WILD BLUEBERRY (*Vaccinium* spp.) - MICROBE INTERACTIONS AND BOTRYTIS BLIGHT MANAGEMENT

by

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ABSTRACT

Botrytis blight is an important disease and a major constraint to wild blueberry (*Vaccinium angustifolium* and *V. myrtilloides*) production. The aim of this research was to address the goals of improving yields, improving disease management, production sustainability, and reducing the cost of production partly through the understanding of molecular and polyphenol consequences of wild blueberry microbe interactions. A similar gene expression pattern was observed with both *Botrytis cinerea* and Serifel® (*Bacillus amyloliquefaciens*) inoculation. Maximum expression of *PR* genes varied and depending on the phenotype and the time of sample collection. Most of the flavonoid genes were suppressed at 12 hpi. The expression of flavonoid pathway genes and accumulation of phenolic compounds were phenotype-specific with their regulation pattern showing a temporal difference among the phenotypes. Flower interaction with microbes did not cause any changes in physiological parameters such as photosynthetic rate and stomatal conductance. For disease management, all the tested chemical fungicides reduced disease development by at least 47 % compared to the untreated control. The mean concentration of fungicides was highest in the corolla compared to the gynoecium and the androecium which is suggestive of limited mobility. Fungicides were sufficiently persistent to inhibit *B. cinerea* at fruit set. No residue was detected in harvested berries. The use of biofungicides provided intermediate levels of disease control with stand-alone applications of biofungicides whereas Switch® in rotation with biofungicides resulted in improved disease control. These results indicate that wild blueberries respond to microbes by the increased expression of *PR* genes and phenolic compounds, specifically *PR3* and *PR4.* Additionally, two dose application of fungicides is adequate to control Botrytis blight with no residue concerns.

LIST OF ABBREVIATIONS AND SYMBOLS USED

% - Percent
< - Less than
> - More than
± - Margin of error of a quantity
® - Registered trademark
µg - Microgram
µl - Microliter
a.i. - Active ingredient
ANOVA – Analysis of Variance
BLAD - Banda de Lupinus albus doce
cDNA - Complementary DNA
DAD - Diode-Array Detection
EC_{50} - Effective concentration that inhibits mycelial growth by 50%
ET - Ethylene
ETI - Effector-triggered immunity
FRAC - Fungicide resistance action committee
FW - Fresh weight
g - Gram
GC-MS - Gas Chromatography - Mass Spectrometer
ha - Hectare
hpi - hours post inoculation
HPLC - High Performance Liquid Chromatography
IDM - Integrated disease management
ISR - Induced systemic resistance
JA - Jasmonic acid
kg - Kilogram
Kow - Octanol/water partition coefficient
kPa - Kilopascal
L - Liter
LOD - Limit of detection
LOQ - Limit of quantification
m - Meter
mg - Milligram
mL - Milliliter
mm - Millimeter
MRL- Maximum residue levels
N - North
ºC - Degrees Celsius
PAMP - Pathogen associated molecular patterns
PCR - Polymerase chain reaction
PR - Pathogenesis related
PRR - Pattern-recognition receptors
PSI - Pound-force per square inch
PTI - PAMP-triggered immunity
QoI - Quinone outside inhibitor
qRT-PCR - Real-Time Quantitative PCR
R^2 - Coefficient of regression
RH - Relative humidity
RSD - Relative standard deviation
SA - Salicylic acid
SAR - Systemic acquired resistance
SDHI - Succinate dehydrogenase inhibitor
spp. - Species
Va- Vaccinium angustifolium
Va- Vaccinium myrtilloides
µg - Microgram

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CHAPTER 1: INTRODUCTION

Blueberries belong to the Ericaceae (Heath) family and are a member of the genus *Vaccinium*. This genus is divided into several sections which include *Cyanococcus*, *Oxycoccus* (Cranberry), *Vitis-idaea* (lingonberry), *Myrtillus* (European bilberry) and *Vaccinium* (Bog bilberry) (Luby et al., 1991; Kron, et al., 2002; Bassil, 2012). Blueberries are the group of species that belong to the *Cyanococcus* section. These include *V. angustifolium, V. myrtilloides, V corymbosum, and V. virgatum.* Generally, blueberries are grouped into either wild lowbush (*V. angustifolium, V. myrtilloides*) or highbush/cultivated blueberries (*V. corymbosum*, *V. virgatum* and *V. corymbosum*/*V. virgatum* hybrid).

The wild blueberry is native to North America and fields are developed from forested areas or abandoned farmland when their vegetation cover is removed. Due to their wild nature and the inherent presence in forest areas, commercial fields are made up of different phenotypes, which are referred to as clones. Commercial fields mostly consist of *Vaccinium angustifolium* (~70-80%), *V. myrtilloides* (~10-20%) on a surface area basis and some other hybrids (Jones and Percival, 2003). Fields are managed on a two-year cycle with the shoot being pruned every other year to maximize berry yield, and ease of mechanical harvest (Hall et al., 1979; Eaton and McIsaac, 1997).

Wild blueberry plants face several challenges which include diseases and pests. Historically, Botrytis blossom blight has been a major problem in wild blueberry production, especially in coastal areas with prolonged wet conditions. The disease is caused by the pathogen *Botrytis cinerea* Pers.: Fr. which typically infects flowers or entire inflorescences at the mid to late bloom stage (Hildebrand et al., 2001). The disease causes over 20% yield loss annually and over the past decade, it has become far more prevalent due to increased canopy densities, longer wetness durations and more susceptible floral tissue (increase in flower densities from 93 million flowers

ha^{-1} in 1994 to over 370 million flowers ha^{-1} in 2012 due to improved practices such as nutrient and weed management) (Percival, 2013). Botrytis blight is a severe fungal disease, however, its effect on commercial fields varies significantly due to differences in susceptibility among the various blueberry phenotypes with the more prevalent *Vaccinium angustifolium* being susceptible, and *V. myrtilloides* being tolerant to the disease (Abbey et al., 2018).

Integrated disease management (IDM) practices have been used over the past two decades for the control of Botrytis blights. However, the unique development and the production system of wild blueberry do not allow the adoption of some IDM practices such as canopy management/pruning during the cropping year. Given this, the disease is managed primarily through fungicide application. An important fungicide presently used for Botrytis blight control in wild blueberry is Switch®, which contains the signal transduction and amino acid inhibitors fludioxonil and cyprodinil, respectively. Other fungicides used include Luna Tranquility® (fluopyram and pyrimethanil), Sercadis® (fluxapyroxad) and Pristine® (pyraclostrobin and boscalid) (Percival, 2013; Burgess, 2020). However, *B. cinerea* is classified as a fungus that poses high-risk of developing resistance to fungicides (FRAC, 2019). Coupled with concerns of fungicide resistance, the high flower densities, pendulous flower orientation, and limited fungicide mobility raises questions about the ability to effectively protect wild blueberry flowers. With Botrytis management products accounting for more than 60% of the fungicide costs in the cropping year and concerns over detectable residues by consumers, an improved understanding of the ability of these fungicides to reach target tissue and their relative persistence is required. Additionally, there is a need to develop disease management approaches that will deliver low/non-detectable levels of fungicide residues in processed fruit. This has given rise to interest in biofungicides including Serifel® (*Bacillus amyloliquefaciens*), Serenade Opti®, Serenade MAX® (*Bacillus*

subtilis) and Fracture® (BLAD polypeptide) (Percival et al, 2016; Abbey et al., 2020; Burgess, 2020).

With variable disease damage observed on fields and increased cost of fungicide application, precision agricultural technologies and strategies including the use of prescription maps and sectional control on sprayers to reduce fungicide applications in areas with tolerant phenotypes would be an important development for the industry. The variable disease damage suggests the possibility of variable phenotype response to *B. cinerea*. Before further reductions in agrochemical applications are made, the universality of Botrytis blight tolerance within wild blueberry phenotypes needs to be verified and the resistance mechanism (s) (i.e., biochemical or avoidance) determined.

Generally, plants have been described to be equipped with a wide range of active defense mechanisms that are expressed when they are challenged by biotic and abiotic stress. Additionally, plants possess pre-existing protective structures/components which include lignified cell walls, cuticular bark and waxes that serve as the first line of defense (Łaźniewska et al., 2010; 2012). Upon pathogen/microbe detection, plants initiate many responses that lead to the formation of broad-spectrum defensive compounds (Parker et al., 2000) that confer some degree of resistance in the plant to various pathogens. Presently two major types of disease resistance are found in plants; basal and R-gene mediated defense which depend on the components of the pathogen involved in the pathogen-host interaction (Gururani et al., 2012). Basal defense (innate) can be a component of both host and non-host resistance. It provides first line of defense against infection by a wide array of pathogens. The basal response occurs through pattern-recognition receptors (PRRs) via pathogen associated molecular patterns (PAMPs) which include lipopolysaccharides, chitins, flagellins and glutens (Boller and Felix, 2009; Gururani et al., 2012; Wirthmueller et al., 2013). This type of defense can be triggered by even non-pathogens such as *Bacillus*

amyloliquefaciens (Li et al., 2015; Dimopoulou et al., 2019). R-gene mediated resistance is based on the ability of a host plant to detect specific pathogen effector proteins that change the physiological state of the host to facilitate infection or interrupt host plant defenses (Collmer, 1998). PAMP's have been observed in blueberry with pathogenesis related genes 3 and 4 (*PR3* and *PR4*) being detected in floral tissues (Thomas, 2012, Jose et al., 2021). Also, a blueberry transcriptome work by Polashock et al. (2014) and Yow (2018) identified genes associated with Monilinia resistance.

Additionally, plants respond to biotic and abiotic stresses via activation of some phenylpropanoid pathway genes and the accumulation of the related compounds known to possess antimicrobial properties (Jasiński et al., 2009; Ganthaler et al., 2017). Blueberry is known to contain a significant amount of flavonoids and other phenolic compounds such as coumaric acids, catechin, procyanidins and chlorogenic acid in different parts of the plant (Rodriguez-Mateos et al., 2012; 2016; Ștefănescu et al., 2020). Aside from their direct effect on pathogens (Tao et al., 2010; Villarino et al., 2011), these constitutive and inducible secondary metabolites have a complex interaction with other plant hormones such as jasmonate, ethylene and abscisic acid, which ultimately regulate gene expression and disease resistance (AbuQamar et al., 2006; Blanco-Ulate et al., 2013; Agudelo-Romero et al., 2015).

Knowledge and understanding of the complex processes of host-microbe interaction during disease development is crucial for proposing new management strategies and important to improving wild blueberry growing practices. While significant studies have been made on the molecular responses of several host pathosystems (Łaźniewska et al., 2010; Dalio et al., 2017), not many studies have been conducted on blueberry. With no reported molecular studies on wild blueberry-microbe interaction, this study seeks to provide into how *B. cinerea* and biofungicide

(*B. amyloliquefaciens*) may affect host metabolism, in addition to finding new disease management tools.

1.1. RESEARCH GOALS AND OBJECTIVES

The research aims to address the strategic goals of improving wild blueberry yields, production sustainability and environmental safety as well as reducing the cost of production partly through the understanding of molecular and polyphenol consequences of Botrytis-wild blueberry and Biofungicide (*Bacillus amyloliquefaciens)* - wild blueberry interactions, the development of reduced risk for Botrytis blight control and the assessment of fungicide mobility, persistence and presence in floral tissues. These aims stemmed from the limited research and knowledge about the molecular mechanisms that occur in wild blueberry plant-fungal pathogen interaction. Furthermore, the basis of this research seeks to reduce the cost of production and the desire to avoid blanket application of fungicides through the reduction in the number of Botrytis fungicide applications. This will help strengthen the aura of wild blueberries as being a low input and "wild" commodity and fortify production sustainability through the integration of biofungicides in Botrytis management systems.

The objectives of this research were to:

- Study the molecular and functional changes in wild blueberries in response to Botrytis blight disease
    - Investigate the molecular response and changes in resistance related genes and flavonoid pathway genes during *Botrytis cinerea* infection and *Bacillus amyloliquefaciens* interaction with wild blueberry.

    - Examine the effect of Botrytis disease on the whole plant and leaf gas exchange which occurs during infection.
- Fungicide mobility, persistence, and efficacy of disease control products
    - Investigate the distribution of fungicides (especially systemic fungicides) within wild blueberry floral tissues and how long they remain present in the tissues.
    - Assess the efficacy of Botrytis blight control products on Botrytis control in wild blueberries.
- Evaluate protection provided by plant- based extracts (BLAD, tea tree oil and polyoxin D) and the biological control agent *Bacillus subtilis* applied alone and in rotation with the reduced risk fungicide Switch® (a. i. cyprodinil and fludioxonil) against Botrytis blossom blight of wild blueberries.

CHAPTER: LITERATURE REVIEW

2.1 Overview of wild blueberry plant and production system

The wild blueberry is an important and economically high-value export crop. The crop is produced on approximately 40,500 ha and accounts for ~30% of Canada's land area in fruit, berries, and nut production (Statistics Canada, 2019a). In 2018, the Atlantic Canadian provinces and Quebec produced approximately 57.3 million kg of wild blueberries with farm gate value ~ $60.9 million (Statistics Canada, 2019b). The crop is the leading horticultural commodity in Nova Scotia, Quebec, New Brunswick, Prince Edward Island, and Newfoundland with about 17,600, 27,800, 13,300, 5,600 and 700 ha, respectively under production (AAFC, 2017).

The production system of wild blueberry focuses on the management of an existing wild population with no tillage and/or planting practices used. On the other hand, the establishment and commercial production of highbush blueberries involve the raising of seedlings from seeds of selected blueberry cultivars. Blueberries can grow on a wide range of soil types; however, they grow best on well-drained, sandy soils with low pH of glacial or alluvial origin (Jensen and Yarborough, 2004; Sanderson et al., 2008).

Wild blueberry production follows a two-year production cycle, thus, the sprout/vegetative and cropping/flowering year. In the sprout year, plants are pruned in the spring/fall near to the ground (Kinsman, 1993). After pruning, plants redevelop naturally from rhizomes and new stems arise from previously mowed stems at the start of each production cycle and grow until tip dieback. The plants are pruned every other year to make the most of floral bud initiation, fruit set, yield and promote harvest efficiency with fewer unbranched stems (Percival and Sanderson, 2004). Blueberry flowers are primarily self-incompatible; hence, cross-pollination is very important in blueberry production. In this context bumblebee and honeybees are employed during bloom to

ensure flower pollination (Yarborough, 2009; Drummond et al., 2010). Berries formed after pollination are then harvested in August/September.

2.2 Wild blueberry plant structure (leaf, flower, berry) and functionality

Wild blueberries are low growing plants with heights ranging from 10 to 60 cm. Shoots or ramets of maturing plants emerge from dormant buds on rhizomes (Kinsman, 1993). Growth and development of the plant in the vegetative or "sprout" year consist of shoot development and vegetative growth until tip dieback which initiates floral bud formation on the upper portion of the shoot (Barker and Collins, 1963; Aalders and Hall, 1964). Given their natural occurrence, wild blueberry fields consist of different types with *V. angustifolium, V. angustifolium* f. *nigrum* and *V. myrtilloides* being of commercial importance. These blueberries are categorized based on their genetic, morphological and phenotypic differences. *V. angustifolium* is a tetraploid and the common and most abundant species representing approximately 80% of wild blueberry species. They are characterized by green or brown stems with white flowers. *Vaccinium angustifolium* f. *nigrum* has deep brown-reddish stem with pink reddish flowers and very dark-colored berries. *V. myrtilloides* is a diploid and characterized by brown or green stems covered with pubescence/ hair-like structures (Strik and Yarbourough, 2005). Rhizomes of *V. angustifolium* mostly spread and develop more horizontally than *V. myrtilloides* rhizomes that grow densely and mostly develop vertically into the soil. This rhizome development difference enables *V. angustifolium* gain more coverage, hence their abundant presence on commercial fields as these fields age.

The leaves develop from small, scale-like buds mostly found on the lower parts of the stem. These vegetative buds produce shoots that bear alternately arranged leaves. Blueberry leaves are broad to elliptic shaped blueish-green shiny/smooth leaves that turn red to purple in the fall (Hall *et al*., 1979). The leaves possess a significantly waxed, thick epicuticular layer with stomata

present on the lower surface (hypostomatous) and minor serration (Figure 2.1b). These features provide the plant with the advantage of withstanding and surviving drought conditions. Furthermore, the thick and rigid nature of the leaves help reduce leaf infection and resistant to some fungal diseases such as Monilinia and Botrytis blight once they are expanded. Leaves of *V. myrtilloides* are covered hair-like structures (pubescence) from which it has obtained its name velvet-leaf blueberry (Figure 2.1a). Functions of trichrome and pubescence have been described to include deterring herbivores (Riddick and Simmons, 2014), retaining water droplets (Brewer et al., 1991) and reducing fungal inoculum load on the leaf surface (Mmbaga et al, 1994). Given this, the presence of pubescence on some leaves could contribute to the variation in disease development among blueberry phenotypes.

Figure 2.1. Fully developed elliptic shaped wild blueberry leaves. A) Leaves *V. myrtilloides* with pubescence (arrowed) on the surface, B) leaves of *V. angustifolium* with minor serration.

Flower bud formation mostly starts at the tip of the shoots above the vegetative bud. Flower buds tend to be larger and produce an inflorescence which consists of at least 8 inferior flowers per bud. Blueberry flowers are perfect pendulous cylindrical to urn-shaped or bell-shaped tube. The flower has four or five lobes which are about 5 mm long and inverted with the opening of the corolla at the bottom, thus edges of the corolla curled back (Eck, 1966, Hall et al., 1979; Kinsman, 1993). The color of the flower ranges from white or pinkish white to deep pink (Figures 2.2a and 2.2b).

The gynoecium of the flower comprises of pale green pistil made up of 5 carpels, a single style and an inferior ovary divided into five locules, each of which bears many ovules in an axile orientation (Palser 1961). *Vaccinium* species have been described to possess varying number of ovules per carpel. For instance, *V. arboruem* and *V. ovalifolium* are reported to have 5-8 and 40-50 ovules per carpel, respectively. In blueberries, each carpel/locule is reported to have 15-25 ovules on the average (Vander Kloet, 1988; Palser 1961). The pistil is long with its stigmatic surface close to the edge of the corolla opening (Figure 2.2c).

The androecium consists of a cluster of 10 relatively short brown stamens with filaments attached to the base of the flower around the nectary area. These filaments carry two poricidal anthers which dehisce by terminal pores from which pollen is released in tetrads (Bell and Burchill, 1955; Vander Kloet, 1988). An anther from blueberry is estimated to hold an average of 100-300 pollen tetrads (Vander Kloet, 1988). Pollen from blueberry flowers is sticky and heavy which makes its movement difficult for pollination (Isaacs, 2018). Additionally, the structure and position of the flower effectively inhibit free movement or wind-dispersed pollen from landing onto the stigma. In this context, the need for pollinator is of utmost importance. Given the sticky and heavy nature of blueberry pollen, vectors that carry out buzz pollination such as bees are the most appropriate pollinators in blueberry production. During anthesis, the stigma in opened blueberry flowers remains receptive for about 5 days with peak receptivity observed between 1 to 2 days

after opening (Brevis et al., 2006; Kirk and Isaacs, 2012). However, the length of days of their viability largely depends on the environmental conditions and the cultivar or phenotype involved. Given the relatively short receptivity time, blueberry flowers tend to be protandrous (Vander Kloet, 1988).

Pollen deposited on stigmatic surfaces are believed to be hydrated by stigmatic fluid/exudates, an event which is the recognition point of pollen-stigma interaction (Dumas and Gaude, 1983; Lang and Parrie, 1992). Over the years, many research activities have studied pollination in blueberry (Lang and Parrie,1992; Moisan-Deserres, et al., 2014; Gibbs et al., 2016). Several of these studies have looked at the number of pollen (pollen load) and viability required for optimum fruit development. It is, however, important to note that, between 100 – 300 pollen tetrads have been reported to optimize fruit development. Lang and Parrie (1992) reported that a stigma required 200 to 300 pollen tetrads to be saturated and cease the production of stigmatic fluid. Also, Dogterom et al. (2000) reported that individual flowers with an average of 106 ovules required 125 pollen tetrads to reach maximum fruit set and mass. Recently, Drummond (2019), demonstrated that the number of fruits set in wild blueberries increased as the number of pollen tetrads increase in a study with varying amount of pollen tetrad (20-100 pollen tetrad per stigma). In addition, the source of pollen also plays a significant role in the number of pollens that can be retained on the stigmatic surfaces (Lang and Parrie,1992; Dogterom et al., 2000). It is important to note that the number of pollen grains needed for optimal fertilization generally outnumber the total number of ovules in the ovary. This is because not every pollination/pollen deposited, or geminated pollen reaches the ovule or results in fertilization. In commercial field, the number of pollen grains deposited on stigmatic surfaces varies significantly and is dependent on the pollinator involved. For instance, Moisan-Deserres et al. (2014) studied the pollen load and specificity of native pollinators and reported that *Bombus* and *Andrena* species carried large amounts pollen. It

has been demonstrated that, as many as four honeybees visit deposited same amount of pollen by a single visit of a bumblebee queen (Javorek et al., 2002). Although pollen number and deposition has been described as an important factor in fruit development, the source of pollen, being self or cross significantly influence the viability of pollen and pollen load on stigmatic surfaces (Lang and Parrie,1992; Dogterom et al., 2000).

Following deposition and pollen germination, pollen tube elongation occurs towards the ovary through the style. Blueberry pollen tetrads start germinating 2-3 hours after deposition on stigmatic surfaces and take 3-5 days to travel down the style into to reach the ovule (Vander Kloet, 1988). Bell (1957) found out that, during the fertilization and seed development process, many ovules do not develop. Thus, about 78% of the average 64 seeds per berry were imperfect seeds. In the study of Bell, he found that the perfect developing ovules/seeds were always clustered around the top of the central axis whereas the non-developing/imperfect seeds were clustered around the basal part of the locules. This phenomenon is thought to be an indication that fertilization occurs on a first come first serve basis, thus the pollen tubes fertilize the ovules they first encounter as they enter the locules. This phenomenon supports the need for more pollen tetrads during pollination compared to the number of ovules to ensure sufficient pollens to fertilize all the ovules. Following syngamy, the ovules develop into mature seeds in an axile orientation. The formation of viable seeds stimulates the development of the ovary wall which becomes the actual berry. This position is supported by the observation that an increasing number of viable seeds is associated with larger berry mass (Drummond, 2019).

Figure 2.2. Urn-shaped flower cluster indicating the different colours of wild blueberry flower. A) white and light pink flowers (*Va*) and B) pink coloured flower (*Va* f nigrum). C) Longitudinal section of wild blueberry corolla showing the pistil surrounded by a cluster of relatively short brown stamen.

Pale green berry clusters are formed from pollinated flowers which increase in size and go through color changes. The blueberry fruit undergoes a double sigmoid growth curve which is grouped into three stages. Stage 1 of the development is characterized by rapid cell division and dry weight gain. This stage occurs between 25-35 days after bloom (Birkhold et al., 1992; Retamales and Hancock, 2012). Stage 2 of fruit development occurs between 30-40 days after bloom. This stage is characterized by active seed development with minimal fruit growth observation (Tamada, 2000). Stage 3 of the fruit development is characterized by rapid cell enlargement that results in rapid fruit growth. This final stage usually lasts between 40-60 days after bloom during which sugars and anthocyanin accumulate (Cano-Medrano, and Darnell, 1997; Tamada, 2000; Retamales and Hancock, 2012). Fruit development may vary from small to large pea-size in the same cluster and the amount of time spent within a particular stage is dependent on

the cultivar, and the environmental conditions. Fruit color changes from green through reddish purple to bluish/indigo at maturity with blue epicuticular coatings (bloom) on the surface. Berries from *V. angustifolium* f. nigrum appear black and lack the epicuticular coating **(**Figure 2.3**)**. The flesh of the blueberry ranges in color from white to light green. This suggests that most of the antioxidants and phenolic compounds found in blueberries are, especially anthocyanin pigments, present in the skin (Figure 2.4b). The fruit is round with a flaring crown (persistent calyx) at its end which arises from the sepals. This flared crown could serve as a suitable den for pathogen inoculum. The fruit of the plant is a false berry which varies in sizes between 5-15 mm in diameter with an average weight of 0.3 grams (AAF, 2010) (Figure 2.3). The fruit holds between 50-70 tiny seeds (average of 64 seed per berry) found in the five (chambers) locules of the developing ovary in axile placentation. Studies have shown that the size of berry, berry weight and fruit set are qualities that are highly dependent on the pollination and fertilization events. For instance, Drummond (2019) reported that an increase in pollen deposition leads to an increase in the fruit set. Thus, for maximum fruit set and fruit to attain its maximum size, nearly all the ovules must be fertilized. Similar to the relationship between fruit set and pollination, many studies have established a positive correlation/relationship between seed number, and fruit size and weight (Vander Kloet,1983; Drummond, 2019).

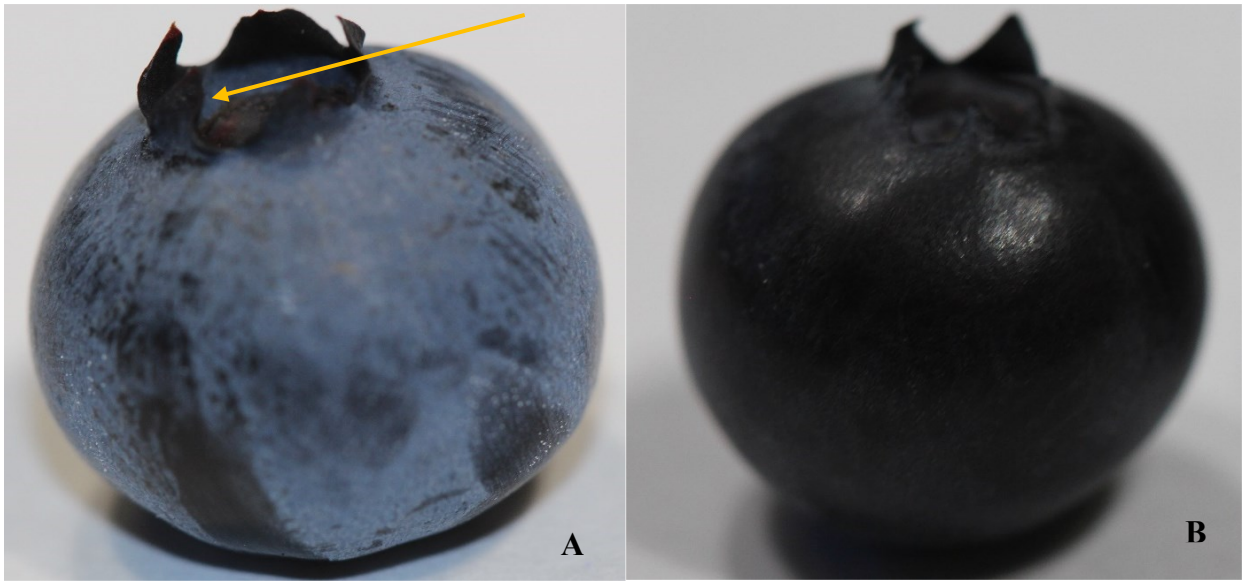

Figure 2.3. Ripe blueberry fruits showing flaring crown (arrowed). A) Fruit from *V. angustifolium* covered with blue epicuticular coatings on the surface. B) Black fruit of *V. angustifolium* f. nigrum with less epicuticular coating.

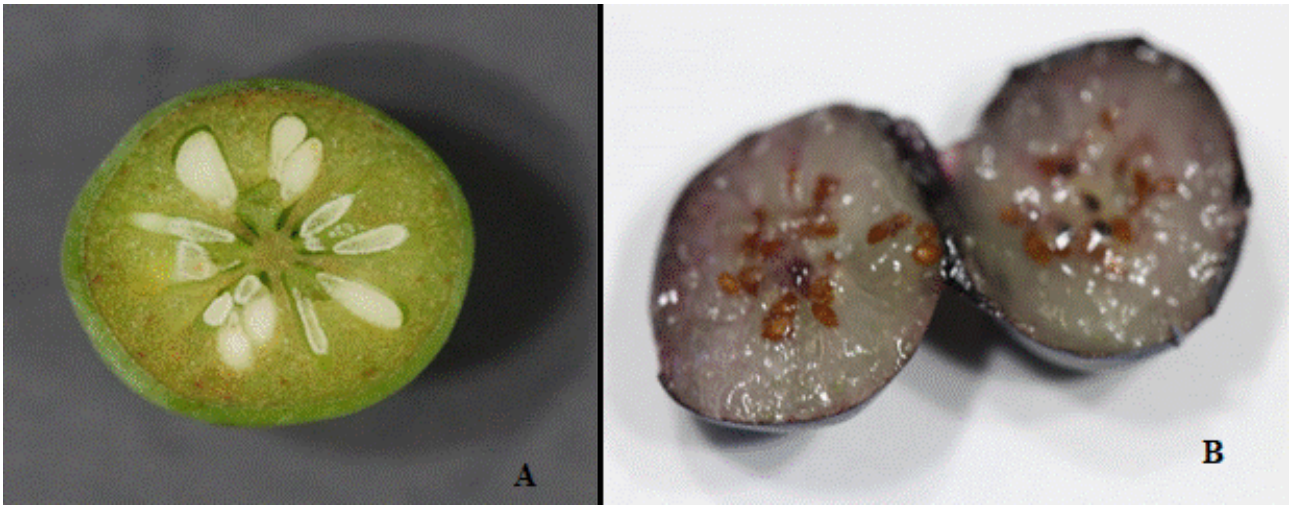

Figure 2.4. A) Cross-section of blueberry fruit, the five ovary chambers (green fruit). B) Cross-section of fully ripe blueberry fruit, showing seeds and white coloured flesh of the berry.

2.3 Morphology and lifecycle of the *Botrytis cinerea*

Botrytis cinerea is an ascomycete fungus that belong to the class of *Leotiomycetes*, order of *Helotiales*, and the *Sclerotiniaceae* family (Williamson et al., 2007). The fungus can easily be identified by their morphological features. *B. cinerea* produces abundant colonies that is at first

white to grayish and turns dark brown as it matures. The mycelia of *B. cinerea* are branched, olive brown, cylindrical, and septate (Mirzaei et al., 2007, 2008). The fungus produces conidia in clusters from enlarged apical cells at the end of branched and slender conidiophores (Elad et al., 2004). The conidia of *B. cinerea* are smooth, single-celled, slightly ash-coloured and oval (Mirzaei et al., 2007; Horst, 2008). The fungus overwinters as sclerotia which contain viable hyphae that serve as primary inoculum for disease development. They are hard and formed in varying shapes and sizes (Chen and Hsieh, 2009; Wang et al., 2011). The sclerotia are whitish in their initial stage then becomes (melanized) black at maturity (Mirzaei et al., 2007, 2008; Zhou et al., 2018). Sclerotia may germinate to produce mycelium, conidiophores and conidia or apothecia and ascospores. *B. cinerea* can also over winter as dormant mycelium or chlamydospores (Dewey and Grant-Downton, 2016).

Asexual reproduction is the commonly observed form of reproduction in *B. cinerea* both in the field and the laboratory. Under favourable conditions, overwintering structures germinate to produce mycelia (Williamson et al., 2007; Dewey and Grant-Downton, 2016). The developed mycelia produce conidiophore, a simple or branched hyphae on which conidia are produced. In spring, when environmental conditions are favourable, conidia (macroconidia) from the overwintering sources provides an abundance of inoculum (Williamson et al., 2007).

The sexual life cycle (teleomorph) of *B. cinerea* (formerly known as *Botryotinia* spp.) occurs through the spermatization of sclerotia that results in the formation of a fruiting body called of apothecium and ascus (Faretra et al., 1988; Dewey and Grant-Downton, 2016). The ascus, a saclike structure contains eight binucleate ascospores (Pöggeler et al., 2006; Rodenburg et al., 2018). Ascospores released from these apothecia can infect plants and thereby serve as source of primary inoculum of the fungus. In ascomycetes, sexual compatibility is determined by two mating

type loci labeled as MAT-1 and MAT-2, hence, making *B. cinerea*, a heterothallic fungus (Dewey and Grant-Downton, 2016; Rodenburg et al., 2018) (Figure 2.5).

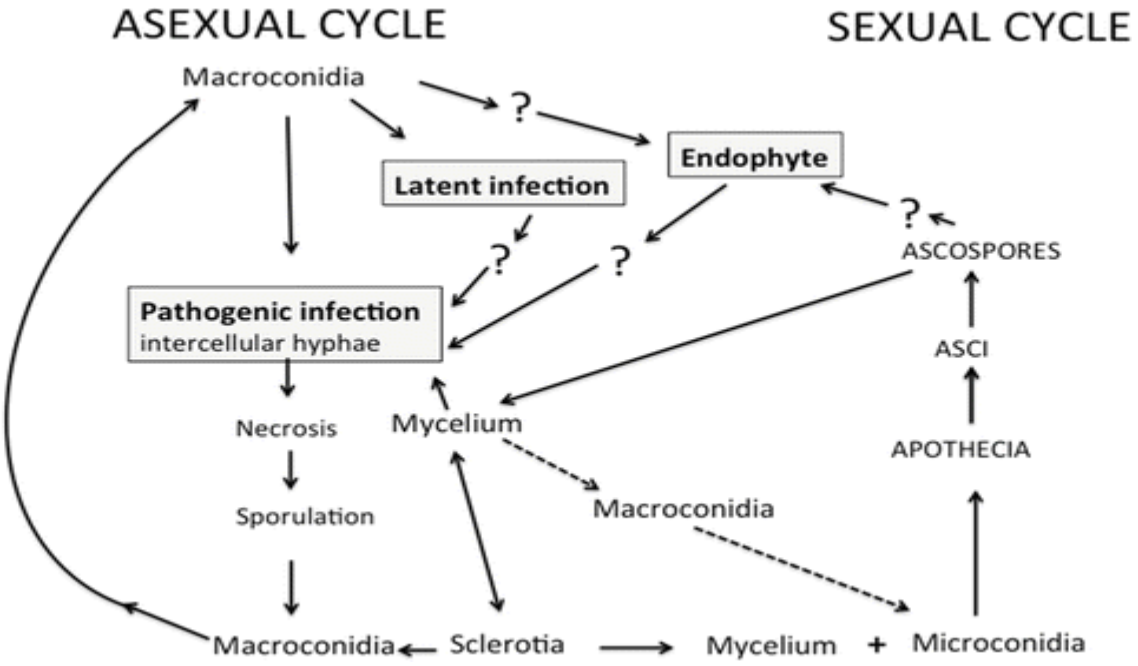

Figure 2.5. Asexual and sexual life cycle of *Botrytis cinerea* and disease cycle. (Dewey and Grant-Downton, 2016).

2.3.1 *Botrytis cinerea* infection of blueberry plant

Botrytis cinerea is an opportunistic fungus that can cause infection in over 500 genera of vascular plants (Elad et al., 2016). The pathogen easily causes infection at wound sites or previously infected sites; however, it can directly enter intact host surfaces through the cuticle (Williamson et al., 1995; González et al., 2015). Pertaining to wounded tissues, one major challenge of blueberry production is frost damage on flowers and tender new growth which may result in wounded tissues. This increases the susceptibility of the blueberry plants to the pathogen. The fungus can also enter its host through stomata and other natural openings (Carisse, 2016). Wet

periods and moderate temperatures are essential factors necessary for conidia germination and infection by *B. cinerea* (Carisse, 2016; Coertze and Holz, 2017). The risk of an infection is dependent on surface wetness and temperature. Though prolong wet periods and moderate temperatures are required for infection, the length of wetness duration is important for infection. Hence, increasing the length of surface wetness and temperature increases the risks of infection (Delbridge and Hildebrand, 2007).

Preceding host invasion, the pathogen kills underlying host cells after penetration of the cuticle (Laluk and Mengiste, 2010). As a necrotrophic pathogen, *B. cinerea* induces host cell collapse possibly by the production of metabolites and toxins which results in necrosis of host tissues (Van Kan, 2006). The fungus is known to secret toxic compounds and cell wall degrading enzymes (CWDEs) such as oxalic acid and cutinase (Hua et al., 2018). Oxalic acid may have direct effects by lowering the pH of the environment and facilitating the pectin breakdown activities of hydrolases in cell walls (González-Fernández et al., 2015). Symptoms of *B. cinerea* infection on blueberry usually start as a small water-soaked light-brown necrotic lesions on the flower (Figure 2.6a). This infection quickly spread to cover the entire flower, and, in most cases, the entire flower clusters may become blighted. At the advanced stage, infected flowers are usually covered with grey mold (Figure 2.6b). Most blueberry flower infection starts from the corolla and spread to the peduncle of the flower clusters. This can be attributed to the bell shape and pendulous structure of the flower and that during bloom, the corolla represents a proportion of the flower tissue and houses the other floral parts (Hildebrand et al., 2001; Abbey et al., 2018). Hence, it is exposed to spore dispersal than the gynoecium and androecium.

Botrytis cinerea has always been labeled as a necrotroph, nonetheless, recent studies have shown that they can also act as endophytes (remain quiescent) and cause latent infection (Sanzani et al., 2012). It is not well known if systemic or latent infection of *B. cinerea* occurs in blueberry,

however, harvested berry may rot and shrivel followed by the growth of gray mold on the berry. Over the years, histological studies have established the systemic floral infection of various crops including blueberries, blackcurrants, and grapes by pathogens such as *B. cinerea* and *Monilinia vaccinii-corymbosi* (McNicol and Williamson, 1989; Ngugi and Scherm, 2004; Viret et al., 2004; Kozhar and Peever, 2018). These studies have demonstrated that conidia of *B. cinerea* germinate in stigmatic fluid and the germ tube grows into the ovary via the stylar canal. The germ tube takes the same route as the pollen tubes within the flower, thus fungal hyphae mimic host pollen tube during gynoecia infection (Viret et al., 2004; Kozhar and Peever, 2018). It is believed that these fungal pathogens take advantage of host mechanisms intended to support reproduction to facilitate infection of the stylar transmitting tract and ovary. In blueberry, studies on systemic/internal infection in flowers have mostly been carried out on *Monilinia vaccinii-corymbosi* due to the occurrence of secondary infections which result in mummy berry formation (Ngugi and Scherm, 2004; Lehman et al., 2007). Lehman et al., (2007) reported the expression of host resistance to *M. vaccinii-corymbosi* in the styles and locules of different blueberry cultivar. They observed that resistance to infection by *M. vaccinii-corymbosi* is initially expressed in the locule, rather than the stigma or style during the infection process. This was also supported by the observation that, there were higher infection frequencies in the styles compared to the locules. None of these histological studies on internal infections has been carried out on *B. cinerea* infection of blueberry. Histological studies on internal infection of flowers by *B. cinerea* on different crops such as strawberry, raspberry, and grapes have reported the observation/growth of germ tube/hyphae in the stylar pathway when stigmatic surfaces were inoculated with *B. cinerea* conidia. However, none of these studies reported the presence of *B. cinerea* in the ovules/inside the ovary (Jarvis, 1962; McNicol et al., 1985; Viret et al., 2004). Generally, latent infections by *B. cinerea* in various crops have been found primarily in the receptacle area and the persistent calyx. Even though post-harvest

Botrytis rot in blueberries exist, it is yet to be studied to ascertain whether the rot is because of flower infection/latent infection or because of the presence of conidia on the berry surface or within the flared crown of the berry.

Although *B. cinerea* infects flowers, they can also infect leaves and stems, especially when they come in contact with infected floral tissues. As an opportunistic pathogen, the thick waxy cuticle of blueberry leaves acts as physical barrier able to resist *B. cinerea* infection. Furthermore, the release of cutin monomers or wax components during plant-pathogen interaction function as signals to activate plant disease responses (Serrano et al., 2014; Aragón et al., 2017). In addition to flower infection, the pathogen can infect young/immature berries, however, immature berries are relatively resistant to the pathogen partly due to higher polyphenols content and antioxidant activities (Arena et al., 2012; Wang et al., 2018).

