19.1bdc	4.53b	9.81c	7.16	5.90ab
Burning	20.4a	4.82b	10.9ab	7.22	5.98a
Lime sulfur -*Trichoderma*	18.2d	4.88b	9.7c	7.18	5.92ab
Lime sulfur	17.7cd	4.67b	10.4abc	7.21	5.88b
Trichoderma	18.2d	4.88b	10.1bc	7.22	5.79c
ANOVA Results[3]	P<0.0001	P=0.0055	P=0.0137	NS	P=0.0015

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.
VB: Vegetative bud, FB: Floral bud

Table 4.4 Effect of burning, Lime sulfur and *Trichoderma* treatment on yield components and harvestable berry yield of wild blueberries from a commercial field at Debert, Nova Scotia.

Treatment	Stem length (cm)	FB number	VB number	Side branches	Set Fruit	Pin heads	Harvestable berry yield (g/m²)
Untreated control	17.7abc	3.73	10.2abc	0.07	13.2	0.780c	696.4abc
Burning - Lime sulfur -*Trichoderma*	18.8a	4.01	10.4ba	0.13	13.5	1.27abc	681.2abc
Burning - Lime sulfur	18.4ab	4.31	10.5ab	0.27	13.2	1.41ab	783.8a
Burning - *Trichoderma*	18.5ab	4.21	9.88bc	0.17	15.8	1.33ab	759.1a
Burning	18.6ab	3.97	11.1a	0.17	14.7	1.12bc	710.0ab
Lime sulfur - *Trichoderma*	17.7abc	4.75	10.2abc	0.12	16.3	1.73a	640.7bc
Lime sulfur	17.6bc	3.97	10.0bc	0.12	13.8	0.890b	590.9c
Trichoderma	16.6c	4.20	9.29c	0.21	14.6	0.77c	774.5a
ANOVA Results[3]	P=0.0033	NS	P=0.0488	NS	NS	P=0.0035	P=0.0068

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (άʹ=0.05). Means in column with the same letters are not significantly different from each other.

VB: Vegetative bud, FB: Floral bud

Table 4.5. Influence of burning, lime sulfur and *Trichoderma* treatment on Botrytis rot incidence after 7 days of berry incubation at 100% RH and 22 0C

Treatment	*Botrytis* incidence on berries (%)[1]
Untreated control	5.60
Burning –Lime sulfur -*Trichoderma*	1.20
Burning - Lime sulfur	2.40
Burning - *Trichoderma*	0.80
Burning	1.20
Lime sulfur -*Trichoderma*	0.40
Lime sulfur	0.40
Trichoderma	2.00
ANOVA Results[3]	NS

[1] % Incidence: % of number of berries where 0 = no fruit showing visual signs of *Botrytis* infection and 100 = all fruits with visual signs of *Botrytis*. [3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05)

Figure 4.3. Growth of Botrytis cinerea (arrowed) on berries incubated for 7 days at 22 0C and 100% RH

4.4. Discussion

In this study, disease reduction was observed on stems before bloom and at full bloom. Disease development observed at mid-bloom were relatively lower than that observed at full bloom. This corroborates with earlier observations that blueberry flowers are more susceptible to Botrytis infection when corolla are fully opened (anthesis) as reported in chapter three of this thesis and Hildebrand, et al., (2001). At mid-bloom stage, majority of floral tissue were at the resistant floral stage, F5 whereas at full bloom, majority of the flowers are at the most susceptible floral stage, F7 (Chapter 3, figure 3.2). The stem Botrytis and Septoria canker observed, is most likely to be stems infected during the sprout year. This could explain why plots treated to burning and lime sulfur had least stem canker because burning and lime sulfur treatments were made during the sprout year.

Field sanitation (burning) reduced the development of Botrytis blight in this study over the two-year production cycle of the crop. However, the reductions were relatively small compared to when burning was augmented with lime sulfur and *Trichoderma*. At the onset of this trial, we postulated that burning would help reduce the development of Botrytis blight. This was based on observations and previous knowledge that burning has the advantage of reducing Botrytis disease on wild blueberry fields (Yarborough, 2004; Delbridge and Hildebrand, 1997). Hence, it is not surprising that stand-alone treatment of burning yielded a significant reduction in Botrytis blossom blight. The reduction in disease by burning is may be due to additive effect of debris destruction as the pathogen is deprived of substrate and reduction of initial inoculum load to initiate primary infections.

Given the results obtained by other studies on the use of lime sulfur and *Trichoderma* for management of Botrytis and other diseases (McGourty et al., 2011; Elad, and Stewart, 2007; Ranasingh et al., 2006; Elad, 2000), it is not surprising that the most disease reductions were achieved with the combination of burning, lime sulfur and *Trichoderma* treatments. Lime sulfur has been found to kill Botrytis sclerotia and significantly reducing inoculum, hence making it a good clean up product (Bettiga, 2013). The importance of the time of application of the factors in this study is that, at every phase of the two-year production cycle, there is a Botrytis management component on the field. It is also important to note that, lime sulphur was applied as a dormant spray to avoid any phytotoxic damage to the blueberry plants.

The suppression of Botrytis infection by burning of prunings and leaf debris, or by applications of lime sulfur, is useful in maintaining the perception of wild blueberries as a low input commodity. With the current low farm-gate price for the crop, these relatively lower cost treatments (~$10/acre) could be very important economically, as compared to conventional control using synthetic fungicides such as Switch® ($64/acre) and Pristine® ($74/acre). The outcome of this study could also have a practical implication on fields with limited fungicide usage, in organic wild blueberry production or on fields with widespread fungicide resistance among *B. cinerea* population.

A non-significant treatment effect observed on vegetative node infection at both mid bloom and full bloom may be due to the very low levels of Botrytis infections observed with the vegetative buds as the fungus characteristically infects floral parts (Hildebrand et al., 2001). Similar trends were also observed with Monilinia blight infection where there was no significant treatment effect due to low level of infection. Though Septoria leaf

spot infections were statistically significant, there were no defined trends in the treatment effects with almost all the treatment having the same impact as the untreated control. This could be an indication that burning, lime sulfur and *Trichoderma* application would not be an effective method for Septoria leaf spot management in wild blueberries.

This study revealed a significant treatment effect on physical development of blueberry plants. Generally, plots that were burnt as a stand-alone treatment or burning combined with lime sulfur and *Trichoderma* had taller stems, and higher number of floral and vegetative buds. This is consistent and supports the observations that the number of sprouts, length of sprouts, the total number of flower buds and number of floral buds per sprout were greater when burning was done in early spring (Eaton and White, 1960).

There was variable outcome from the harvestable berry yields in this study. Plots that were treated with lime sulfur in addition to burning yielded berry which was similar to the yield from *Trichoderma* treatments (highest yield)**.** Although the treatments in this study did not reveal a distinct yield trend, it may partly be attributed to the dry weather conditions experienced from mid to late stages of the growing season and the huge variability in wild blueberry fields. Wild blueberry fields are characterized by excessive plant to plant variability. The variability is so extreme that it is almost impossible to find two morphologically identical clones in the same field (Kinsman, 1993). This variability, combined with the irregularity of plant density and the mixture of two species, makes it very challenging to establish conclusions based on only yield but rather the consideration of several criteria such as stem length and number of floral buds per shoot (Kinsman, 1993; Tower, 1955). This also explains why replicated site trials are important in wild blueberry research to obtain large sample size and data.

The incubation of berries did not show any significant treatment effect on Botrytis fruit rot. In a similar study, Lambert (1990) reported that fields pruned by burning did not show significant difference in Botrytis fruit infection compared to field pruned by mowing. Because more than 95 % of harvested wild blueberries are processed into individually quick frozen (IQF) product within 48 hours after harvest, post-harvest berry rot (infection) is of little importance to the wild blueberry industry.

4.5. Conclusions

The results from this study provide strong evidence that field sanitation and burning practices that reduce field debris and accumulated pathogen inoculum is an important component of management strategies that can be adopted in reducing Botrytis blossom blight infections in wild blueberry fields. The augmentation of burning with lime sulfur and biofungicide applications provides a useful management strategy that takes into consideration the timing of pest, plant development and the susceptible stages of the floral tissues during the 2-year wild blueberry production cycle. Based on the outcome of this study, it can be concluded that Botrytis blossom blights in wild blueberry fields can adequately be managed through the integration of burning, lime sulfur and biofungicides. This approach will help reduce fungicide usage and the dependency on only synthetic fungicides for disease control. This approach could also have practical applications on organic blueberry fields or in fields that require reduced fungicide usage.

CHAPTER 5

CONTROL OF BOTRYTIS BLOSSOM BLIGHT IN WILD BLUEBERRIES BY BIOLOGICAL CONTROL AGENTS UNDER FIELD CONDITIONS.

5.0. Abstract

Botrytis blossom blight is one of the most important diseases affecting wild blueberries in Atlantic Canada with over 20% yield losses recorded annually. Two trials each were conducted in 2015 (Debert and Webb Mountain, NS) and 2016 (Dean and Parrsboro, NS) growing seasons to investigate the use of biofungicides with respect to their efficacy against *Botrytis cinerea* in wild blueberry fields. Botector® (*Aureobasidium pullulans*), Fracture® (Blande de Lupinus Albus Doce, BLAD) and Serenade MAX® (*Bacillus subtilis* strain QST 713) were evaluated alone and in rotation with a reduced risk fungicide Switch® against Botrytis blossom blight with a standard conventional control program [Fontelis® (penthiopyrad), Switch®, Pristine® (boscalid+pyraclostrobin)] and an untreated control. Three applications of each biofungicides (Botector®, Fracture®, Serenade MAX®) was done for stand-alone treatments, and each rotated with Switch® (cyprodinil + fludioxonil) as combined treatment. The products were applied at 7-10-day intervals. In both years, the rotation of Fracture® and Serenade MAX® with Switch® as well as the conventional fungicide program reduced Botrytis blossom blight incidence by over 65% and severity by over 60% compared to the untreated control. The rotation of Botector® with Switch® significantly reduced disease development substantially in 2016 but was not effective in 2015. Improved disease control resulted from the stand-alone application of Botector®, Fracture® and Serenade MAX® in 2016 whereas they were not efficient in 2015. The rotation of Fracture and Serenade MAX® with Switch®, conventional control program as well as Botector® and Serenade MAX® resulted in an

improved harvestable berry yield. The results from this study suggest that the use of biofungicides in tandem with conventional fungicides in an integrated disease management program, can adequately suppress Botrytis blossom blights.