Figure 2.6. A) Symptoms of *B. cinerea* infection on wild blueberry flower as a small water-soaked light-brown necrotic lesion. B) A completely dead wild blueberry flower cluster with the characteristic *B. cinerea* on the tissue.

2.4 Botrytis management strategies

2.4.1 Cultural methods

There are various practices that can help reduce the effect of *B. cinerea* on crop production. Nevertheless, some of these practices are host specific and dependent on the cropping system. Management practices such as hedging, and leaf removal can be used to manage canopy density when suitable. Practices such as planting and harvesting date adjustment, crop rotation, and modification of plant density by row or plant spacing and seeding rate for proper aeration been pointed out to have significant effect on the incidence and severity of Botrytis diseases (Williamson et al., 2007; Agrios, 2005). However, not all these practises are applicable in lowbush blueberry production.

In lowbush blueberry, canopy management is primarily achieved by pruning (thermal pruning and the mower pruning). Studies have shown that the colonization of fruits by *B. cinerea* from sites pruned by mowing was more frequent than by biennial burning (Lambert, 1990). This is because thermal pruning reduces the amount of overwintering fungal spores and overwintering structures such as sclerotia in the field which will serve as primary inoculum source. Conventionally, lowbush blueberry fields were pruned by free burning. Free burning offers a low-cost method of pruning, nevertheless, it is hard to control and often does not burn the field completely (DeGomez, 1988). To help reduce the cost of pruning with straw or fuel oil, flail mower was introduced (Kinsman, 1993; Yarborough, 2004). In addition to killing the blueberry stems, the heat produced from thermal pruning may reduce the incidence of insects, diseases, weeds, and overwintering fungal propagules such as mummy berry, dormant mycelia and sclerotia in the field (DeGomez, 1988; Lambert, 1990).

Also weed management is important to the management of *B. cinerea.* Several weeds have been reported to serve as hosts for the pathogen. In lowbush blueberry fields, these include bunchberry (*Cornus Canadensis* L), sheep sorrel (*Rumex acetosella* L.), goldenrod (*Solidago Canadensis* L.) and Pearly everlasting (*Anaphalis margaritacea*) (Delbridge and Hildebrand, 1997).

Others cultural methods that have been documented to have a significant effect in controlling *B. cinerea* include use of less susceptible cultivars, cultural period, production system, mechanical soil treatment, nitrogen fertilization, defoliation, and sanitation (Daugaard, 1999). Though various methods have been pointed out, it can be concluded that there are no specific or individual practice capable of controlling this pathogen completely.

2.4.2 Biocontrol methods

The management of Botrytis with fungi, bacteria, yeasts, and plant-based compounds has received enormous investigation for over three decades (Blakeman and Fokkema, 1982; Dubos, 1992; Elad and Stewart, 2007). Among the biocontrol methods microbial biocontrol agents, especially fungi have been adopted and investigated for *Botrytis cinerea* management. One major motivation behind the use of microbial and natural compounds is because they are less damaging to the environment, and their complex mode of action decreases the risk of resistance development (Elad et al., 2007).

The search and use of biological control agents for Botrytis disease control begun over half a century ago and several biological agents have proved their worth and ability to control *Botrytis cinerea* (Elad and Stewart, 2007; Sreevidya et al., 2015). *Trichoderma* spp. is one of the key microbes that have been extensively studied and used for Botrytis management on different crops (Baraka et al., 2017; Redda et al., 2018; Aoki et al., 2020). Several modes of action have been

tested including competition, induction of plant resistance (Hanson and Howell, 2004), and mycoparasitism (Papavizas, 1985; Rajani et al., 2021). In mycoparasitism, a microscopic study revealed that *Trichoderma* coils around and penetrates the mycelium of *B. cinerea* (Dubos, 1987).

Several antagonistic yeasts have been good candidates for the management of Botrytis incited disease. Yeast species including *Metschnikowia pulcherrima, Pichia guilliermondii* and *Aureobasidium pullulans*, were able to significantly protect grapes from *B. cinerea* (Raspor et al., 2010). One antagonistic yeast that has been of great benefit and widely used in apple (Vero et al., 2009; Zhang et al., 2010; Mari et al., 2012) and strawberry (Adikaram et al., 2002) production against *B. cinerea* is *Aureobasidium pullulans*. Additionally, the potential of *Aureobasidium pullulans* against *B. cinerea* in wild blueberry has been reported recently (Abbey et al., 2020). Competition for nutrients and the secretion of enzymes such as glucanase and chitinase have been identified as the modes of action of this biocontrol agent (Castoria et al., 2001; Zhang et al., 2010).

Bacillus spp are a group of bacteria that have been extensively studied as biocontrol agents. The antifungal activity of several strains of *Bacillus* against *B. cinerea* have been documented. In both an *in vitro* and field studies, *Bacillus subtilis* significantly reduced gray mould disease on strawberries by more than 85% (Hang et al., 2005). *Bacillus* spp. are well known for their ability to form endospores and produce broad-spectrum antibiotics. These characteristics makes them effective in the management of several plant diseases coupled with extended shelf lives through endospore formation (Emmert and Handelsman, 1999; Khanna et al., 2020). *Bacillus* spp. is one of the biocontrol agents from which different commercial products such as Kodiak HB® (*B. subtilis GB03*), and Serenade® (*B. subtilis* QST 713) have been developed for Botrytis control.

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*. A recent study with essential oils from oregano (*Origanum vulgare* L.) and lemon (*Citrus limon* L.) against

B. cinerea both in vitro and in vivo significantly reduced gray mould severity of infection in tomatoes, strawberries, and cucumbers (Vitoratos et al., 2013). Tea tree oil (TTO) is an essential oil distilled from *Melaleuca alternifolia*, a species that is native to New South Wales, Australia (Swords and Hunter 1978). Studies have reported TTO as a natural antifungal agent that is able to control *Botrytis cinerea* and grey mould in fresh fruit (Cheng and Shao, 2011; Shao et al. 2013).

Like essential oils, polypeptides from plants such as sweet lupine (*Lupinus albus*) known as Banda de Lupinus albus doce (BLAD) have recently been extracted and proved to be effective for Botrytis control. Trials have demonstrated that BLAD is efficacious enough to serve as a substitute for chemical fungicides in *Botrytis* management programs. BLAD was found to bind to the fungal cell wall and interrupted the chitin structure leading to cell wall fracture (Monteiro, et al., 2015). It also degrades chitin by catalyzing and removing N-acetyl-D-glucosamine terminal chitin monomers, leading to the ruin of the cells (APVMA, 2017). This active ingredient is currently marketed as Fracture® and has been shown to provide adequate disease control in blueberry (Percival, 2016; Abbey et al., 2020).

Polyoxin is a peptidyl nucleoside isolated from *Streptomyces cacaoi* (Isono and Suzuki, 1966). It competitively inhibits the activity of chitin synthetase (Li et al, 2012). Polyoxin is an important group of compounds that are well known for their antifungal activities. Following their potential use as biofungicides, several commercial products such as OSO 5% SC, have been developed from and polyoxin D for pathogen control including *B. cinerea*. Unlike many biofungicides where pathogen resistance is of minimal concern, there have been a few reports of Botrytis resistance to polyoxin D on different crops (Mamiev et al., 2013; Dowling et al., 2016)

2.4.3 Chemical control

Presently, most fungicides available for pathogen control including *B. cinerea* are site specific and are grouped based on their mode of action. These groups include anilopyrimidine, phenylpyrroles, SDHIs, QoIs (FRAC, 2019; 2021). Derivatives of anilinopyrimidines such as pyrimethanil and cyprodinil prevent germ-tube elongation and mycelial growth of *B. cinerea* (Avenot et al., 2018; Rosslenbroich and Stuebler, 2000) through the inhibition of methionine biosynthesis (Fritz et al., 1997; FRAC, 2021). Phenylpyrrole fungicides such as fludioxonil inhibits spore germination, germ-tube elongation, and mycelial growth of *B. cinerea* by targeting protein kinase PK-III, which is associated with the osmoregulation signal transmission pathway (Pillonel and Meyer, 1997; Pillonel et al., 2003). Succinate dehydrogenase inhibitors (SDHIs) such as fluxapyroxad, fluopyram, adepidyn, boscalid and penthiopyrad; and the quinone outside inhibitors (QoIs) such as pyraclostrobin are respiration-inhibiting fungicides (Stammler et al., 2008; Avenot and Michailides, 2010; Fernández-Ortuño et al., 2010; FRAC, 2021).

These site-specific fungicides are very potent and effective against fungi. However, resistance development in fungi populations, as well as distribution, mobility, and persistence of the fungicide among other factors can greatly affect their efficacy. For example, anilinopyirimidines have been shown to be effective against *B. cinerea* however, a potential resistance development was noticeable at the preregistration stage in the laboratory (Birchmore and Forster, 1996). Also, cyprodinil resistance was reported by Latorre et al. (2002) in table grapes (*Vitis vinifera* L.) few years after registration and by many recent studies. In recent years, various levels of resistance development among *B. cinerea* to almost all the existing fungicides have been reported (Hauschildt et al., 2020; Shao et al., 2021).

2.5 Fungicide Mobility and Persistence

Fungicides can be either non-systemic or systemic. Non-systemic fungicides remain on the plant surface and do not penetrate the plant whereas systemic fungicides are able to penetrate and move to different points within the plant tissue. Based on mobility and systemicity, fungicides can be classified into local penetrants or translaminar systemicity (e.g., cyprodinil, fluxapyroxad, pydiflumetofen), xylem (acropetal) penetrant (e.g prothioconazole, boscalid, azoxystrobin) and phloem penetrant (basipetal, amphi-mobile) (e.g fosetyl-aluminum, phosphorous acid) (Augusto and Brenneman, 2012; Klittich, 2014). Fungicides classified as local penetrant or translaminar are absorbed by plant tissues and move short distances or do not move significantly away from the point of deposition (McGrath, 2004). They can move and act across a leaf from one surface to the other. Acropetal fungicides are absorbed and translocated over long distance in the direction of the xylem (move upward in plants). Basipetal fungicides are translocated over long distance and have bi-directional mobility. They mostly move in the direction of the phloem stream (McGrath, 2004; Rouabhi, 2010). The mobility and persistence of fungicides is influenced by the physico-chemical properties of the fungicides such as lipophilicity and solubility, and some plant factors. In addition, environmental factors such as temperature, precipitation and air movements also influence persistence (Edwards, 1975; Klittich, 2014; Satchivi, 2014).

For every fungicide applied, the first barrier encountered is the plant cuticular membrane. The fungicide needs to move into the cuticle, diffuse through it, and partition into the apoplast (Edgington, 1981; Klittich, 2014). The penetration through the cuticular membrane is a complex interaction of various factors. The absorption and redistribution of fungicide greatly depends on the partition behaviour of the fungicide between lipophilic and hydrophilic part of the cuticle. Lipophilicity is usually expressed as the log of the partition coefficient (K_{OW}) between octanol and water. Very high lipophilic chemicals may be retained/persist in the cuticle whereas high

hydrophilic compounds tend to be partitioned less rapidly, hence influencing the mobility of these compounds (Edgington, 1981; Satchivi, 2014). Lipophilicity tends to determine the potential of long-distance transportation of fungicides within plant (Bromilow and Chamberlain, 1995). Generally, a log K_{OW} range of 2 to 4 is considered as the preferred range for good fungicide mobility. Water solubility (hydrophilicity) of fungicide is well associated with mobility. The mobility of a fungicide within plant tissue increases with increasing solubility in water.

Though most of the factors that influence fungicide mobility influence persistence, other factors can significantly affect persistence more than mobility. For instance, since contact fungicides remain on the plant surface, they are likely to disappear quite easily through different means such as removal by precipitation, evaporation (for volatile compounds) and photodegradation. The rate at which a fungicide penetrates plant cuticle can significantly influence their persistence. Also, metabolic activities and other plant processes such as excretion, translocation and growth rate can greatly influence the persistence fungicides in the plant (Edwards, 1975). For example, the growth of new shoots will imply the distribution of compounds within the plant to other new areas hence, reducing the persistence within the plant through dilution by increasing biomass. In addition, the rate of fungicide application can significantly influence it persistence within plant tissue.

Fungicide mobility and persistence is very important in achieving good, effective, and extended disease control. Nonetheless, there is a limit to persistence and its resultant chemical residue allowed in agricultural products and the environment. This limitation stems from the increased public concern about contamination of fruits and vegetables with residues from fungicides and their effect on health (Tripathi et al., 2008; Farquhar et al. 2009). Due to these concerns, there have been stringent regulations governing fungicide residues with limited pesticide concentration allowed in fruits.

2.6 Botrytis control challenges in wild blueberry

Integrated disease management (IDM) has been recommended and used over the years for the control of Botrytis incited diseases. However, the unique nature of wild blueberries does not lend itself to the adoption of some of these IDM practices such as pruning during the cropping year. Given this, foliar application of fungicides has been the core of Botrytis control in wild blueberries. The main fungicide presently used for Botrytis blight control in wild blueberry is Switch®, which contains the signal transduction and amino acid inhibitors fludioxonil and cyprodinil, respectively. Other fungicides used include Luna Tranquility® (fluopyram and pyrimethanil) and Sercadis® (fluxapyroxad) (Percival, 2013; Burgess, 2020). Chemical fungicide application for Botrytis management is challenging due to the high-risk nature of the pathogen to resistance development (FRAC, 2019). Coupled with plant factors such as the pendulous orientation of flowers, high flower densities, variable flower progression and limited fungicide mobility within flower, Botrytis control in wild blueberry can be challenging.

The structural and pendulous orientation of the wild blueberry flower posses a challenge with fungicide application. During disease control, applied fungicides are mostly deposited on the upper part of the pendulous flower and mostly on the corolla. This limits the protection of the entire flower with a foliar application as other floral parts do not receive any fungicide. Also, flowers on the lower part of the stem and those within the canopy are not sufficiently exposed to applied fungicides. Furthermore, a significant increase in flower densities (> 370 million flowers per hectare) due to improved management practices (Percival, 2013) increases the amount of susceptible floral tissues on the field. The flower density, limited fungicide coverage/deposition coupled with reduced fungicide efficacy due to resistance development among the fungal population poses a Botrytis management challenge.

For every fungicide applied, their distribution, mobility and persistence are very important. To this effect, the limited coverage/distribution of non-systemic/contact fungicides such as fludioxonil on wild blueberry flowers presents a management challenge. Since some of these fungicides are protectants, they lack mobility, and they are less persistent. Their efficacy is dependent on full coverage of target tissues, therefore in wild blueberries where flower structure/orientation limits foliar application coverage, effective disease management may become a challenge. Also, most fungicides registered for Botrytis control in wild blueberry are locally systemic, hence, their mobility within plant tissues is greatly limited (Beckerman, 2018). Active ingredients are not transported to other parts of the flower that did not receive fungicide during application.

Given some of these challenges encountered during disease management, there is no single solution to mitigate some of these challenges, hence, there is a continuous search for various strategies to achieve effective disease management. These strategies could include the integration of molecular-based approaches such as induced systemic resistance (ISR).

2.7 Plant response to fungal pathogens

2.7.1 General aspects of plant immunity and defense against fungal pathogens

Unlike animals with immune system, plants have developed several mechanisms that enable them to survive biotic and abiotic stresses. The defense against biotic stress in plants are grouped into two, preformed/constitutive and induced defense mechanisms.

2.7.2 Pre-formed/Pre-existing defense mechanisms

The constitutive or preformed defense structures include bark, thick cell wall, and waxy epidermal cuticles. These structures form the first line of defense in every plant which a pathogen needs to

overcome before an infection can occur (Łaźniewska et al., 2010). The polysaccharides such as glycan and pectin as well as the lignin content of plant cell walls strengthen the cell which acts as a physical barrier to prevent infection from pathogens (Freeman and Beattie, 2008). Similarly, the plant cuticle is known to play a major protective role by acting as a physical barrier against pathogens. Furthermore, cuticular and epicuticular waxes which cover the aerial surface of plants act as interference by preventing direct contact between pathogens and the plant surface (upper epidermis) by acting as a physical barrier. Many studies have also described the effect of cuticular waxes on plant-pathogen interaction which includes the reduction of pathogen propagule germination (Hansjakob et al., 2011; Łaźniewska et al., 2012; Aragón et al., 2017). Although there are pre-formed physical barriers, pathogens do not always enter or overcome cuticles through wound sites or natural openings. Some pathogens including *B. cinerea* are well known to produce cell wall degrading enzymes such as lipase, cellulase, cutinase and compounds such as oxalic acid which enable them to overcome these physical barriers (Kars and van Kan, 2007). For instance, lipase has been demonstrated to possess cutinolytic activity through the degradation of unsaturated fatty acid esters, the type that is found in cutin and waxes (Nakajima and Akutsu, 2014). Cellulose and pectin degrading enzymes hydrolyze and cleave the soluble cellodextrin oligomers and the glycosidic bond between the sugar acid in cellulose and pectin respectively (Kubicek et al., 2014). A compound such as oxalic acid secreted by *B. cinerea* enhances the activity of polygalacturonases (PG) by lowering the pH of the host surface. In addition, low pH inhibits the activities of plant enzymes (Favaron et al., 2004; Nakajima and Akutsu, 2014).

In addition to the pre-formed structural defense, plants possess pre-formed chemical defenses. Plant surfaces and tissues contain several antimicrobial compounds that inhibit pathogen infections. For instance, phenols and phenolic glycosides, saponins, and cyanogenic glycosides,

alkaloids and terpenoids are well-known compounds that exist constitutively in plants in high concentrations and been reported to exhibit antifungal activities (Osbourn 1996; Doughari, 2015).

Generally, blueberry plants and fruits are well equipped with functional preformed defense against various biotic stress. Blueberry fruit and plant tissues are coated by a thick and waxy cuticle that present significant level of resistance to pathogen infection and physical damage. Plant cuticles are lipophilic structures covering the aerial surfaces of plants. The cuticle comprises of cutin, polyester polymer and a variety lipids and polysaccharides (Yeats and Rose, 2013; Domínguez et al., 2017). Cuticular wax is a hydrophobic coat on the cuticle which is generally comprised of a complex mixture of very-long-chain (VLC) aliphatic compounds such as fatty acids, alcohols, ketones, esters, and aromatic compounds such as triterpenoids and steroids (Chu et al., 2018; Klavins and Klavins, 2020). The strength of this preformed physical barrier in blueberry is dependent on changes in the composition and structure of the cell wall throughout the development of the plant and berry (Allan-Wojtas et al., 2001). The development of this thick waxy cuticle in blueberry plays an important role in host defense. For instance, blueberry leaves are very susceptible to *Monilinia vacciinii-corymbosum and B. cinerea* infection at the early stage of development when the cells are less thickened, lignified, and waxy compared to when the leaves are fully opened or matured.

Morphologically, leaves and stems of *V. myrtilloides* are covered with hair-like structures (pubescence). These structures have been shown to play an important role in host defense against herbivores and pathogens (Mmbaga et al, 1994; Riddick and Simmons, 2014). In many plants, including strawberries, trichrome and pubescence have been reported to offer resistance to pathogens and herbivores (Dia et al., 2010; Amil-Ruiz et al., 2011), hence these structures are considered important preformed defense mechanism.

With regards to preformed biochemical compounds (phytoanticipins), blueberries are well known to produce phenolic compounds including anthocyanins, and other organic compounds (Lacombe et al., 2012; Shen et al., 2014; Ma et al., 2018), which are known to possess antimicrobial activities.

2.8 Induced defense mechanism

The induced defense mechanisms are activated when a pathogen overcomes the first line of defense and is detected by the plant defense apparatus. Plant induced defense has been classified into two, namely basal and *R*-gene mediated defense (Gururani et al., 2012).

Basal (innate) defense can be a component of both host and non-host resistance (Gill et al., 2015) which provides defense against infection by a wide array of pathogens. Basal defense response occurs through pattern-recognition receptors (PRRs) via pathogen-associated molecular patterns (PAMPs) which include lipopolysaccharides, chitins, flagellins and glutens (Boller and Felix, 2009; Gururani et al., 2012; Wirthmueller et al., 2013). This brings about PAMP-triggered immunity (PTI) which prevents pathogenesis. Given the abundance/the presence of these PAMP in several non-pathogens such as *Bacillus* spp and *Trichoderma* spp, this type of defense can be triggered by non-pathogenic microbes. This explains why the induction of plant defense has been described as part of the modes of action of some biocontrol agents (Li et al. 2015; Rivera-Méndez et al., 2020).

In addition to the PAMP, specific pathogens have evolved effectors to help them evade PTI. In response to pathogen- effectors, plants have also developed receptors identified as resistance (*R*) proteins. This brings about effector-triggered immunity (ETI) which is pathogen-

specific (Gururani et al., 2012). ETI and PTI respond to different pathogen molecules and differ by the strength and scope of their immune responses.

2.8.1 Pattern and Damage–associated molecular pattern (PAMP/DAMP) immunity (PTI)

Recognition of pathogens by the plant cell is dependent on the generation of evolutionarily conserved elicitors PAMP/MAMP (Dixon et al., 1994; Newman et al., 2013). Similarly, endogenous compounds such as peptides or cell wall glycans released upon pathogen attack can act as elicitors known as damage-associated molecular patterns (DAMP) (Abdul Malik et al., 2020). Pattern recognition receptors (PRRs) bind to these PAMP/MAMP/DAMP which serve as an early warning signal to activate PAMP/MAMP/DAMP triggered immunity (PTI/MTI/DTI) (Kushalappa et al., 2016; Bacete et al., 2018). To date, all the PRRs that have been identified in plants are transmembrane proteins which are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs). Both RLKs and RLPs are structurally similar with extracellular binding domain, however, RLPs lack a kinase domain or other identifiable intracellular signal-transducing domains (Couto and Zipfel, 2016; Pandey et al., 2016). The different types of PRRs are classified as Lysine motifs (LysM) or Leucine-Rich Repeat (LRR) according to their domain/motifs (Tang et al., 2017). Generally, LysM-containing PRRs bind to carbohydrates (*N-acetylglucosamine* (GlcNAc)-containing glycans) and peptidoglycan while LRRs interact with proteins or peptides (Zipfel, 2014; Tang et al., 2017). LysM-containing PRRs bind chitin which is the common component of necrotrophic pathogens such as *Botrytis cinerea* (Zipfel, 2014). Chitin elicitor receptor Kinase 1 (CERK1), and LysM-containing RECEPTOR KINASE 5 (LYK5) have been reported to bind to chitin in the pathogen recognition process (Liu et al., 2012). Furthermore, *Botrytis-induced kinase 1* (*BIK1*) which encodes putative RLKs has been identified as a converging point for several PRR

pathways against necrotrophic pathogen including *Alternaria brassicicola* (Veronese et al., 2006; Couto and Zipfel, 2016).

Necrotrophic pathogens are known for their ability to release cell wall degrading enzymes which include cellulases, polygalacturonases, xylanases, and proteinases. Cell wall fragments generated from the activities of these enzymes can act as DAMPs. *Botrytis cinerea* is well known to produced pectinases such as polygalactorunase (PG) to aid cell wall decomposition. In Arabidopsis, PG has been reported to be directly recognized by LRR-RLP receptor known as *Responsiveness to Botrytis polygalacturonases1* (RBPG1) (Hückelhoven et al.,2007; Zhang et al., 2014). Furthermore, LRR PG-inhibiting proteins (PGIPs) associated with the plant cell wall have been observed to interact with PGs to control their activity (Wirthmueller et al., 2013). In addition to the direct recognition of PG, the enzymatic activity of PG generates oligogalacturonides (OG) which act as DAMP. For instance, Zhang et al., (2014), reported that the presence of OG is perceived by cell wall-associated receptor, Wall-Associated Kinase1 (WAK 1) during the plant defense process. Similarly, LRR-RLKs called *PEP RECEPTOR 1 and 2* (PEPR1 and 2) in Arabidopsis have been identified to perceive plant elicitor peptides (Peps), a proteinaceous DAMP (Liu et al., 2013; Yamada et al., 2016).

PTI provides a general defense response against a broad range of pathogens. Following PRR-ELICITOR complex formation, mitogen-activated protein kinases (MAPKs) phosphorylation is believed to be initiated by the receptor (Couto and Zipfel, 2016; Pandey et al., 2016). These phosphorylation activities trigger a cascade of events that activate downstream immune signaling which include oxidative burst, ion fluxes, and transcriptional reprogramming (Bigeard et al., 2015). Also, defense-related hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) which are essential immune signaling molecules are produced in PTI (Mine et al., 2014).

In blueberries, characterization of PRRs that recognize PAMPS/MAMPS/DAMPS and their signal transduction pathways are yet to be fully elucidated. Through indirect experiments, some group receptors common to most microbes have been inferred to operate in blueberries. For instance, the treatment of blueberry fruit with chitosan significantly increased both the total phenolic and anthocyanin contents (Jiang et al., 2016). With total phenolic and anthocyanin contents observed to be accumulated as part of plant defense mechanisms, it can be inferred that blueberry receptors which can seemingly recognize chitin and its derivatives were able to activate PTI in blueberry fruit which improved berry qualities in storage. Also, PGIPs known to act as a receptor to pathogen endo-PG, a defense elicitor has been established in blueberry (Khraiwesh et al., 2013).

2.8.2 Effector-triggered immunity (ETI)

The basal plant defense (PTI) is general and both host and non-host plants can trigger it. In this light, pathogens have evolved and are well adapted to these host, hence pathogens secrete molecules known as effectors which enable them to evade detection by PRRs (Kushalappa et al., 2016). In response to pathogen effectors, plants have evolved proteins known as *R* proteins which recognize these effectors, *avirulence* protein (*Avr*). The direct or indirect interaction of effectors and *R* proteins generate effector-triggered immunity (ETI). The ETI model is based on the *gene-for-gene* hypothesis proposed by Henry Flor (1971). In this defense, the host *R gene* encodes specific receptors that interact with specific effectors encoded by the *Avr gene* of the pathogen (Jia et al., 2000; Deslandes et al., 2003). Plant *R* proteins have been established to be nucleotide-binding, leucine-rich-repeat (NLRs). Immediately downstream of the initial *R* protein–*Avr* interaction, several responses and signaling events are activated in the plant cell (de León et al., 2013). These signals are transmitted into the nucleus where they stimulate expression of defense

response. These network of signaling events lead to localized disease response including the formation of necrotic lesions as a symptom, and production of reactive oxygen species (ROS) as part of hypersensitive response (HR), which is often accompanied by programmed cell death (Wirthmueller et al., 2013; Maqbool et al., 2015). Additionally, the transcription of enzymes essential to produce pathogenesis-related (*PR*) proteins, defense-related hormones such as SA, JA, ET and phenolic compounds may be activated (Oskar et al., 2016). These together result in the formation of a robust defense response to prevent the spread of pathogens.

Given the cell death that accompanies ETI, it has been generally accepted as a good defense mechanism that restricts the growth and colonization of host by biotrophic pathogens. However, HR/cell death contributes significantly to the host colonization by necrotrophic pathogens. Cell death triggered as part of HR was found to enhance the virulence of *B. cinerea* in tobacco (Adachi et al., 2016; Rossi et al., 2017). To enhance their virulence, *B. cinerea* has been reported to possess *BcNoxA* and *BcNoxB* genes which produce NADPH oxidases (ROS generating compound) which play a significant role in pathogenesis (Segmüller et al., 2008; Siegmund et al., 2013). Generally, PTI and ETI are distinguished based on the pathogen component that triggers the response, however, these two immunity systems significantly overlap with their interconnected defense signaling system.

Over the years there have been several studies into ELICITOR-PRR interactions involved in plant immunity using different plant pathogen-interactions. However, no such studies have been conducted in any *Vaccinium* spp. to the best of my knowledge. It is however important to point out the identification of 106 genes that encode nucleotide-binding proteins (including 97 NBS-LRR) in blueberry (Die et al., 2018). This discovery can be an important resource towards the study and identification of *Vaccinium* specific functional *R*-genes and PRRs for the improvement of blueberry molecular studies and breeding programs.

2.9 Localized and systemic induced defense response

2.9.1 Localized defense response

Induced defenses involve an increase in different resistance traits which could be local and/or systemic. The recognition of an effector by the *R*-gene or PAMP by PRR triggers a cascade of signaling which involves protein kinases, G proteins and ion fluxes (Muthamilarasan and Prasad, 2013; Couto and Zipfel, 2016). These signals are transmitted into the nucleus where they stimulate the expression of defense genes that encode transcription factors needed for the transcription of essential enzymes. These enzymes are essential to produce pathogenesis-related (*PR*) proteins or defense-related metabolites such as salicylic acid (SA) and phenolic compounds (Oskar et al., 2016). These signaling events result in the formation of a robust defense response which could be localized and/or systemic defense responses.

Localized response describes an early defense response that is limited to the invaded plant cell and neighboring cells, and in some cases, a response could be in the infected tissue or a single organ such as leave or flower (Hammersschmidt, 2014; David et al., 2019). Localized response is usually associated with the formation of a necrotic lesion as a symptom of the disease or as part of hypersensitive response (HR), and the production of *PR* genes and secondary metabolites (David et al., 2019). The hypersensitive response is part of plant innate immunity which is aimed at limiting the spread of a pathogen, especially biotrophs through programmed cell death (PCD). ROS generation is well understood to precede HR in plant defense. ROS is understood to induce intracellular signaling which includes the production of salicylic acid and activation of MAPK cascades (Mittler et al., 1999; Torres and Dangl, 2005; Hammersschmidt, 2014). Hypersensitive response is important in plant defense mechanisms because it has been associated with increased expression of defense-related genes (*PR* genes), antimicrobial secondary metabolites and PCD at

the site of infection (Torres, 2010; Zurbriggen et al., 2010). In addition to HR development during oxidative burst, ROS can act directly as toxicants to pathogens (Fones and Preston, 2012; Ali et al., 2018). Although HR aims to limit pathogen growth, many studies have reported the relationship between ROS accumulation and the susceptibility or the resistance of plants depending on the pathogen involved. Thus, ROS enhances the susceptibility of plants to necrotrophs and enhances the resistance of plants against biotrophs. For instance, ROS generated in tobacco leaf contributed significantly to *B. cinerea* infection (Rossi et al, 2017) due to the abundance of nutrients for the pathogen from PCD.

2.9.1.1 Pathogenesis Related (PR) Proteins/Genes

Pathogenesis related (PR) proteins are classes of proteins that are not expressed or are maybe constitutively expressed at basal/minimal levels in healthy plants, however, upon pathogen-host or microbe-host interaction or similar situations such as the application of compound (e. g, jamonate, or salicylic acid) that mimic pathogen attack, there is an accumulation and high levels of expression in the host (Van Loon and Van Strien, 1999; Sels et al., 2008; Thomas, 2012). Presently 17 (*PR 1, PR 2, PR 3, ..., PR 17*) families of *PR* proteins have been described (Sels et al., 2008; Van Loon et al., 2006). The role of PR protein in the defense against pathogen is usually identified or associated with their properties. For example. *PR* 3, 4, 8 and 11 are chitinase type of proteins whereas *PR7* is an endoproteinase and *PR6* is proteinase-inhibitor (Van Loon et al., 2006). *PR* genes, over the years, have been used as induced resistance markers in several plant species' interaction with pathogens including *Botrytis* spp.

2.9.1.2 Secondary metabolites

Plants produce many compounds that help them resist pathogen attack and survive stress. One of the most vital plant defenses against pathogens is the production of secondary metabolites (Khare et al., 2020; Zaynab et al., 2018). Secondary metabolites can be either present constitutively in plants known as phytoanticipans or pathogen induced compounds known as phytoalexins (VanEtten et al., 1994). Several phytoalexins exist, however one of the most important of the antimicrobial phytoalexins are phenylpropanoids also known as phenolic compounds (Croteau et al., 2000; Kumar et al., 2020).

2.9.1.2.1 Phenylpropanoids

The core phenylpropanoid biosynthesis starts with conversion of phenylalanine from the shikimate pathway to cinnamate and coumarin derivatives via the activities phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4- coenzyme A ligase (4CL). From the cinnamate and coumarin derivatives, individual pathways are branching leading to the production of compounds such as flavonoid, hydroxycinnamic acids and lignin (Biała and Jasiński, 2018; Dixon et al., 2002; Vogt, 2010). Depending on the modifications involved, thus methylation, reduction, alkylation and hydroxylation, phenylpropanoids can assume different structures and classes (Vogt, 2010; Yadav et al., 2020). The cascade of processes and the metabolons or enzyme complexes involved in the phenylpropanoid pathway are well established and understood (Macoy et al., 2015; Deng and Lu, 2017).

Hydroxycinnamic acid derivatives are simple phenylpropanoids derived from cinnamic acid. The formation of hydroxycinnamic acid derivatives occurs within the first three steps of the core phenylpropanoids pathway (Yadav et al., 2020). Many compounds such as chlorogenic acid,

ferulic acid, p-coumaric acid, caffeic acid and rosmarinic acid belong to this class of phenylpropanoid (Dixon et al., 2002; Vogt, 2010; Biała and Jasiński, 2018).

Flavonoids are one of the largest and complex group of plant phenolics, which accomplish different functions such as pigmentation, chemical messengers, physiological regulators, and defense in plant system (Kondo et al., 1992; Panche et al., 2016). Flavonoids are classified into several group such flavonols, flavanols, isoflavones, anthocyanidins, flavones and flavanones (Deng and Lu, 2017).

Coumarins belong to a general family of plant metabolites known as benzopyranones, with more than 1500 representative in over 800 species Their roles in plants appear to be primarily defense-related, given their antimicrobial, UV-screening, and germination inhibitor properties (Tiwari and Rana, 2015; Yadav et al., 2020). Some coumarin derivatives have been observed to have higher antifungal activity against a range of soil borne plant pathogenic fungi and exhibit more stability as compared to the original coumarin compounds alone (Brooker et al., 2008)

Lignin is a heterogeneous polymer found mainly in the secondary cell walls of plants. They consist of hundreds to thousands of phenolic monomers. They are generally insoluble, rigid, and virtually indigestible, hence, provides an outstanding physical barrier against pathogen attack (Freeman and Beattie, 2008; Yadav et al., 2020). Lignifications have been observed to restrict the growth of pathogens and are frequently produced in response to infection or wounding (Gould, 1983).

The disease and stress combating function of phenylpropanoids is not limited to any class of phenylpropanoid, however many phenylpropanoid posses' broad-spectrum antimicrobial activity help the plant suppress diseases (Koga et al., 1995; Dey, 2016; Zaynab et al., 2018). These

phenylpropanoids employ both multisite or specific mechanism of to counter the disease development either directly or indirectly. These mechanisms include disruption and deformation of microbial cell membrane and membrane proteins, and inhibition of enzyme activity (Zaynab et al., 2018; Simonetti et al., 2020; Yadav et al., 2020).

2.9.3 Systemic defense response

Following localized response, an induced resistance pathway known as systemic acquired resistance (SAR) is activated. SAR activation leads to the development of a long term, broad-spectrum, systemic resistance response towards subsequent pathogen attack (Hunt and Ryals, 1996; Klessig et al., 2018). It is worth noting that a different form of induced resistance known as induced systemic resistance (ISR) exists and this occurs when a plant responds to non-pathogenic microbes such as *Pseudomonas fluorescens* and *Trichoderma* spp. (Pieterse, et al., 1998; Alizadeh et al., 2013). Interestingly, ISR which is similar to SAR can be differentiated by their elicitors and the regulatory pathway. Additionally, the accumulation of *PR* proteins is absent in ISR unlike SAR (Van Loon, 1997; Romera et al., 2019).

Over the years, SAR has been extensively studied and reviewed (Ryals et al., 1996; Fu and Dong, 2013; Gruner et al., 2013). Similar to the localized response, SAR is associated with PR proteins and the production phytoalexins. In this light, SAR can be described as an extension of localized response to uninfected plant tissues through the activities of long-distance signaling molecules. Studies have shown that different molecules and proteins work together for the establishment of SAR. Salicylic acid (SA), an important plant hormone is widely known to be associated with different defense components including PTI, ETI and SAR through signaling (Gaffney, et al, 1993; Lawton, et al., 1995; Lu *et al*., 2016). SAR is associated with increased levels of SA that enhance the expression of *PR* genes through the activation of the transcriptional

co-activator non-expressor of PR-genes (NPR1) (Bektas and Eulgem, 2015). NPR1 acts downstream of SA and interacts with some transcription elements and the loss of this interaction results in loss of systemic acquired resistance (Deprés et al., 2000). Evidence of the role of SA in plant defense was demonstrated by the analysis of transgenic *Arabidopsis* plant expressing *nahG* gene which encodes salicylate hydroxylase, an enzyme that converts SA into catechol. Due to the plant's inability to accumulate SA, they lacked defense response to fungal, bacterial, and viral infection (Lawton, et al., 1995; Wildermuth et al., 2001). Given the remarkable association of SA with SAR, it was initially believed to be a mobile/long-distance signal for SAR. Nonetheless, in a grafting study with transgenic tobacco plant that produces cholera toxin, accumulate high levels of SA and exhibit disease resistance as rootstocks proved otherwise. The wild-type scion was not induced for SAR, when grafted onto the transgenic tobacco rootstock, suggesting that SA is not a mobile signal (Beffa et al., 1995). In a recent study with *Populus tomentosa* - *Botryosphaeria dothidea* pathosystem, Li et al (2018) reported that SA is converted methyl SA by carboxyl methyltransferase at the site of infection. This methyl SA is later converted back to SA in uninfected tissues by SA-binding protein 2, suggesting remote long-distance signaling by SA. In understand the biochemical basis of SAR, many chemical and biochemical elements were found to be involved in the long-distance signalling for the establishment of SAR. These include lipid transfer proteins, methyl salicylic acid, glycerol-3-phosphate (G3P), azelaic acid (AzA), abietane diterpenoid dehydroabietinal (DA) and pipecolic acid (Dempsey and Klessig, 2012; Shah and Zeier, 2013).

Although SAR is mostly associated with SA, other hormones have been found to equally facilitate the establishment of SAR. Depending on the type of pathogen involved, the hormones involved, and PR genes expressed will vary. For instance, SA and the expression of *PR* 1, 2 and 5 are associated with biotrophic and hemibiotrophic pathogens (Liu et al., 2016) whereas plants

activate the jasmonate /ethylene pathway genes and *PR* 3, 4 and 12 against necrotrophic pathogens (Chehab and Braam, 2012, Ali et al., 2017; Zhang et al., 2018). Over the years, JA/ET insensitive mutants have shown a significant reduction in the induction of defense genes against necrotrophic pathogens (Penninckx et al., 1998). For instance, in a study with mutant *Arabidopsis thaliana* which was deficient in JA production, it was observed that this mutant was not able to elicit defense response against *Botrytis cinerea* and *Alternaria brassicicola* (Chehab et al., 2008). Similar to JA, sensitivity to ET has been demonstrated to be a requirement for resistance against necrotrophic pathogens. Tobacco mutants *etr1* which is insensitive to ethylene showed increased susceptibility of different pathogens (Knoester et al. 1998; Geraats et al., 2003). Although many studies have investigated the role of JA and ET in plant defense signaling independently, several other studies have reported a synergistic interaction between JA/ET in plant defense response. This has been reported to be a result of the integration of ethylene response family (*ERF*) transcription factor with signals from JA and ET. The expression of *ERF* which activates defense-related genes, including plant defensin1.2 is known to be dependent on both hormones (Glazebrook, 2005; Broekgaarden et al., 2015; Zhang et al., 2018). Many studies have established that plant hormones SA and JA/ET, play major roles in defense responses and the establishment of systemic resistance against pathogens (de León et al., 2013; Zhang et al., 2018). Although the general signaling and roles of these hormones are well established, a lot more remains to be explored given the complexity and crosstalk that exist among the various signaling pathways.

2.10. Molecular response of *Vaccinium* spp. to plant pathogens and potential application to disease management

Integrated disease management combines different disease control strategies to achieve effective disease control. To add to IDM, molecular techniques have been adopted over the years, especially

in the development of disease-resistant plants which has contributed significantly to disease management in various crops including blueberries.

Over the years, different molecular and genetic engineering approaches such as transcriptomics, specific gene expression analysis and gene transfer has been conducted on different *Vaccinium* spp. for breeding and knowledge generation purposes (Lin et al., 2018; Benevenuto et al., 2019; Qi et al., 2019; Zhang et al., 2020). Although many molecular studies have been conducted on blueberries and other *Vaccinium* species, just a few have focused on their defense response to pathogens (Pehkonen et al., 2008; Koskimäki et al., 2009; Yow et al., 2016, 2018; Jose et al., 2021). It is widely accepted that genetic resistance is an important and effective approach to control plant diseases. Through the elucidation of the molecular mechanisms underlying plant resistance, pathologists, geneticists, and plant breeders can work together to achieve robust host resistance and induce disease resistance in plants as means of disease control. Owing to this, some studies aimed at elucidating the molecular response and identification of defense-related genes in *Vaccinium*-pathogen have been carried out. For instance, through transcriptomic analysis, several genes known to be involved in plant disease resistance were identified in blueberry leaves, flowers, and fruits (mummy berries) when infected with *Monilinia vaccinii-corymbosi* (Yow et al., 2016). In another study, Koskimäki et al., (2009), through gene expression demonstrated the activation and response of selected *PR* gene and flavonoid biosynthesis genes in micropropagated bilberry leaves to *B. cinerea and* fungal endophyte (*Paraphaeosphaeria* sp). Similarly, the defense response of *Vaccinium vitis-idaea* leaves to *Exobasidium species* was also studied and was reported that symptomatic and asymptomatic leaves diseased ramets showed activation of flavonoid biosynthesis at the gene level. Also, *PR 4* was observed to be activated in symptomatic leaves (Pehkonen et al., 2008). In a recent study, several defense-related genes were identified to be activated in a transcriptomic analysis of *Vaccinium*

myrtillus leaves following the application of a volatile derivative of JA, methyl jasmonate which is known to be activated during necrotrophic pathogen-plant interaction (Benevenuto et al., 2019). It is important to note that most of these molecular studies were carried out on cultivated *Vaccinium* species which lend themselves to breeding for desired characteristics and the development of new cultivar. Most molecular studies from wild *Vaccinium* spp. are mostly aimed at generating genetic information that will be useful and can be integrated into *Vaccinium* breeding programs (Ehlenfeldt et al., 2007; Hancock et al., 2008). Nonetheless, molecular studies from wild *Vaccinium* spp. can be useful in disease management in commercial wild species production. Knowledge of the molecular response of defense genes associated with pathogens can be integrated into IDM to induce natural defense through the application of external elicitors.

CHAPTER 3: MOLECULAR AND PHYSIOLOGICAL RESPONSES OF WILD BLUEBERRY TO RESPONSE TO *Botrytis cinerea* AND *Bacillus amyloliquefaciens* UNDER FIELD CONDITIONS

The following have been published as an abstract for a conference and manuscript from this Section.

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3.1 ABSTRACT

Botrytis blight is an important disease of wild blueberry (*Vaccinium angustifolium* and *V. myrtilloides*) with variable symptoms in the field due to differences in susceptibility among blueberry phenotypes. The relative expression of pathogenesis-related genes (*PR3, PR4* and *PR5*), flavonoid biosynthesis genes (*CHS, FLS, ANS, ANR, DFR*), composition of phenolic compounds and physiological changes between *B. cinerea* and *B. amyloliquefaciens* inoculated and uninoculated plants at different time points were analyzed. Gene expression in three wild blueberry phenotypes *Va*, *Va* f. nigrum and *Vm* was studied using qRT-PCR. The results indicated a response of the studied genes to these microbes at either 12, 24 or 48 hours post inoculation (hpi). The highest expression of *PR3* occurred at 24 hpi in all the phenotypes except *Va* f. nigrum. Maximum expression of the *PR* genes occurred at 12 hpi in *Va* f. nigrum. Most of the flavonoid genes were suppressed at 12 hpi. The expression of flavonoid pathway genes was phenotype-specific with their regulation patterns showing temporal differences among the phenotypes. Accumulation of phenolic compounds was temporally regulated at different post-inoculation time points with both microbes. The inoculation of plants with the *B. cinerea* and *B. amyloliquefaciens* did not have a significant effect on photosynthetic rate, transpiration rate and stomatal conductance in the plant. Generally, uninoculated plants had better fruit set and yield than the inoculated plants. Results from this study suggest that the defense response of wild blueberry to *B. cinerea* and *B. amyloliquefaciens* including expression of *PR* genes, flavonoid biosynthetic genes and the

accumulation of phenolic compounds could be phenotype specific. This study provides a starting point for understanding and determining the mechanisms governing the wild blueberry-*B. cinerea* pathosystem and the potential of priming the plant for disease mitigation.

3.2 INTRODUCTION

Botrytis blight can be a severe disease, however, the effect on fields varies significantly due to differences in susceptibility among the various phenotypes. Over the years, minimal damage from Botrytis and Monilinia blights in *Vm* has been reported (Abbey et al., 2018; Ehlenfeldt and Stretch, 2001). *Vm* has been identified as a potential source of blight resistance in breeding programs due to its tolerance as stated in the study by Ehlenfeldt and Stretch (2001). In a recent study, Abbey et al. (2018) indicated that *Va* was the most susceptible to *B. cinerea* followed by *Va* f. nigrum whereas *Vm* was found to be least susceptible.

Presently, Botrytis blight management is primarily dependent on chemical fungicide application. However, growing concerns about environmental safety, the development of fungicides resistance among the pathogen population, and rising production costs make it difficult to rely on this strategy indefinitely. Given this, alternative disease management that reduces the challenges posed by chemical fungicides is critical. Integrating plants' natural defense mechanisms into disease management programs could be a viable and long-term disease management strategy. Therefore, unraveling the molecular basis of wild blueberry response to pathogenic and non-pathogenic microbes would contribute towards understanding the disease resistance mechanism in wild blueberry.

Plants are known to accumulate proteins and biochemical compounds in response to biotic and abiotic stresses to delay or reduce the impact of these stresses on them (Freeman and Beattie, 2008; Abdel-Monaim, 2017). Generally, pathogenesis-related (PR) proteins are induced upon

infection and are associated with host defense machinery to limit pathogen progress (Sudisha et al. 2012). Many studies have been conducted on the host response of various plants to various pathogens including *Botrytis* spp. Cui et al. (2018) reported a high accumulation of transcripts of the genes encoding for various *PR* proteins, phenylpropanoids and lignin in leaves of *Lilium regale* infected with *Botrytis elliptica*. Depending on the type of pathogen involved, PR genes expressed will vary. For instance, the expression of *PR* 1, 2, and 5 are associated with biotrophic and hemibiotrophic pathogens (Liu et al., 2016) whereas *PR* 3, 4, and 12 against necrotrophic pathogens such as *B. cinerea* (Ali et al., 2017; Zhang et al., 2018).

Similar to some *PR* proteins, several genes involved in the phenylpropanoid pathway, as well as their related compounds such as lignin, flavonoids and hydroxycinnamic acid derivatives that possess antimicrobial capabilities and restrict pathogen development are accumulated during pathogen infection (Jasiński et al., 2009; Bi et al., 2011; Falcone Ferreyra et al., 2012; Ganthaler et al., 2017). For instance, an increase in the expression of the flavonoid pathway genes such as chalcone synthase (*CHS*) and anthocyanidin synthase (*ANS*), and elevation in phytoalexin content, such as catechin and quercetin, have been reported in *B. cinerea* and endophyte *Paraphaeosphaeria* sp. inoculated bilberry leaves (Koskimäki et al., 2009). Bi et al., (2011) demonstrated the increased expression of 17 lignin pathway genes, including cinnamoyl CoA reductase (*CCR*), when wheat was challenged with *Fusarium graminearum.* A recent study found that an interaction between grapevine flower and *B. cinerea* resulted in a rapid defense reaction involving the activation of genes associated with the accumulation of antimicrobial proteins, polyphenols, and cell wall reinforcement (Haile et al., 2017). Additionally, non-pathogenic, or beneficial microbes have been reported to equally activate and increase the expression of these defense responses in plants (Conrath et al., 2015; Wang et al., 2020).

Bacillus species are a group of microbes known for their disease/pathogen inhibition ability through the production of secondary metabolites, and competition (Fravel, 2005). Aside from their direct impact on pathogens, they interact with host plants by triggering systemic resistance to counter pathogens (Dimopoulou et al., 2019). *Bacillus amyloliquefaciens* (commercially marketed as Serifel®) is a microbe that has been found to stimulate induced systemic resistance in tomato (Beris et al., 2018; Dimopoulou et al., 2019). Additionally, *B. amyloliquefaciens* was shown to enhance the expression of defense marker genes in lettuce by Chowdhury et al. (2015). Over the years, the use of non-pathogenic, or beneficial microbes such as plant-growth promoting rhizobacteria (PGPR), to prime plants to mitigate disease development have been actively researched (Pieterse et al., 2014). These studies continue as a potentially practical way of priming plants for disease management in agriculture. As potential biofungicide product for disease management in wild blueberry, it is imperative to understand the molecular impact of *B. amyloliquefaciens* on the wild blueberry plant.

There are many studies on plant disease response from different host-pathogen interactions, however, there is no such study on the molecular and biochemical changes induced in wild blueberry during their interaction with *B. cinerea* or a non-pathogenic microbe. In this study, we investigated the wild blueberry defense responses against *B. cinerea* and the potential of *B. amyloliquefaciens* to trigger defense response through the expression levels of selected *PR* and phenylpropanoid biosynthesis pathway genes known to be involved in plant defense responses. We also investigated some biochemical changes that occur in wild blueberry during an interaction with these two microbes.

3.3 MATERIALS AND METHOD

3.3.1 Site selection and experimental design

Trials to study the molecular mechanisms resulting from floral blights was conducted during the 2019 and 2020 growing seasons at Benvie Hill and the Wild Blueberry Research Centre, Debert, NS, respectively. The experiments commenced during the spring of each year.

In 2019, six phenotypes which consisted of 3 *Va* (*Va* brown stem, *Va* green stem, *Va* f. nigrum) and 3 *Vm* (*Vm* short, *Vm* medium, and *Vm* tall stem) were selected from a commercial wild blueberry field, NS, Canada (Figure 3.1). *Vm* plant height was classified as short (< 15 cm), medium (15 – 25 cm), and tall (> 25 cm). In the fields, short stem *Vm* has been observed to be more tolerant to Botrytis blight and Monilinia blight, hence the inclusion of different heights of *Vm*. The response of these phenotypes to *B. cinerea* inoculation at the F7 stage of floral growth (corolla fully opened and most susceptible) was assessed. Three biological replicates (each patch size was 1 m x 2 m area) were selected for each phenotype and each replicate was separated into two, 0.5 × 1 m sample areas. One day before inoculation, one sample area within each replicate was sprayed with the fungicide, Switch® (cyprodinil and fludioxonil, 625g a. i./L) to serve as the control for generating a ΔCt calibrator for the ΔΔCt gene expression analysis (Livak and Schmittgenm, 2001). The fungicide application was to ensure that all the control plants were protected from external inoculum.

In 2020, three phenotypes (*Va* brown stem*, Va* f. nigrum and *Vm*) were used in the study with four replicates. In 2020, the experiment was conducted at Debert, NS on a field with limited *Vm* species, hence the *Vm* plants were combined. Each replicate was separated into three, 0.5 × 1 m sample areas. The molecular response of the phenotypes to *B. cinerea* and *Bacillus amyloliquefaciens* in comparison with non-inoculated flowers and the resulting physiological effects on plants were assessed as described below.

Figure 3.1. *V. angustifolium* green stem (A), *V. angustifolium* brown stem (B), *V. angustifolium* f. nigrum (C) and *V. myrtilloides* (D)

3.3.1.1 Inoculation and sample collection

Distilled water-based spore suspension (10^6 conidia mL^{-1}) was prepared from a two-week-old single spore B. cinerea culture isolated grown on potato dextrose agar (PDA). The *B. cinerea* was isolated from infected *Va* floral tissue and identified based on its morphological characteristics under the microscope (Dowling et al. 2017). The spore concentration was estimated using a hemocytometer (BLAUBRAND® Neubauer) and adjusted to 1×10^6 conidia mL^{-1} and Tween 20 (0.04%) was added to the suspension prior to inoculation. The 10^6 conidia mL^{-1} concentration was tested before the experiment to ensure that the concentration was sufficient to adequately cause infection. The spore suspension was applied to the plants in the remaining sample areas of each plot that did not receive the fungicide (non inoculated) within the replicate using a hand-held pump

sprayer to produce very fine evenly distributed droplets on each plant to the point of runoff. In addition to the *B. cinerea*, Serifel® a WP formulation containing *B. amyloliquefaciens* endospores was applied at a rate of 1.25 g/L of water to one of the sample areas in each replicate in 2020. The plants were immediately covered with a 2 mm plastic film and row cover (DeWitt Plant & Seed Guard, Halifax seed, NS) to provide favorable conditions (100% RH) for 48 hours (Figure 3.2). Prior to inoculation, floral tissues were harvested to represent 0 hours before inoculation or basal expression (0 hbi). Post inoculation, flower tissues were harvested at 12-, 24-, 48-, and 96-hours (hpi). For every sample collection, flowers from 20 plants within each replicate were harvested and pooled together for RNA extraction. The samples were immediately flash frozen in liquid nitrogen and later preserved in -80°C for gene expression and biochemical analyses.

Figure 3.2. Experimental setup on a commercial wild blueberry field. A) Inoculated patch in with a row cover with a 2 mm plastic film to create a humid condition for infection to occur, B) A patch of wild blueberry in their natural growing habit on a commercial field, and C) Infected wild blueberry flower at F7 flower stage (Corolla fully opened).

3.3.1.2 RNA Extraction and cDNA synthesis

Total RNA was isolated from the floral tissue using Qiagen RNeasy Plant kits following the manufacturer's instruction (QIAGEN, Valencia, CA, USA). Genomic DNA was removed by on-column DNase I digestion (Qiagen Inc., Valencia, CA, USA). The concentration and purity of RNA samples were assessed based on an absorbance ratio of 1.8 to 2.0 at 260/280 nm and ≥ 2.0 at 260/230 using the Biotek Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments Inc., Winooski, VT, USA). DNA-free total RNA (1 µg) was used for the cDNA synthesis using MultiScribe™ Reverse Transcriptase from the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) in a 20 µL reaction following the manufacturer's instruction. The MultiScribe™ reaction mix includes random primers to make cDNAs. The final cDNA products were diluted 20-fold before use in real-time PCR.

3.3.1.3 Quantitative real-time PCR (qRT-PCR) analysis

Quantitative RT-PCR (qPCR) analysis of cDNA was carried out in a 96-well rotor in BIO-RAD CFX Connect Real-Time System using BioRAD SsoAdvanced Universal SYBR Green Supermix (BioRad Laboratories Inc., CA, USA) in a 10 uL reaction. Each 10 µL reaction comprised 5 µL SYBR Green supermix, 1 µL H_2O, 2 µL cDNA, and 1 µL forward and reverse primers (10 nM) for each gene of interest. The qPCR parameters used were as follows: 95 °C for 3 min, 35 cycles each at 95 °C for 10 s, and 60 °C for 20 s. Each qPCR reaction was carried out in three technical replicates and a no-template controls (NTC) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene (Jose et al., 2020). Sequences for pathogenesis-related genes (*PR3, PR4 and PR5*), flavonoid biosynthesis genes, (*CHS, FLS, ANS, ANR, DFR*) lignin biosynthesis gene (*CCR*) and lipoxygenase gene (*LOX*)were retrieved from *V. corymbosum* database (www.vaccinium.org) and the National Center for Biotechnology Information (NCBI;

www.ncbi.nlm.nih.gov) to design primers for this study. Specific primers were designed with Primer Premier 5.0 (Premier Biosoft International, California, USA) and analyzed with different bioinformatics tools (BioEdit/ Clustal w/BLAST/ Primer Premier 5.0) (Appendix 3, Table A1). Relative quantification of genes was obtained using the ΔΔCt method. In brief, the Ct values of target genes were normalized to the reference gene (GAPDH) ($\Delta CT = Ct_{target} - Ct_{GAPDH}$) and compared with a calibrator ($\Delta CT = Ct_{sample} - Ct_{control}$). Relative expression (RQ) of the genes was calculated by the formula $2^{-\Delta\Delta CT}$ method using Ct value (Livak and Schmittgenm, 2001).

3.3.2 HPLC-DAD analysis of flavonoids and hydroxycinnamic acids

3.3.2.1 Chemicals and standards preparation

External standards of caffeic acid, neochlorogenic acid, catechin, procyanidin B2, quercetin-3-galactoside, m-coumaric acid, p-coumaric acid, and quercitrin (quercetin 3-rhamnoside) were purchased from Sigma- Aldrich, Inc. (St. Louis, MO, USA). Chlorogenic acid was purchased from MP medicals, France, and kaempferol-3-glucoside was obtained from the HWI group (Rheinzaberner, Germany). Analytical grade methanol, sodium fluoride (NaF), and formic acid (> 95%) were purchased from Merck® (Bengaluru, India). HPLC-grade water was obtained from a Milli-Q System with a resistivity of 18.2 mΩ (Millipore, Billerica, MA, USA).

3.3.2.2 Preparation of Standard Solutions

Calibration standards were prepared by an appropriate dilution of stock solutions with 50% methanol. Nine different concentrations of each compound within 0.01 - 200 µg/mL for all the compounds were prepared to generate calibration curves. Standard curves were generated using linear regression (R^2 of each standard curve was > 0.99).

3.3.2.3 Extraction and analysis of phenolic compounds.

Phenolic compounds were extracted and subsequently analyzed by reverse-phase high performance liquid chromatography – diode array detection (HPLC-DAD) as described by Tomás-Barberán et al. (2001) and Villarino et al. (2011) with modifications. Frozen samples collected at 48- and 96-hours post-inoculation were ground to a fine powder in liquid nitrogen for extraction. Ground material (0.2 g) was extracted with 5.0 mL extraction solution (2% Formic acid 80% methanol containing 2 mM NaF to inactivate polyphenol oxidases and prevent phenolic degradation) for 60 minutes at 8 ºC in the dark. The extract was centrifuged at 4,300 rpm for 15 min at 4 °C and the supernatant was transferred into a clean tube. The extraction was repeated a second time on the residue from the first extraction after which the two supernatants were combined and 1 mL aliquot was filtered through a 0.45 µm nylon filter for analysis.

Phenolic compound compositions were determined from the filtrate using Waters® e2695 HPLC with auto injector equipped with a 2998 photodiode array detector (Waters Corp., Milford, U.S.A.) equipped with a degasser. A Phenomenex Kinetex™ C_{18} column [250 X 4.6 mm (inner diameter); particle size, 5 µm] was used to separate the phenolic compounds at a temperature of 25 ºC. The mobile phases were water (A), and methanol (B) both of which contained 0.5% formic acid to increase peak resolution. The gradient used for eluent A was 100% (0–5 min), 85% (5–20 min), 50% (20-25 min), 30% (25-30 min), 0% (30-40 min), and 100% (40-60 min).

3.3.2.4 Determination and quantification of compounds

The determination was conducted at a flow rate of 1.0 mL/min. Phenolic compounds were identified and quantified by comparing their retention times with those of their respective external standards at wavelengths of 280, 302 and 355 nm (Appendix 3, Table A2).

3.3.2.5 Method validation

The method was validated in accordance with International Conference on Harmonization (ICH) which determined the recovery, linearity, repeatability (precision), LOD, and LOQ (ICH, 1997, 2005; Khattab et al., 2016; Kuppusamy et al., 2018).

The accuracy of method was determined through recovery (%) by preparing spiking 0.2 g quality control sample to 100 µg/mL (chlorogenic acid) 25 µg/mL (kaempferol-3-glucoside) and 50 ug/mL (other 8 standards), then extracted as described previously (Section 3.3.2.3). The percentage of recovery of each standard was calculated based on the ratio of the standard concentration after and before HPLC (changed amount minus original amount). Thus, the recovery was determined using the formula: % Recovery= [Pesticide recovered from fortified sample/ Amount of pesticide] added x 100%

Variations were used to evaluate the precision of the developed method. Variations were expressed as the relative standard deviation (RSD) of the replicates. To determine repeatability (intra-day precision), three (3) independently prepared solutions of lowbush blueberry were analyzed six times in the same day. % RSD was determined for replicate injections on each day (intra-day precision) and for mean values per day (inter-day precision) by considering the respective peak areas.

The detector linearity and detection limits for the reference standards were studied over the wide calibration curve range (0.01-200 µg/ml). Limits of detection (LOD) and limits of quantification (LOQ) were calculated for each compound in triplicates. The mean of the slope (S) and standard deviation of intercept (σ) were calculated from the standard curve of three replicates. LOD and LOQ were calculated with the following equations: LOD = 3.3 x (σ/S), and LOQ = 10 x (σ/S).

All the method validation parameters studies were within the acceptable limits and had appropriate accuracy, precision, and sensitivity for quantification of phenolic compound analysis according to ICH procedures.

3.4 Physiological response of wild blueberry plants

An LCpro-SD portable leaf gas exchange system (ADC Bioscientific, Hoddesdon, UK) equipped with a conifer leaf chamber, a dedicated LED light unit and the ability to control temperature, light intensity and carbon dioxide levels was used. A total of eight stems for each treatment and a dwell time of 3 minutes per measurement was used. Measurements was taken from the upper 10 cm of the shoot between 10:00 and 15:00 hrs. Stem samples containing the leaf area involved in the leaf gas exchange measurements were collected and transported to the lab in a cooler with ice packs for determination of leaf area using a LI-3000 leaf area meter (LI-COR Inc., Lincoln, NE).

3.5 Yield component and berry yield assessment

Ten blueberry stems were collected diagonally along a line transect within each sub plot to examine yield potential (fruit set and small unmarketable berries). Berry yield was assessed by harvesting blueberries with a forty-tine hand rake from two randomly selected 30 × 30 cm quadrats from each sub plot.

3.6 STATISTICAL ANALYSIS

Gene expression, phenolic compound physiological and yield data were analyzed using a two-way ANOVA for the 2019 trial with phenotype and time as fixed factors and replicate as the random factor. In 2020, a three-way ANOVA was used with treatment (*B. cinerea* and *B. amyloquifaciens*), phenotype and time as fixed factors whereas replicate was considered as the random factor. The

PROC GLIMMIX procedure of SAS (version 9.4, SAS Institute, Inc., Cary, NC) was used for the analysis. The least significant difference (LSD) test was used for multiple means separation at α=0.05.

3.5 RESULTS

3.5.1 Pathogenesis-related genes

In 2019, the expression levels of these PR genes varied among the *Va* phenotypes. The expression of *PR* genes was early and maximum in *Va* f. nigrum but delayed in reaching maximum levels in green and brown stem *Va* (Figures 3.3a & 3.3b). The expression of *PR3* and *PR4* in both brown and green stems of *Va* was observed at 12 hpi, however, significant maximum *PR3* expression was observed at 24 hpi in green stem (Figures 3.3a & 3.3b). Similarly, in the green stem of *Va,* significant expression of *PR3* was observed at 24 hpi, but *PR4* showed no significant increment to 48 hpi (Figure 3.3b). In *Va* f. nigrum, both *PR3* and *PR4* were highly expressed, however, *PR4* reached a significant maximum expression at 12 hpi (Figure 3.3b). In the *Vm* phenotypes, the maximum levels of expression varied between the short and medium stem phenotypes. In the short stem of *Vm,* a noticeable expression of *PR3* was observed at 24 hpi whereas, a significantly expression was observed 24 hpi. In the medium stem of *Vm, PR3* expression was maximum at 24 hpi whereas, *PR4* was significantly expressed at both 12 hpi and 48 hpi. There was no upregulation of these *PR* genes in the tall stem *Vm* (Figures 3.3a & 3.3b).

At the phenotype level, the expression pathogenesis related genes revealed a significantly high expression of *PR3* ($p = 0.0119$) and *PR4* ($p < 0.0001$) in *Va* f. nigrum while tall stem *Vm* had the least expression (Appendix 5, Tables A1 & A2). Regarding temporal expression, the expression of both *PR* genes was significantly higher at 24 and 48 hpi (Figures 3.3a & 3.3b).

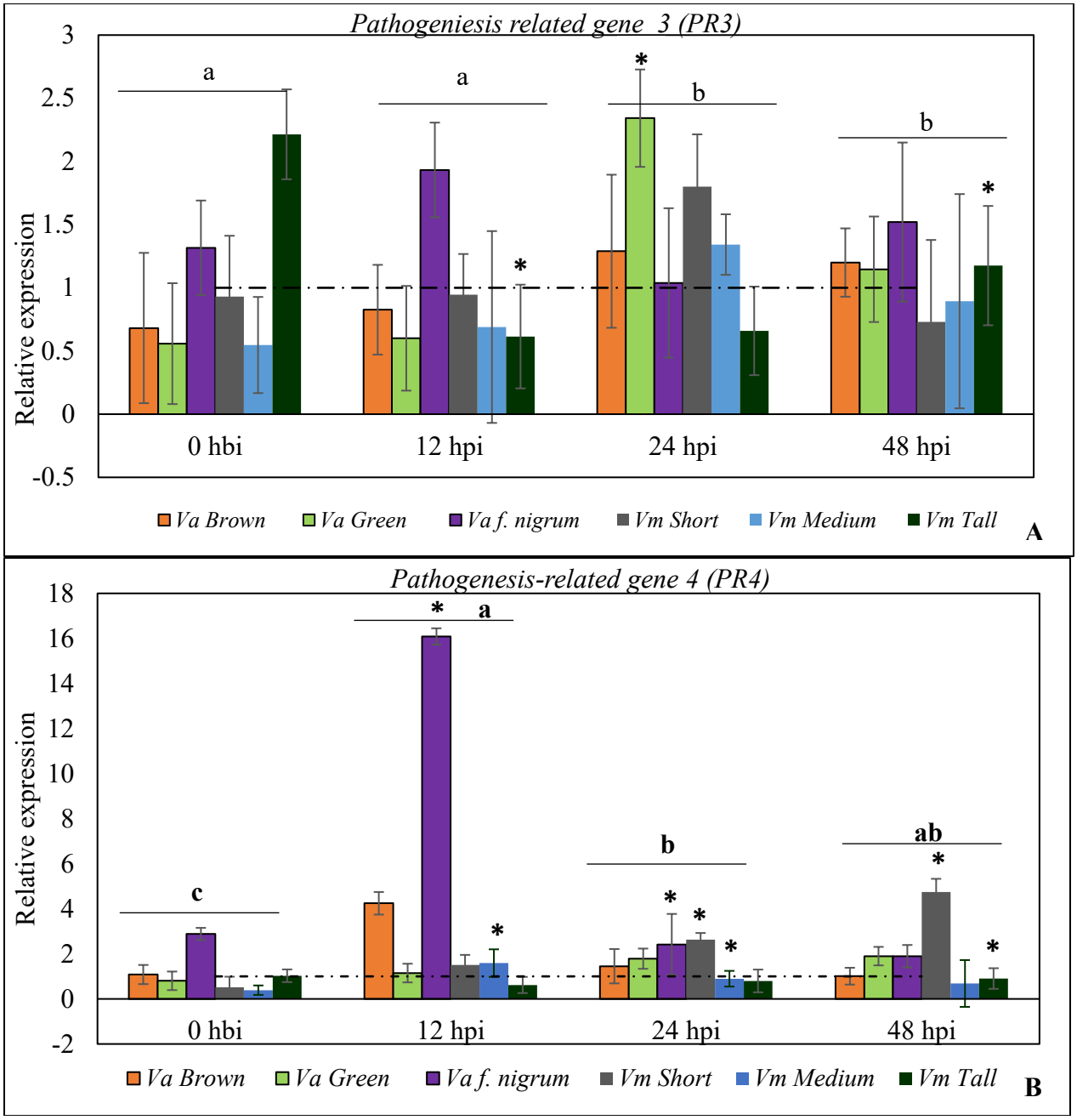

Figure 3.3. Expression pattern of pathogenesis-related genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* infection in 2019. (A) Relative expression of *PR3*. (B) Relative expression of *PR4*. Expression of each gene is shown as a fold change in infected samples relative to their respective uninfected control from the same time point. Results are reported as means ± standard error of three biological replicates. *Significant difference between infected plants and their controls/basal expression (0 hours before inoculation, hbi). Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at $\alpha = 0.05$. Broken horizontal line at 1-fold relative expression represents the calibrator.

In 2020 samples, the expression levels of *PR3* were increased in *B. cinerea* inoculated *Va* and *Va* f. nigrum at 12 hpi. There was a steady increase of *PR3* expression in *Vm* which reached a maximum at 48 hpi. The expression of *PR3* gene inoculated with *B. amyloliquefaciens* did not reveal any early response in *Vm* (Figure 3.4a). The expression of *PR4* increased at both 12 and 24 hpi in *B. cinerea* inoculated *Va* phenotypes (Figure 3.4b). In *Va*, maximum expression was observed at 24 hpi compared to 12 hpi for *PR3*. In contrast, the highest expression of *PR4* was at 12 hpi in *Va* f. nigrum (Figures 3.4a & 3.4b). In *Va*, maximum expression at 24 hpi was observed with *B. amyloliquefaciens*. The expression of *PR4* decreased at 12 hpi in *Va f. nigrum.* However, expression at 24 hpi similar to the basal expression was observed. The expression of *PR4* gene in *B. amyloliquefaciens* inoculated *Vm* was similar to that observed in *PR3* (Figures 3.4a & 3.4b).

The expression of *PR5* were increased in *B. cinerea* inoculated *Va* phenotypes. There was a steady increase of *PR5* in *Va* which was highest at 48 hpi. In *Va* f. nigrum, *PR5* was significantly expressed at 12 hpi and 24 hpi. The expression of *PR5* in *Vm* was similar to that observed in *Va* f. nigrum with significant expression at 24 hpi and 48 hpi. *B. amyloliquefaciens* inoculated showed a significant *PR5* expression in *Va* f. nigrum at 24 hpi and in *Va* at 48 hpi. The expression levels observed in *Vm* after *B. amyloliquefaciens* inoculation was below the basal expression (Figure 3.5a).

At the phenotype level, the expression PR genes revealed a significantly higher expression of *PR3* ($p = 0.0001$) (Appendix 6, Table A1) in *Va* compared to control while *PR5* ($p = 0.0001$) had higher expression in *Va* f. nigrum and *Vm* had the least expression with both genes (Appendix 6, Table A2). Regarding the treatments, *PR4* and *PR5* were more significantly expressed in *B. cinerea* inoculated plants than *B. amyloliquefaciens.*

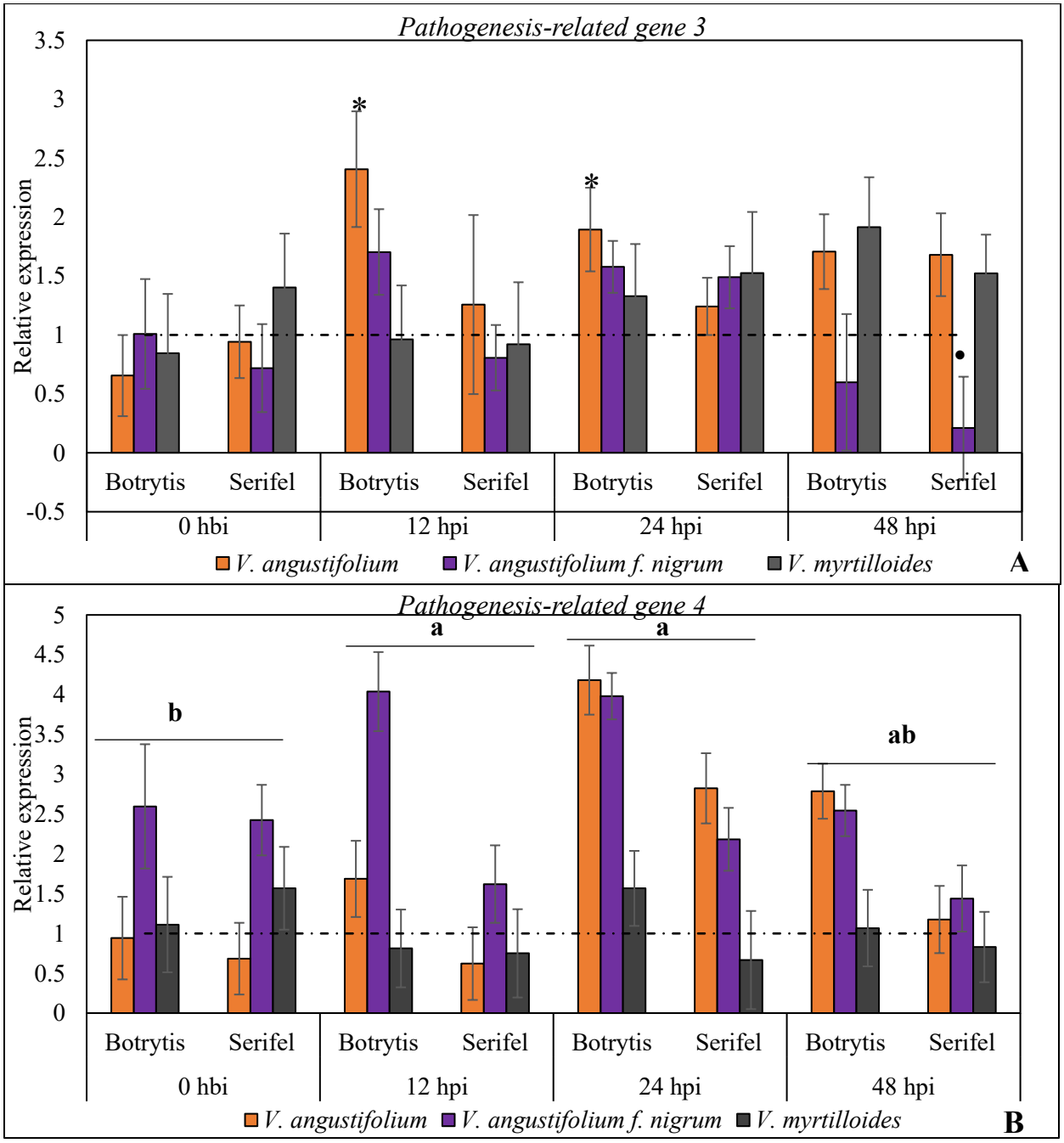

Figure 3.4. Expression pattern of pathogenesis-related genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* and Serifel® (*B. amyloliquefaciens*) inoculation. (A) Relative expression of *PR3* and (B) Relative expression of *PR4*. Results are reported as means ± standard error of three biological replicates. *Significant difference between *B. cinerea* infected plants and their controls/basal expression (0 hours before inoculation, hbi). • Significant difference between *B. amyloliquefaciens* inoculated plants and their controls/basal expression. Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at $\alpha = 0.05$. Broken horizontal line at 1-fold relative expression represents the calibrator.

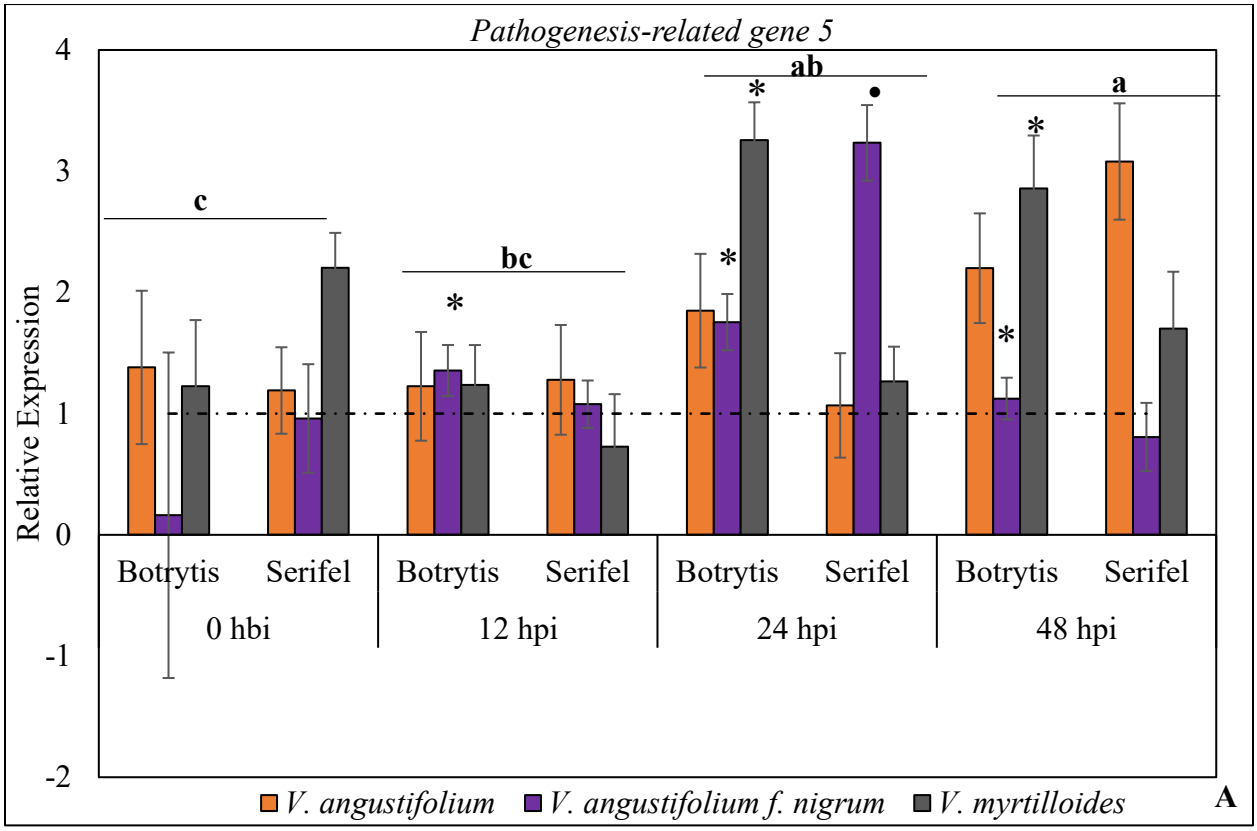

Figure 3.5 Expression pattern of pathogenesis-related genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* and Serifel® (*B. amyloliquefaciens*) inoculation. (A) Relative expression of *PR5*. Expression of each gene is shown as a fold change in infected samples relative to their respective uninfected control from the same time point. Results are reported as means ± standard error of three biological replicates. *Significant difference between *B. cinerea* infected plants and their controls/basal expression (0 hours before inoculation, hbi). • Significant difference between *B. amyloliquefaciens* inoculated plants and their controls/basal expression (0 hours before inoculation, hbi). Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at $\alpha = 0.05$. Broken horizontal line at 1-fold relative expression represents the calibrator.

3.5.2 Phenylpropanoid pathway genes

The expression of the flavonoid pathway genes *CHS*, *FLS* and *ANS* decreased in the early stages (12 hpi) of infection in all three *Va* phenotypes followed by a rise in expression. Although there was an increase in expression levels of *CHS* at 24 hpi, it was not significantly different from the basal expression (0 hbi) in the brown and green stem of *Va* (Figure 3.6a). The expression of *FLS* was significantly higher in the brown stem at 24 hpi whereas it was not significantly different from

the basal expression in *Va* f. nigrum. The expression of *FLS* in the green stem of *Va* was similar to the basal expression at 48 hpi (Figure 3.6b). An increased expression of *ANR* in *Va* f. nigrum up to 48 hpi was observed (Figure 3.7a). *ANS* expression was maximum at 24 hpi in the green stem of *Va* and *Va* f. nigrum (Figure 3.7b). *DFR* expression was slightly increased early (12 hpi) in brown stem *Va* and *Va* f. nigrum with the maximum expressions at 24 hpi (Figure 3.8a).