5.1. Introduction

Botrytis cinerea Pers.: Fr., is one of the most destructive pathogens in wild blueberries causing Botrytis blight on blueberry flowers. The fungus is a ubiquitous pathogen found under wide range of environmental conditions and infects a wide range of economically important plant species such as vegetables, ornamentals and fruits (Pollastro et al., 1996). The pathogen can infect different parts of the plant. However, the flowers are the main tissues infected (Lambert, 1995). *B. cinerea* is an opportunistic fungus that causes infection at wounds or previously infected sites. Nevertheless, it can directly penetrate intact host surfaces (Williamson et al., 1995; Cole et al., 1996), or enter its host through natural openings (Hsieh et al., 2001) to cause infection. Infection of host by pathogen is enhanced by free surface water, high humidity and low temperatures (Williamson et al., 1995).

Botrytis blossom blight has become of concern over the years, especially across coastal wild blueberry fields. This can partly be attributed to the significant increase in flower densities from 38 million flowers·acre^{-1} in 1994 to more than 150 million flowers·acre^{-1} due to improved practices such as nutrient management, weed management and leaf spot disease control (Percival, 2013). The increase in floral densities provides abundant

susceptible tissues for infection and proliferation of the pathogen. Following the development of *B. cinerea* on infected flowers, conidia are produced on infected tissues which are disseminated by wind and pollinators to healthy blossoms where secondary infections occur. The fungus is estimated to produce over 15 million conidia in seven (7) days on a 2cm long stem segment (Nicot et al., 1996). These together with cool and wet conditions during bloom, and increased leaf and berry debris present suitable conditions for the rapid escalation of this disease.

Over the past decades, management of *B. cinerea* has relied greatly on the use of chemical fungicides (Rosslenbroich and Stuebler 2000). The main active ingredients of fungicides registered for Botrytis blight control in wild blueberries are cyprodinil, fludioxonil, boscalid and pyraclostrobin (Agriculture, Aquaculture and Fisheries, 2016; Percival, 2013). Although these fungicides remain the principal method of Botrytis control, the pathogen is a polycyclic fungus that is classified as a high-risk pathogen (FRAC, 2014). Therefore, it's able to develop resistance to fungicides easily. In view of this, the increase and frequent use of chemical fungicide to control this pathogen is increasingly questioned in crop production due to the occurrence of fungicide resistance of *B. cinerea* populations (Leroch et al. 2013; Delbridge and Hildebrand, 2007), and the prospective environmental and health threats (Hauschild et al., 2012; Farquhar et al. 2009). Presently, up to three fungicide applications are made on wild blueberry fields during bloom for Botrytis blight control. Botrytis blight is the costliest disease to be controlled in wild blueberry production with over 75% of total expenditure on fungicides in wild blueberry production going into Botrytis control (Percival, 2013).

In addition to the increased cost of production, the limited pesticide concentration in berries allowed especially in Europe (Munitz et al, 2013), and stringent regulations governing botryticide residues has posed a major challenge to the wild blueberry industry because most of the fruits are exported to several countries in Europe and Asia. This therefore restricts the use of most fungicides in wild blueberry production. These together with the rising public interest for organic fruit and products have urged the search for innovative and safer replacements for chemical fungicides that aim at reducing the costs of production and the effect on the environment.

In the last two decades, substantial consideration and research has been placed on the use of microbes (Elad, and Freeman, 2002; Ippolito et al. 2000) and naturally occurring compounds to control or suppress Botrytis and other plant diseases (Adebayo et al., 2013; Gatto et al. 2011; Elad et al., 1996). The antifungal activities of several fungi against *B. cinerea* have been investigated over the years. Most commonly occurring fungi tested against *B. cinerea* include *Trichoderma* spp. (Elad, 2000; Freeman et al., 2004), *Ulocladium* spp. (Kohl et al., 2001) and *Gliocladium roseum* (Burgess et al, 1997). Among all the biological control agents, *Trichoderma* spp. are the most studied group. Yeast species such as *Aureobasidium pullulans* is viewed as an important microbe for biocontrol or supression of *B. cinerea* due to its adaptation to the plant phyllosphere (Chi et al. 2009; Blakeman and Fokkema 1982) and strong competition for nutrients (Lima et al. 1997). These characteristics of *Aureobasidium pullulans* has been shown to be essential for the control of necrotrophs (Blakeman and Fokkema 1982). In strawberries, it was shown that *A. pullulans* can effectively suppress *B. cinerea* development under field conditions (Sylla et al., 2015; Adikaram et al. 2002). Other yeast species *Metschnikowia*

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pulcherrima and *Pichia guilliermondii* have been reported to provide significant *B. cinerea* control (Raspor et al., 2010). Many studies have explored the use of bacteria as potential biocontrol agents. The antifungal activity of several strains in the genus Bacillus against *B. cinerea* have been described (Lee et al., 2006; Walker et al., 1998). In both *in vitro* and field trials, *Bacillus subtilis* S1-0210 significantly reduced gray mould infection in strawberries (Hang et al., 2005). The strain J9 of *B. subtilis* was able to reduce gray mould disease in strawberry under field conditions with efficacy comparable to those of chemical fungicides (Essghaier et al., 2012). Various commercial products including Kodiak HB, *B. subtilis* GB03 (Mahaffee and Blackman, 1993) and Serenade, *B. subtilis* QST-713 (Marrone, 2002) have been formulated from *Bacillus* spp. and tested for their Botrytis control abilities.

Plant extracts and other plant based compounds are good alternatives or complementary control methods due to their antifungal activities, biodegradability and non-phytotoxicity (Gatto et al., 2011). Wilson et al. (1997) identified 13 plant extracts that exhibited high levels of antifungal activity against *B. cinerea* with species of *Allium* and *Capsicum* dominating. Several essential oils and volatile constituents of plants such as *Zingiber* spp. have shown great antifungal activity (Tripathi et al., 2008). In an *in vivo* study, protein hydrolysates, *LupP* from lupin seeds showed significant effect in controlling gray mould of grapes (Lachhab et al., 2016). Outcome of some of these researches have led to the development of commercial biofungicides for Botrytis control. However, their potential for suppression of Botrytis blossom blight in wild blueberry fields remain unknown given the diversity and uniqueness of wild blueberry fields. Therefore, the objective of this

study was to evaluate the potential of selected biofungicides applied alone and in rotation with selected reduced risk fungicide used in wild blueberry production.

5.2. Materials and Methods
5.2.1. Site selection and experimental design

Field trials were conducted during the crop year of 2015 and 2016 in commercial wild blueberry fields in Nova Scotia, Canada. Two trials were set up at Debert, NS (coordinates = 45°26'35.65 N, 63°27'5.69 W) and Web Mountain, NS (coordinates = 45°26'34.99 N, 63°41'11.90 W) in 2015. Two other trials were located at Dean, NS (coordinates = 45°12'40.63 N, 62°52'4.46 W) and Parrsboro, NS (coordinates = 45°25'40.18 N, 64°19'45.39 W) in 2016. The trial was set up in June in both year and followed a randomized complete block design (RCBD) with 5 replications, a plot size of 4 × 6 m with 2 m buffers between plots and eight (8) treatments. The plots were staked out and treatment tags were installed. The fields for the experiments were equipped with Watchdog® weather station model 2700 (Aurora, IL, USA) to monitor air temperature, relative humidity, wind speed and direction and leaf wetness and logged data every 15 minute for the duration of the trial.

5.2.2. Treatments and treatment applications

Treatments included: **1)** untreated control, **2)** 3 applications of Botector® (*Aureobasidium pullulans*) applied at 0.7 kg product·ha^{-1}, (Bio-ferm, Austria) **3)** 3 applications of Fracture® (a.i. Banda de Lupinus albus doce, BLAD) applied at 2.56 L·ha^{-1}, (FMC Agricultural Solutions, SK, Canada), **4)** 3 applications of Serenade MAX® (a.i. *Bacillus subtilis* strain QST 713) applied at 6 kg product·ha^{-1}, (Bayer Crop Science, Canada), **5)**

Botector®, Switch® (a.i. cyprodinil and fludioxonil) applied at 0.975 kg product·ha⁻¹ (Syngenta, Canada), Botector®, **6)** Fracture®, Switch®, Fracture®, **7)** Serenade MAX®, Switch®, Serenade MAX®, and **8)** Fontelis® (a.i. penthiopyrad) applied at 1.75 L·ha⁻¹ (DuPont, Canada), Switch®, Pristine® (a.i. boscalid and pyraclostrobin) applied at 1.5 kg·ha⁻¹ (BASF Canada Inc.).

First fungicide applications were done at 10% bloom prior to visual symptoms of Botrytis blight. The second fungicide applications were done 7 to 10 days after the first application and the third application 7 to 10 days after the second application. The fungicides were applied using a Bell spray Inc.® hand-held research sprayer with 2m carbon dioxide propelled boom with 4 Tee Jet Visiflow 8003VS nozzles at a pressure of 220 kPa. The nozzle discharge rate was 12.5 mL·s⁻¹ and application ground speed was approximately 1.2 m·s⁻¹. In 2015, fungicides were applied on 5 June, 11 June, and 18 June, at both Debert and Web Mountain. In 2016 fungicide applications were done on 7 June, 17 June, and 22 June, at Dean and on 10 June, 18 June, and 23 June, at Parrsboro.

5.2.3. Disease assessment, yield components and berry yield

Fifteen randomly selected blueberry stems were collected from the field 7 to 10 days after the second fungicide application and 14 to 17 days after third fungicide application. Fifteen stems were randomly selected before harvest to assess yield component. Disease development, yield component and berry yield were assessed as described previously in section 4.2.3 (Chapter 4).

5.2.4. Statistical analysis

Analysis of all the data collected in the experiment were carried out as described previously in **section** 4.2.4 (Chapter 4).

5.3. Results

The extent of tissue infection on plants exposed to the prevailing environmental conditions during June of the growing seasons in 2015 and 2016, in a field known to contain inoculum is described below. The month of June is presented because that is the period of the season within which Botrytis flower infections is important. There was no infection period observed in the month of June, 2015 at both Debert and Webb Mountain (Figure A-1, Table A-1). In Parrsboro, no infection period was observed at the early and in the middle of June, however, two distinct periods of infection were observed towards the end of the month (Figure A-2, Table A-2). Contrary to conditions in 2015 and Parrsboro in 2016, there were four moderate and two high periods of infection at Dean, in 2016 within the month which coincided with the beginning of susceptible flower stages. One moderate infection period at the beginning of the month and one high infection period in the middle of the month with the other infection periods toward the end of the month (Figure A-3, Table A-3).

In this experiment, disease development (incidence and severity) varied among the treatments. Single and rotational treatments were able to suppress Botrytis floral incidence and severity. Generally, low levels of *Botrytis* infections were recorded at both sites during the 2015 field trials with 0.17% and 2.50% of the total stem collected showing symptoms of Botrytis blossom blight after the 2nd fungicide applications at

Debert and Web Mountain, respectively. Nonetheless, there was higher Botrytis infection during the 2016 growing season at both Dean and Parrsboro with respective 15.7% and 13.8% of total stem collected displaying symptoms of Botrytis blossom blight after the 2nd fungicide applications. After the 3rd fungicide applications, 2.85% and 6.83% of total stems collected in 2015 had symptoms of Botrytis blossom blight at Debert and Web Mountain, respectively. In 2016 after 3rd fungicide application, 18.2% and 17.2% of total stems collected were affected Botrytis blossom blight at Dean and Parrsboro, respectively. Less than 0.70% of the total stems collected from all the four sites in 2015 and 2016 had symptoms of Botrytis vegetative bud blight. There was no phytotoxicity symptoms associated with any of the treatments used (Table 5.1a, 5.2a and 5.3a).

In 2015, Botrytis blossom blight incidence and severity ranged from 0 to 0.33% and 0 to 1.07%, respectively at Debert after the 2nd fungicide application. At the same site, disease incidence and severity ranged from 0 to 4.16% and 0 to 2.93% respectively after the 3rd fungicide application. None of the stems assessed had any symptom on vegetative buds after both 2nd and 3rd fungicide applications. Though some diseased blossoms were observed, no significant treatment effects on disease development was observed on both floral and vegetative buds in this trial (Table 5.1a). At Web Mountain, disease incidence and severity observed on floral buds ranged from 0 to 2.13% and 0 to 4.13%, respectively after the 2nd fungicides application. There was not significant treatment effect among treatment on incidence, however, there was significant difference in severity with Fracture® and Serenade MAX® rotation with Switch®, and standard fungicide program (Fontelis®, Switch®, Pristine®) with no disease symptoms (100% less severity than the untreated control) (Table 5.2a). After the 3rd fungicide application, incidence and severity

ranged from 0 to 5.58% and 0 to 10.0%, respectively. Disease assessment revealed that Botrytis floral incidence and severity were significantly reduced with the applications of Fracture® and Serenade MAX® in rotation with Switch®, likewise Fontelis®, Switch®, Pristine®. Fracture® in rotation with Switch®, Serenade® MAX in rotation with Switch®, and conventional fungicide program effectively suppressed Botrytis blossom with 95.1%, 96.9%, and 100% less floral bud incidence respectively than control (Table 5.2a). Similarly, Fracture® in rotation with Switch®, Serenade MAX® in rotation with Switch®, and conventional fungicide program reduced severity by 97.4%, 92.0% and 100% respectively, compared to the untreated control. The stand-alone treatment of Botector®, Fracture® and Serenade MAX® did not reveal any significant effect on both incidence and severity compared to the untreated control (Table 5.2a). No significant treatment effect was observed on both floral and vegetative buds after the 2nd and 3rd fungicide applications (Table 5.2a).

In the 2016 trial at Dean, there was significant treatment effect on floral bud infection after the 2nd fungicide application with incidence and severity ranging from 0.27 to 10.8% and 1.33 to 20.5%, respectively. Only Botector®-Switch® rotation and the conventional fungicide program (Fontelis®-Switch®-Pristine®) reduced disease development with 87.6% and 97.4% less incidence, and 81.0% and 93.5% less severity, respectively compared to the untreated control (Table 3a). Incidence and severity observed on stems after the 3rd fungicide application, ranged from 1.80 to 19.9% and 5.33 to 35.1%, respectively. All treatments reduced Botrytis blossom blight significantly compared to the untreated control with over 70% less incidence and 60% less severity (except Serenade MAX® with 57.7% less incidence and 40.1% less severity) compared to

the untreated control. Disease suppression from all the treatments were comparable to the efficacy of the conventional fungicide program except stand-alone application of Serenade MAX® (Table 5.3a). In this trial, vegetative bud infections did not show any significant difference with incidence and severity ranging from 0.0 to 0.8% and 0.0 to 1.21%, respectively after 2nd fungicide application, and 0.0 to 0.67% and 0.0 to 2.67% after 3rd fungicide application, respectively (Table 5.3a). Unlike Dean where significant treatment effect was observed with flower infection, no significant treatment effect was observed with both incidence and severity at Parrsboro after the 2nd fungicide application. Disease incidence range from 0.77 to 4.85%, and severity from 4.45 to 13.96% after the 2nd fungicide application. Results obtained after the 3rd fungicide application was similar to that obtained at Dean with incidence and severity ranging from 0.99 to 18.1% and 4.27 to 37.6%. All treatments reduced Botrytis blossom blight significantly compared to the untreated control with over 72% less incidence and over 60% less severity (except Botector® 57.39% less incidence) compared to the untreated control. Disease suppression achieved all treatments but stand-alone application of Botector® and Fracture®-Switch rotation were comparable to disease suppression obtained with the conventional program (Table 5. 4a).

Similar to all the other sites in both 2015 and 2016, no significant treatments effect on disease incidence and severity were observed with vegetative bud infections after both 2nd and 3rd fungicide applications.

In both 2015 and 2016 trials and in all the stem collections, there were significant treatment effects on physical development (stem length, floral bud and vegetative bud numbers) from some sample collections dates whiles others were not significantly

different (Table 5.1b, 5. 2b, 5. 3b and 5.4b). Similar results were obtained with yield component (stem length, number of vegetative buds and floral buds per stem, number of pinhead, set fruit and side branches per stem). Though some parameters varied significantly, there were no distinct trend of treatment effect on physical development of plants and yield components among those that were significant. Though significant, most of the treatments were no different from the untreated control (Table 5.1c, 5.2c, 5.3c and 5.4c).

In the 2015 trials, harvestable berry yield from Debert varied significantly among treatment (Table 5.1c) whiles the trial at Webb Mountain did not reveal any significant treatment effect (Table 5.2c). Similarly, in 2016, significant treatment effect was observed in the trial at Dean while at Parrsboro there was no significant treatment effect (Table 5.3c). At Debert, stand-alone treatment of Serenade MAX®, Fracture®-Switch® rotation, and the conventional fungicide program yielded 11.8%, 28.3% and 38.5% more berries respectively than the untreated control (Table 5.4c). At Dean, Serenade MAX®, Serenade MAX®-Switch® rotation, and the conventional fungicide program also yielded 40.7%, 33.0% and 97.4% more berries respectively than the untreated control (Table 5.4c).

Table 5.1a. Incidence and severity of Botrytis observed from a commercial wild blueberry field at Debert, Nova Scotia after biofungicide application in 2015

| Treatment | After 2nd fungicide application | | | | | After 3rd fungicide application | | | | |
| | Disease incidence (%)[1] | | Disease severity (%)[2] | | Phytotoxicity (Yes=1, No=0) | Disease incidence (%)[1] | | Disease severity (%)[2] | | Phytotoxicity (Yes=1, No=0) |
	FB	VB	FB	VB		FB	VB	FB	VB	
Control	0.33	0	1.07	0	0	0	0	0	0	0
Botector®	0	0	0	0	0	3.05	0	2.93	0	0
Fracture®	0	0	0	0	0	1.33	0	2.27	0	0
Serenade MAX®	0	0	0	0	0	4.16	0	2.86	0	0
Botector®, Switch®, Botector®	0	0	0	0	0	2.85	0	1.33	0	0
Fracture®, Switch®, Fracture®	0	0	0	0	0	3.33	0	0.67	0	0
Serenade MAX®, Switch®, Serenade MAX®	0	0	0	0	0	0	0	0	0	0
Fontelis®, Switch® Pristine®	0	0	0	0	0	1.00	0	1.07	0	0
ANOVA Results[3]	NS	NS	NS	NS		NS	NS	NS	NS	

[1] % Disease incidence: % of number of floral/vegetative buds showing visual signs and symptom of *Botrytis* infection.
[2] Disease severity: 0 to 9 rating scale where 0 = no disease and 9 = 90-100% where infected whole blossom/leaf tissue area is affected.
[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05).
VB: Vegetative bud, FB: Floral bud

Table 5.1b. Effects of biofungicide treatment on physical development of wild blueberries observed from a commercial wild blueberry field at Debert, Nova Scotia in 2015

Treatment	After 2nd fungicide application					After 3rd fungicide application				
	Stem length (cm)	FB Number	VB Number	FB Stage	VB Stage	Stem length (cm)	FB Number	VB Number	FB Stage	VB Stage
Control	15.3bc	4.52bcd	12.1a	7.81	5.99a	15.2ab	2.71	10.7	8.67c	6.0
Botector®	14.3c	4.42cd	8.70bc	7.64	4.80b	13.6c	3.35	10.5	8.69d	5.0
Fracture®	16.3b	6.23a	10.4b	7.78	6.00a	14.0bc	3.68	10.6	8.94a	6.0
Serenade MAX®	15.5bc	4.70bc	10.4b	7.68	6.00a	15.6a	3.27	11.1	8.82ab	6.0
Botector®, Switch®, Botector®	18.4a	5.48ab	12.1a	7.87	6.00a	14.0bc	3.13	10.8	8.87ab	6.0
Fracture®, Switch®, Fracture®	16.7ab	3.69d	9.9cb	7.56	6.00a	16.4a	2.64	11.3	8.85ab	6.0
Serenade MAX®, Switch®, Serenade MAX®	15.5bc	4.95bc	10.1cb	7.88	6.00a	15.7a	3.09	12.0	8.82bc	6.0
Fontelis®, Switch® Pristine®	15.2bc	4.41cd	10.3b	7.67	6.00a	16.3a	3.09	10.5	8.92ab	6.0
ANOVA Results[3]	P=0.0034	P<0.0001	P=0.0001	NS	P<0.0001	P=0.0003	NS	NS	P<0.0001	NS

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.