In the three *Vm* phenotypes, there was a decrease in *CHS* expression at 12 hpi (Figure 3.6a). A steady decrease in the expression of *FLS* in short and medium stem *Vm* at all time points was observed. Similar to *CHS*, a decrease in *FLS* expression in tall stem *Vm* at 12 hpi followed a steady rise in expression up to 48 hpi was observed (Figure 3.6b). *ANR* exhibited an increased expression in all three *Vm* phenotypes. There was an early response (12 hpi) of *ANR* in short and medium stem *Vm*. However, the *ANR* expression in the medium stem of *Vm* peaked at 12 hpi while the expression in the short stem of *Vm* peaked at 48 hpi. A steady increase in expression of *ANR* which peaked at 48 hpi was observed in the tall stem of *Vm* (Figure 3.7a). The expression of *ANS* and *DFR* were decreased at 12 hpi in short stem *Vm*, nonetheless, there was an increase in the expression of both genes at 24 and 48 hpi (Figures 3.7b & 3.8a). On the contrary, there was an increase in *ANS* and *DFR* expression in the medium stem of *Vm* at 12 hpi. The expression of *ANS* in tall stem *Vm* was similar to the *ANS* expression pattern in medium stem *Vm*. However, the expression at 12 hpi was not significantly different from the basal expression (Figures 3.7b & 3.8a). Interestingly, there was a decrease of *DFR* in tall stem *Vm* at 12 hpi, followed by a steady increase up to 48 hpi (Figure 3.8a).

At the phenotype level, no significant difference was observed with *ANR*, *ANS* and *DFR*. However, *Va* f. nigrum had a significantly high expression of *CHS* ($p = 0.0041$) while the brown stem *Va* had a significantly higher expression of *FLS* ($p = 0.0031$) (Appendix 5, Tables A3 & A4). Regarding temporal expression of flavonoid genes, *CHS* ($p = 0.0001$) and *ANS* ($p = 0.028$) were

significantly higher at 24 hpi whiles *ANR* (p = 0.049) and *DFR* (p = 0.0110) were significantly expressed at 48 hpi.

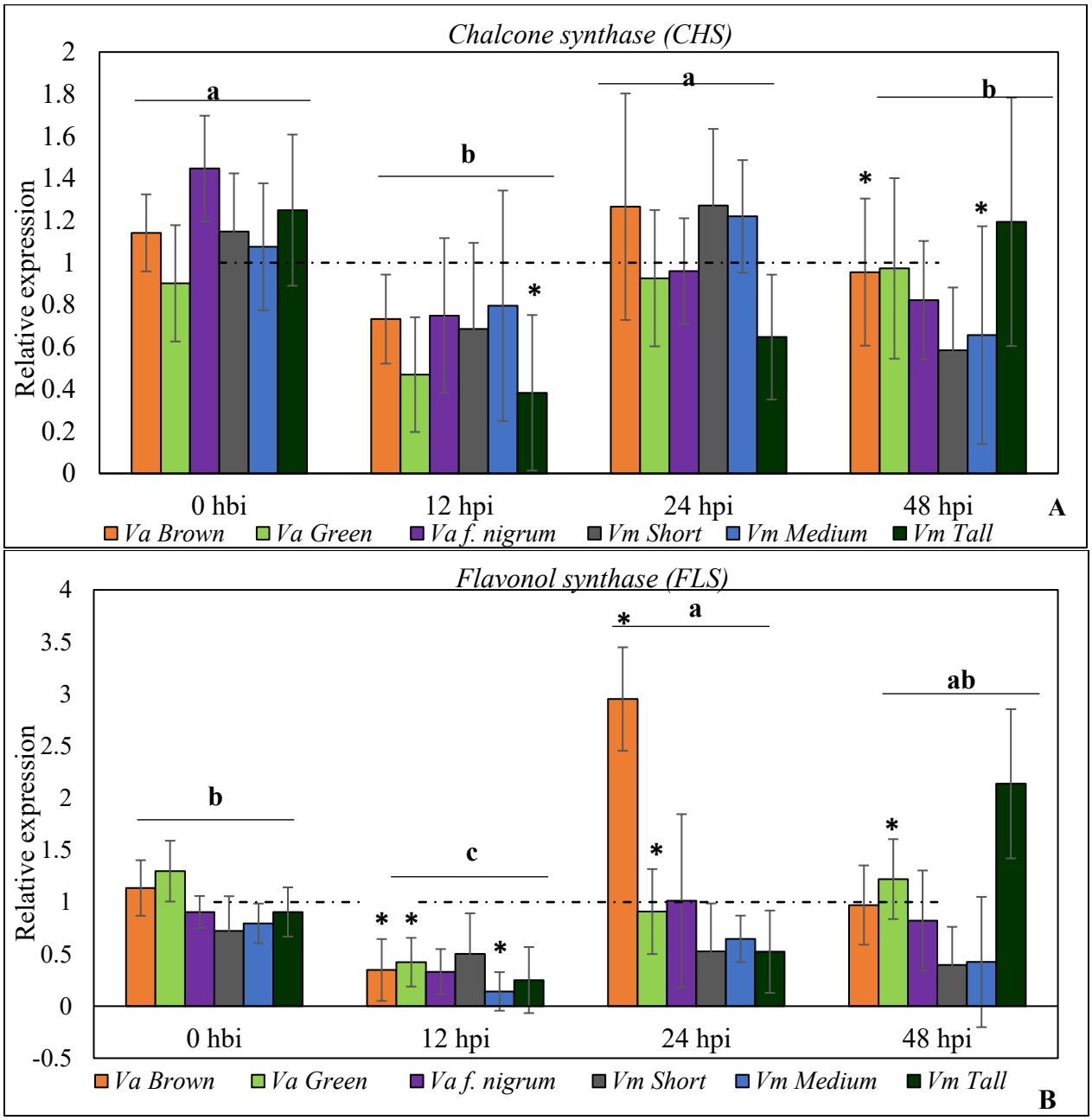

Figure 3.6. Expression pattern of flavonoid biosynthesis genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* infection. (A) Chalcone synthase (*CHS*) and (B) Flavonol synthase *(FLS)*. Results are reported as means ± standard error of three biological replicates. *Significant difference between infected plants and their controls/basal expression (0 hours before inoculation, hbi). Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at α = 0.05. Broken horizontal line at 1-fold relative expression represents the calibrator.

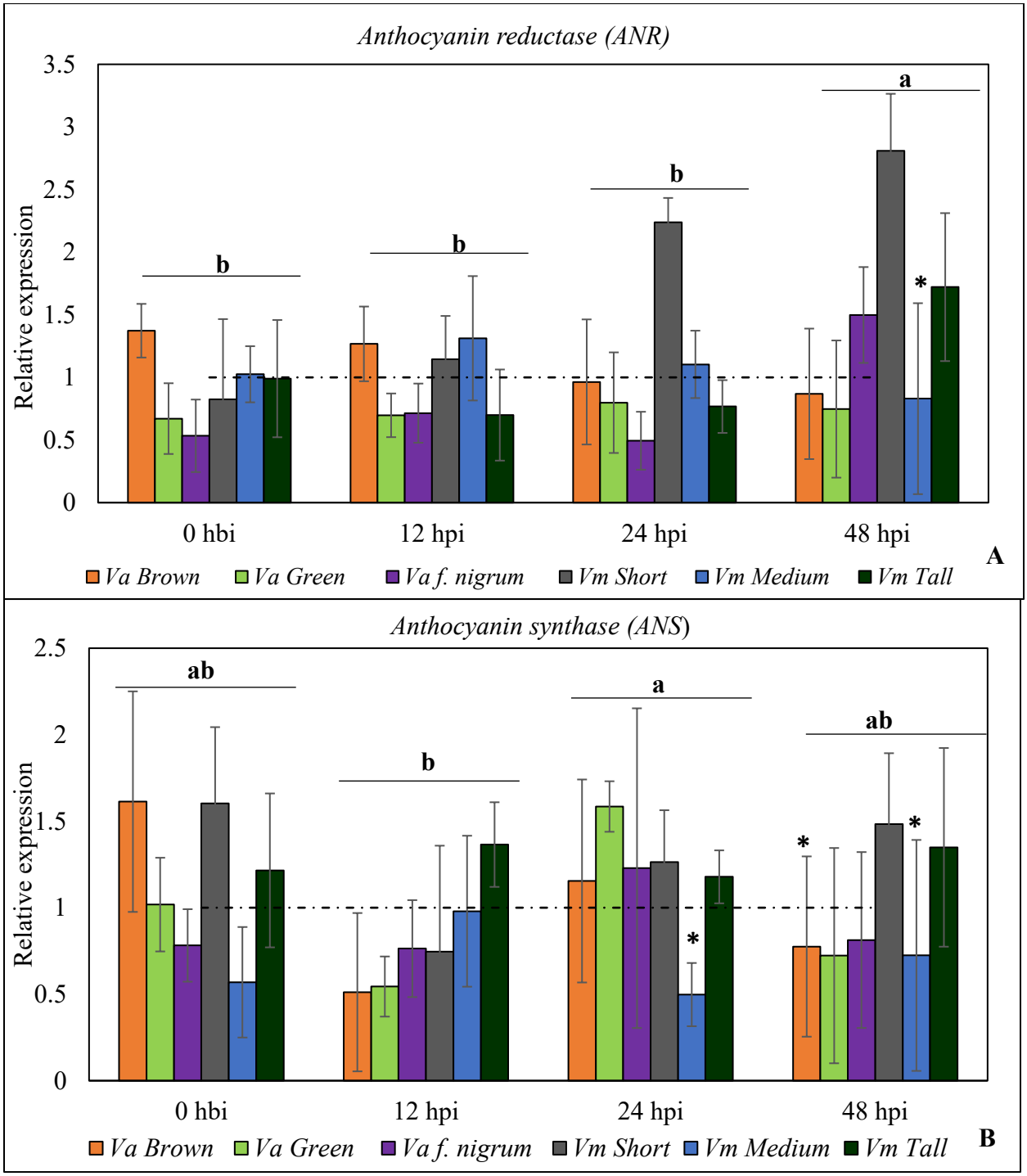

Figure 3.7. Expression pattern of flavonoid biosynthesis genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* infection. (A) Anthocyanin reductase (*ANR*) and (B) Anthocyanin synthase (*ANS*). Results are reported as means ± standard error of three biological replicates. *Significant difference between infected plants and their controls/basal expression (0 hours before inoculation, hbi). Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at $\alpha = 0.05$. Broken horizontal line at 1-fold relative expression represents the calibrator.

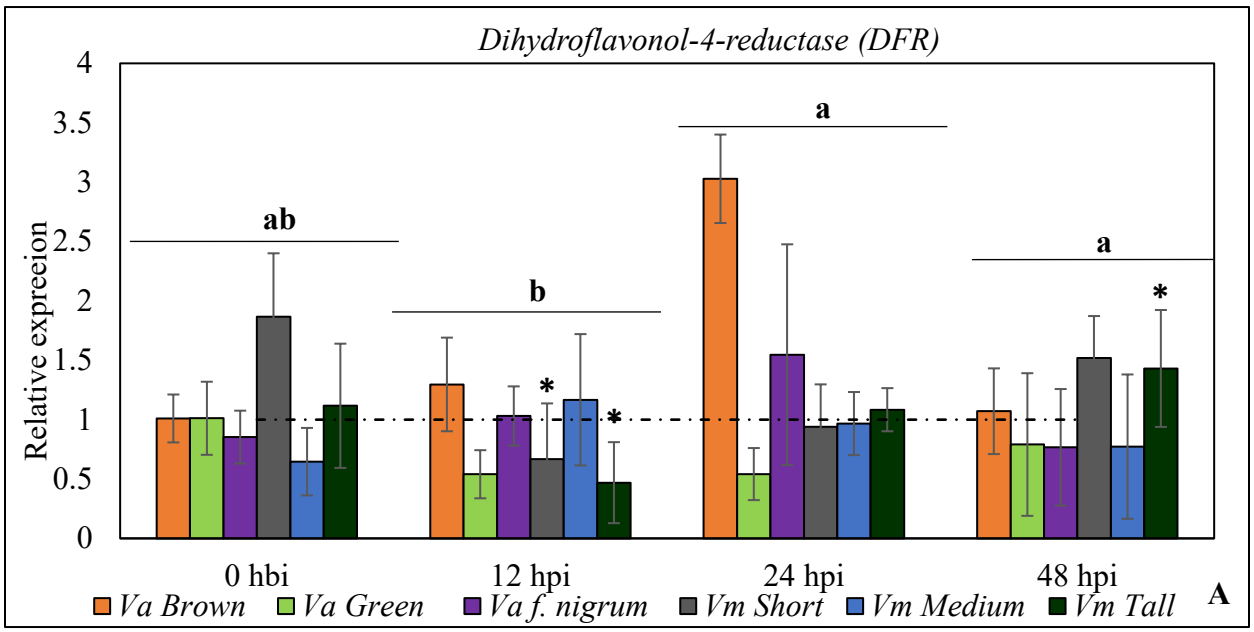

Figure 3.8. Expression pattern of flavonoid biosynthesis genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* infection. Dihydroflavonol-4-reductase (DFR). Expression of each gene is shown as a fold change in infected samples relative to their respective uninfected control from the same time point. Results are reported as means ± standard error of three biological replicates. *Significant difference between infected plants and their controls/basal expression (0 hours before inoculation, hbi). Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at $\alpha = 0.05$. Broken horizontal line at 1-fold relative expression represents the calibrator.

In 2020, the expression pattern of the flavonoid pathway genes in *B. cinerea* inoculated *Va* was similar to that observed in 2019; where there was a decreased in most of the studied genes at 12 hpi (Figures 3.6 - 3.11). This decrease was followed by mostly an increase in expression at 24 or 48 hpi. Although there was an increase after the initial decrease, most of these increments was comparable to the basal expression. For instance, the basal expression in of *CCR* at 0 hpi was 1.23-fold whereas the highest expression at 48 hpi was 1.69-fold (Figure 3.11b). *ANR* and *DFR* did not show any remarkable changes in their expression (Figures 3.10a & 3.11a). On the contrary, *CHS* and *CCR* had their highest expression levels at 24 and 12 hpi, respectively (Figures 3.9a & 3.11b). In *Va* f. nigrum, the initial decrease in *ANR*, *ANS* and CCR at 12 hpi was followed by an increase

in their expression which were comparable to the basal expression (Figures 3.10a, 3.10b & 3.11b). Interestingly, there was an early response of *CHS*, *FLS* and *DFR* in *Va* f. nigrum at 12 hpi (Figures 3.9a, 3.9b & 3.11a). In *Vm*, there was steady decrease in *CCR* and *DFR* up 48 hpi whereas *CHS* and *FLS* did not reveal any remarkable changes in their expression. There was no early expression of *ANS* in *Vm* until 48 hpi where there was an increase in *ANS* transcript (Figure 3.10b). There was an expression of *ANR* in the early stages of infection at 12 hpi. Similar to *B. cinerea* inoculated *Vm*, the expression pattern of the flavonoid pathway genes in *B. amyloliquefaciens* inoculated *Vm* revealed a decreased in all these genes at 12 hpi, except *CCR.* Following the decrease, the rise in the gene expression at 24 and 48 hpi was comparable to the basal expression. There was early expression and a highest expression of *CCR* at 12 hpi (Figure 3.11b). In *Va* f. nigrum, there was an initial decrease in *ANR*, *CHS* and *FLS* at 12 hpi followed by a rise in expression which were comparable to the basal expression (Figures 3.9 & 10). On the contrary, *ANS* and *CCR* showed and early response (12 hpi) with highest expression levels at 48 and 24 hpi, respectively (Figures 3.10b & 3.11b). There was an early response of *DFR* at 12 hpi with the highest expression observed at 48 hpi. Interestingly, there was no remarkable changes in the expression of *CHS*, *FLS*, *DFR* and *CCR* in *Vm*. On the contrary, there was a decrease in *ANR* and *ANS* expression at 12 hpi (Figures 3.9 – 3.11).

At the phenotype level, no significant difference was observed with *ANR* and *DFR*. However, *Va* had a significantly high expression of *ANS* ($p = 0.0057$), *FLS* ($p < 0.0001$) and *CHS* ($p < 0.0001$) whiles the *Va* had a significantly high expression of *CHS* ($p < 0.0001$), CCR ($p < 0.0001$) *ANS* ($p < 0.0001$) and *LOX* ($p < 0.0001$). *Vm* had a significantly high expression of *CCR* ($p < 0.0001$) (Appendix 6, Tables A3-A6). Regarding the treatments, *ANR* and *FLS* were significantly expressed in *B. amyloliquefaciens* than *B. cinerea* inoculated plants whereas there were not significant different in the other genes.

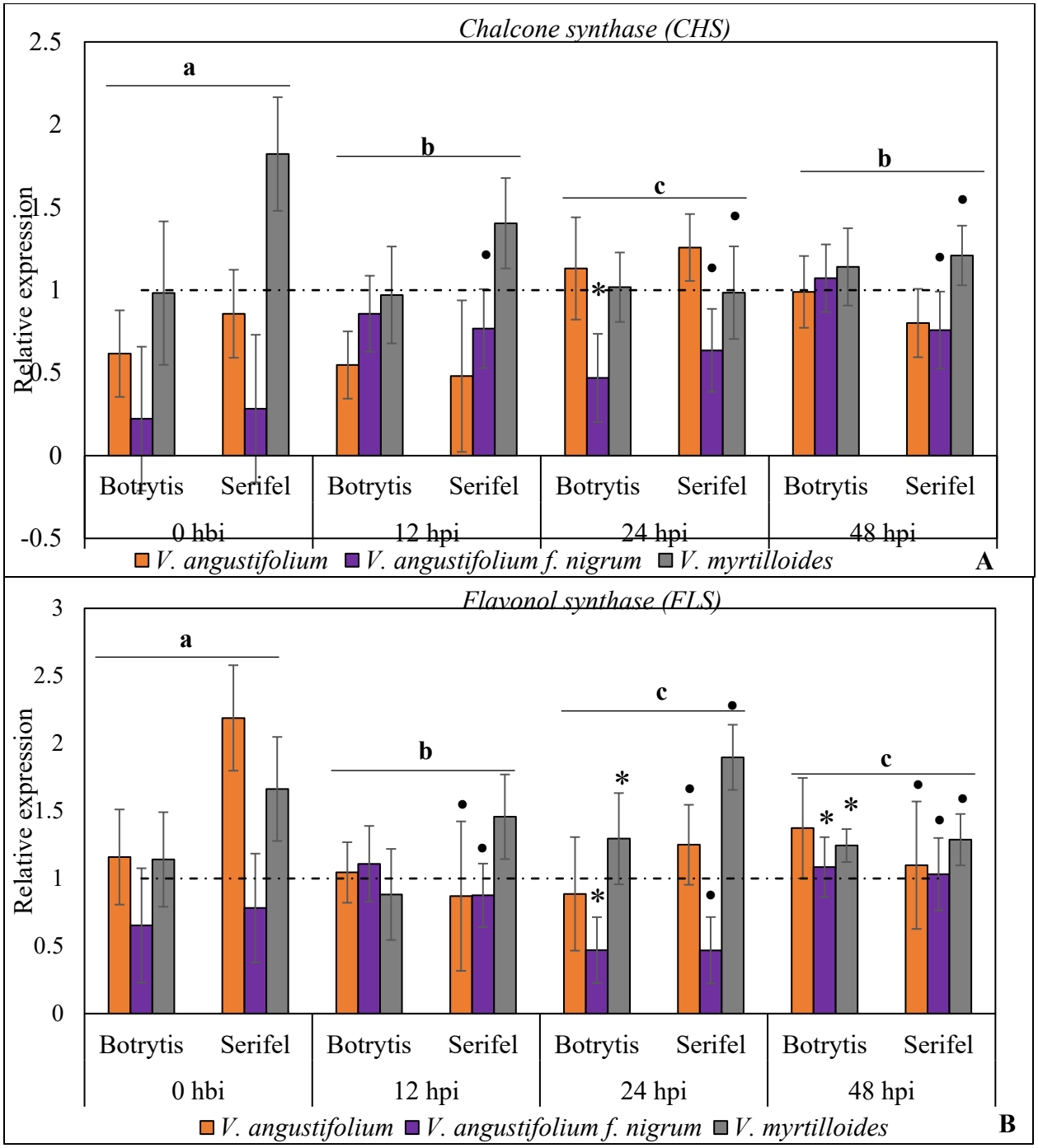

Figure 3.9. Expression pattern of flavonoid biosynthesis genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* and *Bacillus amyloliquefaciens* inoculation. (A) Chalcone synthase (*CHS*) and (B) Flavonol synthase *(FLS*. Results are reported as means ± standard error of three biological replicates. *Significant difference between infected plants and their controls/basal expression (0 hours before inoculation, hbi). • Significant difference between *B. amyloliquefaciens* inoculated plants and their controls/basal expression. Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at α = 0.05. Broken horizontal line at 1-fold relative expression represents the calibrator.

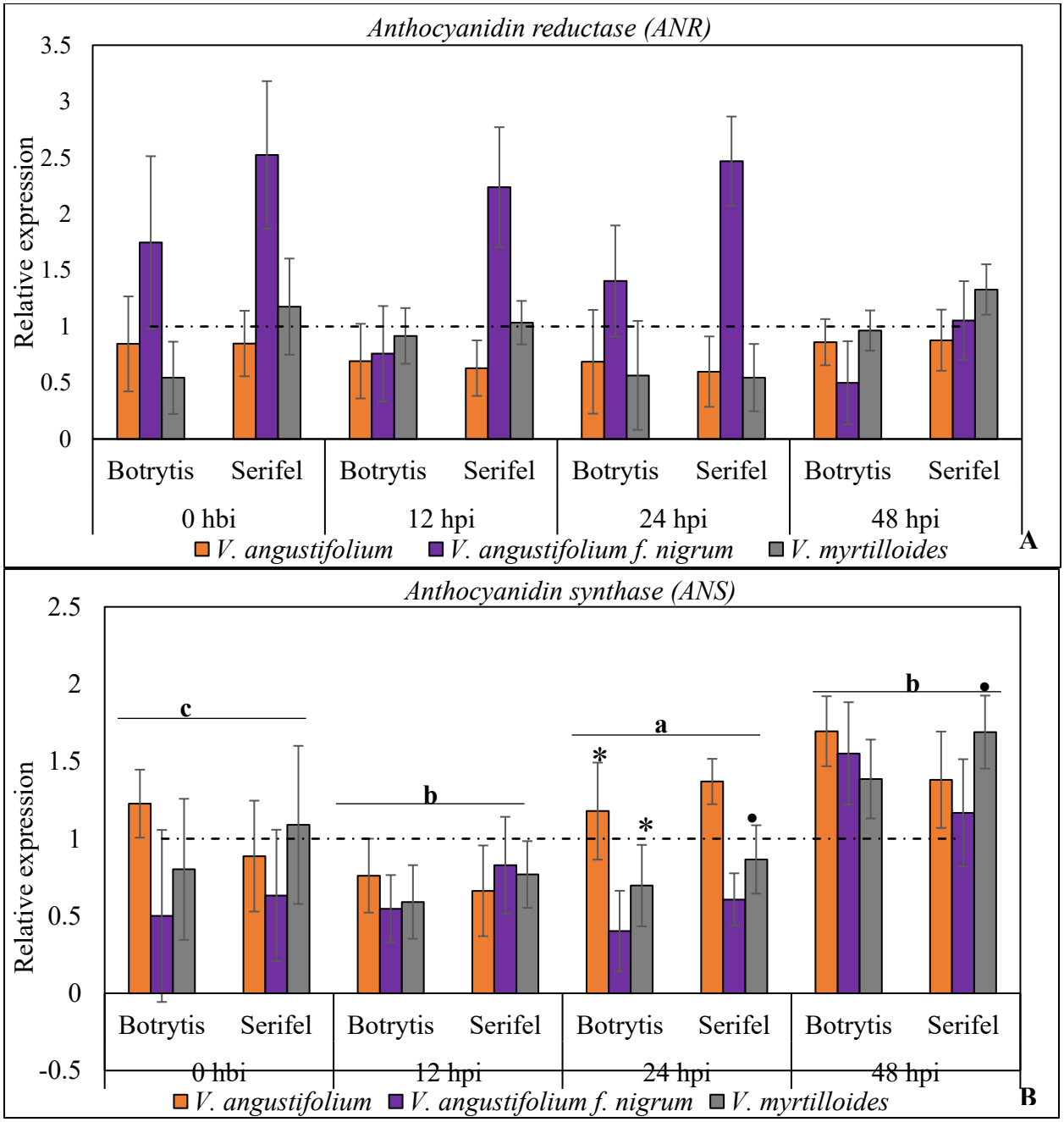

Figure 3.10. Expression pattern of flavonoid biosynthesis genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* and *Bacillus amyloliquefaciens* inoculation. (A) Anthocyanin reductase (*ANR*) and (B) Anthocyanin synthase (*ANS*). Expression of each gene is shown as a fold change in infected samples relative to their respective uninfected control from the same time point. Results are reported as means ± standard error of three biological replicates. *Significant difference between infected plants and their controls/basal expression (0 hours before inoculation, hbi). • Significant difference between *B. amyloliquefaciens* inoculated plants and their controls/basal expression. Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at $\alpha = 0.05$. Broken horizontal line at 1-fold relative expression represents the calibrator.

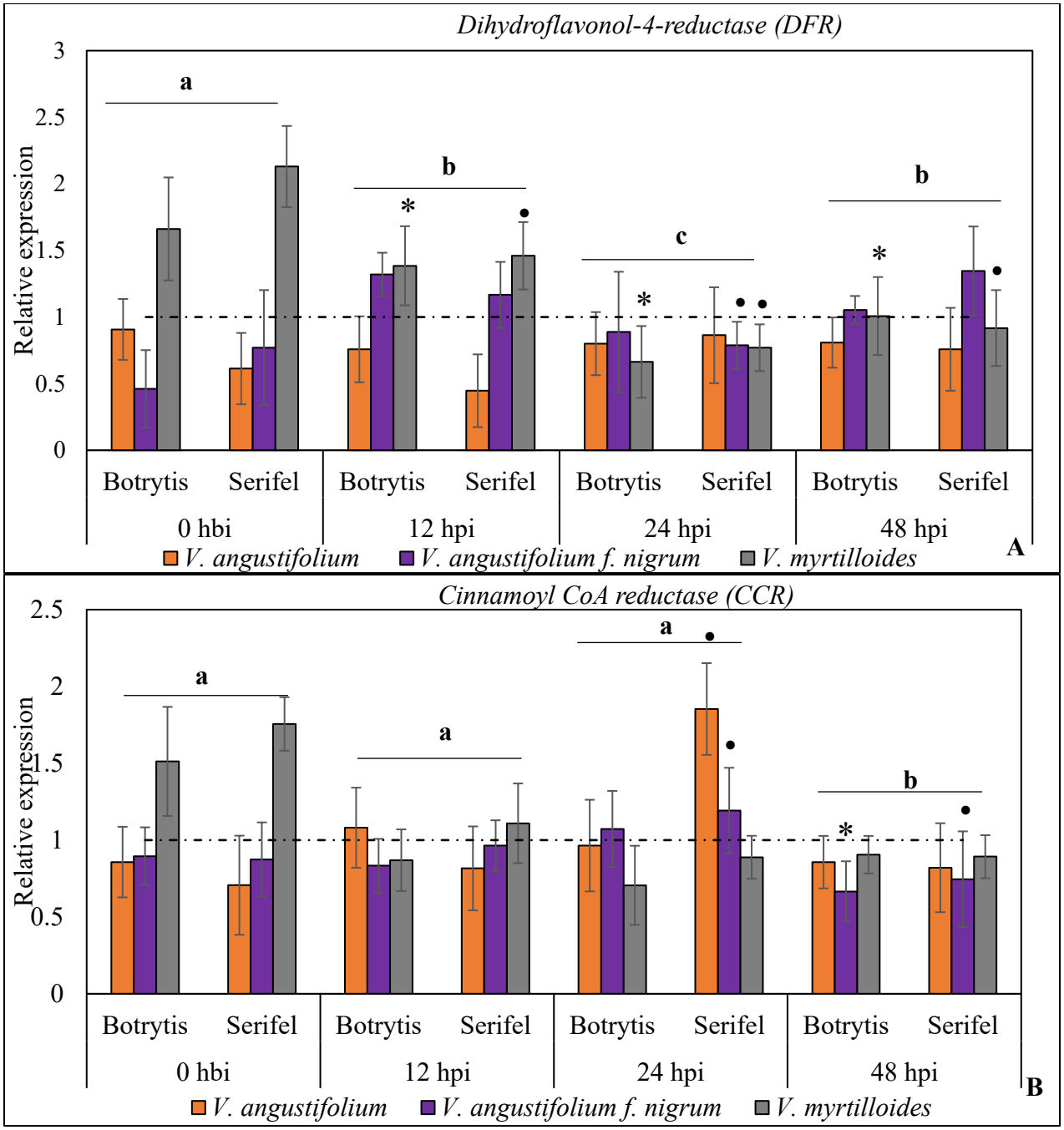

Figure 3.11. Expression pattern of flavonoid genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* and *Bacillus amyloliquefaciens* inoculation. A) Dihydroflavonol-4-reductase (*DFR*) and Cinnamoyl CoA reductase (*CCR*). Expression of each gene is shown as a fold change in infected samples relative to their respective uninfected control from the same time point. *Significant difference between infected plants and their controls/basal expression (0 hours before inoculation, hbi). • Significant difference between *B. amyloliquefaciens* inoculated plants and their controls/basal expression. Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at $\alpha = 0.05$. Broken horizontal line at 1-fold relative expression represents the calibrator.

3.5.3 *LOX* – Jasmonate pathway gene

In 2019, the jasmonate pathway gene (*LOX*) showed and early response in brown stem *Va* with maximum expression at 12 hpi. Similar brown stem *Va,* the response of *LOX* in *Va* f. nigrum started at 12 hpi, but maximum expression was attained at 24 hpi. Interestingly, the was no *LOX* induction in green stem *Va*. In short and medium stem *Vm,* there was an early *LOX* at 12 hpi with the highest expressions at 24 hpi. Interestingly, *LOX* in tall stem *Vm* decreased at 24 hpi followed by an up regulation at 48 hpi (Figure 3.12a).

In 2020, *Botrytis cinerea* inoculation, resulted in early response of *LOX* in all the three phenotypes at 12 hpi. The highest expression of this gene occurred at 48 hpi in *Va* and *Vm*. In *B. amyloliquefaciens* inoculated *Va* phenotypes, there was an early response of *LOX*. *LOX* reached significant maximum expression in *Va* at 48 hpi. On the contrary, there was a decrease in expression of *LOX* in *Vm* at 12 hpi (Figure 3.12b).

At the phenotype level *Va* and *Vm* had significantly high expression of *LOX* ($p < 0.0001$) (Appendix 5, Table A7). Regarding the treatments, there was no significant expression of *LOX* in both *B. amyloliquefaciens* and *B. cinerea* inoculated plants.

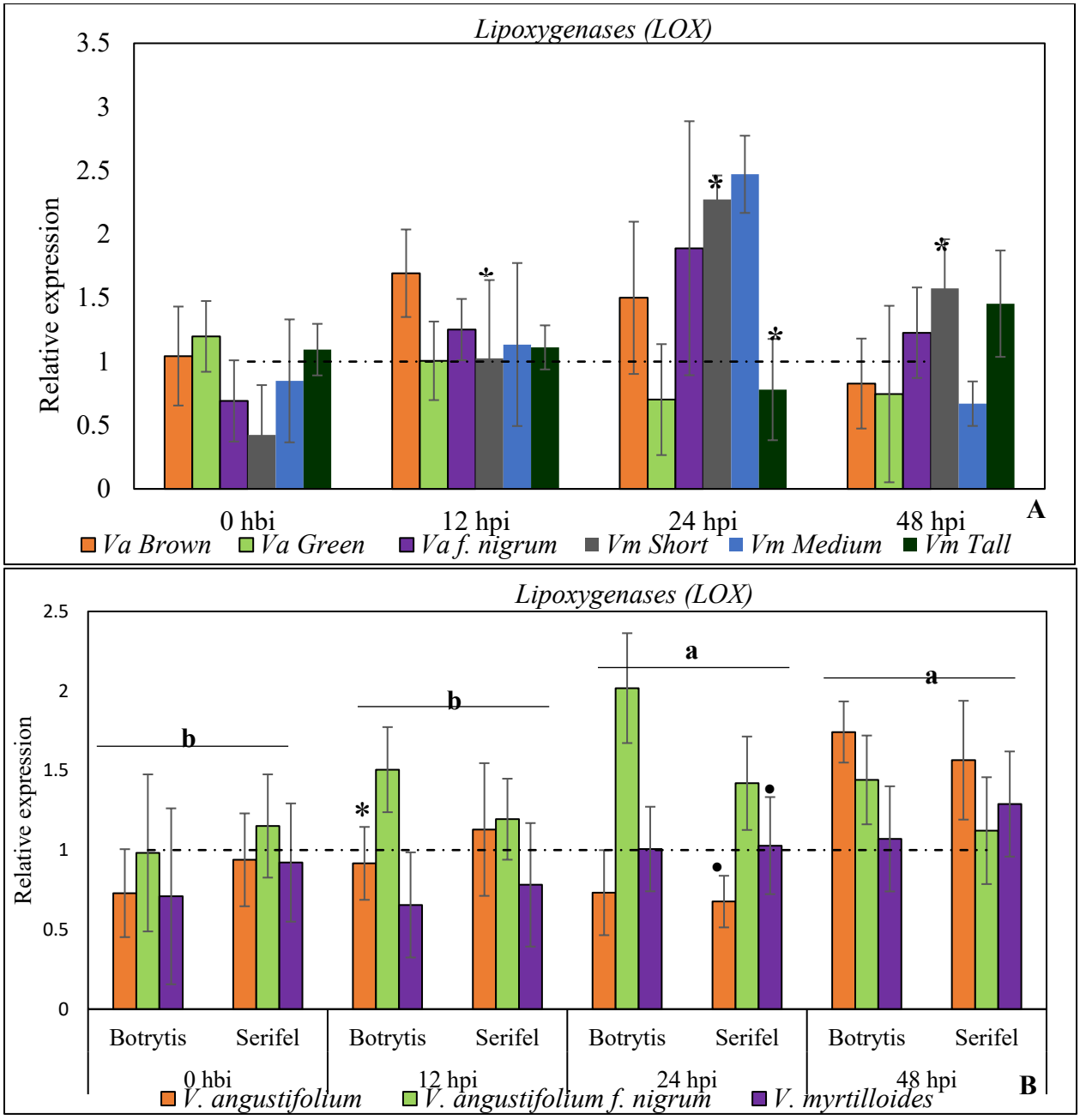

Figure 3.12. Expression pattern of flavonoid biosynthesis genes in wild blueberry phenotypes (*V. angustifolium* and *V. myrtilloides*) in response to *Botrytis cinerea* and Serifel® (*Bacillus amyloliquefaciens*) inoculation. (A) Lipoxygenases in 2019 and (B) Lipoxygenases in 2020 Expression of each gene is shown as a fold change in infected samples relative to their respective uninfected control from the same time point. Results are reported as means ± standard error of three biological replicates. *Significant difference between infected plants and their controls/basal expression (0 hours before inoculation, hbi). • Significant difference between *B. amyloliquefaciens* inoculated plants and their controls/basal expression (0 hours before inoculation, hbi). Horizontal bars represent mean of expression at specific post inoculation time points (hbi/hpi). Horizontal bars with the same letters on the horizontal bar are not significantly different from each other at α = 0.05. Broken horizontal line at 1-fold relative expression represents the calibrator.

3.5.4 Method validation

The method validation parameters were studied using the authentic standards. The recovery values of all the investigated compounds were were between 91–110%. The intraday precision (repeatability) and interday (intermediate) precision of the method were verified by analyzing the extracts six times a day for three days within a week repetitively. The relative standard deviation (% RSD) values of all the compounds were < 3 % for repeatability and < 4.5 % for intermediate precision (Table 3.1). Limit of detection (LOD) and quantification (LOQ) ranged from 0.03 to 0.95 µg/mL and from 0.09 to 2.87 µg/mL, respectively. The linearity of each phenolic standard was assessed over 0.01-200 µg/ml concentration range. All the ten phenolic compounds demonstrated excellent linearity over the evaluated concentration range with correlation coefficients > 0.98 (Table 3.1).

Table 3.1. Parameters of the calibration curves, precision, and recovery studies for different phenolic standards

Compound	Linearity (R^2)	LOD (µg/mL)	LOQ (µg/mL)	Intraday precision %RSD	Intermediate precision %RSD	Recovery (%)
Catechin	1.00	0.40	1.23	0.94	0.62	92.76
Procyanidin	1.00	0.84	2.54	1.19	0.66	106.57
m-Coumaric acid	1.00	0.03	0.09	3.76	4.43	91.68
Neochlorogenic acid	1.00	0.87	2.65	5.73	2.24	94.73
Chlorogenic acid	0.99	0.95	2.87	0.32	0.30	92.12
Caffeic acid	1.00	0.52	1.58	1.94	1.44	95.63
p-Coumaric acid	1.00	0.05	0.15	2.99	2.03	102.15
Quercitin-3-Galactoside	1.00	0.47	1.43	0.38	0.39	94.59
Quercitrin	1.00	0.66	1.99	0.47	0.39	109.23
Kaempferol-3-Glucoside	1.00	0.10	0.30	1.36	0.78	106.78

LOD: Limit of detection, LOQ: limit of quantification.

3.5.5 Total flavanols, hydroxycinnamic acids and flavonols

Total flavanols

In 2019, total flavanols which represents the sum of catechin and procyanidin B2 in this study was significantly (p= 0.0011) affected by *B. cinerea* infection (Table 3.2). Brown stem *Va* had a significantly higher flavanol content after 96 hpi compared to its control. Although there was a significant effect among the phenotypes, there was a wide variation in flavanol concentration between the healthy and inoculated plants among the various phenotypes. Given this, the flavanol concentrations in most of the phenotypes at the two time points were not significantly different from each other and their respective controls (Table 3.2).

In 2020, however, there was no significant difference among *B. cinerea*, *B. amyloliquefaciens* inoculated and uninoculated plants with regards to total flavanols (Table 3.3).

Total hydroxycinnamic acids

In 2019, hydroxycinnamic acid derivatives, which comprised the sum of caffeic, chlorogenic, neochlorogenic acids, m-coumaric acid, and p-coumaric acid were significantly affected by *B. cinerea* inoculation (p = 0.0010) (Table 3.2). Interestingly, the healthy *Va* f. nigrum had numerically the highest concentration of hydroxycinnamic acids at 48 hpi although it was not significantly different from most of the phenotypes either inoculated or uninoculated.

In 2020, hydroxycinnamic acid levels were significantly affected by *B. cinerea* and *B. amyloliquefaciens* (p < 0.0001) (Table 3.3). *V. angustifolium* species had significantly higher hydroxycinnamic acid levels compared to *V. myrtilloides*. *Bacillus amyloliquefaciens* inoculated *V. angustifolium* resulted in higher hydroxycinnamic acid after 48 hpi followed by *B. cinerea* inoculated an *V. angustifolium* and *V. angustifolium* f. nigrum after 48 hpi (Table 3.3).