VB: Vegetative bud, FB: Floral bud

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Table 5.1c. Effect biofungicide treatment on yield components and harvested berry yield of wild blueberries from a commercial field at Debert, Nova Scotia in 2015

Treatment	Stem length (cm)	FB number	VB number	Side branches	Set Fruit	Pin heads	Harvestable berry yield (g/m^2)
Control	13.8bc	4.49cd	11.0abc	0.34	18.8b	0.57cd	1708.0cd
Botector®	12.8d	4.57d	11.4ab	0.41	17.8b	0.57cd	1576.6d
Fracture®	13.4cd	6.17a	11.1abc	0.64	25.0a	1.81acd	1811.8cd
Serenade MAX®	14.2ab	4.96bcd	10.3bcd	0.45	18.8b	0.57cd	1909.6bc
Botector®, Switch®, Botector®	12.9d	4.35d	9.92cd	0.21	18.1b	0.43d	1731.0cd
Fracture®, Switch®, Fracture®	13.8bc	4.31d	11.7a	0.57	19.5b	0.63cd	2192.6ab
Serenade MAX®, Switch®, Serenade MAX®	14.8a	5.55abc	9.57d	0.33	21.6ab	1.39ab	1682.8cd
Fontelis®, Switch® Pristine®	14.1abc	5.82ab	12.0a	0.60	24.9a	1.15bc	2365.8a
ANOVA Results[3]	P<0.0001	P=0.0006	P=0.0015	NS	P=0.0040	P<0.0001	P<0.0001

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.
VB: Vegetative bud, FB: Floral bud

Table 5.2a. Incidence and severity of Botrytis observed from a commercial wild blueberry field at Web Mountain, Nova Scotia after biofungicide application in 2015

| | After 2nd fungicide application | | | | | After 3rd fungicide application | | | | |
| | Disease incidence (%)[1] | | Disease severity (%)[2] | | Phytotoxicity (Yes=1, No=0) | Disease incidence (%)[1] | | Disease severity (%)[2] | | Phytotoxicity (Yes=1, No=0) |
Treatment	FB	VB	FB	VB		FB	VB	FB	VB	
Control	1.39	0	3.47ab	0	0	5.58b	1.33	10.0a	1.20	0
Botector®	1.88	0	1.47abc	0	0	4.15abc	1.33	3.47bcd	1.20	0
Fracture®	1.47	0.40	1.33abc	0.67	0	3.17abcd	0	6.13ab	1.20	0
Serenade MAX®	0.19	0	0.67bc	0	0	2.59bcd	0	5.33abc	0	0
Botector®, Switch®, Botector®	2.13	0	4.13a	0	0	6.61a	0	2.13cbd	0	0
Fracture®, Switch®, Fracture®	0	0	0c	0	0	0.27cd	0.08	0.26d	0	0
Serenade MAX®, Switch®, Serenade MAX®	0	0	0c	0	0	0.17d	0.29	0.80cd	0.80	0
Fontelis®, Switch® Pristine®	0	0	0c	0	0	0.00d	0	0d	1.07	0
ANOVA Results[3]	NS	NS	P=0.024	NS		P=0.0021	NS	P=0.0002	NS	

[1] % Disease incidence: % of number of floral/vegetative buds showing visual signs and symptom of *Botrytis* infection.
[2] Disease severity: 0 to 9 rating scale where 0 = no disease and 9 = 90-100% where infected whole blossom/leaf tissue area is affected.
[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other
VB: Vegetative bud, FB: Floral bud

Table 5.2b. Effects of biofungicide treatment on physical development of wild blueberries observed from a commercial wild blueberry field at Web Mountain, Nova Scotia in 2015

Treatment	After 2nd fungicide application					After 3rd fungicide application				
	Stem length (cm)	FB Number	VB Number	FB Stage	VB Stage	Stem length (cm)	FB Number	VB Number	FB Stage	VB Stage
Control	16.5	6.45	12.2cde	7.62bc	6.00	16.2bcd	5.69	12.5a	8.58c	6.00
Botector®	16.3	6.44	13.8ab	7.65bc	6.00	16.9ab	6.16	10.3c	8.68bc	6.00
Fracture®	17.1	6.48	13.5abc	7.33e	6.00	16.9ab	6.32	12.5a	8.75ab	5.97
Serenade MAX®	16.7	7.56	10.8e	7.89a	6.00	15.1de	6.57	10.7bc	8.64bc	6.00
Botector®, Switch®, Botector®	16.6	5.71	14.2a	7.29e	6.00	15.7cd	6.12	12.6a	8.59c	6.04
Fracture®, Switch®, Fracture®	16.4	6.51	14.9a	7.72b	6.00	14.4e	5.18	11.9ab	8.62bc	5.91
Serenade MAX®, Switch®, Serenade MAX®	16.9	7.56	12.6bcd	7.50cd	6.00	17.5a	5.63	10.8bc	8.89a	6.00
Fontelis®, Switch® Pristine®	16.8	5.72	11.5de	7.39de	6.00	16.3bc	5.05	11.4abc	8.56c	5.81
ANOVA Results[3]	NS	NS	P<0.0001	P<0.0001	NS	P<0.0001	NS	P=0.0092	P=0.0005	NS

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.
VB: Vegetative bud, FB: Floral bud

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Table 5.2c. Effect biofungicide treatment on yield components and harvested berry yield of wild blueberries from a commercial field at Web Mountain, Nova Scotia in 2015

Treatment	Stem length (cm)	FB number	VB number	Side branches	Set Fruit	Pin heads	Harvestable berry yield (g/m^2)
Control	14.9	3.89b	10.9bc	0.77	11.3d	1.19d	1001.3
Botector®	15.9	5.83a	12.6a	1.35	13.0bcd	2.80bc	934.6
Fracture®	15.6	5.61a	10.9bc	0.91	16.4ab	1.36d	1207.9
Serenade MAX®	14.6	5.13ab	9.8c	0.89	13.0cd	3.39b	1065.9
Botector®, Switch®, Botector®	14.9	5.65a	11.9ab	1.23	14.4abcd	5.67a	996.3
Fracture®, Switch®, Fracture®	15.7	5.40a	12.1ab	1.27	15.8abc	2.00cd	1060.6
Serenade MAX®, Switch®, Serenade MAX®	15.8	6.35a	10.8bc	1.03	14.3abcd	2.79bc	1128.1
Fontelis®, Switch® Pristine®	19.3	5.34a	11.2abc	0.89	17.1a	1.48d	1209.4
ANOVA Results[3]	NS	P=0.0318	P=0.0083	NS	P=0.0131	P<0.0001	NS

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at $p<0.05$. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.

VB: Vegetative bud, FB: Floral bud

Table 5.3a. Incidence and severity of Botrytis observed from a commercial wild blueberry field at Dean, Nova Scotia after biofungicide application in 2016

Treatment	After 2nd fungicide application					After 3rd fungicide application				
	Disease incidence (%)[1]		Disease severity (%)[2]		Phytotoxicity (Yes=1 No=0)	Disease incidence (%)[1]		Disease severity (%)[2]		Phytotoxicity (Yes=1 No=0)
	FB	VB	FB	VB		FB	VB	FB	VB	
Control	10.6a	0.19	20.5a	1.21	0	19.9a	0.67	35.1a	1.33	0
Botector®	5.57abc	0.80	11.0ab	0.89	0	5.9bc	0.30	12.8bc	2.67	0
Fracture®	7.74a	0	13.5a	0	0	3.1c	0	7.69c	0	0
Serenade MAX®	10.8a	0	16.8a	0	0	8.4b	0.13	21.0b	1.33	0
Botector®, Switch®, Botector®	1.31bc	0	3.8bc	0	0	1.82c	0.22	6.56c	1.33	0
Fracture®, Switch®, Fracture®	8.30a	0	14.6a	0	0	3.58c	0	10.0c	0	0
Serenade MAX®, Switch®, Serenade MAX®	6.44ab	0	12.8ab	0	0	4.86bc	0	13.2bc	0	0
Fontelis®, Switch® Pristine®	0.27c	0	1.33c	0	0	1.80c	0	5.33c	0	0
ANOVA Results[3]	P=0.0040	NS	NS	NS		P =0.0001	NS	P =0.0344	NS	

[1] % Disease incidence: % of number of floral/vegetative buds showing visual signs and symptom of *Botrytis* infection.
[2] Disease severity: 0 to 9 rating scale where 0 = no disease and 9 = 90-100% where infected whole blossom/leaf tissue area is affected.
[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other
VB: Vegetative bud, FB: Floral bud

Table 5.3b. Effects of biofungicide treatment on physical development of wild blueberries observed from a commercial wild blueberry field at Dean, Nova Scotia in 2016

Treatment	After 2nd fungicide application					After 3rd fungicide application				
	Stem length (cm)	FB Number	VB Number	FB Stage	VB Stage	Stem length (cm)	FB Number	VB Number	FB Stage	VB Stage
Control	14.7c	3.10	9.57cd	7.47bc	6.00	16.8	4.36b	9.81bc	8.77	6.00b
Botector®	16.8a	3.23	12.1ab	7.61ab	5.96	16.7	3.89b	10.9a	8.96	5.97b
Fracture®	15.4bc	3.10	8.97d	7.55bc	6.00	16.4	3.92b	9.19c	8.85	6.57a
Serenade MAX®	16.1ab	3.66	9.57cd	7.76ab	5.97	16.4	4.24b	9.92bc	8.86	6.00b
Botector®, Switch®, Botector®	16.5ab	3.49	10.6bcd	7.73ab	5.97	16.7	4.21b	10.4abc	8.91	5.99b
Fracture®, Switch®, Fracture®	16.2ab	3.51	10.1dc	7.64ab	6.00	16.1	3.91b	9.21c	8.94	6.00b
Serenade MAX®, Switch®, Serenade MAX®	17.3a	3.71	13.3a	7.29c	6.00	17.4	4.55ab	10.3abc	8.87	5.99b
Fontelis®, Switch® Pristine®	17.2a	3.96	11.4abc	7.88a	5.97	17.3	5.24a	11.6a	8.89	5.99b
ANOVA Results[3]	P=0.0004	NS	P=0.0004	P=0.0041	NS	NS	P=0.0128	P=0.0061	NS	P<0.0001

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ɑ=0.05). Means in a column with the same letters are not significantly different from each other.

VB: Vegetative bud, FB: Floral bud

Table 5.3c. Effect biofungicide treatment on yield components and harvested berry yield of wild blueberries from a commercial field at Dean, Nova Scotia in 2016

Treatment	Stem length (cm)	FB number	VB number	Side branches	Set Fruit	Pin heads	Harvestable berry yield (g/m^2)
Control	1.37c	4.03	10.7b	0.53b	10.8cd	1.65	388.8d
Botector®	18.4a	5.14	12.8b	1.14a	13.9abc	2.31	546.9bc
Fracture®	17.6abc	4.07	9.88b	0.58b	11.2bcd	1.33	487.4bcd
Serenade MAX®	16.5c	3.73	10.1b	0.61b	10.1d	1.13	587.4b
Botector®, Switch®, Botector®	18.8a	4.80	16.9a	1.17a	13.8abc	1.58	463.7cd
Fracture®, Switch®, Fracture®	17.7abc	3.76	11.7b	0.39b	11.6bcd	1.34	444.9cd
Serenade MAX®, Switch®, Serenade MAX®	17.9ab	4.27	10.5b	0.74b	14.6ab	1.93	516.9cb
Fontelis®, Switch® Pristine®	16.9bc	4.54	11.4b	0.68b	15.1a	1.95	767.4a
ANOVA Results[3]	P=0.0030	NS	P=0.009	P=0.0002	P=0.0152	NS	P<0.0001

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.
VB: Vegetative bud, FB: Floral bud

Table 5.4a. Incidence and severity of Botrytis observed from a commercial wild blueberry field at Parrsboro, Nova Scotia after biofungicide application in 2016

| | After 2nd fungicide application | | | | | After 3rd fungicide application | | | | |
| Treatment | Disease incidence (%)[1] | | Disease severity (%)[2] | | Phytotoxicity (Yes=1, No=0) | Disease incidence (%)[1] | | Disease severity (%)[2] | | Phytotoxicity (Yes=1, No=0) |
	FB	VB	FB	VB		FB	VB	FB	VB	
Control	4.85	0	13.9	0	0	18.1a	0.23	37.6a	1.43	0
Botector®	2.50	0	9.23	0	0	7.75b	0	14.5b	0	0
Fracture®	3.65	0	7.45	0	0	2.00cd	0	7.56bc	0	0
Serenade MAX®	1.58	0	7.04	0	0	2.71cd	0	9.67bc	0	0
Botector®, Switch®, Botector®	2.61	0	7.91	0	0	5.07bcd	0	10.9bc	0	0
Fracture®, Switch®, Fracture®	5.42	0	16.9	0	0	6.36bc	0	15.0b	0	0
Serenade MAX®, Switch®, Serenade MAX®	4.02	0	14.4	0	0	4.33bcd	0	13.6bc	0	0
Fontelis®, Switch® Pristine®	0.77	0	4.45	0	0	0.99d	0	4.27c	0	0
ANOVA Results[3]	NS	NS	NS	NS		P<0.0001	NS	P<0.0001	NS	

[1] % Disease incidence: % of number of floral/vegetative buds showing visual signs and symptom of *Botrytis* infection.
[2] Disease severity: 0 to 9 rating scale where 0 = no disease and 9 = 90-100% where infected whole blossom/leaf tissue area is affected.
[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other
VB: Vegetative bud, FB: Floral bud

Table 5.4b. Effects of biofungicide treatment on physical development of wild blueberries observed from a commercial wild blueberry field at Parrsboro, Nova Scotia in 2016

Treatment	After 2nd fungicide application					After 3rd fungicide application				
	Stem length (cm)	FB Number	VB Number	FB Stage	VB Stage	Stem length (cm)	FB Number	VB Number	FB Stage	VB Stage
Control	15.2abc	7.70a	11.7	8.47a	5.95	15.2ab	5.00	10.6a	8.96	6.00b
Botector®	15.9a	5.18c	11.6	8.02b	5.90	16.1a	4.91	10.9a	8.96	5.97b
Fracture®	15.6ab	6.60ab	11.3	8.17bcd	5.95	15.2ab	5.59	10.1ab	9.00	6.00b
Serenade MAX®	14.3cde	5.10c	11.0	8.20bcd	5.83	15.1ab	3.86	10.0ab	8.99	5.96b
Botector®, Switch®, Botector®	13.5e	5.63bc	10.4	8.40ab	5.82	15.7a	4.52	7.56d	8.99	6.59a
Fracture®, Switch®, Fracture®	14.8abc	5.35bc	11.3	8.15cd	5.90	14.5b	4.67	9.02bc	8.99	5.99b
Serenade MAX®, Switch®, Serenade MAX®	14.7cbd	6.03bc	10.0	8.35abc	5.93	14.3b	4.88	8.23cd	9.00	5.96b
Fontelis®, Switch® Pristine®	13.9de	6.35abc	9.05	8.37abc	5.91	16.b	4.61	9.05bc	9.00	5.99b
ANOVA Results[3]	P=0.0005	P=0.0028	NS	P=0.001	NS	P=0.010	NS	P<0.0001	NS	P<0.0001

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.

VB: Vegetative bud, FB: Floral bud

Table 5.4c. Effect biofungicide treatment on yield components and harvested berry yield of wild blueberries from a commercial field at Parrsboro, Nova Scotia in 2016

Treatment	Stem length (cm)	FB number	VB number	Side branches	Set Fruit	Pin heads	Harvestable berry yield (g/m^2)
Control	15.7	6.13	9.42b	1.27	13.9	2.71bc	510.5
Botector®	16.3	6.29	10.5b	1.37	14.4	2.79bc	585.0
Fracture®	15.4	5.86	9.91b	1.43	12.3	3.24bc	648.0
Serenade MAX®	15.1	6.28	10.9b	1.08	17.1	2.95bc	625.5
Botector®, Switch®, Botector®	15.8	6.16	10.9b	1.22	15.8	2.83bc	540.0
Fracture®, Switch®, Fracture®	16.3	6.07	13.8a	1.72	13.7	3.62ab	573.5
Serenade MAX®, Switch®, Serenade MAX®	16.0	6.21	10.4b	1.35	17.4	2.13c	569.0
Fontelis®, Switch® Pristine®	15.2	6.00	9.83b	0.84	14.5	4.68a	561.5
ANOVA Results[3]	NS	NS	P<0.0001	NS	NS	P=0.0090	NS

[3] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at p<0.05. Mean separation was completed using LSD test procedure (ά=0.05). Means in a column with the same letters are not significantly different from each other.

VB: Vegetative bud, FB: Floral bud

5.4. Discussion

Botrytis infections follows the disease triangle (favourable weather, susceptible host and virulent pathogen). Therefore, any epidemiological study or field infection study is greatly influenced by weather conditions. In this study, like any other multiple year field experiment, weather conditions varied between the two years of the trials. Environmental conditions observed at the research sites in 2015 were relatively dry with less rainfall and this might have contributed to the low Botrytis blight disease pressure observed at both Debert and Webb Mountain. This was in contrast with the significant disease pressure observed in 2016. In 2016, there were appreciably high *Botrytis* infection periods at Parrsboro and Dean which coincided with the susceptible stages of the blueberry flowers and this could be a major contributor to the different levels of infection between the two years (Appendices, Figures A-2 & A-3; Tables A-2 & A-3).

The low levels of Botrytis flower infection in 2015, could account for the insignificant effects observed among all the treatments at Debert. In comparison, the highest disease incidence observed for both experiments in 2015 was 6.61% whereas in 2016, it was 19.9%. Similarly, low levels of infection on vegetative buds could be attributed to the insignificant effect of the treatments. This is because, the pathogen typically infects floral tissues while vegetative buds get infected when they come in contact with an infected blossom.

The findings of this study demonstrate that, Biological Control Agents (BCAs) (*Aureobasidium pullulans* and *Bacillus subtilis*) and plant derived polypeptide (Fracture® a. i. BLAD) can effectively suppress Botrytis disease development in wild blueberry fields. Results obtained from this trial confirms previous studies that investigated the

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suppression of *B. cinerea* by *Aureobasidium pullulans* (Pertot et al., 2017; Sylla et al., 2015; Adikaram et al., 2002), *Bacillus subtilis* (Pertot et al, 2017; Elmhirst et al., 2011), BLAD (Monteiro et al., 2015) and other plant derived protein (Lachhab et al., 2016) on other crops. Following the discovery of BCAs and plant based compounds as potential disease control agents, several studies have investigated the possible mode of action of BCAs and natural compounds. Biological control agents, have been found to inhibit pathogen growth and control plant diseases through parasitism, production of metabolites, induction of host resistance, and competition for space and nutrients (Elad et al., 2007; Jacobson et al., 2004). *Bacillus* spp. have been described to be effective in the control of several plant diseases owing to their ability to produce numerous broad-spectrum antibiotics and their extended shelf lives through the formation of endospore (Emmert and Handelsman, 1999). *Aureobasidium pullulans* is an important microbe in the phyllosphere that has a unique ability to proliferate and colonize host surfaces especially wounded surfaces (Andrew et al., 2002; Dik et al., 1999). Some of these abilities of BCAs could account for the effective disease reduction observed in this study. Fracture® is a new Botrytis control product that has the unique ability to deform the chitin structure of fungus. BLAD has been found to possess a plethora of biochemical characteristics. With the presence of a wide range of chemical characteristics, antifungal activity is likely to arise spontaneously (Monteiro et al., 2015). This active ingredient is relatively new with novel mode of action. Multiple effects on cell wall and ion membrane transporters have been indicated as a means of disease suppression (FRAC, 2017). Though many natural compounds and peptides are sensitive to the sun UV radiation, BLAD has been found to be remarkably stable to the UV radiation (Monteiro et al.,

2015). Given the novelty of the active ingredient in Fracture®, its ability to suppress Botrytis disease to levels comparable to the conventional control program in this study is not surprising.