Total flavonols

In 2019, total flavonols, which is comprised of the sum of quercitin-3-galactoside, quercitrin (quercetin-3-rhamnoside) and kaempferol-3-glucoside, were also significantly affected by *B. cinerea* inoculation ($p = 0.0156$) with inoculated brown stem *Va* at 96 hpi having the highest concentration (Table 3.2).

In 2020, *B. cinerea* and *B. amyloliquefaciens* both resulted in significant induction of flavonols ($p < 0.0001$) (Table 3.3). Flavonols in *V. myrtilloides* were higher whereas the concentrations in *V. angustifolium* f. nigrum were the lowest. *Botrytis cinerea* and *B. amyloliquefaciens* inoculated *V. myrtilloides* had the highest flavonol concentration at 48 hpi (Table 3.3).

Table 3.2. Total flavanols, hydroxycinnamic and flavonols subclasses of phenolic compounds (mg/g FW) in *B. cinerea* inoculated and healthy wild blueberry flower tissues in 2019

	***Va* brown stem**		***Va* green stem**		***Va f.* nigrum**		***Vm* short**		***Vm* medium**		***Vm* tall**	
	48 hpi	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**
Total flavanols												
Control	67.0±10.2 abc	44.9±8.1 efg	44.3±3.7 efg	43.9±6.3 efg	52.1±16.2 b-e	54.6±15.4 b-e	64.3±12.3 a-d	67.9±21.9 ab	59.8±10.0 a-e	53.5±7.1 b-e	57.5±8.9 b-e	47.7±7.7 b-f
Botrytis cinerea	60.8±9.4 a-e	69.3±9.8 ab	47.8±4.3 d-g	31.3±4.4 fg	46.1±9.9 d-g	30.1±15.0 g	76.7±21.1 a	53.1±4.8 b-e	45.2±12.4 efg	49.0±12.3 c-g	53.7±13.4 b-e	46.2±5.7 d-g
Total hydroxycinnamic acid												
Control	375.9±113 abc	396.4±120 abc	337.8±13 a-d	361.6±34.3 abc	408.7±108 a	358.3±19.1 a-d	270.1±37.9 b-h	240.6±65.7 d-h	266.3±160 c-h	196.1±122 e-h	195.6±80 e-h	227.9±60.8 d-h
Botrytis cinerea	324.5±54.3 a-e	398.1±62.7 ab	392.1±26.7 abc	291.9±95.7 a-g	348.2±63.5 a-d	311.9±91.6 a-f	328.8±64.9 a-d	243.0±54.5 d-h	189.3±37.5 fgh	240.4±32.3 d-h	179.1±98.6 hg	157.1±79.9 h
Total flavonols												
Control	113.1±29.9 a-d	77.0±30.3 b-f	112.2±6.2 a-d	103.3±7.7 a-e	71.6±20.4 c-f	101.2±10.7 a-f	101.9±41.8 a-f	88.6±52.3 b-f	93.6±10.1 b-f	89.8±20.2 b-f	66.3±24.3 def	65.4±22 def
Botrytis cinerea	117.8±39.5 abc	147.2±64.8 a	121.6±3.2 ab	114.7±10.6 a-d	67.9±25.9 def	69.9±22.2 def	110.4±33.7 a-d	86.9±23.1 b-f	56.1±13.5 f	72.5±10.9 c-f	60.6±19.8 ef	67.3±27.4 def

Mean value (n = 3) ± standard deviation. Mean separation was completed using LSD test procedure. For each compound, mean with the same letters are not significantly different from each other at α=0.05. Total flavanols: P=0.0011, hydroxycinnamic acid: P= 0.0010, total flavonols P= 0.0156

Total flavanols is the sum of catechin and procyanidin B2

Total hydroxycinnamic acids is the sum of caffeic, chlorogenic neochlorogenic acids, m-coumaric acid and p-coumaric acid

Total flavonols is the sum of quercetin-3-galactoside, quercitrin and kaempferol-3-glucoside

Table 3.3. Total flavanols, hydroxycinnamic and flavonols subclasses of phenolic compounds (mg/g FW) in *Botrytis cinerea, Bacillus amyloliquefaciens* inoculated and healthy wild blueberry flower tissues in 2020

	V. angustifolium			***V. angustifolium* f. nigrum**			***V. myrtilloides***		
	48 hpi	**96 hpi**	**6 dpi**	**48 hpi**	**96 hpi**	**6 dpi**	**48 hpi**	**96 hpi**	**6 dpi**
Total Flavanols									
Control	39.8±12.0	51.9±11.2	52.1±19.6	52.7±7.9	30.0±5.4	41.1±12.1	46.2±10.6	44.4±12.1	58.5±7.2
Botrytis cinerea	50.7±21.4	55.7±17.1	54.8±17.6	53.0±10.5	45.8±11.2	52.2±13.2	50.0±16.7	54.0±20.4	63.8±8.8
Bacillus amyloliquefaciens	64.3±13.8	47.7±15.5	55.6±18.0	51.0±4.2	35.0±8.5	47.4±11.6	56.6±15.4	48.0±18.5	58.2±14.4
Total Hydroxycinnamic acid									
Control	413.6±63.0 ab	410.3±60.7 ab	422.2±93.8 ab	442.8±69.3 a	337.0±36.1 b-e	389.1±59.3 abc	267.5±115.3 d-g	250.1±67.7 efg	309.5±47.8 c-f
Botrytis cinerea	437.6±53.7 a	389.5±88.1 abc	430.9±42.4 a	440.1±52.8 a	353.1±39.5 a-d	406.1±51.8 abc	212.6±54.8 fg	179.5±27.5 g	264.2±70.6 d-g
Bacillus amyloliquefaciens	444.9±75.7 a	360.9±91.7 a-d	392.1±106.6 abc	411.3±63.1 ab	357.9±68.2 a-d	401.6±41.1 abc	263.9±66.5 d-g	179.6±18.5 g	276.6±38.5 d-g
Total Flavonols									
Control	80.9±15.0 ab	63.1±9.2 a-f	65.3±18.5 a-f	33.9±4.8 fg	28.4±9.6 g	31.0±9.3 g	79.5±19.4 ab	65.1±20.6 a-f	63.9±24.7 a-f
Botrytis cinerea	78.8±13.5 ab	70.0±16.4 a-d	72.7±11.3 ab	41.3±6.8 c-g	37.1±10.5 efg	56.9±43.1 b-g	92.6±19.7 a	70.5±26.0 abc	84.8±21.4 a
Bacillus amyloliquefaciens	69.9±12.5 a-d	65.4±16.6 a-e	54.0±20.8 b-g	40.7±5.6 d-g	37.1±7.5 efg	37.2±10.7 efg	90.2±18.9 a	74.7±28.9 ab	68.6±25.1 a-d

Mean value (n = 4) ± standard deviation. Mean separation was completed using LSD test procedure. For each compound, mean with the same letters are not significantly different from each other at α=0.05. Total flavanols: P=0.0011, hydroxycinnamic acid: P= 0.0010, total flavonols P= 0.0156

Total flavanols is the sum of catechin and procyanidin B2

Total hydroxycinnamic acids is the sum of caffeic, chlorogenic neochlorogenic acids, m-coumaric acid and p-coumaric acid

Total flavonols is the sum of quercitin-3-galactoside, quercitrin and kaempferol-3-glucoside

3.5.6 Individual flavanols

In 2019, a significant difference in the concentrations of catechin ($p = 0.0009$) and procyanidin B2 ($p = 0.0041$) among the inoculated and healthy plants was observed (Table 3.4). Similar to the total flavanol, there were significantly higher concentrations of catechin in brown stem *Va* at 96 hpi, in the inoculated plants (Table 3.4). Like the total flavanol, most of the phenotypes either healthy or inoculated were not significantly different from each other, thus treatment and temporal concentrations were phenotype dependent

In 2020, there was no significant difference in the concentration of catechin and procyanidin B2 among the phenotypes after the application of *B. cinerea* and *B. amyloliquefaciens* in 2020 (Table 3.5). At the phenotypic level, there was significantly higher ($p < 0.05$) individual flavanols in *Vm* and *Va* than *Va* f. nigrum. However, there was no significant difference among the time points.

Table 3.4. Concentration of individual flavanol compounds (mg/g FW) in *B. cinerea* inoculated and healthy wild blueberry flower tissues in 2019

	***Va* brown stem**		***Va* green stem**		***Va f.* nigrum**		***Vm* short**		***Vm* medium**		***Vm* tall**	
	48 hpi	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**
Catechin												
Control	33.2±4.3a b	21.1±5.0 c-f	18.5±2. 0 def	19.7±3. 0 def	21.8±7.6 c-f	28.6±10. 7 a-d	30.7±6.4 abc	32.1±13. 2 ab	27.6±2. 7 a-e	28.5±5. 7 a-d	23.0±3. 4 b-f	21.7±3. 1 b-f
Botrytis cinerea	30.8±5.3 abc	35.2±2.7 a	20.3±2. 2 def	15.9±2. 5 f	20.0±7.1 def	14.5±12. 8 f	35.5±8.5 a	24.1±0.9 b-f	17.8±4. 4 ef	24.0±3. 2 b-f	21.5±5. 9 c-f	18.7±3. 5 def
Procyanidin B2												
Control	33.8±6.3 a-d	23.7±4.3 def	25.8±1. 7 b-f	24.2±3. 3 c-f	30.3±8.9bc d	26.0±5.1 b-e	33.6±6.1 a-d	35.8±8.9 ab	32.1±8. 8 a-d	25.0±1. 4 c-f	34.5±5. 7 abc	26.0±5. 1 bcd
Botrytis cinerea	30.0±5.3 bcd	34.1±7.1a -d	27.5±2. 2 bcd	15.3±2. 0 f	26.1±3.8 b-e	15.6±4.0 ef	41.2±13. 3 a	28.9±4.7 bcd	27.4±8. 9 bcd	24.9±9. 2 c-f	32.2±7. 5 a-d	27.5±4. 9 bcd

Mean value (n = 3) ± standard deviation. Mean separation was completed using LSD test procedure. For each compound, mean with the same letters are not significantly different from each other at α=0.05. Catechin: P=0.0009, and Procyanidin B2: P=0.0041

Table 3.5. Concentration of individual flavanol compounds (mg/g FW) in *Botrytis cinerea, Bacillus amyloliquefaciens* inoculated and healthy wild blueberry flower tissues in 2020

	V. angustifolium			***V. angustifolium f. nigrum***			***V. myrtilloides***		
	48 hpi	**96 hpi**	**6 dpi**	**48 hpi**	**96 hpi**	**6 dpi**	**48 hpi**	**96 hpi**	**6 dpi**
Catechin									
Control	24.4±1.8	28.3±6.7	29.6±10.7	25.7±7.6	15.7±5.2	21.8±10.0	26.4±2.2	25.3±1.2	31.3±5.3
Botrytis cinerea	29.5±7.5	27.5±9.7	31.2±7.3	27.1±9.8	22.8±10.7	26.9±12.4	28.1±2.6	32.1±7.9	33.6±2.5
Bacillus amyloliquefaciens	33.9±6.9	23.9±7.1	25.6±10.9	25.3±6.2	17.8±4.0	24.7±0.2	33.7±2.9	26.1±6.8	31.3±5.4
Procyanidin B2									
Control	28.7±9.1	23.6±4.6	22.5±9.1	26.9±2.8	18.2±3.9	19.3±3.3	26.4±3.1	25.4±3.2	27.2±5.1
Botrytis cinerea	28.2±3.6	28.2±7.9	23.6±10.6	25.9±4.5	23.0±2.2	25.2±2.9	28.9±5.2	30.0±5.6	30.2±7.2
Bacillus amyloliquefaciens	30.4±7.2	23.8±8.6	26.7±7.5	25.6±4.9	21.6±5.7	22.7±6.2	31.3±3.8	28.4±6.0	26.9±9.2

Mean value (n = 4) ± standard deviation. Mean separation was completed using LSD test procedure. For each compound, mean with the same letters are not significantly different from each other at α=0.05. Catechin: NS, and Procyanidin B2: NS

3.5.7 Individual hydroxycinnamic acids

In 2019, chlorogenic acid characterized the majority (> 95%) of hydroxycinnamic acids measured in both years. Significant changes in the concentration of chlorogenic acid ($p = 0.0009$), neochlorogenic acid ($p = 0.0335$) and m-coumaric acids ($p < 0.0001$) were detected among the treatments and phenotypes (Table 3.6). Although significant differences were observed, almost all the phenotypes were not different from each other. It is however worth noting that short *Vm* had a higher content of neochlorogenic acid in inoculated plants at 48 and 96 hpi (Table 3.6). The concentration of m-coumaric acid was significantly higher in all inoculated *Va* phenotypes at different times of assessment except *Va f.* nigrum at 48 hpi. A significantly higher concentration of m-coumaric acid was observed in inoculated short stem *Vm* and tall stem *Vm* at 48 and 96 hpi, respectively. No significant changes in the concentrations of caffeic acid and p-coumaric acid were observed.

In 2020, significant differences in the concentration of chlorogenic ($p = 0.0009$) and neochlorogenic acids ($p = 0.0335$) were detected among the treatments. Almost all the phenotypes were not different from each other (Table 3.7). The concentration of m-coumaric acid was higher in *V. myrtilloides* compared to the *V. angustifolium* species. Both *B. cinerea* and *B. amyloliquefaciens* inoculated *V. myrtilloides* (48 hpi ,96 hpi and 6 dpi) had significantly higher level of m-coumaric acid compared to *V. angustifolium* species (Table 3.7). At the phenotypic level, there was significantly ($p < 0.0001$) higher concentration of m-coumaric acid and neochlorogenic acid in *Vm* whereas chlorogenic acid, caffeic acid and p-coumaric acid were significantly ($p < 0.0001$) higher in *Va* f. nigrum. Regarding temporal concentration, the hydroxycinnamic acids were significantly higher ($p <0.05$) at 48 hpi followed by 6 days post inoculation.

Table 3.6. Concentration of individual hydroxycinnamic acid derivatives (mg/g FW) in *B. cinerea* inoculated and healthy wild blueberry flower tissues in 2019

	***Va* brown stem**		***Va* green stem**		***Va f.* nigrum**		***Vm* short**		***Vm* medium**		***Vm* tall**	
	48 hpi	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**
m-Coumaric acid												
Control	0.80±0.3 efg	0.65±0.2 fg	0.69±0.3 fg	0.45±0.1 g	0.95±0.2 d-g	0.51±0.1 g	0.70±0.2 fg	0.82±0.2 efg	0.94±0.3 d-g	1.16±0.5 b-f	1.53±0.7 abc	0.63±0.2 fg
Botrytis cinerea	1.38±0.1 a-d	1.55±0.5 abc	1.51±0.4 abc	1.11±0.2 c-f	1.48±0.1 a-d	1.68±0.1 ab	1.81±0.5 a	1.34±0.a-e	1.44±0.3 a-d	1.32±0.2a -e	1.60±0.4 abc	1.80±0.5 a
Neochlorogenic acid												
Control	3.87±2.1 b-g	2.76±0.2 g	3.10±0.2 efg	2.86±0.1 fg	3.48±2.3 c-g	4.18±2.1 b-g	5.74±2.0 abc	5.45±2.6 a-e	4.50±2.2 b-g	5.67±0.5 a-d	4.94±0.6a -g	4.51±0.8 b-g
Botrytis cinerea	3.21±0.6 d-g	4.47±2.4 b-g	1.95±0.2 efg	2.36±0.5 c-g	2.97±1.1 efg	3.46±1.8 c-g	7.38±0.8 a	6.53±0.1 ab	4.39±1.5 b-g	5.28±2.3 a-f	4.76±0.6 b-g	5.02±1.0 a-g
Chlorogenic acid												
Control	368.0±114 abc	389.3±121 ab	331.2±13.2 a-d	355.6±34.3 a-d	400.7±109 a	349.6±17.5a-d	259.9±35.3 b-h	231.1±62.7 d-h	257.8±162 c-h	186.7±122 e-h	185.8±79.4e-h	219.9.6± 89.8 d-h
Botrytis cinerea	316.9±54.4a-e	387.4±64 abc	384.6±26.8abc	283.8±95.3a-g	340.3±64.2a-d	302.7±92.1 a-f	315.9±63.2 a-e	233.0±52.5 d-h	180.7±37.0fgh	229.9±34.5d-h	169.4±96.8 gh	146.4±78.3 h
Caffeic acid												
Control	2.01±0.4	2.71±1.2	1.95±0.1	2.05±0.1	2.60±0.8	2.66±1.1	2.09±0.3	1.87±0.1	1.93±0.2	1.77±0.2	1.99±0.3	1.72±0.2
Botrytis cinerea	1.93±0.3	2.65±0.5	2.10±0.3	2.34±0.3	2.58±0.7	2.74±1.2	2.15±0.2	2.36±0.4	1.83±0.1	2.39±0.5	1.90±0.4	2.09±0.2
p-Coumaric acid												
Control	1.19±0.6	0.92±0.6	0.86±0.0	0.69±0.0	1.00±0.5	1.31±1.1	1.68±0.7	1.35±0.2	1.06±0.0	0.83±0.1	1.40±1.2	0.91±0.8
Botrytis cinerea	1.12±0.6	1.96±1.0	0.78±0.1	1.35±0.1	0.94±0.5	1.35±0.5	1.58±0.5	1.91±0.2	0.97±0.4	1.53±0.5	1.39±1.5	1.78±1.3

Mean value (n = 3) ± standard deviation. Mean separation was completed using LSD test procedure. For each compound, mean with the same letters are not significantly different from each other at α=0.05.m-Coumaric acid: P<0.0001, Neochlorogenic acid: P=0.0335, and Chlorogenic acid: P=0.0009.

Table 3.7. Concentration of individual hydroxycinnamic acid derivatives (mg/g FW) in *Botrytis cinerea, Bacillus amyloliquefaciens* inoculated and healthy wild blueberry flower tissues in 2020.

	V. angustifolium			***V. angustifolium f. nigrum***			***V. myrtilloides***		
	48 hpi	**96 hpi**	**6 dpi**	**48 hpi**	**96 hpi**	**6 dpi**	**48 hpi**	**96 hpi**	**6 dpi**
m-Coumaric acid									
Control	0.74±0.5 de	0.40±0.1 ef	0.57±0.4 def	0.43±0.02 ef	0.34±0.1 f	0.36±0.1 f	1.09±0.4 bc	1.27±0.1 ab	1.46±0.3 a
Botrytis cinerea	0.52±0.3 ef	0.55±0.2 def	0.62±0.4 def	0.57±0.2 def	0.53±0.2 ef	0.38±0.1 f	1.34±0.2 ab	1.34±0.2 ab	1.46±0.2 a
Bacillus amyloliquefaciens	0.59±0.4 def	1.09±0.3 bc	0.87±0.3 cd	0.41±0.1 ef	0.42±0.05 ef	0.34±0.1 f	1.35±0.2 ab	1.31±0.2 ab	1.28±0.1 ab
Neochlorogenic acid									
Control	2.35±0.3 f	2.46±0.3 f	2.53±0.2 f	2.82±0.7 f	2.57±0.3 f	2.66±0.4 f	5.32±1.8 e	6.83±1.5 bc	6.82±1.2 bc
Botrytis cinerea	2.43±0.4 f	2.18±0.2 f	2.72±0.3 f	2.93±1.0 f	2.63±0.7 f	2.57±0.3 f	6.60±1.0 cd	5.52±0.7 de	8.16±1.6 a
Bacillus amyloliquefaciens	2.47±0.3 f	2.36±0.3 f	2.68±0.8 f	2.72±0.7 f	2.65±0.4 f	2.91±0.8 f	7.85±1.1 ab	6.23±0.8 cde	6.89±1.1 bc
Chlorogenic acid									
Control	407.9±63.2 ab	405.2±60.6 ab	416.7±93.6 ab	436.3±69.9 a	331.4±36.5 b-f	383.1±59.0 abc	258.6±116.1 e-h	240.3±69.1 fgh	298.8±48.3 c-g
Botrytis cinerea	432.0±54.1 a	384.4±88.1 abc	425.1±42.6 a	433.7±53.4 a	346.9±39.6 a-e	400.4±51.2 ab	202.3±55.3 gh	170.5±28.2 h	251.6±70.3 e-h
Bacillus amyloliquefaciens	439.0±75.2 a	355.1±91.5 a-d	386.3±106.5 abc	405.0±62.9 ab	351.9±68.5 a-e	395.4±48.1 abc	252.5±66.2 e-h	169.8±19.1 h	265.9±38.8 d-h
Caffeic acid									
Control	1.92±0.1 d-i	1.76±0.1 hi	1.75±0.1 hi	2.33±0.3 ab	2.03±0.3 b-h	2.27±0.2 abc	1.84±0.3 f-i	1.77±0.1 hi	1.67±0.1 hi
Botrytis cinerea	1.96±0.2 c-i	1.82±0.2 g-i	1.82±0.2 g-i	2.27±0.3 abc	2.19±0.2 a-e	2.13±0.4 a-g	1.88±0.2 e-i	1.68±0.1 hi	1.75±0.1 hi
Bacillus amyloliquefaciens	1.98±0.1 c-i	1.74±0.1 hi	1.70±0.1 hi	2.36±0.5 a	2.16±0.2 a-f	2.22±0.4 a-d	1.85±0.1	1.75±0.1 hi	1.68±0.1 hi
p-Coumaric acid									
Control	0.74±0.2 a-f	0.63±0.2 a-g	0.66±0.2 a-h	0.88±0.1 a	0.71±0.1 a-g	0.75±10.6 a-f	0.66±0.5 a-h	0.32±0.1 i	0.52±0.1 d-i
Botrytis cinerea	0.77±0.2 a-e	0.70±0.2 a-g	0.60±0.2 b-h	0.77±0.2 a-e	0.84±0.2 abc	0.73±0.2 a-f	0.45±01 ghi	0.44±0.1 ghi	0.44±0.2 hi
Bacillus amyloliquefaciens	0.80±0.2 a-e	0.65±0.1 a-g	0.58±0.1 c-i	0.79±0.1 a-e	0.85±0.3 ab	0.83±0.2 a-d	0.54±0.2 e-i	0.48±0.1 f-i	0.60±0.2 b-h

Mean value (n = 4) ± standard deviation. Mean separation was completed using LSD test procedure. For each compound, mean with the same letters are not significantly different from each other at α=0.05.M-Coumaric acid: P<0.0001, Neochlorogenic acid: P<0.0001, Chlorogenic acid: P<0.0001, Caffeic acid: P<0.0001 and P-Coumaric acid: P= 0.001

3.5.8 Individual flavonols

In 2019, among the individual flavonols, no significant changes in the concentrations of quercetin-3-galactoside and quercetin-3-rhamnoside were observed. Kaempferol-3-glucoside concentration was significantly higher in inoculated brown stem *Va* at 96 hpi. Although changes in the kaempferol-3-glucoside concentration were statistically significant, most of the phenotypes were not different from each other, where inoculated plants did not indicate significant differences when compared to their respective healthy plants (Table 3.8).

In 2020, all the individual flavonols measured indicated significant concentration among the treatments ($p < 0.0155$) (Table 3.9). Although quercetin-3-galactoside, quercetin 3-rhamnoside and kaempferol-3-glucoside concentrations were statistically significant, most of the treatments were not different from each other including the uninfected plants. At the phenotypic level, there was significantly ($p < 0.0001$) higher concentration of quercitin-3-galactoside in *Vm* whereas quercetin 3-rhamnoside and kaempferol-3-glucoside were significantly ($p < 0.0001$) higher in *Va*. Similar to the other phenolic compounds, flavonol concentrations were significantly higher ($p < 0.05$) at 48 hpi followed by 6 dpi.

Table 3.8. Concentration of individual flavonol compounds (mg/g FW) in *B. cinerea* inoculated and healthy wild blueberry flower tissues in 2019

	***Va* brown stem**		***Va* green stem**		***Va* f. nigrum**		***Vm* short**		***Vm* medium**		***Vm* tall stem**	
	48 hpi	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**	**48 hpi**	**96 hpi**
Quercitin-3-Galactoside												
Control	63.5±35.3	65.7±29.1	106.5±6.1	94.9±10.8	63.5±20.1	57.6±32.2	79.8±34.9	67.2±42.1	74.2±11.6	69.5±9.9	49.5±20.9	49.9±21.2
Botrytis cinerea	67.7±49.9	99.2±91.3	115.8±3.1	108.2±10.9	60.3±25.4	62.9±24.5	90.3±23.6	67.5±15.6	40.3±11.7	52.2±6.9	44.7±18.3	49.9±24.3
Quercitrin												
Control	38.1±39.3	7.01±4.3	3.34±0.1	5.89±4.5	4.65±1.1	32.0±28.2	19.7±8.3	19.7 ±9.2	15.9±4.3	14.4±10.8	14.5±9.6	13.4±8.5
Botrytis cinerea	36.8±34.2	36.2±34.3	3.39±0.1	3.81±0.1	4.40±1.4	4.71±1.5	17.7±9.7	16.2±7.6	10.6±1.1	11.6±1.4	13.8±10.0	14.1±8.5
Kaempferol-3-Glucoside												
Control	11.5±6.1 a	4.26±1.7bc	2.35±0.2 c	2.50±1.1 c	3.49±1.3 c	11.5±3.0 c	2.46±1.0 c	1.73±1.3 c	3.52±1.4 c	5.87±3.8bc	2.23±1.5c	2.08±1.3 c
Botrytis cinerea	13.4±6.4 a	11.7±6.6 a	2.35±0.1 c	2.62±0.1 c	3.15±1.6 c	2.33±1.1 c	2.39±0.7 c	3.26±1.0 c	5.29±2.9bc	8.68±4.1ab	2.15±1.4 c	3.28±1.4 c

Mean value (n = 3) ± standard deviation. Mean separation was completed using LSD test procedure. For each compound, mean with the same letters are not significantly different from each other at α=0.05. Kaempferol-3-Glucoside: P<0.0001

Table 3.9. Concentration of individual flavonol compounds (mg/g FW) in *Botrytis cinerea, Bacillus amyloliquefaciens* inoculated and healthy wild blueberry flower tissues in 2020

	V. angustifolium			***V. angustifolium f. nigrum***			***V. myrtilloides***		
	48 hpi	**96 hpi**	**6 dpi**	**48 hpi**	**96 hpi**	**6 dpi**	**48 hpi**	**96 hpi**	**6 dpi**
Quercitin-3-Galactoside									
Control	67.1±7.1 a-d	48.7±8.6 b-i	52.0±11 a-h	19.5±7.2 ij	16.9±10.6 j	18.5±12.2 ij	66.8±20 a-d	53.5±21 a-g	52.6±25 a-h
Botrytis cinerea	65.7±8.9 a-d	55.0±13.6 a-f	56.9±12.2 a-e	27.3±10.1 e-j	24.6±12.6 g-j	22.6±11 hij	80.0±21.9 a	59.2±26. a-d	73.3±22 abc
Bacillus amyloliquefaciens	54.0±12.1 a-g	51.1±14.7 a-h	40.6±16.3 d-j	26.3±8.9 e-j	25.3±8.8 f-j	23.2±12 h-j	76.5±20.1 ab	63.1±30 a-d	55.8±25 a-e
Quercitrin									
Control	12.1±3.4 a-f	11.1±3.5 def	14.4±1.1 a-d	13.1±3.0 a-f	10.3±1.2 ef	11.2±1.5 e-f	11.6±1.5 b-f	10.2±2.7 f	10.0±2.6 f
Botrytis cinerea	15.3±3.7 ab	15.0±2.2 abc	15.7±2.0 a	12.7±3.5 a-f	11.2±2.8 c-f	11.5±2.3 b-f	11.5±1.8 b-f	10.0±1.2 f	10.3±1.1 ef
Bacillus amyloliquefaciens	15.8±2.2 a	14.3±3.2 a-d	13.9±3.4 a-e	13.0±3.5 a-f	10.4±1.5 ef	12.5±2.1 a-f	12.2±1.3 a-f	10.4±1.6 ef	11.6±1.9 b-f
Kaempferol-3-3Glucoside									
Control	4.42±1.5 a	3.34±1.0 ab	3.55±1.3 ab	1.31±0.2 d	1.19±0.02 d	1.26±0.2d	1.30±0.1 d	1.43±0.3 d	1.37±0.4 d
Botrytis cinerea	3.85±1.4 ab	3.73±1.4 ab	4.04±2.0 ab	1.30±0.1 d	1.35±0.2 d	1.68±0.3 cd	1.47±0.2 d	1.19±0.4 d	1.22±0.2 d
Bacillus amyloliquefaciens	3.98±1.5 ab	3.56±1.3 ab	2.99±1.7 bc	1.40±0.1 d	1.40±0.2 d	1.59±0.4 d	1.52±0.4 d	1.22±0.2 d	1.18±0.4 d

Mean value (n = 4) ± standard deviation. Mean separation was completed using LSD test procedure. For each compound, mean with the same letters are not significantly different from each other at α=0.05. Quercetin-3-galactoside: P =0.0002, Quercitrin: P =0.0155 and Kaempferol-3-glucoside: P<0.0001

3.5.9 Physiological responses

In this study, two photosynthetic and related parameters were measured at 6 days (Table 3.10) and 14 days (Table 3.11) after inoculation. Parameters that were recorded were photosynthetic rate, transpiration rate and stomatal conductance.

On the 6th day after inoculation, there was no significant phenotype, treatment or interaction effect on photosynthetic rate, transpiration rate and stomatal conductance. However, treatment and phenotype * treatment interaction revealed a significant effect on transpiration rate and stomatal conductance. *Botrytis cinerea* inoculated plots had the highest transpiration rate and stomatal conductance compared to untreated. Phenotype * treatment interaction was significant, *Va* interaction with *B. cinerea* inoculated had the highest transpiration rate and stomatal conductance (Table 3.10).

Similar to the 6th day, there was no significant phenotype, treatment or interaction effect on transpiration rate and stomatal conductance on the 14th day after inoculation. There was significantly marginal ($p = 0.054$) phenotype effect on photosynthetic rate. *Vaccinium angustifolium* had the highest photosynthetic rate whereas there was no difference between the *Va* f. nigrum and *Vm* plots (Table 3.10).

Table 3.10. Physiological response of different wild blueberry phenotypes 6 days after *Botrytis cinerea* inoculation in 2020.

Photosynthetic rate (A) (μmol m^{-2} s^{-1})			
	Control	***Botrytis cinerea***	Main effect (Phenotypes)
Vaccinium angustifolium	5.45 ns	6.25 ns	**5.85 ns**
***Vaccinium angustifolium* f. nigrum**	5.41 ns	4.31 ns	**4.86 ns**
Vaccinium myrtilloides	6.37 ns	4.75 ns	**5.56 ns**
Main effect (treatment)	**5.75 ns**	**5.11 ns**	
Transpiration rate (E) (mmol m^{-2} s^{-1})			
Vaccinium angustifolium	1.17c	2.03a	**n/a**
***Vaccinium angustifolium* f. nigrum**	1.69ab	1.86ab	**n/a**
Vaccinium myrtilloides	1.46bc	1.47bc	**n/a**
Main effect (treatment)	**n/a**	**n/a**	
Stomatal conductance of CO_2 (gs) (mol m^{-2} s^{-1})			
Vaccinium angustifolium	0.054b	0.138a	**n/a**
***Vaccinium angustifolium* f. nigrum**	0.077b	0.071b	**n/a**
Vaccinium myrtilloides	0.069b	0.077b	**n/a**
Main effect (treatment)	**n/a**	**n/a**	

*Photosynthetic rate (A) p-values: Phenotype- NS, Treatment-NS, Phenotype * Treatment- NS*
*Transpiration rate (E) p-value: Phenotype-NS, Treatment p=0.0160, Phenotype * Treatment p=0.0434*
*Stomatal conductance of CO_2 (Gs) p-value: Phenotype-NS, Treatment- p=0.0076, Phenotype * Treatment- p=0.0018*
ns: not significant (not statistically significant), na: not applicable when there is significant interaction effect.

Table 3.11. Physiological response of different wild blueberry phenotypes 14 days after *Botrytis cinerea* and *B. amyloliquefaciens* inoculation in 2020.

Photosynthetic rate (A) (µmol m^{-2} s^{-1})				
	Control	***Botrytis cinerea***	***B. amyloliquefaciens***	Main effect (Phenotypes)
Vaccinium angustifolium	6.13 ns	8.85 ns	8.96 ns	**7.98a**
***Vaccinium angustifolium* f. nigrum**	7.06 ns	7.07 ns	7.55 ns	**7.23ab**
Vaccinium myrtilloides	5.63 ns	6.37 ns	6.44 ns	**6.14b**
Main effect (treatment)	**6.27 ns**	**7.43 ns**	**7.65 ns**	

Transpiration rate (E) (mmol m^{-2} s^{-1})				
Vaccinium angustifolium	0.736 ns	0.882 ns	0.953 ns	**0.857 ns**
***Vaccinium angustifolium* f. nigrum**	0.756 ns	0.830 ns	0.817 ns	**0.801 ns**
Vaccinium myrtilloides	0.845 ns	1.08 ns	0.792 ns	**0.907 ns**
Main effect (treatment)	**0.779 ns**	**0.932 ns**	**0.854 ns**	

Stomatal conductance of CO_2 (gs) (mol m^{-2} s^{-1})				
Vaccinium angustifolium	0.047 ns	0.062 ns	0.053 ns	**0.054 ns**
***Vaccinium angustifolium* f. nigrum**	0.048 ns	0.050 ns	0.054 ns	**0.051 ns**
Vaccinium myrtilloides	0.044 ns	0.059 ns	0.042 ns	**0.048 ns**
Main effect (treatment)	**0.047 ns**	**0.057 ns**	**0.049 ns**	

*Photosynthetic rate (A) p-values: Phenotype p= NS, Treatment-NS, Phenotype * Treatment -NS*
*Transpiration rate (E) p-value: Phenotype-NS, Treatment – NS, Phenotype * Treatment- NS*
*Stomatal conductance of CO_2 (Gs) p-value: Phenotype-NS, Treatment- NS, Phenotype * Treatment- NS*
ns: not significant (not statistically significant), na: not applicable when there is significant interaction effect.

3.5.10 Yield component and harvestable berry yield

In 2019, significant phenotype and phenotype * treatment interaction effects were observed on yield component (fruit set) in the study (Table 3.12). *Va* phenotypes had the most fruit compared to *Vm* phenotypes except *Vm* tall. Phenotype interaction with untreated control plots had higher fruit set compared to the *B. cinerea* inoculated plots in 2 of the 6 treatments. Unlike fruit set, significant difference was observed in the number of pinheads/unmarketable berries on only phenotype (Table 3.12). *Va* f. nigrum had the most pinheads followed by *Va* green. Significant phenotype * treatment interaction effect was observed on berry yield from experimental plots. Inoculated *Vm* medium and *Va* brown yielded the most berries whereas *Va* green had the least yield. Although phenotype * treatment interaction was significant, most of the treatments were not different from each other (Table 3.12).

In 2020, significant phenotype * treatment interaction effect was observed on fruit set. Phenotypes interaction with untreated control had the most fruit set (Table 3.13). Contrary to fruit set, only phenotype had significant effect on the number of pinheads on. *Va* f. nigrum had the most pinheads whereas there was no difference between the *Va* and *Vm* plots. *Va* yielded the most berries with over 18% more yield compared to *Va* f nigrum and *Vm*. No significant phenotype*treatment interaction effect was observed on yield (Table 3.13). With respect to the treatment applications, untreated control and Botrytis inoculated had similar yield which was higher compared to the *B. amyloliquefaciens* inoculated plots.

Table 3.12. Yield component (fruit set and pinhead) and harvestable berry yield from different wild blueberry phenotypes after *Botrytis cinerea* inoculation in 2019.

Yield component (Fruit set: Number of viable/marketable berries per shoot/stem)			
	Control	**Botrytis inoculated**	Main effect (Phenotypes)
***Va* Brown**	15.2a	8.20cd	**na**
***Va* Green**	13.3ab	11.7abc	**na**
***Va* f. nigrum**	13.5ab	9.50bc	**na**
***Vm* Short**	4.67de	3.70e	**na**
***Vm* Medium**	1.93e	9.86bc	**na**
***Vm* Tall**	15.3a	10.8bc	**na**
Main effect (treatment-value)	**na**	**na**	

Yield component (Pinhead: small, unmarketable berries per shoot/stem)			
***Va* Brown**	0.866 ns	0.800 ns	**0.83c**
***Va* Green**	1.50 ns	2.56 ns	**2.03b**
***Va* f. nigrum**	4.90 ns	4.67 ns	**4.79a**
***Vm* Short**	0.600 ns	0.667 ns	**0.63c**
***Vm* Medium**	0.200 ns	0.733 ns	**0.47c**
***Vm* Tall**	1.03 ns	1.800 ns	**1.42bc**
Main effect (treatment)	**1.52 ns**	**1.87 ns**	

Harvestable yield ($g \cdot m^{-2}$)			
***Va* Brown**	402.2b	631.1a	**na**
***Va* Green**	155.6d	116.7d	**na**
***Va* f. nigrum**	385.6bc	396.7bc	**na**
***Vm* Short**	691.1a	424.4b	**na**
***Vm* Medium**	650.0a	718.9a	**na**
***Vm* Tall**	225.1cd	285.6bcd	**na**
Main effect (treatment)	**na**	**na**	

*Fruit set p-values: Phenotype * Treatment- p <0.0001*
Pinhead p-values: Phenotype-p<0.0001
*Harvestable yield p-values: Phenotype * Treatment. P = 0.0094*
ns: not significant (not statistically significant), na: not applicable when there is significant interaction effect.

Table 3.13. Yield component (fruit set and pinhead) and harvestable berry yield from different wild blueberry phenotypes after *B. cinerea* and *B. amyloliquefaciens* (Serifel®) inoculation in 2020.

Yield component (Fruit set: Number of viable/marketable berries per shoot/stem)				
	Control	**Botrytis inoculated**	***B. amyloliquefaciens* inoculated**	Main effect (Phenotypes)
Vaccinium angustifolium	13.1ab	11.6bc	9.28c	**na**
***Vaccinium angustifolium* f. nigrum**	16.1a	8.60c	11.1bc	**na**
Vaccinium myrtilloides	11.9bc	10.3bc	12.9ab	**na**
Main effect (treatment)	**na**	**na**	**na**	
Yield component (Pinhead: small, unmarketable berries per shoot/stem)				
Vaccinium angustifolium	1.48 ns	0.400 ns	0.650 ns	**0.841b**
***Vaccinium angustifolium* f. nigrum**	2.61 ns	2.88 ns	1.80 ns	**2.43a**
Vaccinium myrtilloides	0.950 ns	0.950 ns	1.85 ns	**1.25b**
Main effect (treatment)	**1.68 ns**	**1.41 ns**	**1.43 ns**	
Harvestable yield ($g \cdot m^{-2}$)				
Vaccinium angustifolium	830.0 ns	640.0 ns	536.7 ns	**670.0a**
***Vaccinium angustifolium* f. nigrum**	540.0 ns	523.3 ns	420.0 ns	**494.4b**
Vaccinium myrtilloides	634.4 ns	656.7 ns	410.0 ns	**566.7b**
Main effect (treatment)	**667.8a**	**607.8a**	**455.6b**	

*Fruit set p-values: Phenotype * Treatment- p=0.0108*

Pinhead p-values: Phenotype-p=0.0001

Harvestable yield p-values: Phenotype p= 0.0014, Treatment p <0.0001

ns: not significant (not statistically significant), na: not applicable when there is significant interaction effect.

3.6 DISCUSSION

3.6.1 Molecular response

In this study, we examined selected candidate genes that had previously been reported in literature to be expressed after pathogen infection, and host interaction with other non-pathogenic microbes. Generally, PR proteins have been reported to be induced in plants during pathogen attacks to improve host plants defense capacity (González et al., 2013; Oliveira et al., 2015; Eichmann et al., 2016). Similarly non-pathogenic microbes including *Bacillus* sp. and plant growth promoting rhizobacteria have been documented to induce several PR proteins in different host plants (Gond et al., 2015; Kim et al., 2015).