Although stand-alone treatments reduced disease development in the 2016 trials, they however, were unable to reduce disease development in both trials in 2015. The finding of the present study is not surprising because variable results have been reported from biofungicides trials over the years by many studies. Therefore, this corroborates the inconsistent efficacies of biofungicides under field conditions with reference to more than one growing season. The use of BCAs as main Botrytis control method has been found to be challenging. This challenge rest mainly on the inconsistency of field trial efficacies as observed in this study. The inconsistency is thought to arise from the complex interactions between the BCAs and the various factors such as changes in the environmental conditions (Elad and Stewart, 2004), chemical composition of the environment and plant microbiomes (Jacobsen, 2006), plant architecture (Andrews, 1992) and accuracy of biofungicide deposition on flowers (Kovach et al., 2000). These factors influence the establishment of BCAs in the field, hence, plays a significant role in biological control programs. The inability of biofungicides to provide consistent disease control could also be attributed to product formulation and storage. As living organisms, special formulation and storage conditions are crucial to keep the microbes alive and active (Usta, 2013). Natural compounds, inherently are unstable especially when exposed to environmental conditions such as sunlight hence may not be adequately persistent on the field for effective disease control (Martinez, 2012). This may account for why stand-alone treatment of Fracture® did not show consistent disease control in this study in 2015.

Due to the inconsistency and/ or less efficacy of biofungicides compared to fungicides, combining biofungicides in an integrated strategy with reduced rates or applications of synthetic and other biofungicides presents the opportunity to achieve disease control that is competitive to chemical control programs (Jacobsen, 2006). It is therefore not surprising that the rotation of biofungicides with synthetic fungicide were effective as the conventional fungicide control program in both 2015 and 2016. Our results also confirm the findings of previous studies involving the combination of biofungicides and synthetic fungicides (Buck, 2004; Korsten et al., 1997). The effectiveness of biofungicide rotated with fungicide could be due to the compensatory effect of the two products; where biofungicides may colonize and inhibit pathogen multiplication or synthetic fungicide may weaken pathogen to augment biofungicide activity. Alternating of biofungicides with or in a mixture with chemical fungicides or other biofungicides have been proposed and tested. However, in combining or alternating, the compatibility of the products must be known as reduction in disease control have been reported due to antagonism and incompatibility (Xu et al., 2010; Robinson-Boyer et al., 2009). Though good disease suppression was obtained with stand-alone application of biofungicides, it is important to acknowledge the effect of field conditions (host/pathogen/environment interactions) on biofungicides as reported in literature (Jacobsen, 2006; Elad and Stewart, 2004; Andrews, 1992). In view of this, the rotation or combination of biofungicides with chemical fungicides, is highly useful in obtaining dependable and effective disease control when biofungicides fail to establish.

In this study, the reduced risk fungicide was applied during the second application which coincides with the peak of the bloom at which blossoms are more susceptible. The

chemical application in this study may also act as a safeguard when weather conditions do not favor establishment and antifungal activity of biofungicides as described by Cal et al. (1990). Alternating treatments of biofungicides with chemical fungicide in this study may have the advantage of reducing the total amount of chemical fungicide application used. This also helps increase the preharvest interval of the chemical fungicide, hence, reducing the event of detectable fungicide residues in berries. There is also no doubt that the rotation of biofungicides with chemical fungicide is an important tool for resistance management programs. Since the biofungicides used in this study have mode of action different from that of the chemical fungicides, it is rational that this can be adopted in a fungicide resistance management program. In resistance management, biofungicides especially BCAs would be an excellent resistance management tool. This is because biofungicides, especially BCAs that act through competition, induced resistance and parasitism barely affect the metabolic processes in their target pathogen, therefore the tendency of pathogen developing resistance is of minimal concern. However, resistance to BCAs that act through antibiotics production and plant based compounds could be of interest in the future owing to the high-risk nature of the *Botrytis cinerea.*

Since Botrytis blight control represents over 60% of the total expenditure on fungicides in wild blueberry disease management (Percival, 2013), it is important to note the economic impact of the effective disease suppression obtained using biofungicides and their rotation with conventional chemical fungicides. As biofungicides are available at a relatively lower cost than the chemical fungicides being replaced, this will help sustain production in the face of low and unstable farm-gate prices. Minimizing the use of chemical biofungicides by rotation with biofungicides will also help maintain the

perception of the crop as a healthy, low input commodity. This strategy will also have a practical implication on organic wild blueberry production.

Another implication of using biofungicides in wild blueberry production is reduction (if not elimination) in agrochemical residue on harvestable berries. Unlike most chemical fungicides whose pre-harvest interval (PHI) range between 7 and 10 days, PHIs for biofungicides in some cases is 0 day (can be applied on the day of harvest) and leave practically no detectable residue on harvested produce. Through the elimination of detectable agrochemical residue using biofungicides, there would be decrease concern with maximum residue limit (MRL) and an increased preference by consumers for residue free berries.

The differences in the physical development of blueberry plants observed in this study could be attributed to the inherent variability among plants in wild blueberry fields. Given the growth stage of the plants at the time of treatment applications, it is unlikely that the treatments would have significant effect on the plant growth such as stem height, number of vegetative and floral buds. The ability to use biofungicides in this study to manage Botrytis blights without causing any obvious damage to the blueberry flowers and plant is of importance given the susceptibility of the flowers to damage associated with agrochemical application during bloom.

Given the inherent variability in wild blueberry fields and the dry weather conditions experienced from mid to late stages of the growing seasons, it is important to note the increased in yield by the stand-alone application of Botector® and Serenade MAX® (Table 5.3c), and rotational treatment of Fracture and Serenade MAX® (Table 5.1c).

5.5. Conclusion

The results from this study indicates that, the biofungicides and their rotation with reduced risk fungicides is an effective means of Botrytis blight suppression in the field. The rotation of biofungicides with reduced risk fungicides was effective as the conventional Botrytis control program involving all chemical fungicides. Similarly, stand-alone biofungicide application was important, and their applications achieved consistent and effective as the conventional control program on the separate fields. From the outcome of this study, it can be concluded that, the rotation of Botector®, Fracture® and Serenade MAX® with switch® will be an effective and a reliable Botrytis control strategy. This approach to disease management will help reduce the usage and dependency on chemical fungicides for disease control and could have practical applications on organic blueberry fields or in fields that require reduced fungicide usage.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.0 Overview

Wild blueberry is an important commodity in North America specifically Maine, US and the Maritime provinces of Canada. Wild blueberry production is faced with several challenges ranging from weeds, insects and diseases. Over the years, Botrytis blights have been one of the major blueberry diseases of concern to the blueberry industry. Botrytis infection is common in most crop production and once Botrytis infection is observed, it is always assumed to be present in the field due to the production of abundant conidia and the formation of sclerotia to withstand harsh conditions. In wild blueberry fields, Botrytis infections tends to begin in late May to early June and usually reach peak infection during full bloom under wet conditions which corresponds to mid-late June. Several products especially chemical fungicides and activities such as burning have been employed to help manage Botrytis blight on wild blueberry fields. Due to the complex and polycyclic nature of Botrytis, resistance development and public concern on chemical residues and environmental impact of chemical fungicides, new and effective management strategies are needed to address the challenges encountered by the present management activities. The scope of this study encompasses the development of an integrated disease management program for Botrytis blight in wild blueberry fields. Of interest was how to incorporate different biofungicides into Botrytis control programs and also assess the state of Botrytis resistance in Nova Scotia.

The specific objectives were to: 1) Examine the *in vitro* sensitivity of *Botrytis cinerea* to active ingredients fludioxinil, cyprodinil, pyraclostrobin, boscalid and penthiopyrad in

fungicides used for the management of Botrytis blight.; 2) Determine the relative susceptibility of four wild blueberry phenotypes at different floral stages to Botrytis blight, and verify the specific location of infection (floral part); 3) Examine the main and interactive effects of thermal pruning, dormant lime sulfur and *Trichoderma* spray applications; 4) Evaluate the efficacy of biofungicide applications used alone and in rotation with conventional fungicides used in the management of Botrytis blight.

6.1. Overall Conclusions

One objective of this study was to examine whether the *Botrytis cinerea* population in the commercial wild blueberry fields in Nova Scotia has developed resistance under the selection pressure of commonly used active ingredients. 15 single-spore isolates were tested *in vitro* using mycelial growth assay. From the result of this experiment, it can be concluded that the *Botrytis cinerea* population tested have developed resistance to, cyprodinil, pyraclostrobin, and boscalid, however, *Botrytis cinerea* population have not developed resistance to penthiopyrad and fludioxonil.

Owing to the variability of plants on wild blueberry fields, a study designed to test the differences in the susceptibility/resistance response exhibited by four wild blueberry phenotypes, *V. angustifolium, angustifolium* f. nigrum, *V. angustifolium* var. 'Fundy' and *V. myrtilloides* was conducted. The relative susceptibility of wild blueberry to Botrytis blight varied among phenotype and floral development stages. Phenotypes belonging to the *angustifolium* species were more susceptible than *V. myrtilloides*. In the study comparing the flower stages of four phenotypes, fully open blossom (F7) were the most susceptible whiles buds break (F5) and senesced corolla (F8) were least susceptible. It

can be concluded that *V. myrtilloides* is least susceptible whiles *V. angustifolium* is more susceptible among the phenotypes found on wild blueberry fields. Flower stages, F5, bud break is least susceptible whiles F7, fully opened blossom is most susceptible to Botrytis infection. Corolla was the floral part mostly infected.

In this study, field trial examining the potential of alternative disease control methods which do not involve the use of chemical fungicides but the application of biofungicide (*Trichoderma harzianum*), lime sulfur together with burning over the two-year production cycle, all treatment combinations were able to suppress Botrytis infection. Treatment combinations which involved burning and lime sulfur yielded the most effective disease suppression. The single application of burning, lime sulfur and *Trichoderma* were not as effective as their combined effect. It can therefore be concluded that, a combination of spring application of burning after pruning, lime sulfur application in the fall of the vegetative year and *Trichoderma harzianum* at 10% bloom is an effective tool for Botrytis blight suppression wild blueberry fields.

In the use of selected biofungicides together with chemical fungicide, our findings indicated that the stand-alone application of biofungicides (Botector®, Serenade MAX® and Fracture®) had variable results, thus they were able to suppress disease development in two out of the four fields. However, the rotation of these biofungicides with chemical fungicides (Switch®) was able to suppress disease development in all the four fields. It can therefore be concluded that, the rotation of biofungicides (Botector®, Serenade MAX® and Fracture®) with a chemical fungicide (Switch®) is an effective and potential strategy for Botrytis blight suppression in wild blueberry production.