PR3 and *PR4* are genes that encode chitinases. Chitinases are known to play an important role in plant defense machinery (Xi et al., 2015) by catalyzing the hydrolysis of chitin, a key structural component of fungal cell walls (Hamid et al., 2013; Patel and Goyal, 2017). In plants, chitinases play a role in the development through their involvement in combating environmental stresses (Kikuchi and Masuda, 2009; Kumar et al., 2018). Given the functions of chitinases, it is not surprising that many studies have reported that chitinase encoding genes (*PR3* and *PR4*) are up-regulated during host-pathogen interaction and some host-nonpathogenic microbe interaction (Cui et al., 2018; Koskimäki et al., 2009; Sridevi et al., 2008; Xayphakatsa et al., 2008). The early expression of *PR3* and *PR4* genes in the *Va* phenotypes, as well as the short and medium stem *Vm* phenotypes in this study agrees with previous studies (Koskimäki et al., 2009; Jose et al., 2021). For instance, Koskimäki et al. (2009) reported the accumulation of *PR4* genes in *V. myrtillus* 12 hours after inoculation with *B. cinerea*. Similarly, Gond et al. (2015) reported the high induction of *PR1* and *PR4* 24 hours after maize seedlings were treated with *B. subtilis.* Also, the expression of defense genes such as *PR10* have been observed in Lilium plants treated with *B.*

amyloliquefaciens (Nakkeeran et al., 2020). Although both *PR3* and *PR4* were weakly induced in this study, the expression of *PR4* was relatively high suggesting that *PR4* might play an important role in the defense of wild blueberry, especially *Va* f. nigrum against *B. cinerea*. The early and relatively high expression of these *PR* genes in *Va* f. nigrum among the phenotypes could partly explain the tolerance of *Va* f. nigrum to Botrytis blight compared to the other *Va* phenotypes (Abbey et al. 2018). Additionally, *PR5* proteins are a group of defense-related proteins known to be induced during host pathogen interaction through the SA pathway (El-Kereamy et al., 2011; Li et al., 2015). These proteins are known as thaumatin-like proteins (TLPs) due to their structural similarity with the fungal inhibiting protein thaumatin isolated from *Thaumatococcus daniellii* (Sudisha et al., 2012). *PR5* proteins are known to exhibit antifungal activity by disrupting the lipid bi-layer and creating trans-membrane pores. Hence, increase in the permeability of the fungal plasma membrane (Jain and Khurana, 2018). The increased expression of *PR5* in this study, agrees with the many studies that have reported increased in the expression of *PR5* in plants (El-Kereamy et al., 2011; Li et al., 2015). Compared to the *PR3* and *PR4*, the *PR5* genes were delayed in their expression. One reason for this could be due to the association of *PR5* genes with biotrophic pathogen through the salicylic acid (SA) signalling pathway while *PR3* and *PR4* are associated with necrotrophic pathogens such as *B. cinerea* through the jasmonate-Ethylene (JA-ET) signaling pathway (Liu et al., 2016).

The inoculation of wild blueberry plants with *B. cinerea* and *B. amyloliquefaciens* resulted in the up regulation of *LOX* genes except with the *V. angustifolium* green stem phenotype. Lipoxygenases (*LOX*) are ubiquitous enzymes commonly found in plants where they play many developmental and plant defense roles. The host defense role of *LOX* is related to the biosynthesis of signaling compounds such as Jasmonic acid (JA), a well-known signaling compound in

necrotrophic pathogen attack in plants (Porta and Rocha-Sosa, 2002; Jannoey et al., 2017). *LOX* catalyzes the oxygenation of polyunsaturated fatty acids to produce unsaturated fatty acid hydroperoxide, using linoleic acid as substrate in the JA biosynthesis pathway. Given the roles of *LOX* in plant defense, it is not surprising that there was an increase in *LOX* mRNA transcripts in most of the phenotypes. Although evidence of LOXs gene responses in plants to nonpathogenic microbes is scarce, this study agrees with Balthazar et al. (2020), who reported that *Pseudomonas* and *Bacillus* sp. strains priming of cannabis seedlings failed to upregulate or enhance the expression of the putative defense genes including *LOX* gene. The findings of this study also agree with many studies that have shown an induction of *LOX* genes during plant-pathogen interactions. For instance, several *LOX* genes in rice plants were found to be induced when inoculated with virulent strains of *Magnaporthe grisea* (Marla and Singh, 2012). Similarly, Oh et al. (2014) reported an increased expression of *LOX* in cucumber after infection with *Sphaerotheca fuliginea.*

Blueberry plants are rich in flavonoids and hydroxycinnamic acids such as flavonols, kaempferol, quercetin, catechins, and caffeic acid and chlorogenic acid respectively. These compounds perform several functions including the protection of plants against harmful radiation and plant defense against pathogens (Cheynier et al., 2013). The biosynthesis of these compounds occurs in the phenylpropanoid pathway and changes in their accumulation are affected by the transcription profiles of the enzyme genes such as *CHS*, *FLS*, *DFR*, *ANR*, *ANS and CCR*. This study reveals that most of the flavonoid biosynthesis genes had similar expression patterns upon *B. cinerea* and *B. amyloliquefaciens*. Many studies have investigated the response of these flavonoid pathway genes in different plants to pathogens (Koskimäki et al., 2009; Jose et al., 2021) and nonpathogenic microbes (Abdelkhalek et al., 2020; Gutiérrez-Albanchez et al., 2020). Rose plant infected with *Podosphaera pannosa* and *Diplocarpon rosae* led to the upregulation of *CHS*,

FLS, *DFR* and *ANS* (Neu et al., 2019). Also, Cedar plants infected with *Gymnosporangium yamadai* resulted in the upregulation of *CHS*, *FLS*, *DFR* and *ANS* (Lu et al., 2017). Up-regulation of *CHS*, *DFR*, *ANS* and *ANR* was reported in *B. cinerea* infected bilberry (Koskimäki et al., 2009). Similarly, Abdelkhalek et al. (2020) revealed that *Bacillus licheniformis* induced *CHS, FLS* and *DFR* in potato plants. Dupont et al. (2015) observed that there was an upregulation of several *CCR* gene isoforms in an endophyte infected rye plant. Results from this study were in some cases consistent with these previous studies. For instance, compared to Koskimäki et al. (2009) the up-regulation of *CHS, FLS*, *DFR, ANS* and *ANR* in this study was minimal, thus the up-regulation following a downregulation in some cases were below or like the basal expression levels. Also, the expression of the flavonol-anthocyanin pathway genes such as *CHS, FLS, ANS, ANR* and *DFR* were found to be higher in controls plants than *B. amyloliquefaciens* treated blackberry plants (Gutiérrez-Albanchez et al., 2020). Similarly, the expression *CCR* was found to be downregulated when endophytic *Bacillus altitudinis* WR10 was applied to wheat plants (Yue et al., 2021). Likewise, when the endophytic bacterium *Paraburkholderia phytofirmans* was applied to leaves of rice, a decrease in the expression of *CCR* was reported (King, 2019). Similarly to Lu et al. (2017), there was an initial decrease in transcript levels of *CHS*, *FLS* and *ANS* in almost all the phenotypes at 12 hpi. The early decrease in the expression of flavonoid genes in this study could partly be attributed to the circadian rhythm in the plants (Backes et al., 2021). Backes et al. (2021) found that several genes including *CHS*, flavonoid 3′-hydroxylase (*F3'H*), and *CCR* were subjected to a circadian regulation. Ni et al. (2018) indicated that circadian rhythms affected the flavonoid contents in Ginkgo leaves, where transcriptome results revealed a decrease in flavonoid gene expression in samples collected in the night. In this study, it is important to note that the 12 hpi

samples were collected in the night (9 -10 pm), which could potentially explain the consistent decrease in the expression of the flavonoid genes at 12 hpi.

In addition to the flavonoid pathway genes, this study aimed to explore whether *B. cinerea* infection leads to significant changes in phenolics as part of the wild blueberry defense mechanism. Significant variation in the concentration of phenolic compounds in inoculated (either *B. cinerea* or *B. amyloliquefaciens*) and control plants revealed differential behavior which is compound and phenotype dependent. The accumulation of phenolic compounds in plants, especially flavonoids as a component of defense mechanism against pathogens has been described by many studies (Koskimäki et al., 2009; Wallis and Galarneau, 2020). Mikulic-Petkovsek et al. (2014) found that *Didymella applanata* Sacc. and *Leptosphaeria coniothyrium* Sacc. infected raspberry increased specific phenolic compounds, such as flavanols. In Santin et al. (2018), *Monilinia fructicola* infected peach resulted in increased total phenolics and flavonols. Additionally, Koskimäki et al. (2009) reported that *B. cinerea* infected bilberry plants contained higher levels of flavanols, flavonols and hydroxycinnamic acids. Regarding nonpathogenic microbes, *B. subtilis* was recently documented to enhance the production of flavonoids and other phytoalexins in tomato plants (Pretali et al., 2016). Flavonoids are important compounds in blueberries (Borges et al., 2010; Guofang et al., 2019), and many studies have reported their accumulation and role as physiological regulators, chemical messengers, and inhibitors against biotic and abiotic stress (Zhou et al., 2014; Lu et al., 2017). Inoculation of wild blueberry flowers with *B. cinerea* and *B. amyloliquefaciens* resulted in the accumulation of some flavanols, flavonols and hydroxycinnamic acid in this study. As to the individual phenolic compounds, particularly *m*-coumaric acid, and kaempferol-3-glucoside were increased with *B. cinerea* inoculation. The results of this study agree with previous findings of the accumulation of phenolic compounds in infected plants, particularly flavonols and

flavanols (Taware et al., 2010; Chowdhury et al., 2015; Vagiri et al., 2017; Wallis and Galarneau 2020). Also, the lower contents of these flavonoids and in some cases lower that the control plants agree with Gutiérrez-Albanchez et al. (2020). In their study, Gutiérrez-Albanchez et al. (2020) reported higher flavonoid and phenolic acids such as catechin, kaempferol, kempherol-3-O-glucoside, quercetin, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, epicatechin in controls than in *B. amyloliquefaciens* QV15 treated blackberries, except for kaempherol-3-O-rutinoside. Interestingly, some of the hydroxycinnamic acids had decreased concentration in both inoculated plants. Nonetheless, these finding corroborates the report of some previous studies (Mikulic-Petkovsek et al., 2014; Kunej et al., 2020). This observation in hydroxycinnamic acids may be due to their naturally high abundance in blueberry or their role as a substrate in the biosynthesis of some complex phenolics, such as lignin and suberin (Vanholme et al., 2019). Hydroxycinnamic acids, particularly chlorogenic acid was the most abundant phenolic observed in this study which may suggest that they form part of pre-formed biochemical defense in wild blueberry. Given their abundance, a further increase in their concentration during pathogen attacks might not be essential.

Molecular and plant defense response events can be triggered by a variety of abiotic or biotic factors. Given that this study was conducted under field conditions and on a perennial plant, the wild blueberry plants were in constant interaction with the environment, which may account for the relatively low levels of differences in gene expression levels and seeming fluctuation pattern for some of the genes and phenolic compounds. Studies have demonstrated that in the field, plants are partly induced through their interaction with both biotic and abiotic factors. Pasquer et al. (2005) found that the expression of defense genes was already at a high level in wheat plants before the application of defense elicitors (benzo (1,2,3) thiadiazole-7-carbothioic acid s-methylester, BTH) under field conditions. Also, Herman et al. (2007) found that different

cultivars exhibited near-baseline expression levels of defense genes when plants were initially induced with acibenzolar-*S*-methyl (ASM). Furthermore, given the induction of flavonoid genes in bilberry by the endophyte, *Paraphaeosphaeria* sp. (Koskimäki et al., 2009), one will not rule out their potential contribution to the variation in flavonoid genes expression on the field. Additionally, environmental factors such as light and temperature have been reported as important elements that affect flavonoid pathway genes (Zoratti et al., 2014; Schulz et al., 2015). Azuma et al. (2012) reported that low temperature and light intensity have a synergistic effect on the expression of genes that are involved in flavonoid biosynthesis. Given the complexity of the environment and the perennial nature of the plants, the major determinant of this variation cannot be easily identified. Nonetheless, it is worth noting that despite the basal expression of these defense and flavonoid genes, some of the genes were significantly upregulated over the different time points, suggesting the potential involvement of these genes in wild blueberry plant defense against *B. cinerea*.

The variation in the phenolic response in this study could be due to natural variation in the field and environmental conditions. Environmental factors such as light, radiation and temperature have been reported to affect secondary metabolism in fruits including *Vaccinium* spp. (Karppinen et al., 2016). The variation in phenolic compounds is not surprising because many studies have also reported significant phenolic variation within and among different cultivars (Mikulic-Petkovsek et al., 2014; Guofang et al., 2019). Although the difference between inoculated and control plants was observed for some compounds, phenolic changes among the various phenotypes mostly did not show any statistical significance as observed with the expression of the flavonoid genes. The accumulation of flavonoids is governed by a complex network of genes in the phenylpropanoid pathway and regulatory genes (Falcone Ferreyra et al., 2012), hence, under such

complex study conditions, similarity in the variation between the flavonoid genes and the flavonoid compounds is noteworthy.

Results from this study reveal a difference between the expression levels and response time among the phenotypes, indicating a phenotype-specific response mechanism to the *B. cinerea* and *B. amyloliquefaciens*. The more susceptible *Va* phenotypes responded to both *B. cinerea* and *B. amyloliquefaciens* inoculation earlier (mostly at 12 hpi) than *Vm*, which mostly showed upregulation at 24 hpi. Interestingly, this finding contradicts previous research, which have found that resistant cultivars exhibit early responses with mostly high expression levels of defense-related genes upon pathogen infection (Silvar et al., 2008; Sun et al., 2019). The reason for this is unknown, however, this could partly be related to *Vm's* morphological and physical features. *Vm* is covered with pubescence/hair-like structures (Kinsman, 1993), which have the potential to interfere with direct plant surface contact by conidia. This could potentially delay pathogen perception and defense response activation in *Vm*. Although there was a difference in the gene expression pattern, the transcript levels among the various phenotypes did not indicate any statistical significance. One reason might be the low expression levels observed. In addition, the wide variation observed on wild blueberry fields, even within the same phenotypes could contribute to the non-significance observed among the phenotypes. Although *Vm* and *Va* phenotypes had similar expression values, it is worth noting the difference in ploidy between the two groups. Polyploid species tend to have greater outcomes during genome duplication (Zhang et al., 2019; Zhang et al., 2020). Hence, coupled with its unique morphological features and late flower bud development, theoretically doubling the expression levels in the *Vm* phenotypes could show strong up-regulation of the various genes to possibly explain why *Vm* is less susceptible to pathogens.

3.6.2 Physiological response

To detect whether the biological interactions between the studied microbes and wild blueberry causes physiological changes we evaluated three physiological measurements which included photosynthetic rate (A), transpiration rate (E), and stomatal conductance (Gs). Disease development has been reported to reduce net photosynthetic rate due to reduction in leaf surface area (Mandal et al., 2009). Also, other physiological processes such as stomatal conductance, respiration and transpiration are mostly known to increase during disease development (Withers et al., 2011). In contrast, some studies have also reported a decrease in these physiological parameters (Zhao et al., 2011). These contradictions suggest that some of these physiological processes maybe dependent on factors such as the type of pathogen (necrotroph or biotroph) and temperature (Lindenthal et al., 2005) and sometimes other physiological processes. Although the physiological measurements in this study at 6 dpi and 14 days after inoculation were not significant, except transpiration rate at 6dpi, our results were largely consistent with the study of Endeshaw et al. (2012). The stable or insignificant changes in physiological changes observed in this study could be due to the asymptomatic nature of the wild blueberry leaves. *Botrytis cinerea* hardly infects leaves and in this study, none of the leaves from the infected plant showed any disease symptom or leaf destruction which may explain the insignificant differences. This result suggests that Botrytis floral blight might not impact these physiological processes investigated.

3.6.3 Yield Component and Harvestable berry yield

In this study, the inoculation of wild blueberry *B. cinerea* and *B. amyloliquefaciens* generally had a significant impact on set fruit and berry yield. Untreated control and its interaction with phenotype had the most set fruit and berry yield compared to the inoculated plants. This can be

attributed partly to the effect of disease development which reduces yield through flower destruction. The activation of defense mechanisms beyond basal/constitutive levels comes at the expense of other plant developmental features. Previous studies have demonstrated the existence growth–defense trade-off associated with host defense activation (Huot et al., 2014). For instance, priming for defense in Arabidopsis strongly affected some fitness parameters (relative growth rate and seed production) (van Hultenet al., 2006). This growth-defense trade-off could potentially explain why untreated control plants had the most yield compared to the *B. cinerea and B. amyloliquefaciens* inoculated plots.

3.7 CONCLUSION

Understanding the molecular mechanism employed by wild blueberry against *B. cinerea* infection is important for sustained wild blueberry production and the development of disease control tools. In this study, the inoculation of wild blueberry by *B. cinerea* and *B. amyloliquefaciens* was characterized by phenotype-specific increased expression of *PR* genes which suggests their potential involvement in wild blueberry defense machinery. Additionally, a most common response of downregulation of flavonoid genes was observed followed by a weak upregulation. Also, our results indicate that the induction and accumulation of phenolic compounds in *B. cinerea* and *B. amyloliquefaciens* inoculated flowers might be temporal and phenotype dependent. This study may provide insight into the wild blueberry defense mechanism and serve as a starting point for achieving a better understanding of the wild blueberry-*B. cinerea* pathosystem and the path to incorporate induced resistance as defense strategies in wild blueberry production.

CHAPTER 4: BOTRYTIS BLIGHT MANAGEMENT AND FUNGICIDE MOBILITY AND PERSISTENCE IN FLORAL TISSUE OF WILD BLUEBERRY

4.1 ABSTRACT

Botrytis blossom blight disease is one of the major constraints to wild blueberry production. In this study, the effect of different fungicide treatments on Botrytis blight development, and fungicide mobility and persistence within the flower tissues, and berry from wild blueberry were evaluated under field conditions. Multi-year field trials which involved the applications of Switch®, Luna Tranquility®, Merivon® Xenium, Propulse®, and Miravis® Prime at 7-10-day interval were conducted and fungicide residues in plant samples were assessed. Quantification of the fungicide in floral and berry tissues was conducted using a modification of the QuEChErs extraction method and GC-MS analysis. All the treatments except Switch® reduced the final disease incidence and severity by at least 78 and 40 %, respectively. Similarly, Switch® and Miravis® Prime treatments reduced disease incidence and severity by at least 64 and 67 %. Luna Tranquility®, Merivon® Xenium, and Propulse® reduced incidence and severity by over 47 and 51 %, respectively. Berry yields were higher in plots treated to Switch®, Luna Tranquility® and Miravis® Prime with at least a 19% increase in yield compared to the untreated control. The developed QuEChERS method for the GC-MS analysis was suitable and could satisfactorily be applied for the analysis of blueberry matrix. The mean concentration of all the fungicides was higher in corolla than in gynoecium and the androecium, an indication of limited mobility. Fungicides were persistent and concentrations were sufficient to suppress *B. cinerea* at fruit set. No residue was detected in harvested berries.

4.2 INTRODUCTION

Botrytis blight (caused by *Botrytis cinerea*) is one of the major constraints to wild blueberry production with over 20% yield loss recorded annually. The management of the disease is highly dependent on fungicide application. The main fungicide presently used for Botrytis blight control in wild blueberry is Switch®, which contains the signal transduction and amino acid inhibitors fludioxonil and cyprodinil, respectively. Other fungicides known for Botrytis disease management include Luna Tranquility® (fluopyram and pyrimethanil), Fontelis® (penthiopyrad) and Pristine® (pyraclostrobin and boscalid). Despite the wide usage of fungicides, the ability to effectively protect blueberry flowers with the present fungicides remains challenging. *Botrytis cinerea* is a high-risk fungus regarding resistance development (FRAC, 2019), hence, loss of fungicide efficacy has been reported in several crops (Latorre and Torres, 2012; Hahn, 2014; Grabke et al., 2013). Similarly, resistant *B. cinerea* isolates from wild blueberry fields have also been detected for some of the commonly used fungicides (Abbey et al., 2018). These coupled with the pendulous flower orientation, and limited fungicide mobility contribute significantly to the challenge of effectively protecting wild blueberry flowers. Additionally, the inferior and the bell structure of the wild blueberry flower prevents direct contact of the androecium and the pistil of sprayed fungicides. Also, with the inferior nature of the flower, the corolla which receives the applied fungicides does not form part of the resulting berry but senescence after pollination. Given these limitations associated with the flower and existing disease management strategies, the search for an improved disease management approach that can adequately protect the entire blueberry flower very is important.

New fungicides are being developed to enhance disease management in various crops. Adepidyn (trademark for the active ingredient pydiflumetofen, FRAC 7) is the new carboxamide

fungicide discovered by Syngenta and is the first member of a new chemical group among the succinate dehydrogenase inhibitor (SDHI) fungicides, the phenyl-ethyl pyrazole carboxamides (FRAC, 2020). The compound is found to be highly effective against difficult pathogens such as *Botrytis cinerea* and *Sclerotia sclerotiorum* (Sierotzki et al., 2017). Also, fluxapyroxad, an SDHI which belongs to the carboxamides group was recently introduced into the wild blueberry production system. As new additions to disease management in wild blueberries, adepidyn is co-formulated with fludioxonil while fluxapyroxad is co-formulated with pyraclostrobin as commercial products. As new products, their efficacy for disease control in commercial wild blueberry fields has not been reported.

For every fungicide applied, its distribution, mobility and persistence are very important. Their efficacy is dependent on full coverage of target tissues, therefore, in wild blueberries where flower structure/orientation limits foliar application coverage, effective disease management may become a challenge. Most fungicides registered for Botrytis control in wild blueberry are locally systemic, hence, their mobility within plant tissues is greatly limited (Beckerman, 2018). The degree of active ingredient redistribution, persistence and the efficacy of the common fungicides including the new ones such as pydiflumetofen has not been investigated in wild blueberry plants. Additionally, food safety is a major public concern and with the potential harmful effect of pesticides on humans and the environment, countries have established specific maximum residue levels (MRL) of pesticides allowed in fruits and vegetables. Reports on the presence of pesticide residues in different fruit/vegetables including blueberries from previous studies are available (Yang et al., 2011; Munitz et al., 2013, 2014). With more than 90% of wild blueberry exported to Europe, United States, and Asia, assessing the residues status of the commonly used and newly registered Botrytis control fungicide products in harvested berries is important.

The analysis of pesticides in food involves sample preparation and instrumental detection/quantitation. In recent times, the QuEChERS (quick, easy, cheap, effective, rugged and safe) method has been accepted by the scientific community due to its ability to extract multi residues from foods and environmental samples with little modification (Banerjee et al., 2012; Wang et al., 2014; He et al., 2015). The QuEChERS method is flexible and lends itself to several instrumental analyses. Many studies have used different instruments and detectors ranging from LC-UV to GC-MS/MS. However, the majority of recent QuEChERS related research employed LC–MS/MS and GC–MS/MS due to the high selectivity provided by MS/MS (Gilbert-López et al., 2010; Melo et al., 2020). The objectives of this study were to evaluate the efficacy of selected fungicides on Botrytis control in wild blueberries and to investigate the distribution, mobility and persistence of these fungicides within wild blueberry floral tissues. Additionally, we assessed the presence of these fungicides and their metabolites in harvested berries.

4.3 MATERIALS AND METHODS

4.3.1 Site selection and experimental design

Two research trials were conducted in 2018 at Debert, Murray Siding and Fox Point, NS. Two more research trials were established in 2019 at two commercial wild blueberry fields at Debert and Mount Thom, NS. Fields for the experiments were equipped with Watchdog® model 2700 weather station (Aurora, IL, USA) to monitor air temperature, relative humidity, leaf wetness, wind speed, and direction every 15 minutes for the duration of the trial. A randomized complete block design with six replications was used. The plot size is 4 × 6 m with 2 m buffers between plots. Six treatments and two application of each treatment was made: (1) untreated control; (2) Switch® (a.i. cyprodinil and fludioxonil); (3) Luna Tranquility® (a.i. fluopyram and pyrimethanil);

(4) Merivon® Xenium (a.i. fluxapyroxad and pyraclostrobin); (5) Propulse (a.i. fluopyram and prothioconazole); and (6) Miravis® Prime (a.i. pydiflumetofen and fludioxonil).

4.3.2 Treatment application

First fungicide applications were made at 10% bloom prior to visual symptoms of Botrytis and the second fungicide applications were made 7-10 days after the first applications. Fungicides were applied using a hand-held CO_2 research sprayer (Bell spray Inc.) with a 2 m boom equipped with 4 Tee Jet Visiflow 8002VS nozzles at a pressure of 32 PSI (220 kPa). The volume application rate used was 250 L/ha. The recommended doses of each fungicide used are listed in Table 4.1.

Table 4.1. Fungicides and their application rate

Products	**Active ingredients**	**Product application Rates**	**Amount of active ingredient applied**
Untreated Control			
Merivon® Xenium	Fluxapyroxad & pyraclostrobin	350 ml/ha	Fluxapyroxad 87.5g/ha Pyraclostrobin, 87.5g/ha
Propulse®	Fluopyram & prothioconazole	750 ml/ha	Fluopyram, 150 g/ha Prothioconazole, 150 g/ha
Miravis® Prime	Pydiflumetofen & fludioxonil	800 ml/ha	Pydiflumetofen 120 g/ha Fludioxonil, 200 g/ha
Switch ®	Cyprodinil & fludioxonil	975 g/ha	Cyprodinil, 156 g/ha Fludioxonil, 234 g/ha
Luna Tranquility®	Fluopyram & Pyrimethanil	1.2 L/ha	Fluopyram, 150 g/ha Pyrimethanil, 450 g/ha

4.3.3 Sample collection, disease assessment, yield component and berry yield

Samples for fungicide mobility and persistence was done by collecting 50 stems at 15 cm intervals across each plot 24 hours after the initial and last fungicide applications and 10 days after the last

fungicide application (fruit set). Stems were kept in a cooler with ice and then brought to the lab where floral tissues were separated into corolla and gynoecium (ovary, style, stigma), and androecium (anther, filament).

Fifteen blueberry stems were randomly selected seven days after the first fungicide application and 14 days after the second fungicide application for disease assessment. The stems were cut diagonally at 20 cm intervals along a 4 m line transect in each plot. The stem samples were placed in plastic bags and taken to the laboratory for assessment of Botrytis disease development (incidence and severity). Disease incidence was determined as the proportion of floral buds with visual symptoms of Botrytis blight within a stem expressed as a percentage. Disease severity was assessed as the percentage of floral tissue area infected with visual symptoms of Botrytis blight on a stem. A 0–7 disease severity rating scale was used where 0 = no symptoms, healthy plants; 1 = 0–5% affected flower area; 2 = 5–15% affected flower area: 3 = 15–35% affected flower area; 4 = 35–65% affected flower area; 5 = 65–85% affected flower area; 6 = 85–95%; 7 = 95–100% affected flower area (Smith, 1998). The data were expressed as a percentage of affected flower area (disease severity).

Yield components (number of fruit set per stem) were measured in early August by randomly selecting 15 stems per plot. Berries were harvested in August with a forty-time 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 were recorded.

4.3.4 Analysis of fungicide residue using the 'quick, easy, cheap, effective, rugged, and safe' (QuEChERS) method and detection by GC-MS

4.3.4.1 Chemicals and standards preparation

External standards of cyprodinil, fludioxonil and pydiflumetofen were obtained from Syngenta, Canada, fluxapyroxad and pyraclostrobin from BASF Canada, fluopyram, pyrimethanil, prothioconazole, and prothioconazole-desthio were obtained from Bayer Crop Science (Kansas City, USA), and triphenyl phosphate was obtained from Acros Organics, Germany. Analytical grade acetonitrile, and toluene (Fisher Scientific, ON, Canada), and formic acid (> 98%) were purchased from Sigma- Aldrich. Anhydrous magnesium sulphate ($MgSO_4$) (Sigma- Aldrich), sodium acetate (NaOAc), (Fisher Scientific), and primary-secondary amine (PSA, Cole Parmer, USA) for this analysis.

4.3.4.2 Preparation of standard solutions and calibration curves

At least 5 mg of the chemical standards were dissolved in toluene to make a 1 mg/ml stock solution and stored at -18 ºC. Calibration standards were prepared by an appropriate dilution of stock solution in toluene. Six different concentrations (0.005, 0.01, 0.02, 0.05, 0.5 and 1.0 µg/mL) of each compound were prepared to generate calibration curves. Another set of calibration standards was prepared in blank extracts of wild blueberry flower. Standard curves were generated using linear regression (R^2 of each standard curve was > 0.98).

4.3.4.3 Extraction and analysis of phenolic compounds.

Sample preparation was carried out according to the method described by AOAC 2007.1 with modification (Lehotay, 2007; Walorczyk, 2014; David et al., 2016). Homogenized berry sample (ripped berry and set fruit at 10 days post fungicide application) 15 g was weighed into a 50 ml

centrifuge tube and 15 ml of 1% formic acid in acetonitrile was added. Internal standard (TPP), 75 µl at 150 µg/ml was then added and the tube was vigorously shaken by hand and vortex for 5 min. A buffer-salt mixture consisting of 6 g $MgSO_4$ and 1.5 g NaOAc was added to the tube and vigorously for 5 min. The tube was then centrifuged for 5 min at > 4300 rcf and the supernatant was transferred to a new tube for dispersive SPE clean up.

For the flower sample, 100 mg of ground sample was weighed into a 2 ml Eppendorf tube and 0.5 ml of distilled water was added to hydrate the sample for 5 mins.0.6 ml of 1% acetic acid in acetonitrile was added. Internal standard (TPP), 9 µl at 50 µg/ml was then added to the tube and vortexed for 2 mins. A buffer-salt mixture consisting of 200 mg $MgSO_4$ and 50 mg NaOAc was added and vortexed for 2 mins. The tube was then centrifuged for 2 min at > 15000 rcf and the supernatant was transferred to a new tube for dispersive SPE clean up.

4.3.4.4 Clean up: Dispersive SPE with PSA (amino sorbent)

For berry and fruit set samples, acetonitrile extract (4 ml) was transferred to a polypropylene disposable centrifuge tube containing 200 mg PSA (50 mg/ml extract), 600 mg $MgSO_4$ (150 mg/ml extract) and 200 mg GCB (50 mg/ml extract). The tube was shaken for 30 sec. and centrifuged for 1 min at > 4300 rcf. An aliquot (2 ml) of the supernatant was transferred into a clean tube. The sample was evaporated under a gentle stream of nitrogen to approx. 0.3 µl at 30 ºC. The sample extract was reconstituted 1 ml with toluene and passed through a 0.45 µm nylon filter for analysis on GC-MS.

For flower samples, acetonitrile extract (0.4 ml) was transferred to a 1.5 ml centrifuge tube containing 20 mg PSA, 60 mg $MgSO_4$ and 20 mg activated carbon (for samples containing cyprodinil and pyrimethanil, activated carbon was excluded to prevent their adsorption by the

carbon). The tube was shaken for 30 sec. and centrifuged for 1 min at >15000 rcf and an aliquot (0.35 ml) of the supernatant was transferred into a clean tube. The extract was evaporated under a gentle stream of nitrogen at 30 ºC reconstituted in 0.5 ml of toluene and passed through a 0.45 µm nylon filter for analysis on GC-MS.

4.3.4.5 Gas chromatographic mass spectral conditions

Using similar conditions as described previously (David et al., 2015; Cho et al., 2016), the analysis was performed on a Scion 456A GC–triple-quadrupole mass spectrometer (Bruker, Scion Instrument, Amundsenweg The Netherlands). Injection of 2 µl (split of 1:20) was made using a Bruker autosampler (Bruker, Scion Instrument, Amundsenweg The Netherlands). The GC separation was conducted on a 30 m × 0.25 mm × 0.5 µm capillary column. Helium (99.9% purity) at a flow rate of 1.2 mL/min was used. The oven temperature was programmed as follows: Start at 80 °C (hold for 1 min), increase to 180 °C at the rate of 25 °C/min (hold for 1 min), then increase to 310 °C at 10 °C/min (hold for 5 min). The ion source and MS transfer line temperatures of 280°C were used. Electron ionisation energy of 70 eV was used. Selected ion monitoring (SIM) mode detecting 2-3 ions for each analyte was used.

4.3.4.6 Method validation

The method validation was performed according to the European Union guidance criteria on analytical quality control and validation procedures for pesticide residues analysis in food and feed (SANTE/12682/2019 (2020)

Recovery study

Blank/control samples were spiked at three levels 0.01, 0.05 and 0.25 mg/kg and extracted according to the QuEChERS procedure described above. Average recovery and relative standard deviation (% RSD) values per spiking level and the overall value will be calculated for each fungicide. Matrix matched calibration standard curve was used to calculate the analyte recoveries (Ly et al., 2020; Mahdavi et al., 2021).

Linearity, limits of quantification (LOQ) and limits of quantification (LOQ)

Linearity of calibration curves was studied by analyzing six calibration solutions concentrations of 0.005, 0.01, 0.02, 0.05, 0.2 and 0.5 μg ml^{-1} (n=3), both in pure solvent and in blueberry extracts. The LOD and LOQ were determined from the signal-to-noise ratio (S/N). They were determined as the lowest concentration of the analytes of a chromatographic peak, where LOD = 3 x S/N and LOQ = 10 x S/N (Hrouzková and Szarka, 2021; Kang et al., 2020).

Precision

Precision was analyzed as intra- (repeatability) and inter-day (intermediate) and expressed as relative standard deviation (RSD %). The three spiked samples from the recovery study were analyzed for repeatability (six replicates). Inter-day precision was studied at the same concentration levels by processing spiked samples on three different days within a week.

Evaluation of matrix effects

The effect of the matrix of tissue extracts will be evaluated by comparison of the slopes of the calibration curves in solvent only and in the matrix. The % increase or decrease of the matrix

matched calibration curve was measured in relation to the solvent-only curve. In terms of slope ratios: $\%ME=100\% \times (1 - \text{slope}_{\text{pure solvent}}/\text{slope}_{\text{extracts}})$

4.3.5 STATISTICAL ANALYSIS

Data collected on disease development, yield components, and harvested berry were analyzed using the PROC GLIMMIX procedure of SAS (version 9.4, SAS institute, Inc., Cary, NC). Minitab version 19 was used for the analysis of residue concentration in samples using one-way ANOVA and repeated measures for flower samples. LSD was used for multiple means comparison at $\alpha=0.05$. Prior to the analysis, the data set was subjected to normality test. Residue concentrations from flower samples were transformed using square root.

4.4 RESULTS

Botrytis blight pressure was low in the two trials in 2018 with 8.70, and 11.20 % of the total stems (n=540) collected from Fox Point and Murray siding respectively after 1st fungicide application was infected whereas 6.40 and 2.89 % of the total stems were infected after 2nd fungicide application. In contrast to 2018, high disease pressures were observed in 2019 with 14.4 and 38.8 % of stems infected at Debert and Mt Thom, respectively after the 1st application and 3.01 and 7.0 % infected after 2nd application.

In 2018 after the 1st fungicide application at Fox Point, Switch®, Luna Tranquility® and Miravis® Prime significantly suppressed incidence and severity by over 92 and 90 %, respectively whereas Propulse® reduced incidence by 67.9 % and severity by 65.3 % (Table 4.2). Merivon® Xenium provided complete disease control.

After the 2nd fungicide application, Switch®, Luna Tranquility®, Merivon® Xenium and Miravis® Prime reduced incidence and severity by over 78 and 40 % (Table 4.2). At Murray siding Propulse® and Miravis® Prime reduced incidence by 80.2 and 69.3% respectively. Disease severity was reduced by all the treatments by more than 64 % compared to the untreated control (Table 4.2). After the 2nd fungicide application, all the fungicide treatments reduced disease incidence and severity by more than 80% except Switch® (Table 4.2).

In 2019, all the treatments reduced disease incidence and severity by over 76 and 57 %, respectively after the 1st fungicide application at Debert (Table 4.3). All the treatments significantly reduced disease incidence and severity by over 64 and 67 %, respectively compared to the untreated control after the 2nd fungicide application. At Mt Thom, all the treatments reduced disease incidence and severity by more than 69 and 80 %, respectively compared to the untreated (Table 4.3). After the 2nd fungicide application, Miravis® Prime and Switch® significantly reduced incidence by 78.7 and 71.6 %, and severity by 81.3 and 76.2 %, respectively. Luna Tranquility®, Merivon® Xenium, and Propulse® reduced disease incidence and severity by over 47 and 51 %, respectively (Table 4.3).

At Fox Point, there was a significant treatment effect on set fruit per stem, however, Luna Tranquility®, Merivon® Xenium and Propulse® had fewer set fruit and no treatment increased fruit set compared to the untreated control (Table 4.4). At Murray Siding, all the fungicide treatments had more pinheads per stem than the untreated control except Propulse® (Table 4.5). Although there was a significant treatment effect on berry yield at Murray Siding, most of the treatments had lower yield than the untreated control except Miravis® Prime which improved yield by 19.3 % (Table 4.6).

In 2019, there was a significant treatment effect on yield components and harvestable berry yield in both trials except for set fruit at Mt Thom. At Debert, Switch® resulted in higher set fruit (Table 4.4). Interestingly, Switch®, Luna Tranquility®, Miravis® Prime and Propulse® resulted in high pinheads per stem compared to the untreated control (Table 4.5). Switch® and Luna Tranquility® applications resulted in a 36.3 and 32 % yield increase, respectively compared to the untreated control at Debert (Table 4.6). At MT Thom, Switch®, Luna Tranquility®, Propulse®, and Miravis® Prime increased yield by 22, 25.7, 43.5 and 20.2 %, respectively (Table 4.6).

Table 4.2. Botrytis blight incidence and severity observed in wild blueberries at Fox Point and Murray Siding, Nova Scotia, after fungicide applications in 2018.

	Fox Point				**Murray Siding**			
	1st application		**2nd application**		**1st application**		**2nd application**	
Treatment	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**
Untreated Control	12.7 a	6.66 a	4.22 b	1.86 ab	11.9 a	7.44 a	3.35 a	2.16 a
Switch®	0.15 c	0.15 c	0 c	0 c	6.21 ab	2.61 b	3.18 a	1.33 ab
Luna Tranquility®	0.69 c	0.44 c	1.58 c	1.11 bc	6.10 ab	1.98 b	0 b	0 b
Merivon® Xenium	0 c	0 c	0 c	0.00 c	7.99 ab	2.60 b	0 b	0 b
Propulse®	4.07 b	2.31 b	6.80 a	3.51 a	2.37 b	1.22 b	0 b	0 b
Miravis® Prime	0.95 bc	0.61 bc	0.13 c	0 c	3.67 b	0.59 b	0.66 b	0.16 b
ANOVA [a]	**P < 0.0001**	**P < 0.0001**	**P < 0.0001**	**P < 0.0001**	**P = 0.05**	**P< 0.0001**	**P =0.002**	**P =0.003**

[a] 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. Data in a column with the same letters are not significantly different at α=0.05. Inc (%): Percent disease incidence - determined as the proportion of floral buds with visual symptoms of Botrytis blight within a stem/shoot. Sev (%): Percent disease severity- assessed as the percentage of floral tissue area infected with visual symptoms of Botrytis blight on a stem/shoot.

Table 4.3. Botrytis blight incidence and severity observed in wild blueberries at Mount Thom and Debert, Nova Scotia, after fungicide applications in 2019.

	Mount Thom				Debert			
	1st application		2nd application		1st application		2nd application	
Treatment	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**
Untreated Control	45.4 a	43.4 a	11.5 a	10.6 a	20.2 a	9.90 a	3.57 a	1.31 a
Switch®	12.9 cd	10.3 c	0.92 b	0.46 b	4.42 b	1.32 b	0.60 b	0.05 b
Luna Tranquility®	21.0 bc	19.3 b	0.37 b	0.06 b	6.26 b	3.20 b	0.32 b	0.17 b
Merivon® Xenium	23.9 b	20.9 b	0.53 b	0.09 b	7.12 b	2.05 b	0.56 b	0.56 ab
Propulse®	16.9 bcd	15.1 bc	3.56 b	2.04 b	3.20 b	0.91 b	0.83 b	0.11 b
Miravis® Prime	9.66 d	8.09 c	0.96 b	0.24 b	3.87 b	1.14 b	0.64 b	0.04 b
ANOVA [a]	**P < 0.0001**	**P < 0.0001**	**P < 0.0001**	**P < 0.0001**	**P <0.0001**	**P < 0.0001**	**P=0.041**	**P=0.05**

[a] 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. Data in a column with the same letters are not significantly different at α=0.05.
Inc (%): Percent disease incidence - determined as the proportion of floral buds with visual symptoms of Botrytis blight within a stem/shoot. Sev (%): Percent disease severity- assessed as the percentage of floral tissue area infected with visual symptoms of Botrytis blight on a stem/shoot

Table 4.4. Yield component (fruit set: number of viable/marketable berries per shoot/stem) observed from wild blueberry field after fungicide applications.

	2018		2019	
Treatment	**Fox Point**	**Murray Siding**	**Mount Thom**	**Debert**
Untreated Control	6.64 a	3.96	6.11	7.21 b
Switch®	5.80 ab	3.54	8.08	9.04 a
Luna Tranquility®	4.93 b	4.74	8.59	7.04 b
Merivon® Xenium	4.47 b	4.02	6.17	7.10 b
Propulse®	4.51 b	3.17	6.90	5.95 b
Miravis® Prime	5.15 ab	4.56	7.86	6.53 b
ANOVA[a]	**P**=0.0274	NS	**NS**	**P**=0.0162

[a] 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. Data in a column with the same letters are not significantly different at α=0.05.

Table 4.5. Yield component (Pinheads: number of small unmarketable berries per shoot/stem) observed from wild blueberry field after fungicide applications.

	2018		2019	
Treatment	**Fox Point**	**Murray Siding**	**Mount Thom**	**Debert**
Untreated Control	2.48 bc	0.65 b	2.61ab	0.77 c
Switch®	2.57 bc	1.49 a	2.21bcd	1.51 ab
Luna Tranquility®	4.35 a	1.52 a	1.29cd	1.67 a
Merivon® Xenium	2.79 bc	1.62 a	1.08d	0.84 bc
Propulse®	2.02 c	0.58 b	3.55a	1.29 abc
Miravis® Prime	3.34 ab	1.78 a	2.50abc	1.85 a
ANOVA[a]	**P = 0.0044**	**P = 0.0022**	**P** = 0.0009	**P = 0.0121**

[a] 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. Data in a column with the same letters are not significantly different at α=0.05.

Table 4.6. Harvestable berry yield ($g.m^{-2}$) observed from wild blueberry field after fungicide applications.

	2018		2019	
Treatment	**Fox Point**	**Murray Siding**	**Mount Thom**	**Debert**
Untreated Control	499.08	284.91 ab	238.09 bc	529.75 b
Switch®	360.09	254.26 bc	291.66 ab	722.00 a
Luna Tranquility®	327.23	214.16 cd	299.31 ab	699.35 a
Merivon® Xenium	327.37	212.32 cd	213.59 c	576.43 b
Propulse®	335.68	191.63 d	341.63 a	537.41 b
Miravis® Prime	369.21	339.86 a	286.27 ab	508.27 b
ANOVA[a]	**NS**	P < 0.0001	**P**=0.0046	**P<0.0001**

[a] 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. Data in a column with the same letters are not significantly different at α=0.05.

4.4.1 Method development and validation

Limits of detection (LOD) and limit of quantitation (LOQ)

The retention times and the selective ion monitoring *m/z* used for the identification, quantitation and qualification of the fungicides studied with the developed method are presented (Tables 4.7 & 4.8). In this study, the LOD and LOQ derived from the matrix-matched calibration curve ranged from 0.007 - 0.021 mg/kg and 0.023 - 0.071 mg/kg respectively for the berry samples (Table 4.7). For flower samples, LOD and LOQ ranged from 0.008 - 0.031 and 0.027 - 0.105 mg/kg respectively (Table 4.7 & 4.8).

The linearity was checked using a matrix-matched calibration curve in the 0.005 - 1.0 µg/mL concentration range for all the fungicides. Excellent linearity was achieved for all the compounds with a coefficient of determination (R^2) better than 0.99 (Tables 4.7, 4.9, 4.10).

There was a significant matrix effect (> 20 %) when the slope of the matrix-matched calibration curve was compared to that of the solvent curve. Matrix effects were observed for all the 8 fungicides in this study. A signal enhancement (positive ME value) in the response was obtained for cyprodinil, fludioxonil, fluopyram, fluxapyroxad, pydiflumetofen, and pyrimethanil whereas a significant suppression was observed for prothioconazole-desthio and pyraclostrobin (Table 4.9 & 4.10). Regarding the method modification for the flower samples, matrix enhancement was observed for prothioconazole, prothioconazole-desthio and pyraclostrobin (Table 4.7).

Recovery studies and method precision

The means recoveries of the various fungicides ranged from 59.9 - 121.6 % for the lowest spiking concentration of 0.01 mg/kg, 97.4 - 118.8% for 0.05 mg/kg and 90.4 - 108% for the highest concentration (0.25 mg/kg) (Tables 4.7 – 4.10).

Method precision which is represented by the relative standard deviation (RSD %) ranged between 0.5% and 15% for all three-spiking levels for intraday and the interday precision (Tables 4.7 – 4.10).

Table 4.7. Analytical standard showing retention time, regression coefficient (R^2), limit of detection (LOD), limit of quantification (LOQ) and Matrix effect for analysis on flower samples.

	Retention time (min)	R^2	LOD (mg/kg)	LOQ (mg/kg)	Matrix effect (%)
Prothioconazole	n/a	n/a	n/a	n/a	n/a
Cyprodinil	11.3	0.996	0.009	0.030	15.3
Prothioconazole-desthio	n/a	n/a	n/a	n/a	n/a
Fludioxonil	12.3	0.993	0.012	0.039	-3.7
Fluopyram	11.5	0.995	0.010	0.032	-7.3
Fluxapyroxad	14.8	0.989	0.015	0.050	-9.6
Pydiflumetofen	17.0	0.991	0.013	0.043	-6.2
Pyraclostrobin	18.5	0.992	0.019	0.062	107.9
Pyrimethanil	9.04	0.996	0.008	0.027	3.8

Table 4.8. Mean recoveries at three blank spiking levels (0.01, 0.05, 0.25 mg/kg) and relative standard deviation (RSD %). Values are means from six replications

	Recovery (%) (*n = 6*)			**Precision (RSD %) (*n = 6*)**			
				Intraday			**Interday**
	0.01 mg/kg	**0.05 mg/kg**	**0.25 mg/kg**	**0.01 mg/kg**	**0.05 mg/kg**	**0.25 mg/kg**	
Prothioconazole	n/a	n/a	n/a	n/a	n/a	- n/a	n/a
Cyprodinil	85	109.	96.1	8.6	7.2	5.6	6.4
Prothioconazole-desthio	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Fludioxonil	n/a	118.8	83.9	n/a	10.8	11.8	0.5
Fluopyram	102.6	111.2	107.1	11.6	7.0	8.3	7.6
Fluxapyroxad	n/a	100.8	84.6	n/a	9.4	7.2	8.3
Pydiflumetofen	n/a	59.9	88.5	n/a	26.1	9.4	7.8
Pyraclostrobin	n/a	112.4	114.7	n/a	9.7	7.9	8.8
Pyrimethanil	95.5	110	121.6	7.3	8.5	5.1	6.8

Table 4.9. Analytical standard showing retention time, regression coefficient (R^2), limit of detection (LOD), limit of quantification (LOQ) and Matrix effect for analysis on fruit set and harvested berry.

	Retention time (min)	R^2	LOD (mg/kg)	LOQ (mg/kg)	Matrix effect (%)
Prothioconazole	n/a	n/a	n/a	n/a	n/a
Cyprodinil	11.3	0.999	0.010	0.033	-71.6
Prothioconazole-desthio	n/a	n/a	n/a	n/a	n/a
Fludioxonil	12.3	0.998	0.013	0.043	66.9
Fluopyram	11.5	0.999	0.007	0.023	88.6
Fluxapyroxad	14.8	0.995	0.021	0.072	65.3
Pydiflumetofen	17.0	0.999	0.012	0.040	79.9
Pyraclostrobin	18.5	0.998	0.013	0.044	-38.5
Pyrimethanil	9.04	0.999	0.008	0.025	59.5

Table 4.10. Mean recoveries at three blank spiking levels (0.01, 0.05, 0.25 mg/kg) and relative standard deviation (RSD). Values are means from six replications

	Recovery (%) *(n = 6)*			**Precision (RSD %) *(n = 6)***			
				Intraday			**Interday**
	0.01 mg/kg	**0.05 mg/kg**	**0.25 mg/kg**	**0.01 mg/kg**	**0.05 mg/kg**	**0.25 mg/kg**	
Prothioconazole	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Cyprodinil	84.7	109.2	108.3	18.2	9.5	7.3	14.5
Prothioconazole-desthio	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Fludioxonil	92.0	118.8	103.6	8.2	6.1	9.2	9.9
Fluopyram	102.5	111.1	101.1	16.9	6.1	8.0	12.0
Fluxapyroxad	113.1	105.5	92.9	17.2	3.3	9.3	13.4
Pydiflumetofen	98.3	98.5	90.4	9.0	4.4	7.8	9.3
Pyraclostrobin	88.7	97.4	105.6	17.3	5.0	7.2	15.3
Pyrimethanil	95.5	110.1	101.3	15.2	7.0	7.0	11.6

4.4.2. Residue in flower samples and berry samples

After method validation, the developed procedures were used to evaluate the presence of the active ingredients/residue in the applied disease control product for the samples collected from the 2019 trial in Mount Thom, NS.

The concentration of all fungicides in flower samples after the first and second fungicide applications was higher than their respective LOQ (Table 4.11). Mean concentrations of all the fungicides in the flower tissues were higher in samples collected 24 hours after the second fungicide application (Table 4.11). The concentrations of all the fungicides were significantly higher in the corolla samples except fludioxonil in Miravis® Prime and prothioconazole. Residue concentrations were consistently similar (not significant) between the gynoecium and androecium for all the fungicides. Depending on the time of sample collection and analysis (average of the collection times, the concentration value varied, however, the fungicide distribution pattern among the three flower parts were consistent and similar in all the analysis conducted on flower samples (Table 4.11). Interestingly, there was no significant difference in the residue concentrations among the three flower parts with regards to fludioxonil contained in Miravis® Prime.

In the fruit set samples collected 10 days after the last fungicide application, all the other fungicides were above the detection limit. However, prothioconazole-desthio and fluxapyroxad were found to be below the LOQ whereas cyprodinil, fludioxonil, fluopyram, pydiflumetofen, pyrimethanil, and pyraclostrobin were significantly higher ($p < 0.05$) than the LOQ (Table 4.12). All the fungicides were below the MRL except pydiflumetofen (Table 4.12). For the ripe berries harvested 59 days after the last fungicide application, none of the fungicides were detected (below the detection limited) in the berry harvested (Table 4.12)

Table 4.11. Fungicide residue detected in different flower parts of wild blueberry plants treated with commercial fungicides 24 hours after foliar application. Concentration values are means of six replications (standard deviation). The average residue is the mean of the two flower sampling times analyzed as repeated measures.

Fungicide		1st fungicide application (mg/kg)	2nd fungicide application (mg/kg)	Average residue (mg/kg)
Prothioconazole	**Corolla**	n/a	n/a	n/a
	Gynoecium	n/a	n/a	n/a
	Androecium	n/a	n/a	n/a
Cyprodinil	**Corolla**	11.1 (4.2) a*	30.5 (7.4) a	17.2 (1.8) a
	Gynoecium	2.84 (1.2) b*	15.8 (3.8) b	7.78 (1.7) b
	Androecium	3.02 (2.1) b*	13.6 (5.0) b	7.01 (1.4) b
Prothioconazole-Desthio	**Corolla**	n/a	n/a	n/a
	Gynoecium	n/a	n/a	n/a
	Androecium	n/a	n/a	n/a
Fludioxonil (Switch®)	**Corolla**	7.52 (2.8) a*	16.74 (4.3) a	10.5 (0.8) a
	Gynoecium	1.59 (1.0) b*	11.8 (3.2) b	5.24 (1.6) b
	Androecium	2.4 (3.5) b*	11.0 (4.2) b	5.29 (1.6) b
Fludioxonil (Miravis ® Prime)	**Corolla**	5.56 (1.6)*	28.8 (17.4)	13.1 (3.2)
	Gynoecium	6.02 (1.2)*	17.2 (10.0)	10.2 (1.5)
	Androecium	5.78 (2.7)*	31 (6.9)	15.5 (3.1)
Fluopyram (Luna Trianquilty ®)	**Corolla**	5.09 (2.7) a*	14.7 (3.6) a	8.91 (1.1) a
	Gynoecium	1.23 (0.7) b*	9.39 (1.6) b	4.25 (1.2) b
	Androecium	0.652 (0.3) b*	7.8 (2.6) b	3.15 (1.2) b
Fluopyram (Propulse®)	**Corolla**	10.73 (2.3) a*	26.4 (5.3)	17.1 (1.6) a
	Gynoecium	7.27 (2.6) b*	14.7 (6.5)	10.3 (0.8) b
	Androecium	6.55 (3.4) b*	22.8 (12.4)	12.6 (2.2) ab
Fluxapyroxad	**Corolla**	11.15 (3.4) a	15.2 (6.5)	12.7 (0.5) a
	Gynoecium	6.88 (2.2) b*	8.15 (4.5)	7.16 (0.4) b
	Androecium	6.06 (3.0) b	15.0 (6.0)	9.57 (1.1) ab
Pydiflumetofen	**Corolla**	7.34 (2.3) a*	29.2 (16.6) a	14.8 (2.7) a
	Gynoecium	5.03 (1.4) ab*	11.8 (7.2) b	8.45 (0.8) b
	Androecium	4.71 (2.2) b*	25.4 (4.5) a	12.7 (2.5) ab
Pyraclostrobin	**Corolla**	5.09 (3.0)*	10.0 (4.3) a	6.98 (0.6) a
	Gynoecium	3.28 (1.8)	3.93 (2.5) b	3.26 (0.4) b
	Androecium	2.45 (1.2)*	9.79 (3.7) a	5.28 (0.9) ab
Pyrimethanil	**Corolla**	3.79 (1.8) a*	22.8 (3.6) a	8.09 (2.3) a
	Gynoecium	1.22 (0.5) b*	12.9 (3.7) b	3.65 (1.7) b
	Androecium	1.23 (0.4) b*	12.4 (3.1) b	3.61 (1.5) b

* Asterisks indicate a significant difference between **1st fungicide application and 2nd fungicide application at α =0.05**

Table 4.12. Fungicide residue detected in fruit set (10 days post second fungicide application) and harvested berries from wild blueberry plants treated with commercial fungicides. Concentration values are means of six replications (standard deviation).

Fungicides	MRL (mg/kg)	Fruit set (mg/kg)	Harvested berry
Prothioconazole	n/a	n/a	n/a
Cyprodinil	8.0	0.764 (0.15)	ND
Prothioconazole-desthio	n/a	n/a	n/a
Fludioxonil (Switch®)	4.0	0.314 (0.18)	ND
Fludioxonil (Miravis ® Prime)	4.0	0.034 (0.01)	ND
Fluopyram (Luna Trianquilty®)	7.0	0.122 (0.04)	ND
Fluopyram (Propulse®)	7.0	0.145 (0.02)	ND
Fluxapyroxad	7.0	0.071 (0.02)	ND
Pydiflumetofen	0.01	0.290 (0.03)	ND
Pyraclostrobin	4.0	0.155 (0.04)	ND
Pyrimethanil	8.0	0.467 (0.06)	ND

ND: Not detected
MRL: maximum residue limit

4.5. DISCUSSION

4.5.1. Fungicide efficacy

Fungicide application may not always offer desirable disease control due to factors such as fungicide resistance among pathogen population (Liu et al., 2016; Weber and Hahn, 2019), inadequate application rates, and wrong timing (Sylvester and Kleczewski, 2018). Nevertheless, fungicide control of plant pathogens has been an effective means of disease control over the years. As a result, new products are being developed for the management of plant diseases.

General disease development observed in this study varied between the two years. Botrytis blight in wild blueberry is significantly influenced by environmental conditions to which the variations in disease development can be attributed. In 2019, there were many infection periods (based on weather conditions) that favored Botrytis blight development during the flowering period than in 2018, consequently, the higher levels of disease development in 2019. Additionally,

the occurrence of frost in 2018 destroyed many floral tissues which contributed to the low disease development in 2018 (Appendix 4, Tables A3-A4, Figures A1).

This study has demonstrated that the application of fungicides can be an effective means of reducing disease development in wild blueberries. However, disease development varied between the two years and among the various trials. Fungicide applications in this study mostly resulted in low Botrytis development on floral tissues compared to the untreated control. This suggests that the application of Merivon® Xenium and Miravis® Prime may be an effective means of Botrytis blight control in wild blueberry. Although Miravis® Prime is a relatively new product, its effectiveness in this study is consistent with similar studies by Abramians and Gubler, (2017) and Blundell et al., (2019) who obtained significant Botrytis disease reduction in grapes through the application of Miravis® Prime. Similarly, high efficacy of Merivon® Xenium in strawberries against *B. cinerea* was demonstrated by (Cordova et al., 2017). Additionally, the effective disease control obtained with these products is not surprising because these active ingredients have labelled for use in various crops and on various pathogens. These active ingredients have demonstrated high efficacy both individually or in co-formulation with other active ingredients (Uppala and Zhou, 2018). For instance, Achala et al., (2017) reported that Merivon® provided season-long control of Anthracnose on pomegranate. Also, pydiflumetofen (Miravis®), has been demonstrated to be effective against *Fusarium* head blight and foliar diseases such as *Septoria* sp., *Pyrenophora* sp. and powdery mildew in wheat (Glynn et al., 2018). Although Switch®, Luna Tranquility® and Propulse® are known products in the industry, their suppression of disease development is encouraging because efficacy loss has been observed on some commercial fields.

Fungicide resistance is a widespread problem in plant disease management and as such, it is generally recommended that fungicides with different modes of action be used. It is in this light

that these products contain co-formulated active ingredients. Thus, Miravis® Prime contains pydiflumetofen, an SDHI and fludioxonil, a sterol biosynthesis inhibitor (Grabke and Stammler, 2015). Also, Merivon® Xenium, is co-formulated as fluxapyroxad, an SDHI and pyraclostrobin, a quinone outside inhibitor (Bardas et al., 2010). The co-formulation offers high fungi control activity and presents an in-built resistance management strategy. The effectiveness of these products in Botrytis blight control is important because at least one of the two co-formulated active ingredients in the fungicides presently used in wild blueberries has demonstrated reduced sensitivity to *B. cinerea* isolates. For instance, cyprodinil in Switch® and boscalid in Pristine® have been demonstrated to have reduced sensitivity to *B. cinerea* from wild blueberry (Abbey et al., 2018). It is therefore, important that new active ingredients that are effective against *B. cinerea* are introduced in wild blueberry production. Although the different fungicides present different modes of action, all these active ingredients are classified as medium to high-risk fungicides, except fludioxonil which is classified as a low - medium risk fungicide (FRAC, 2021). Given this, it will be prudent that these fungicides are applied in rotation/mixed with fungicides from other groups to minimize the development of resistance in the pathogen population.

Berry yield and yield components (set fruit and pinheads/unmarketable berries) from this study varied among the various trials. However, no consistent pattern was observed among the various fungicide treatments on yield components. This inconsistency may partly be attributed to variations that exist from plant to plant. It is important to note that the application of Switch®, Luna Tranquility® and Miravis® Prime increased berry yields by at least 19% compared to the untreated control. Although berry yield and yield component increase were not statistically significant in some trials, disease development together with, other parameters such as berry yield

and fruit set are important in determining efficacy due to plant-to-plant variation and variation in factors such as disease incidence, severity, and environmental conditions.

4.5.2. Method development and validation

Over the years, several sample preparation methods such as liquid-liquid extraction, and solid-phase extraction have been used for pesticide analysis in food and environmental samples (Zhang et al., 2012; Dimpe and Nomngongo, 2016). In recent times, a satisfactory and adaptable sample preparation technique known as QuEChERS (quick, easy, cheap, effective, rugged and safe) has been well accepted for pesticide analysis due to its ability to extract multi residues from foods and environmental samples with little modification (Banerjee et al., 2012; Wang et al., 2014; He et al., 2015), and its ability to be applied to different instrumental analysis (Gilbert-López et al., 2010; Melo et al., 2020). In this study, a modified QuEChERS method was adopted and validated according to the European Union guidance criteria on analytical quality control and validation procedures (SANTE/12682/2019, 2020). In this study, the parameters for the validation of method included limits of detection and quantification, recovery (trueness, accuracy), within laboratory repeatability (intraday precision), reproducibility (interday precision) and matrix effect.

The matrix-matched calibration curves were used to establish the linearity for all the study fungicides, with coefficients of determination higher than 0.99. Limits of quantification (LODs), calculated as the lowest concentration of compound giving a response 3 times higher than the background noise and LOQs was given as a response 10 times higher than the background noise. The LODs and LOQs values of the various fungicides showed that the sensitivity of the method was below the MRLs set by the EU and as such, the developed method was effective and appropriate for monitoring the fungicide residues studied in blueberry samples.

The recovery of fungicides was within the range of 70 - 120% for the analytes, except for pydiflumetofen in the flower sample modification. According to the SANTE/12682/2019 guidelines, the acceptable trueness/accuracy range is 70 - 120. As observed in this study, the sample preparation method yielded recovery values within the range 70 - 120%, which complies with SANTE/12682/2019 guidelines. The developed method was validated in terms of precision and accuracy. Using the RSD from the recovery studies, the precision values ranged between 3% and 15% which demonstrates good methodology according to SANTE/12682/2019 guidelines and literature (Maestroni et al., 2018; Constantinou et al., 2021). Although the method for suitable and acceptable for the analysis, the spiking level of 0.01 mg/kg was extremely close to the LOD for most of the fungicides, hence in the modification for the flower samples, the 0.01mg/kg spiking were below the detection limit in some cases.

Analytes in a sample are often co-extracted with other compounds or components of the sample (matrix) which may interfere with the baseline of the chromatogram (Tripathy et al., 2019). This interference may alter the response of the analytes, and subsequently, affect the results of the analysis. In this study, significant matrix effect (ME%) was observed on all the fungicides. ME could be in the form of ion enhancement (positive value) or suppression (negative value). The level of ME can be categorize into 3; low matrix effect $< \pm 20$ %, medium $> \pm 20$ and $< \pm 50$% and high $> \pm 50$% (Mohammed et al., 2020). The ME observed in this study was largely positive with only cyprodinil and pyraclostrobin having a negative value. Interestingly, the matrix had a positive impact on pyraclostrobin in the modified flower method. Studies have shown that signal enhancement tends to be common and unavoidable in GC-MS/MS (Kwon et al., 2012; Rahman et al., 2013). This phenomenon is believed to occur when matrix components interact with active sites on the GC column and injector resulting in more analytes reaching the detector (Ly et al.,

2020). The ME challenge in pesticide analysis can be compensated by dilution, use of standard addition, or matrix match calibration (Ly et al., 2020; Mahdavi et al., 2021). Additionally, ME can be reduced through extensive sample cleanup. This explains why there was minimal ME on all the fungicides except pyraclostrobin in the flower sample due to the addition of activated carbon as a component of the clean-up phase. To compensate for the ME on the response signals in this study, the matrix-matched calibration curve and dilutions were used for the quantification of the fungicides in this study.

4.5.3. Residues in flowers and berries

In the present study, residues of all the fungicides in the ripe berries and fruit set were either below detection limits or detected only in traces with mean concentrations close to zero, however, significant residues were detected in the flower samples. The high-level residue detected in the flowers after the second fungicide application is expected due to the second dose of the fungicides which adds to the residue from the first application.

At 24 h after the foliar application, higher GC-MS analysis showed the presence of all the studied fungicides in all the flower parts sampled. Generally, a higher concentration of all fungicides was detected in corolla compared with gynoecium and the androecium. This is not surprising considering the corolla presumably intercepted the fungicide spray droplets from where they move to the other part of the flower if they are systemic. The structural and pendulous orientation of the wild blueberry flower poses a challenge with fungicide application. Applied fungicides are mostly deposited on the pendulous flower and mostly on the corolla which completely houses the androecium and the pistil with its stigmatic surface. Interestingly, all the fungicides, regardless of their physicochemical properties showed a similar pattern of residue

distribution among the three flower parts from both sampling times, except the contact fungicide fludioxonil in Miravis® Prime and prothioconazole in Propulse® (https://pubchem.ncbi.nlm.nih.gov; https://sitem.herts.ac.uk). The detection of these fungicides in gynoecium and androecium could be an indication of a potential mobility within the wild blueberry flower However, the concentration of these fungicides in the corolla compared to the gynoecium and androecium suggests that the mobility of these fungicides might be limited. Given the structure of the flower and the location of the androecium (not exposed to direct fungicide contact) the detection of fungicides in this component of the flower could be due to the activities of the pollinating insects may have also been a vector for these fungicides. Also, the volatilization of some fungicides such as pyrimethanil may have contributed to the presence of these fungicides in the androecium (Green et al, 1999). The similarity of the concentration between the gynoecium and androecium suggests there might not be a preferred location for the fungicides within the flower. Interestingly and unexplained is why a similar concentration was observed for fludioxonil in Miravis® Prime. This could be so considering that fludioxonil is a contact fungicide and flower samples from each plot were put together in one sampling bag and brought to the lab before the flowers were separated into the various components. There is therefore the possibility of cross-contamination of the gynoecium and the androecium from the corolla.

Although this study included Propulse®, the residue analysis did not focus on prothioconazole and its metabolite prothioconazole-desthio. Prothioconazole is not registered for Botrytis control (Stehmann, 1995). However, the commercial product Propulse® contains fluopyram, a Botrytis control, hence it is mostly used as a bridge between the *Monilinia* infection, and the *Botrytis* infection windows. Also, prothioconazole is not well suited for GC-MS instrumentation but can appropriately be analysed by LC-MS, hence the levels of residues detected

for prothioconazole could be inaccurate (Pizzutti et al, 2012; Kiet Ly, 2020; Hergueta-Castillo et al., 2022).

Residues of all the applied fungicides were detected in set fruits during the 10-day post-application analysis. The residue concentration varied depending on the product applied. However, all the residues were below their corresponding MRL except pydiflumetofen (https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database). Aside from fungicide degradation which can cause a decrease in residue in floral tissues, plant biomass as the flower develops and corolla senesces, tend to dilute the residue more than the initial amount of active ingredient applied (Lawson et al., 2020). During fruit set, the corolla on which fungicides are applied drops off and with the seaming limited mobility of these fungicides within the flower tissue, it is not surprising that very low levels of fungicides were detected just 10 days after the second fungicide application. From a pathogen control point of view, all the fungicides residue detected were at, or above the EC_{50} concentrations for *B. cinerea* in previous studies with different resistance/susceptibility statuses. For instance, the residue of fludioxonil in the 10-day fruit set is 0.314 mg/kg, however, the previous studies have reported 0.0047 -0.0073 µg/ml and 0.1 -0.2 µg/ml different *B. cinerea* isolates (Fernández-Ortuño et al., 2013; Abbey, 2017). Also, 0.314 mg/kg residue of pydiflumetofen observed was way more than the *B. cinerea* EC_{50} between 0.003 to 0.028 µg/ml reported (He et al., 2020). This suggests that the fungicides residues are persistent enough and still have the potential to inhibit *B. cinerea* growth up to fruit set.

While monitoring the degradation of fungicide residue, a decrease in residue to levels not only below the maximum residue level (MRL) but even below LOD was observed at harvest (65 days fungicide application). To our knowledge, no data regarding the tested fungicides on wild blueberry exist in literature, however, residue testing must have been conducted by the Canadian

Food Inspection Agency (CFIA) on wild blueberries for the purposes of registering these fungicides for use in wild blueberry. Sadło et al., (2018) reported that residues of cyprodinil, pyraclostrobin, and pyrimethanil in ripe raspberries were well below their corresponding MRLs even on day zero of picking ripe raspberries. Gabriolotto et al., (2009) reported residues of fludioxonil and cyprodinil at harvest were below the MRL in grapes. Wang et al., (2015) also reported residue levels in strawberries below MRL for pyraclostrobin 14 days after a second dose application. Finally, non-detectable levels of these fungicides were expected given the half-lives of these compounds reported in literature. The pre-harvest interval for wild blueberry is approx. 65 days which is more than twice the half-lives of these compounds. The half-life of pyrimethanil has been reported to be between 11-22 days in different crops including apples, table grapes and strawberries (Angioni et al., 2006; Szpyrka and Walorczyk, 2013). In a similar study, cyprodinil was reported to have a half-life ranging between 9- 20 days (Zhang, et al., 2015). Fluopyram and pyraclostrobin have been reported to have a half-life of less than 10 days in different crops even when double dose application is made (Fantke et al., 2014). Among all the fungicides, fludioxonil has been reported to have the longest half-life between 33-44 days in cherry and tomato leaves (Szpyrka and Sadło, 2009; Yao et al., 2021;). Although fludioxonil has a high half-life, it is a contact fungicide and the corolla which receive most of the fungicides drops during fruit set, hence its absence in the ripe berries.

5.6. CONCLUSION

The results from this study provide strong evidence of the effectiveness of Switch®, Luna Tranquility®, Propulse® and newly introduced products, Miravis® Prime and Merivon® Xenium for Botrytis blight control in wild blueberry fields. The application of Miravis® Prime and

Merivon® Xenium was effective as the already known industry standard Switch®. The application Luna Tranquility® resulted in increased berry yield. The data presented here for the control of Botrytis blight control in wild blueberry indicate that Miravis® Prime and Merivon® Xenium can provide an alternative disease control option for growers.

The developed method and instrument parameters for the analysis were suitable for fungicide residue analysis in blueberry matrix. The method was validated by the required data of linearity, recovery, and precision and the data obtained were within acceptable range of validation limits for in-house method development and validation.

While residues were consistently detected in flower and fruit set samples, the concentrations of fungicides were low in fruit set but high enough to adversely suppress *B. cinerea*. Fungicide concentrations were higher in corolla than in gynoecium and the androecium which is suggestive of limited mobility.

CHAPTER 5: USE OF BIOFUNGICIDES AND CONVENTIONAL FOR THE MANAGEMENT OF BOTRYTIS BLOSSOM BLIGHT IN WILD BLUEBERRIES

The following have been published as manuscript from this Section.

Abbey, J. A., Percival, D., Jaakola, L., and Asiedu, S. K. (2021). Potential use of biofungicides and conventional fungicide for the management of Botrytis blossom blight in lowbush blueberries. Canadian Journal of Plant Pathology, 1-10.

5.1. ABSTRACT

Botrytis blight is an economically important disease of wild blueberry that causes significant yield loss annually. In this study, the biofungicides, Diplomat 5SC® (polyoxin D), Timorex Gold® (tea tree oil), Fracture® (BLAD) and Serenade MAX® (*Bacillus subtilis*) were evaluated for their disease suppression potential against *B. cinerea* individually and in rotation with Switch® under field conditions. Three applications of each biofungicide were made for the stand-alone treatment at 7-10 days' interval with Switch® replacing the 2nd application in the combined treatment. The results from the study indicated that the products have potential for use as a biofungicide in wild blueberries. All the stand-alone and rotational applications brought about significant reduction in disease development, especially in 2019. The application of Diplomat 5SC® and Fracture®-Switch® rotation decreased disease development by over 63% in 2018. In 2019, all stand-alone treatments reduced disease development by more than 42% whereas their rotation with Switch® reduced disease by over 69% at Earltown and at least 30% at Farmington. The outcome from this study suggests that the biofungicides and their integration with chemical fungicides have the potential as an alternative management strategy against Botrytis blossom blight to reduce the use of conventional fungicides.

5.2. INTRODUCTION

Botrytis blossom blight has been a major problem in wild blueberry production, especially in coastal areas with prolonged wet conditions. The disease is caused by the pathogen *Botrytis cinerea* Pers.: Fr. and it typically infects flowers or entire inflorescences at the mid to late bloom stage (Hildebrand et al., 2001). The disease causes over 20% yield loss annually and over the past decade, it has become far more prevalent due to increased canopy densities, longer wetness durations and more susceptible floral tissue (increase in flower densities from 93 million flowers ha^{-1} in 1994 to over 150 million flowers ha^{-1} due to improved practices such as nutrient and weed management) (Percival, 2013).

Given that Botrytis blight is caused by a fungus classified as posing high risk of developing fungicide resistance (FRAC, 2019), fungicides with different modes of actions are used in controlling the disease (FRAC, 2010). The main fungicide presently used for Botrytis blight control in wild blueberry is Switch®, which contains the signal transduction and amino acid inhibitors fludioxonil and cyprodinil, respectively. Other fungicides used include Luna Tranquility® and Merivon® (Burgess, 2020; Percival, 2013). Although there are several fungicides available, the management of the pathogen is challenging due to its high-risk nature, fungicide cost, concerns about fungicide residue and strict maximum residue limits (MRL) allowed on the international market. With Botrytis management products accounting for more than 60% of the fungicide costs and concerns over detectable residues by consumers, there is a need to develop disease management approaches that will help address some of these challenges. This has given rise to the interest in biofungicides including Serifel® (*Bacillus amyloliquefaciens*), Serenade MAX® (*Bacillus subtilis*) and Fracture® (BLAD polypeptide) (Percival et al, 2016). Studies have shown that some biological control agents, plant extracts and biologically active natural products

can serve as alternatives to the chemical (conventional) fungicides presently being used (Abbey et al., 2019; Calvo-Garrido et al., 2019; Jiang et al., 2018; Li et al., 2017; Monteiro et al., 2015; Shao et al., 2013). Some essential oils including *Melaleuca alternifolia* tree oil, have been reported to be effective against plant pathogens including *B. cinerea* (Nguyen et al., 2013; Potočnik et al., 2010). Similarly, polyoxin extracted from the soil bacterium (*Streptomyces cacaoi* var.*asoensis*) (Mamiev et al., 2013), has been demonstrated to effectively suppress *B. cinerea* in strawberry (Dowling et al., 2016; Nguyen et al., 2013).

Several biofungicides have recently been developed, but their efficacy against Botrytis blossom blight in the wild blueberry production system have not been evaluated. Preliminary studies have demonstrated that adequate disease suppression can be achieved when biofungicides are combined with the conventional fungicide by way of rotation (Percival et al, 2016, Abbey et al., 2020) and/or when conventional fungicides are used during peak disease pressures. In view of the continuous search for more economically and environmentally friendly alternative for conventional fungicides, it is important to evaluate new products that have been shown to suppress *Botrytis cinerea* in other crops. The objective of this research was to determine the efficacy of commercially formulated tea tree essential oil, polyoxin D, BLAD and *Bacillus subtilis* used alone and in combination with conventional fungicide for the control of Botrytis blossom blight in wild blueberry fields.

5.3. MATERIALS AND METHODS

5.3.1. Site selection and experimental design

Four field trials using biofungicide treatments against Botrytis blossom blight were carried out in two consecutive years at two different locations in each year. In 2018, experiments were conducted at Pigeon Hill (coordinates = 45°34′35.03 N, 63°51′54.84 W) and Blue Mountain, NS (coordinates = 45°28′53.29 N, 62°25′27.26 W), and at Farmington (coordinates = 45°34′24.20 N, 63°53′37.84 W) and Earltown, NS (coordinates = 45°34′50.58 N, 63°06′05.15 W) in 2019. A randomized complete block design (RCBD) with five replications was used. Plot size was 4 × 6 m with 2 m buffers between plots. Fields for the experiments were equipped with Watchdog® model 2700 weather station (Aurora, IL) to monitor air temperature, relative humidity, leaf wetness, wind speed and direction every 15 min for the duration of the trial.

5.3.2 Fungicide products and treatment application

Ten treatments were included: (1) untreated control; (2) Diplomat 5SC®; (3) Timorex Gold®; (4) Fracture®; (5) Serenade MAX®; (6) Diplomat 5SC® - Switch® - Diplomat 5SC®; (DSD) (7) Timorex Gold® - Switch® - Timorex Gold® (TST); (8) Fracture® - Switch® - Fracture® (FSF); (9) Serenade MAX® - Switch® - Serenade MAX® (SSS); and (10) Luna Tranquility® - Switch® - Pristine® (LSP). The active ingredients and the application rate of products are indicated (Table 5.1).

Table 5.1. Product application rates and active ingredients of fungicides used for Botrytis blight control in wild blueberries.

Products	Active ingredients	Product application rates
Diplomat 5SC ®	Polyoxin D zinc salt (5 %)	0.926 L ha ^{1}
Timorex Gold®	Tea Tree Oil (23.8 %)	1.5 L ha ^{1}
Fracture®	Banda de Lupinus albus doce, BLAD (20 %)	2.6 L ha ^{1}
Serenade MAX®	*Bacillus subtilis* strain QST 713 (7.3 x 10^{9} CFU/g)	6 kg ha ^{1}
Switch®	Cyprodinil and fludioxonil	0.975 kg ha^{-1}
Pristine®	Boscalid (25.2 %) and pyraclostrobin (12.8 %)	1.3 kg ha^{-1}
Luna Tranquility®	Fluopyram and pyrimethanil	1.5 kg ha^{-1}

5.3.3 Fungicide applications

First fungicide application was made at 10% bloom stage prior to visual symptoms of Botrytis blight. The second application was made 7 to 10 days after the first application, and the third application was made 14 to 17 days after the first application. Fungicides were applied as described in **Section 4.3.2 of Chapter four**.

5.3.4. Disease assessment, yield component, berry yield and statistical analysis

Assessment of yield components, and berry yield was done as described in Section 4.3.3 in Chapter four.

5.3.6 STATISTICAL ANALYSIS

Data collected on disease development and harvested berries were checked for normality and constant variance on the residuals. Harvested berries were square root [$\sqrt{(\times)}$] transformed to ensure normality. All the data were analyzed using the PROC GLIMMIX procedure of SAS (version 9.4,

SAS institute, Inc., Cary, NC). The fixed effect was treatments, and the replication was the random effect. Least Significance Differences (LSD) was used for multiple means comparison at $\alpha=0.05$ when the P-value in ANOVA indicates a significant difference ($p < 0.05$) among the treatment means.

5.4 RESULTS

Botrytis blight disease pressure was low in the two trials in 2018 with 1.3 and 10.7 % of the total stems assessed (n=750) showing Botrytis blight symptoms and signs at Blue Mountain and Pigeon Hill, respectively after 2nd fungicide application. After the 3rd fungicide application, 0.35 and 1.1 % of the total stems assessed showed Botrytis blight at Blue Mountain and Pigeon Hill, respectively. Contrary to 2018, high disease pressures were observed in 2019 with 30.4 and 32.2 % of assessed stems showing disease symptoms at Earltown and Farmington, respectively after the 2nd application. After the 3rd fungicide application, 9.20 and 26.3 % of total stem samples assessed showed disease symptoms at Earltown and Farmington, respectively.