Bringing together the findings in this study, the shift in *B. cinerea* population towards resistance to the common fungicides used in the wild blueberry industry is of great concern. This is because, among the active ingredients in Botrytis control products used in wild blueberry, only fludioxonil, which is one of the two active ingredients in Switch® and penthiopyrad are effective on *Botrytis cinerea*. This suggests potential inefficacy of Botrytis blight control products in wild blueberry field. Fungicide resistant *B. cinerea* population coupled with the presence of the most susceptible phenotype, *V. angustifolium,* which dominate commercial wild blueberry fields, presents an avenue for widespread Botrytis blight development. In view of fungicides ineffectiveness due to resistant development as observed in this study, the adoption of a new disease management strategy is important. The reliable disease suppression by burning, lime sulfur and *Trichoderma harzianum* as well as biofungicides and their rotation with chemical fungicide without any effects on the blueberry plant tissues makes these strategies effective options for sustainable Botrytis blight management. This will help address the major challenges (pathogen resistance, residue on fruits and negative environmental impact) whiles effective disease suppression as chemical fungicides.

6.3. Recommendation

The first step in Botrytis blight management should always be frequent monitoring for signs of disease pressures in the field. In so doing, it is important to observe the growth stages of the plant, thus when blueberry plants reach F5 to F7 stages and the weather conditions, thus duration of wetness and temperature. It is essential to know that *Botrytis cinerea* inoculum, is always present in fields with a history of Botrytis blight. Monitoring

plant growth stages and weather conditions may give an indication of potential disease development. Early detection of signs of infection may lead to the application of preventive fungicides as biofungicides because most biofungicides are preventive. Once some basic phenology of the plant and the weather are known, a management plan can be developed. In developing a disease management plan, the risk of resistance development and the status of pathogen resistance to a particular product must be considered since much cultural practices aimed at disease management is not available in wild blueberry production.

According to the result from the sensitivity study of *B. cinerea* isolates to different active ingredients, *B. cinerea* population had developed reduced sensitivity or resistance to cyprodinil, pyraclostrobin, and boscalid. However, the isolates are sensitive to fludioxonil and penthiopyrad. This implies high possibility of poor disease control in the near future which calls for other Botrytis control alternatives. Switch® being an important Botrytis control product in the wild blueberry industry, the loss of sensitivity by *B. cinerea* isolates to cyprodinil may influence the effectiveness of Switch®. In the perspective of resistance management, it is important to rotate fungicides with different modes of action, since repeated usage of one class of fungicides, (in this case, fludioxonil since cyprodinil is becoming less effective) will increase the risk of resistance development. Due to the absence of comprehensive baseline sensitivity data for the active ingredients used in the wild blueberry industry and difficulty in obtaining baseline isolates after a product has been for used in wild blueberries, it is important that new products are tested to obtain a baseline data which can be used for future monitoring and evaluation of resistance development.

The combine effect of burning, lime sulfur and Biofungicide, Trianum P (*Trichoderma harzianum*) was found to be effective in suppressing Botrytis blight on the field. The suppression of disease without the application of any chemical fungicide for Botrytis control suggests a good opportunity for development of an integrated disease management program for *B. cinerea* management in wild blueberry. Though Trianum P was used in this experiment, other biofungicides such as, Fracture® and serenade® are potential products that can be exploited in combination with burning and lime sulfur. Although, effective disease suppression was observed, however, for the purposes of this study, only one field trial was conducted and as a result, data from this study alone cannot be used to conclude the disease suppression capacity of burning, lime sulfur and biofungicide combination. Therefore, further research to evaluate the capacity of this technique is important.

The biofungicides (Botector®, Serenade MAX® and Fracture®) were found to be effective, organically compatible, option for suppression of Botrytis blight in wild blueberry plants. The outcome of this study suggests a good opportunity for development of a disease control program for *B. cinerea* management in wild blueberry. Although this research focused on Botrytis blight, further research to evaluate the capacity of these products to suppress other important diseases such as Monilinia blight may increase the usefulness of these products for the blueberry industry. In this study, the biofungicides were rotated with the chemical fungicides, however, mixing/combining biofungicides with chemical fungicides may also produce a synergistic effect (Gilardi et al., 2008; Elad et al., 1993). In combining of biofungicides with chemical fungicides, interactions may vary between fungicides and biofungicides, hence, a preliminary research to ensure the

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safety and efficacy of such combinations should be conducted before use on the field. Given that separate spray application of biofungicides and chemical fungicides were made, spray application of biofungicides, Botector®, Serenade MAX® and Fracture® or with Switch® possibly as tank mix, particularly earlier in the season as a preventive measure could be an opportunity worth exploring.

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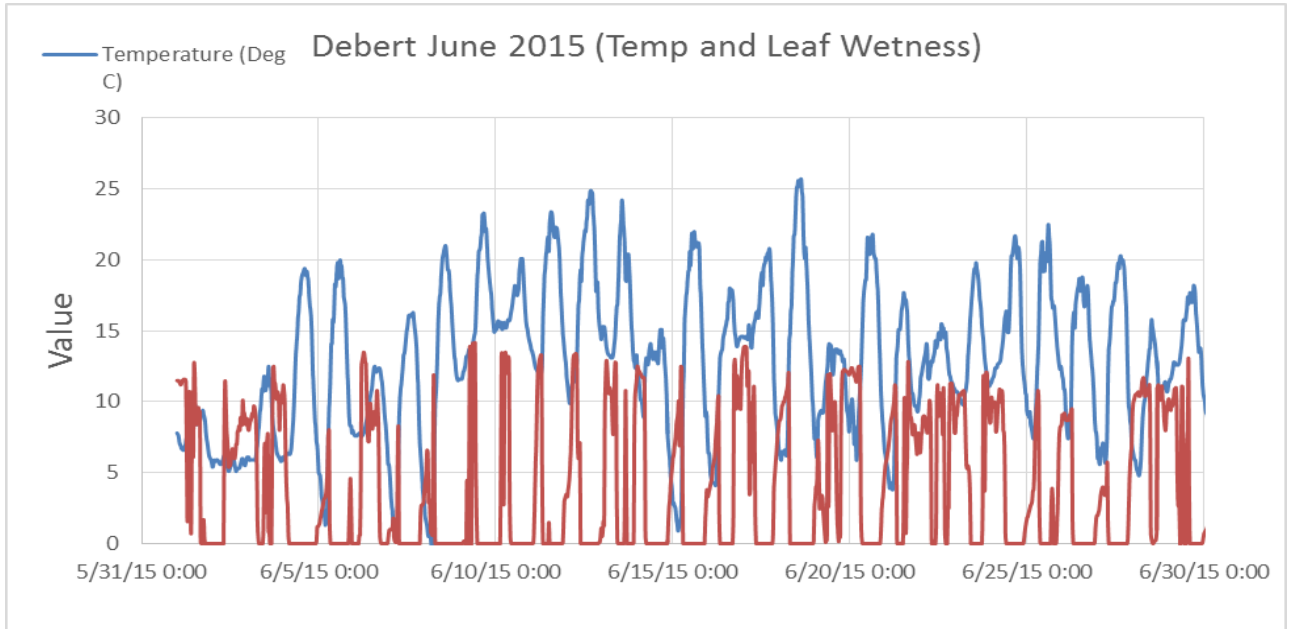
Figure A-1. Environmental conditions (Leaf wetness and temperature) observed in Debert and Webb Mountain (17km away), NS Botrytis blight field trial in 2015. Data was collected using a Spectrum Technologies Watchdog 2700 (Plainfield, Illinois) weather station located at the field site.
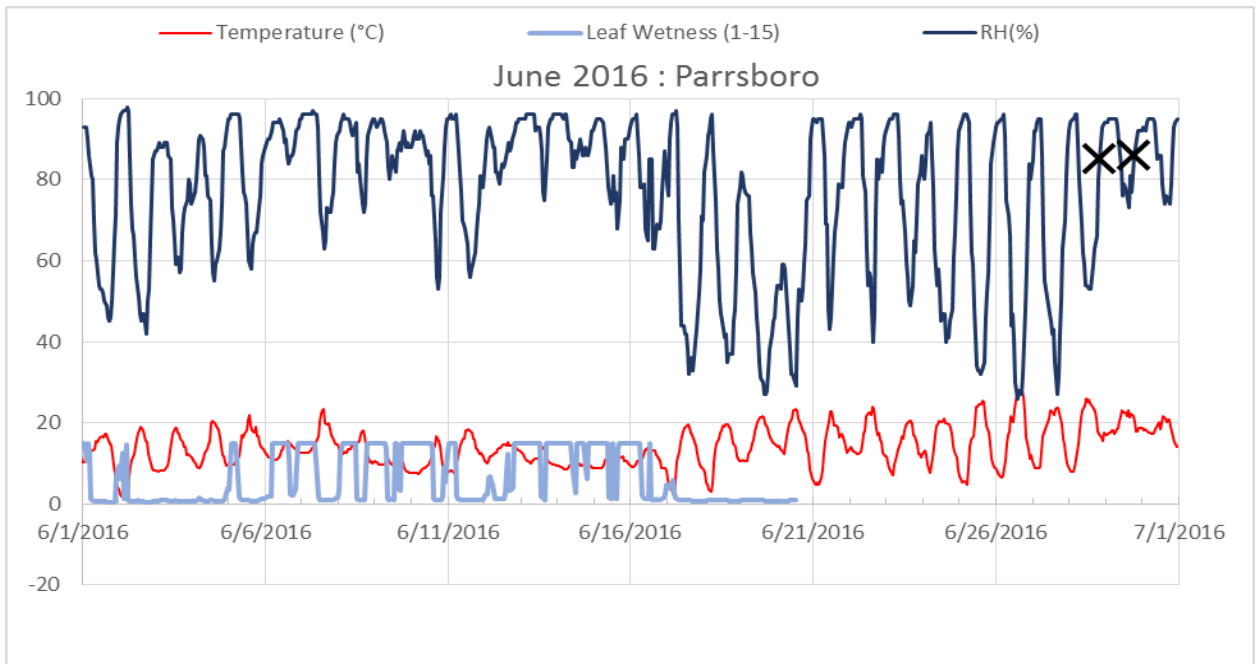

Figure A-2. Environmental conditions observed in Parrsboro, NS Botrytis blight field trial in 2016. Data was collected using a Spectrum Technologies Watchdog 2700 (Plainfield, Illinois) weather station located at the field site.
X: Start of high risk Botrytis infection period

Figure A-3. Environmental conditions observed in Dean, NS Botrytis blight field trial in 2016. Data was collected using a Spectrum Technologies Watchdog 2700 (Plainfield, Illinois) weather station located at the field site.

X: Start of high risk Botrytis infection period, + moderate risk infection period

Figure A-4. Environmental conditions observed in Debert, NS Botrytis blight field trial in June, 2016. Data was collected using a Spectrum Technologies Watchdog 2700 (Plainfield, Illinois) weather station located at the field site.
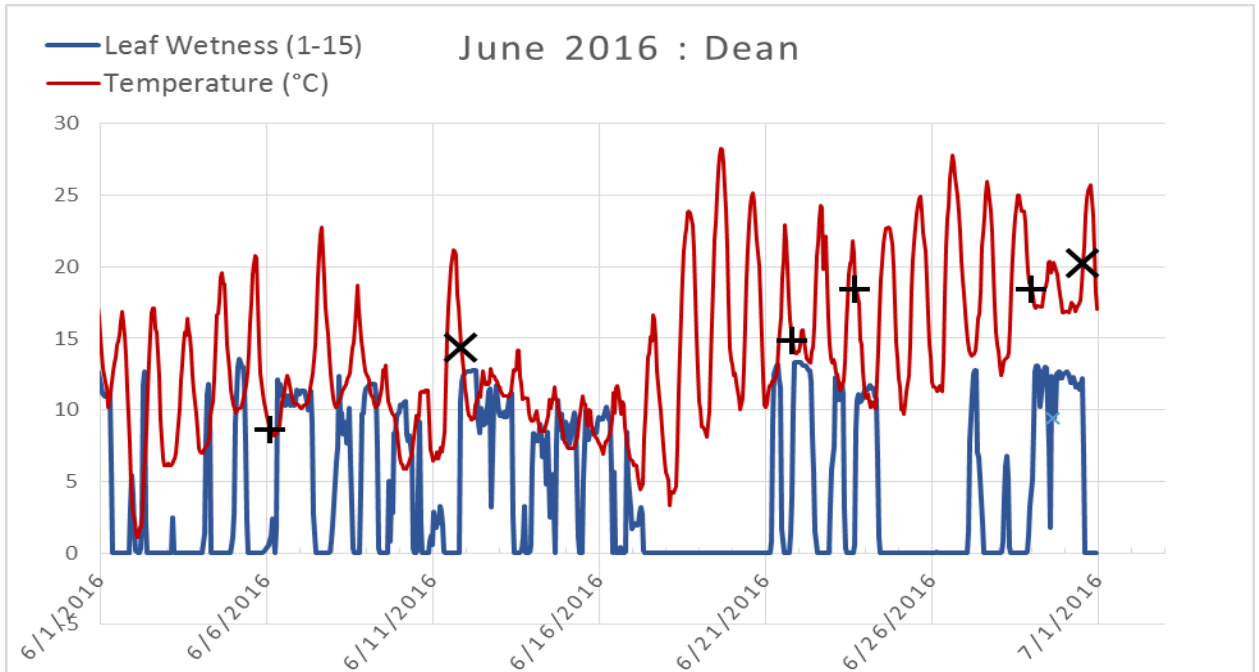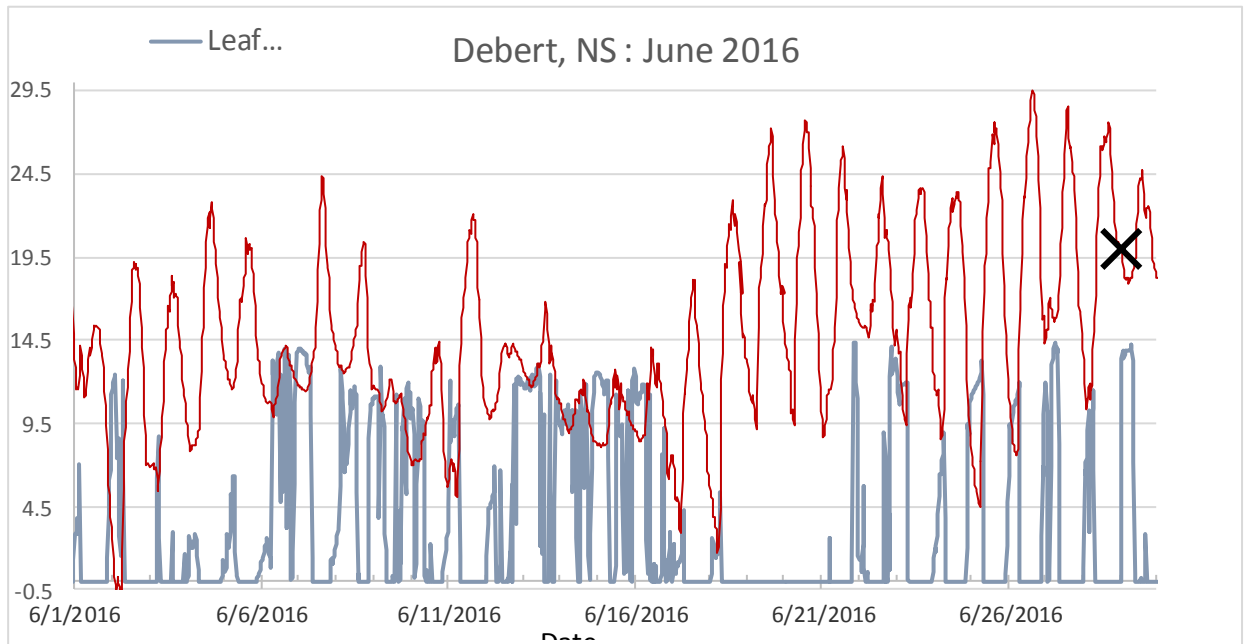X: Start of high risk Botrytis infection period.

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Table A-1. Infection periods for Botrytis Blight observed at Debert in June 2015

Start of infection period (Date, time)	Wetness duration (hours)	Mean Temperature (^{0}C)	Infection period Rating
6/1/15 6:30	7.2	7.09	Low
6/1/15 15:30	9.3	9.19	Low
6/3/15 6:00	6.1	5.79	Low
6/4/15 1:30	7.0	6.66	Low
6/6/15 16:30	8.3	10.13	Low
6/12/15 9:00	7.1	13.22	Low
6/13/15 9:30	12.7	13.83	Low
6/14/15 5:00	10	11.14	Low
6/17/15 3:30	12.1	14.57	Low
6/18/15 6:30	12	7.12	Low
6/19/15 14:30	7.7	13.29	Low
6/20/15 7:00	10.8	9.80	Low
6/21/15 7:30	7.6	5.84	Low
6/22/15 7:00	6.7	11.76	Low
6/22/15 16:00	7.3	14.80	Low
6/23/15 6:30	7.4	10.71	Low
6/24/15 8:30	7.8	12.42	Low
6/26/15 6:30	9.5	10.27	Low
6/28/15 11:30	9.3	8.27	Low
6/29/15 5:30	9.8	11.54	Low

Table A-2. Infection periods for Botrytis Blight observed at Parrsboro in June 2016

Start of infection period (Date, time)	Wetness duration (hours)	Mean Temperature (^{0}C)	Infection period Rating
6/11/16 4:40	5.0	8.02	Low
6/13/16 12:40	17.0	11.77	Low
6/14/16 10:40	18.0	9.56	Low
6/15/16 8:40	20.0	9.38	Low
6/16/16 6:40	15.0	10.40	Low
6/15/16 7:00	7.0	8.98	Low
6/16/16 5:00	12.0	10.07	Low
6/17/16 6:00	5.0	3.50	Low
6/18/16 6:00	5.0	3.73	Low
6/21/16 7:00	9.0	6.76	Low
6/22/16 8:00	12.0	13.29	Low
6/23/16 8:00	10.0	10.58	Low
6/25/16 6:00	7.0	6.41	Low
6/26/16 6:00	8.0	8.17	Low
6/27/16 6:00	6.0	9.79	Low
6/28/16 6:00	7.0	9.88	Low
6/29/16 9:00	13.0	17.66	High
6/30/16 12:00	17.0	18.34	High

Table A-3. Infection periods for Botrytis Blight observed at Dean in June 2016

Start of infection period (Date, time)	Wetness duration (hours)	Mean Temperature (^{0}C)	Infection period Rating
6/4/16 4:00	3	7.7	Low
6/5/16 2:00	7	10.4	Low
6/6/16 8:00	25	10.7	Moderate
6/8/16 2:00	11	11.5	Low
6/8/16 21:00	11	11.8	Low
6/9/16 20:00	13	7.0	Low
6/11/16 20:00	21	11.3	High
6/12/16 19:00	14	11.5	Low
6/14/16 0:00	9	9.1	Low
6/14/16 17:00	15	8.3	Low
6/15/16 13:00	20	8.5	Low
6/21/16 5:00	6	13.6	Low
6/21/16 20:00	15	14.4	Moderate
6/23/16 0:00	8	12.5	Low
6/23/16 17:00	16	12.9	Moderate
6/27/16 3:00	8	15.2	Low
6/29/16 0:00	12	18.0	Moderate
6/29/16 14:00	23	18.0	High

Table A-4. Infection periods for Botrytis Blight observed at Debert in June 2016

Start of infection period (Date, time)	Wetness duration (hours)	Mean Temperature (^{0}C)	Infection period Rating
6/6/16 21:00	11.0	11.82	Low
6/8/16 2:30	11.0	13.42	Low
6/8/16 21:30	6.0	11.93	Low
6/9/16 18:30	7.5	8.48	Low
6/10/16 4:00	5.0	7.82	Low
6/11/16 1:30	5.5	6.53	Low
6/12/16 20:00	15.0	12.62	Low
6/13/16 21:30	12.5	9.99	Low
6/14/16 13:00	5.0	11.17	Low
6/14/16 20:30	11.0	8.39	Low
6/15/16 12:30	4.0	11.30	Low
6/15/16 22:30	9.0	8.96	Low
6/22/16 20:30	10.5	13.50	Low
6/24/16 22:00	10.0	7.66	Low
6/26/16 0:30	7.0	9.33	Low
6/27/16 3:30	5.5	16.20	Low
6/29/16 1:00	8.0	18.54	Moderate
6/30/16 3:30	5.5	18.02	Low