In 2018, disease incidence and severity ranged from 1.65 to 11.7% and 1.65 to 11.6%, respectively, after the 2nd application, and 0 to 1.81 for both incidence and severity after the 3rd application at Pigeon Hill (Table 5.2). After the 2nd fungicide applications, there was a significant treatment effect on disease development. The application of Diplomat 5SC® and FSF significantly lowered incidence by 76 and 68 %, and severity by 69.4 and 63 %, respectively compared to the untreated control. On the contrary, there was no significant treatment effect on disease development after the 3rd fungicide application with zero to very low disease occurrence (Table 5.2). In the trial at Blue Mountain, there was no significant treatment effect ($P > 0.05$) on disease

development after 2nd and 3rd fungicide applications probably due to the very low disease levels (Table 5.2).

In 2019, significant treatment effect was observed at Earltown with disease incidence and severity ranging from 6.90 to 41.9 % and 2.97 to 34.6 %, respectively after the 2nd application. An incidence range of 1.10 to 16.7 % and severity of 0.33 to 13.0 % were observed after the 3rd application at Earltown **(**Table 5.3). After the second fungicide application, stand-alone Diplomat 5SC®, Timorex Gold®, Facture and Serenade MAX® significantly reduced disease incidence by 78.7, 43.3, 42.3 and 60.5%, respectively and severity by 83, 71.7, 46.7 and 71.2 %, respectively compared to the untreated control. The rotation of all the biofungicides with Switch® (DSD, TST, FSF, SSS) and conventional control program (LSP) highly suppressed disease development with over 69 and 81 % less incidence and severity (Table 5.3). All the stand-alone treatments reduced disease incidence by more than 50 % and severity by over 42 % after the 3rd fungicide application (Table 5.3). The rotation of all the biofungicides with Switch® significantly reduced disease incidence and severity by more than 78 and 77 % compared to the untreated control. Interestingly, disease suppression provided by Diplomat 5SC®, DSD, TST, FSF and SSS were comparable to that of the LSP (Table 5.3). At Farmington, disease incidence and severity ranged from 7.59 to 32.9 % and 3.41 to 23.2 %, respectively after the 2nd application. After the 3rd fungicide application, incidence and severity ranged from 4.09 to 23.8 % and 1.92 to 10.8 %, respectively (Table 5.3). After the 2nd fungicide application, both stand-alone and their rotation with Switch® significantly reduced disease with over 55 and 66 % less incidence and severity, respectively compared to the untreated control. Disease control achieved by stand-alone treatments and their rotation with Switch® were comparable to the disease suppression achieved by LSP. After the 3rd fungicide

application, Diplomat 5SC®, TST and FSF significantly reduced disease incidence and severity by over 30 and 37 %, respectively which were comparable to the LSP (Table 5.3).

Table 5.2. Botrytis blight incidence (inci %) and severity (sev %) observed in wild blueberries at Pigeon Hill and Blue Mountain, Nova Scotia, following biofungicide and fungicide applications in 2018.

	Pigeon Hill				Blue Mountain			
	2nd application		3rd application		2nd application		3rd application	
Treatment	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**
Control	6.90 abc	5.40 bc	1.81	1.81	1.82	1.68	0	0
Diplomat 5SC®	1.65 c	1.65 c	0.59	0.59	1.25	0.87	0	0
Timorex Gold®	9.12 ab	9.18 ab	0	0	0.22	0	0	0
Fracture®	5.81 bc	5.54 bc	0	0	0	0	0	0
Serenade MAX®	5.72 bc	5.27 bc	0.36	0.36	0	0	0	0
DSD	5.87 bc	5.14 bc	0.79	0.64	2.67	0.02	0	0
TST	11.7 a	11.7 a	0	0	0	0	0	0
FSF	2.21 c	1.98 c	0	0	0.14	0.08	1.74	1.63
SSS	5.02 bc	3.44 c	0	0	0	0	0	0
LSP	5.71bc	5.71 c	0	0	0	0	0	0
ANOVA [a]	P=0.0337	P=0.0102	NS	NS	**NS**	**NS**	**NS**	**NS**

[a] 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. Data in a column with the same letters are not significantly different at $\alpha=0.05$. Diplomat 5SC® - Switch® - Diplomat 5SC® (DSD), Timorex Gold®- Switch® - Timorex Gold® (TST), Fracture® - Switch® - Fracture® (FSF), Serenade MAX® - Switch® - Serenade MAX® (SSS), Luna Tranquility® - Switch® - Pristine® (LSP)

Table 5.3. Botrytis blight incidence (inc %) and severity (sev %) observed in wild blueberries at Earltown and Farmington, Nova Scotia, following biofungicide and fungicide applications in 2019.

	Earltown				Farmington			
	2nd application		3rd application		2nd application		3rd application	
Treatment	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**	**Inci (%)**	**Sev (%)**
Control	41.9 a	34.6 a	16.7 a	13.01 a	32.9 a	23.2 a	14.5 bc	7.37 ab
Diplomat 5SC®	8.95 c	5.87 c	1.67 bc	1.00 cd	9.3 b	6.81 b	10.1 bcd	4.62 bcd
Timorex Gold®	23.8 b	9.79 bc	7.57 b	4.58 bcd	14.2 b	6.17 b	14.1 bc	8.11 ab
Fracture®	24.2 b	18.4 b	8.32 b	7.50 b	11.3 b	4.50 b	23.9 a	10.6 a
Serenade MAX®	16.6 bc	9.97 bc	8.01 b	5.55 bc	10.2 b	4.69 b	14.9 bc	6.75 abc
DSD	6.90 c	3.67 c	2.27 bc	2.06 cd	11.5 b	4.33 b	16.9 ab	10.0 a
TST	8.95 c	5.12 c	3.66 bc	2.93 bcd	11.6 b	7.84 b	8.23 cd	2.55 cd
FSF	11.1 c	6.37 c	1.11 c	0.94 cd	14.7 b	4.89 b	16.9 ab	4.63 bcd
SSS	12.9 bc	3.99 c	2.79 bc	0.97 cd	12.7 b	3.41 b	16.5 abc	9.40 a
LSP	8.13 c	2.97 c	1.10 c	0.33 d	7.59 b	3.41 b	4.09 d	1.92 d
ANOVA [a]	P<0.0001	P<0.0001	P<0.0001	P<0.0001	**p=0.0008**	**p<0.0001**	P=0.0004	P=0.0002

[a] 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. Data in a column with the same letters are not significantly different at α=0.05. Diplomat 5SC ®- Switch® - Diplomat 5SC® (DSD), Timorex Gold®- Switch® - Timorex Gold® (TST), Fracture® - Switch® - Fracture® (FSF), Serenade MAX® - Switch® - Serenade MAX® (SSS), Luna Tranquility® - Switch® - Pristine® (LSP).

There was a significant treatment effect on yield components at Pigeon Hill and Blue Mountain in 2018. At Pigeon Hill, Fracture®, TST and FSF resulted in significantly high set fruit per stem with over 30% more set fruit than untreated control and LSP (Table 5.4). At Blue Mountain, the convention control program resulted in the highest number of set fruit (5.04).

In 2019, there was no significant treatment effect ($P > 0.05$) yield components at Earltown. At Farmington, Diplomat 5SC®, Fracture®, FSF, SSS and LSP resulted in higher set fruit per stem with over 28 % more set fruit compared to the untreated control. Among all the treatments, LSP had the highest fruit set per stem (Table 5.4).

There was a significant treatment effect in harvestable berry yield at Pigeon Hill and Blue Mountain in 2018. TST, FSF and SSS resulted in improved berry yield compared to the untreated control with over 34.7, 26.0 and 33.2 % more berry yield, respectively at Pigeon Hill. At Blue Mountain, none of the treatments increased yield compared to the control and some treatments had lower yield than the control (Table 5.4). Diplomat 5SC®, Timorex Gold®, and FSF resulted in improved berry yield 14, 8 and 7%, respectively.

In 2019, there was also a significant treatment effect on berry yield. Although significant, most of the treatments were not different from the untreated control and among each other (Table 5.6). At Earltown, no treatments increased yield compared to the control. However, at Farmington, several treatments increased yield. Yield in the Diplomat treatment was equivalent to LSP. (Table 5.4).

Table 5.4. Yield component (fruit set per shoot/stem) observed from wild blueberry field following biofungicide and fungicide applications.

	2018		2019	
Treatment	**Pigeon Hill**	**Blue Mountain**	**Earltown**	**Farming ton**
Control	5.55 de	3.09 bcd	7.34	7.90 d
Diplomat 5SC®	4.59 e	3.98 abc	3.80	11.1 bc
Timorex Gold®	5.83 de	3.62 abcd	7.87	8.91 cd
Fracture®	7.93 bc	2.32 d	4.79	11.2 bc
Serenade MAX®	2.16 f	4.65 ab	6.65	9.77 bcd
DSD	4.74 e	2.76 cd	8.53	10.5 bcd
TST	8.20 ab	3.84 abc	7.76	9.65 bcd
FSF	9.63 a	3.58 abcd	7.45	12.4 b
SSS	6.56 cd	2.42 d	8.03	12.3 b
LSP	4.82 e	5.04 a	5.61	15.6 a
ANOVA[a]	P<0.0001	P=0.0111	NS	P=0.0001

[a] 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. Data in a column with the same letters are not significantly different at α=0.05.
Diplomat 5SC® - Switch® - Diplomat 5SC® (DSD), Timorex Gold®- Switch® - Timorex Gold® (TST), Fracture® - Switch® - Fracture® (FSF), Serenade MAX® - Switch® - Serenade MAX® (SSS), Luna Tranquility® - Switch® - Pristine® (LSP)

Table 5.5. Harvestable berry yield ($g \cdot m^{-2}$) observed from wild blueberry field after biofungicide and fungicide applications.

	2018		2019	
Treatment	**Pigeon Hill**	**Blue Mountain**	**Earltown**	**Farmington**
Control	252.8 de	383.3 ab	252.3 a	566.7 c
Diplomat 5SC®	317.9 abcd	437.2 a	118.9 f	779.0 ab
Timorex Gold®	295.1 abcd	414.5 a	181.3 bcde	763.5 b
Fracture®	289.6 bcd	294.1 bc	137.6 ef	710.0 bc
Serenade MAX®	186.3 e	273.5 c	167.5 def	710.4 bc
DSD	283.5 cd	382.9 ab	183.4 abcde	812.3 ab
TST	386.9 a	282.2 c	248.2 ab	562.2 c
FSF	341.8 ac	409.6 a	206.8 abcd	682.4 bc
SSS	378.6 a	284.9 c	171.6 cdef	733.1 b
LSP	287.9 bcd	283.9 c	237.8 abc	953.0 a
ANOVA[a]	P=0.0003	P=0.0003	P=0.0001	P=0.0002

[a] 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. Data in a column with the same letters are not significantly different at $\alpha=0.05$.
Diplomat 5SC® - Switch® - Diplomat 5SC® (DSD), Timorex Gold®- Switch® - Timorex Gold® (TST), Fracture® - Switch® - Fracture® (FSF), Serenade MAX® - Switch® - Serenade MAX® (SSS), Luna Tranquility® - Switch® - Pristine® (LSP)

5.5 DISCUSSION

Botrytis cinerea is a high-risk polycyclic pathogen which causes floral blight disease in blueberries under conducive environmental conditions. In view of this, frequent application of control products, especially chemical fungicides, are carried out to maintain high crop value and reduce yield losses in fields. Despite this, *B. cinerea* continues to cause significant losses in wild blueberry fields due to the

development of resistance among the pathogen population (Abbey, 2017). To this effect, the implementation of an integrated disease management strategy which involves the use of both biofungicides, and conventional fungicides would be beneficial for the successful control of Botrytis blossom blight in wild blueberry fields.

In this study, the application of biofungicides were able to suppress Botrytis blight infection in both years, however, disease pressures varied between the two years and the time of disease assessment. Although there were significant Botrytis infection periods throughout the flowering period in both years, environmental conditions played a significant role in this variation in disease pressures (Appendix 4, Tables 6-9, Figures A2-A3). A significant frost occurrence (-3.3 ^{0}C) (Appendix 4, Figure A1 and Appendix 4, Figure A2-A3) affected the flower tissues which explains the low disease pressures in 2018 compared to 2019. Also, flower tissues assessed after the 2nd fungicide applications had higher disease level because the 2nd application occurred at full bloom (stages F6 - F7), a stage at which flower tissues are most susceptible to disease infection (Hildebrand et al., 2001; Abbey et al., 2018).

This study demonstrated that the application of biofungicides significantly reduced disease development in most situations. Generally, many natural compounds including polyoxin D (Dowling et al., 2016; Brannen et al., 2020), banda de lupinus albus doce (BLAD) (Monteiro et al., 2015; Abbey et al., 2020) and tea tree oil (Cheng and Shao 2011; Shao et al. 2013) as well as biocontrol agents such as *Bacillus* spp. (Lee et al., 2006; Martínez-Absalón et al., 2014) are well known to be effective in the management of *B. cinerea* and several plant pathogens. Due to the extensive biofungicide research, several modes of action are known to exist and are well understood (Lahlali et al., 2011; Nie et al., 2017; Sarrocco et al., 2017). While some are known to have simple and direct modes of action, others such as *Bacillus* spp. have complex modes of action (Cawoy et al., 2011). Polyoxin D interferes

with the activities of chitin synthetase which results in the inhibition of chitin formation in the fungal cell wall (Becker et al., 1983; Adaskaveg et al., 2011). Similarly, BLAD is also known to interfere with fungal chitin by binding and degrading chitin through the removal of the N-acetyl-D-glucosamine terminal in chitin (Monteiro et al., 2015; APVMA, 2017). Also, the terpenoids (terpinen-4-ol) content of tea tree oil has been reported to act on cell membranes and alter the permeability of fungal cells including *B. cinerea* (Carson et al., 2006; Yu et al., 2015). *Bacillus* spp. has been identified to suppress pathogens through the production of antibiotics and induction of host resistance (Niu et al., 2011; Pathma et al., 2011; Chowdappa et al., 2013; Ji et al., 2013). Given the extensive reports on the use of biofungcides and their modes of action, it is not surprising that Diplomat 5SC ®, Timorex Gold®, Fracture® and Serenade MAX® suppressed Botrytis blossom blight in wild blueberry, and in some cases were comparable to the convention control program in this study.

Generally, the combination of different biofungicides have been touted to be an effective way of disease control. This is to help address the inconsistencies in disease control experienced with stand-alone application of biofungicide. The effectiveness of biofungicides is well known to be greatly influenced by environmental conditions such as temperature (Xu et al., 2011; De Cal et al., 2012; Sylla et al., 2015). As a part of addressing this challenge, many studies have achieved significant disease control when biofungicides are combined as a tank mix or in alternation with compatible chemical fungicides (Elad et al., 1993; Gilardi et al., 2008; Boukaew et al., 2013; Ons et al., 2020). In Elad et al. (1993), a potential synergistic effect was observed when a mixture of *T. harzianum* (T-39) with a dicarboximide fungicide resulted in up to 96% control of grey mould in cucumber. In another study, the mixed application of *T. harzianum* (Jn14) with cyprodinil, fludioxonil and pyrimethanil provided an absolute inhibition gray mould disease severity strawberry (Barakat et al., 2017).

In this study, it was hypothesized that the integration of biofungicide treatments with a chemical fungicide would have the potential to improve efficacy and reduce variability of biofungicides. It is therefore not surprising that the rotation of Diplomat 5SC ®, Timorex Gold®, Fracture® and Serenade MAX® with Switch® also resulted in significant disease control, especially at the Earltown site. It is also noteworthy that biofungicides and their rotation with the chemical fungicides provided disease control similar to the conventional control program of three chemical fungicide applications. This is important because harvested fruit from fields treated with biofungicide/or their rotation with a conventional are far less likely to have chemical residues.

The combination of biofungicide and Switch® (a.i fludioxonil, FRAC group 12), a signal transduction inhibitor and cyprodinil (FRAC group 9), an amino acid and protein synthesis inhibitor) with different modes of action falls in line with the concept of integrated pest management. This approach protects the various components of the management strategy from total failure. For instance, the application of Switch® will help to prevent total disease control failure in the event that the environmental conditions do not favour the establishment of biocontrol agents or biodegradation of natural compounds. In addition, the rotation of biofungicide with Switch® helps to reduce the number of chemical fungicide applications from 3 to 1. This will have a practical implication on growers and the environment as they will have to use less amount of chemical fungicides and environmental pollution from excessive use of chemical fungicides. Also, the timing of the Switch® application in these trials was based on the desire to ensure good disease control and avoid or completely reduce fungicide residue in harvested berries. This is possible because the chemical fungicide was applied at full bloom when disease pressures were most likely to be high (Hildebrand et al., 2001). It has been reported that biofungicides are less effective when disease pressures are high (Hofstein and Chapple, 1999; Reiss and Jørgensen, 2017), hence rotating with conventional fungicides is critical in achieving

adequate disease control. The timing of Switch® treatment helps to extend the pre-harvest interval which can contribute significantly to residue reduction. Also, the Switch® application was made while the flowers are in bloom and not as much of the developing ovary is exposed to the chemical fungicides. Being able to produce residue free berries to meet international MRLs is very important, as most wild blueberries are exported to Europe and Asia with strict and limited MRLs.

Although the biofungicides used in this study have shown great potential as alternatives for conventional fungicides, there are some reports of resistance development among *B. cinerea* populations from strawberry fields. For instance, *B. cinerea* isolates with reduced sensitivity to polyoxin have been reported from commercial strawberry fields in the USA (Dowling et al., 2016) and sweet basil in Israel (Mamiev et al., 2013). This could be because these natural compounds act directly on the fungi and given the genetic diversity and high-risk nature of *B. cinerea*, there is the potential for resistance development (Dowling et al., 2016). In view of this, resistance management may be necessary for control strategies which include polyoxin D.

In this study, the application of biofungicides and their rotation led to improved fruit set (fruit per stem) in two of the four trials. This can partly be attributed to the effective disease suppression obtained from the application of biofungicides. Although not all the fungicides resulted in consistent harvestable berry yield, it is worth mentioning that treatments such as Diplomat 5SC®, Timorex Gold®, TST, FSF and SSS resulted in ~20% more berries in two of the four trials. The inconsistency observed among some of the treatments can partly be attributed to the variability in plant populations in wild blueberry fields. Wild blueberries are native, naturally occurring and not planted. Hence, significant variations exist among phenotypes and plants density from field to field (Hepler and Yarborough, 1991).

5.6 CONCLUSION

In conclusion, the outcome from this study provides information on the potential of introducing biofungicides into Botrytis blight management programs. The application of biofungicides alone and their rotation with Switch® yielded a promising result. Significant disease suppression was obtained with stand-alone applications of biofungicides as well as their rotation with Switch® in some trials when disease pressure was high. This study revealed that biofungicides can stand alone in the control of Botrytis blight, however, their integration with low-risk fungicides is a more promising approach.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION

Botrytis blossom blight has been one of the major blueberry diseases of concern to the blueberry industry. 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 that can withstand harsh conditions. Sclerotia and dormant mycelia within field debris and neighbouring bushes germinate to produce macroconidia and ascospores which serves as primary inoculum source (Dewey and Grant-Downton, 2016). Many products especially chemical fungicides and the use of biofungicides have been employed to help manage Botrytis blight on wild blueberry fields. Regardless of these products, there are Botrytis control challenges faced by the industry. These include concerns regarding fungicide resistance and loss of product efficacy, pesticide residue in berries, and limited fungicide mobility within flower tissues. Given these challenges, new, cost effective and internationally acceptable management strategies are needed to help address the challenges encountered by the present management activities.

The overall aim of this project was to address the strategic goals of improving yields, production sustainability, environmental safety and reducing the cost of production partly through the understanding of molecular and polyphenol outcome of plant-pathogen interaction. The objective of the study presented in this thesis was to understand the molecular and biochemical response between microbes (*Botrytis cinerea* and *Bacillus amyloliquefaciens*) and wild blueberry flower and their physiological impact on the plant. This thesis also assessed the efficacy, mobility, and persistence of fungicides in floral tissues and berries. Finally, this thesis investigated the use of a reduced risk Botrytis blight control strategy through the combination of chemical and biofungicides.

In this study, new understanding and knowledge have been obtained regarding the response of wild blueberry to both pathogenic and non-pathogenic microbes under field conditions from the experiments in Chapter 3. Wild blueberry plants respond similarly to both *B. cinerea* and *B. amyloliquefaciens* which changed overtime and phenotype dependent. This study may serve as a starting point for achieving a better understanding of the wild blueberry-*B. cinerea* pathosystem and the path to incorporate induced resistance as defense strategies in wild blueberry production. Molecular, biochemical, and physiological changes during plant-microbe interactions have long been recognized as a plant defense/stress response (Huang 2013; Kim et al., 2015; Kumar and Verma, 2018). When plants perceive the presence of microbes or infection by a pathogen, a cascade of signal transduction events occurs which results in the reprogramming of their transcriptome. These events lead to increased expression of different families of genes involved in antimicrobial protein and polyphenolic compound production (Figure 6.1) (Huang 2013; Kumar and Verma, 2018). Although the molecular and biochemical analysis in this study revealed mixed results with regards to response over the time points and phenotypes, it is worth noting the field conditions under which the study was conducted. Wild blueberries are native and perennial plants of North America, hence are always in constant interaction with the environment (both biotic and abiotic factors). Beneficial and pathogenic microbes induce molecular and biochemical changes in plants, hence the mixed result in this study is not surprising (Fujita et al., 2006; Dangi et al., 2018). This was also evident in the basal expression of the studied genes observed in this study. Notwithstanding the potential impact of these external factors, this study revealed that some of the genes, especially the *PR* genes were notably expressed, an indication of their potential involvement in wild blueberry plant defense response.

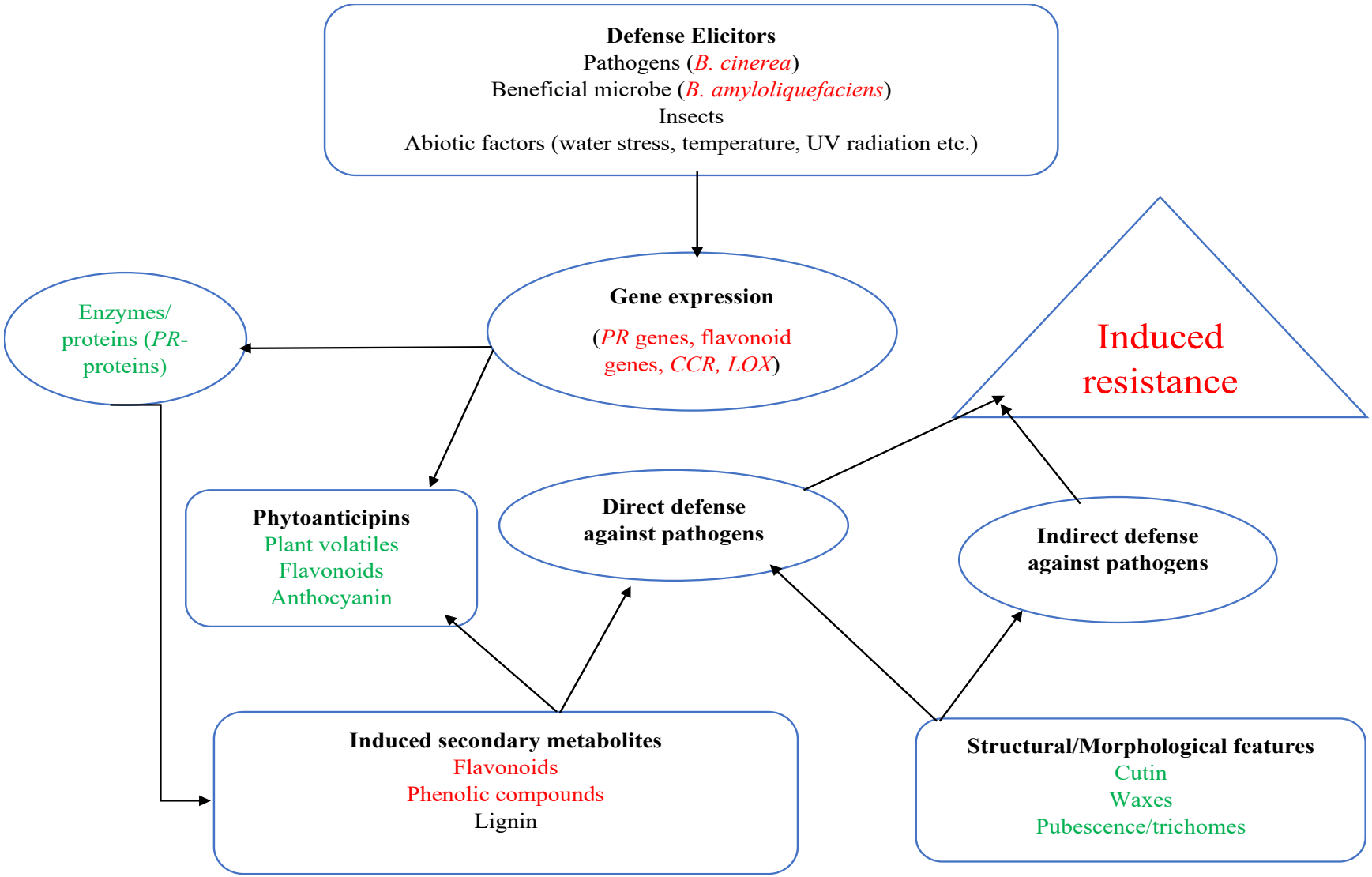

Figure 6.1. Illustration of the generalized defense mechanisms in plants against pathogens. Red fonts represent key factors and responses discussed in this thesis. Green fonts represent preformed and constitutive defense mechanisms in *Vaccinium* species.

The chapter 3 of this thesis also established that the studied microbes had no significant effect on photosynthetic rate (A), transpiration rate (E), and stomatal conductance (Gs) in wild blueberries. Changes in physiological processes in relation to pathogen infection have long been recognized as a plant stress indicator (Mandal et al., 2009; Withers et al., 2011; Zhao et al., 2011). A decrease in photosynthetic activities has mostly been linked to a reduction in photosynthetic surface area due to necrosis or due to lost/breakdown of chlorophyll (Berger et al., 2007; Shtienberg, 1992). Also, the contrasting effect of pathogen infection on photosynthesis related parameters such as transpiration rate (E), and stomatal conductance (Gs) have been reported. These mixed results are observed due to differences in the type of pathogen (necrotrophic or biotrophic) and the resistance status of the host plant (Shtienberg, 1992; Yang et al., 2016). For example, in wheat and soybean, infection of resistant varieties led to a significant reduction in photosynthesis than susceptible varieties (Zou et al., 2005; Yang et al., 2016). Contrary to these reports, the inoculation of *Va* and *Vm* resulted in similar values for physiological parameters, thus no significant difference among the phenotypes and microbes. The reason for this phenomenon could be due to the inability of *B. cinerea* to infect the photosynthetic tissues of the wild blueberry plant. Unlike *Monilinia vaccinii-corymbosi* and other leaf damaging pathogens such as *Septoria* spp. and rust (*Thekopsora minima*) which can destroy leaves, *B. cinerea* hardly infects wild blueberry leaves due to their thick and waxy cuticle. None of the leaves from the inoculated plants showed any disease symptom/necrosis which is known to negatively affect these physiological parameters. In highbush blueberry leaves infected with *Septoria albopunctata* resulted in decreased net assimilation rate (NAR) (CO2 assimilation and leaf conductance). A negative effect of increasing necrosis was observed on photosynthesis, with NAR decreasing exponentially as disease severity increased (Roloff et al., 2004). Similarly, Dawson (2009) reported a negative correlation between Septoria leaf spot and leaf rust severity, and net carbon dioxide exchange rates (NCER).

Finally, a negative correlation was reported by Gruber et al. (2012) when they studied the effect of leaf spot (*Blumeriella jaapii*) on tart cherry. As disease severity increased, leaf-level physiological parameters including photosynthesis and stomatal conductance (gs) decreased significantly. *Bacillus amyloliquefaciens* is not pathogenic, hence the photosynthetic tissues of the wild blueberry plants were intact.

From a host resistance and disease management point of view, the induction of defense response is very essential. External elicitors (biofungicides or chemical elicitors) can be incorporated into fungicide spray programs to prime/activate sufficient defense response in the plant against pathogen. Regardless of the basal expression, a further increase in the expression levels of defense genes can be achieved with external elicitors (Herman et al., 2007). For instance, in a recent study, the plant-defense eliciting fungal protease from *Acremonium strictum* (PSP1) was demonstrated to induce pathogen defense in soybean under field conditions. In the multi-year, different locations and different genotypes study, foliar application of PSP1 enhanced pathogen defense which effectively reduced late season diseases development in soybean caused by *Septoria glycines*, *Cercospora kikuchii* and *Cercospora sojina* (Chalfoun et al., 2018). Similarly, Yi et al., (2012) demonstrated that pepper seedling treated with benzothiadiazole (0.5 mM BTH) under field conditions resulted in less disease symptoms during a natural bacterial spot and *Cucumber mosaic virus* disease outbreaks.

Plants have long possessed their defense tools to protect themselves and reduce the impact of pest and pathogen attacks. These tools in most situations are not adequate to completely prevent and resist pathogen attacks, hence for adequate and complete protection, plants need external tools and strategies to support them which over the year has been delivered in the form of fungicides, especially in commercial fields. That notwithstanding, these additional support plants receive to survive pathogen attacks is not without challenges. In the quest to improve disease management and address some of the

current challenges regarding fungicide resistance among pathogen population, this study provides evidence to support the adoption of some relatively new fungicides in wild blueberry production. This study (chapter 4) established that Miravis® Prime (pydiflumetofen & fludioxonil) and Merivon® Xenium (fluxapyroxad & pyraclostrobin) can be a potential alternative to the already existing Botrytis control products. In both years, Miravis® Prime and Merivon® Xenium consistently provided significant disease control. These two products were effective and, in some cases, better than the already existing Botrytis control products (Luna Tranquility® and Switch®) registered for use in wild blueberries.

Although these relatively new products were equally effective as the existing products, previous studies have shown some level of resistance development among the pathogen population to the registered fungicides used in wild blueberry production. For instance, *B. cinerea* isolated from wild blueberry fields was found to be developing resistance to the active cyprodinil found in Switch® (Abbey, 2017). Although pyrimethanil which is one of the active ingredients in Luna Tranquility® has not been tested on *B. cinerea* isolates from wild blueberry fields, it belongs to the same group (FRAC 9) and are classified as a medium risk with known resistance among *B. cinerea* population (FRAC, 2021). Therefore, there is an increased potential of experiencing reduced efficacy with these Botrytis blight registered products over time. Given this, the addition of Miravis® Prime and Merivon® Xenium as new products for Botrytis blight management in wild blueberries will be essential. With different modes of action represented in these products, there would be an opportunity to implement fungicide programs that would provide acceptable levels of disease control as well as resistance management *B. cinerea* population.

While monitoring the distribution and persistence of fungicides in plant samples, a relatively important concentrations of all the fungicides were observed in the samples collected. A higher

concentration of fungicides in corolla is of critical importance regarding disease control. The corolla represents a large proportion of the flower and is the most susceptible and exposed part of the flower (Hildebrand et al., 2001; Abbey et al., 2018). In this regard, it must be mentioned that the very high residue concentrations of fungicides in corolla is important for disease management.

The concentrations between the gynoecium and androecium were similar for all the fungicides and thus there was no mobility preference for any of the organs. A comparison of the concentrations between the corolla, and the gynoecium and androecium, suggests limited mobility of these fungicides within the wild blueberry flower. This finding confirms the local systemicity (translaminar redistribution) of these fungicides (Manaresi and Coatti, 2002; Walter et al., 2007; Warneke et al., 2020). Although the concentration of the tested fungicides in the gynoecium and androecium was lower than in the corolla, they were significantly high to inhibit pathogen development with respect to the EC_{50} of various *B. cinerea* isolates reported in literature (Fernández-Ortuño et al., 2013; Abbey, 2017; He et al., 2020).

All the fungicides in this study were found to be persistent 10 days after the second fungicide application. Generally, fungicides breakdown or dissipation has been reported to be dependent on the plant parts, type of plants, cultivar, and environmental conditions (Fenoll et al., 2009; Zhang et al., 2015; Yogendraiah Matadha et al., 2021). Marín et al., (2003) reported the half lives of cyprodinil and fludioxonil to be 2.46 and 1.68 days respectively in lettuce and 4.68 and 4.53 days, respectively in table grapes. Regardless of the fungicide and the recommended rate applied in this study, they were persistent in levels greater than the EC_{50} of *B. cinerea* reported in literature including some resistant isolates (Kim and Xiao, 2010; Vitale et al, 2016). This implies that, when the corolla senesces and drops as the flower develops, there will be adequate concentration of fungicides to inhibit *B. cinerea* development, especially in the persistent calyx where latent infections are observed close to harvest.

With these levels of fungicide residue at fruit set, two doses of each fungicide would be adequate for Botrytis blight control.

In the ripe berries, the fungicides were not just below their corresponding MRLs but below the detection limits of the developed method. This is the first report of these fungicides in wild blueberry; however, the presence of these fungicides has been studied in different crops which corroborates with our findings. For instance, non detectable levels of fludioxonil and low persistence of cyprodinil in grape at 0.03 mg/kg were reported by Marín et al., (2003). Regardless of the MRL allowed for the tested fungicides in blueberries, the non detection of all these fungicides in harvested berries is very significant from a dietary risk (consumer preference for consuming fruit with no detectable residues) and international market point of view.

Results from this study are important because they support the potential of adopting these relatively new control products for Botrytis blight management in wild blueberries. In addition, the two applications of these products for disease control as demonstrated in this study is important towards a reduction in the cost of fungicides and a reduction in the use of chemical fungicides. The implication of minimal to no detectable fungicide residue on berry is important since about 90% of lowbush blueberries are exported to countries with strict MRLs. Therefore, being able to produce residue free berries to meet international MRL is of utmost importance.

Regardless of the effectiveness chemical fungicides, there is always the tendency for fungicide resistance among pathogen population to occur with time. Additionally, dietary, and environmental risks associated with fungicides are reviewed periodically. Through these reviews, some fungicides can be deregistered or their use in specific crops discontinued. Considering this, the identification of alternative disease control products such as biofungicides has become a research priority. Chapter 5 of this study established that biofungicides applied alone or their rotation with chemical fungicide

(Switch®) have the potential of reducing disease development in wild blueberry fields, however, the integration with low-risk chemical fungicides is a more promising approach. The use of biofungicides, be it biocontrol agents or extract from plants/microbes in disease control has been in existence for years (Eilenberg et al., 2001; Shafi, et al., 2017; Sood et al., 2020). Extensive studies into the modes of action of these biofungicides have been conducted over the years. Biocontrol agents such as *Bacillus* spp. and *Trichoderma* spp. and their derivatives are known to act directly against plant pathogens through competition for nutrients or space parasitism, or antibiosis and indirectly through the induction of host resistance machinery (Eilenberg et al., 2001; Dimopoulou et al., 2019). Even though the exact mode of action of plant extracts/essential oils against plant pathogens are not clear, some modes of action that have been reported include cytoplasmic membrane disruption and inhibition of microbial enzymes (Marchese et al., 2017). It is believed that essential oils accumulate in the cell membrane and disrupt the cell structure thereby leading to leakage of cell constituents (Diao et al., 2014). Given these, one can expect that the use of Diplomat 5SC® (polyoxin D), Timorex Gold® (tea tree oil), Fracture® (BLAD), and Serenade MAX® (*Bacillus subtilis*) will result in sufficient disease control. This study has indeed shown that the use of these biofungicides has the potential to reduce disease development in wild blueberry in some situations.

This study also found that the rotation of these biofungicides with the chemical fungicide Switch® improved disease control in two of the four trials. The use of biofungicide alone especially biological control agents such as *Bacillus* spp. may be vulnerable to the weather, therefore, limiting their establishment and efficacy against pathogens, therefore affecting their consistency (Xu et al., 2011; Sylla et al., 2015; Wei et al., 2016). In addressing this challenge, the combination of different biofungicides or with compatible chemical fungicides has always been explored and previous studies have shown that this approach improves and ensure consistent disease control (Droby et al., 2009;

Hahn, 2014; Abd-El-Khair et al., 2019). The results from the rotation of the biofungicides with Switch® (chapter 5) revealed the ability of this strategy to suppress disease development in wild blueberry, especially Tea tree oil. This result partly agrees with a previous study by Dorighello et al., (2020) who showed that the stand-alone applications of coffee oil and *Bacillus subtilis* were able to reduce the development of Asian soybean rust (*Phakopsora pachyrizhi*), however, the rotation of these biofungicides with commercial fungicide (pyraclostrobin + epoxiconazole) resulted in better and consistent disease control.

The results of this study indicate that the use of Diplomat 5SC®, Timorex Gold®, Fracture®, and Serenade MAX® certainly can be considered an alternative to conventional fungicides, however, their combination with chemical fungicides would most likely provide significant and consistent disease control. Although biofungicides are known to provide significant disease control, they generally have low to moderate innate efficacy, hence the high efficacy chemical fungicides act as a guard to ensure disease control in a situation when the biofungicides fail. Additionally, the concept of rotating the biofungicides with chemical fungicides may delay the onset of fungicide resistance by reducing the amount of chemical fungicide usage. With wild blueberry mostly exported to countries with strict MRL regulations, the reduction in chemical fungicides and increased pre-harvest interval from the time of Switch® will produce berries with no to minimal fungicide residue.

This study revealed a variable and inconsistent outcome of the different treatments on yield components and harvestable yield from Chapters 3, 4 and 5. Regardless of the significant disease reduction obtained, the impacts of the treatments seem not to translate directly into yield. This suggests other factors likely played a role in the yield data. It should be noted that commercial wild blueberry fields are extremely variable and diverse with multiple *Vaccinium* spp., plant density, and plant coverage. Plant density and coverage within an area is a key determinant of berry yield per specific

area, hence given the randomness of these variability of the field, yield data tend to be often variable (Hepler and Yarborough, 1991). Wild blueberry plants are inherently variable that it is difficult to find two identical clones within a patch. Nonetheless, a recent study by Barai et al, (2022) revealed that the number of leaf and stem functional characteristics are linked to yield related traits. These leaf and stem functional traits can be vital in predicting wild blueberry yields in specific parts of the fields. With this predictive ability, specific fields or parts of field can benefit from targeted management practices.

This diversity in commercial fields makes it very challenging to establish conclusions based on only yield parameters in scaled down field experiments but a consideration of several criteria such as stem length, disease severity and incidence, fruit set, and pin heads among others (Kinsman, 1993).

6.2 CONCLUSIONS

This study investigated gene expression changes and physiological alterations during *B. cinerea* and *Bacillus amyloliquefaciens* interaction with different wild blueberry phenotypes. Alterations in pathogenesis-related, flavonoid pathway and jasmonate pathway gene transcripts, and alterations of polyphenolic compounds were caused by these microbes. This study provides evidence that the PR genes (*PR3, PR4* and *PR5*) and some of the studied flavonoid pathway genes which were upregulated following infection might play an important role in wild blueberry defense against *B. cinerea.* The *B. amyloliquefaciens* demonstrated the potential of inducing defense related genes such as *PR4*, *FLS, ANS* and *CCR* in wild blueberry which is suggestive of induced resistance as a mode of action for this biocontrol product. Infection of wild blueberry flowers had no significant impact on photosynthetic rate (A), transpiration rate (E), and stomatal conductance (Gs) which mostly take place within the leaves of the plant. This study provides an insight into the molecular and biochemical mechanisms of plant-microbe interaction in wild blueberry under field conditions. Wild blueberries interact with microbes

through an increased production of pathogenesis related genes and some flavonoid genes, and the selective accumulation of polyphenolic compounds as part of its defense mechanism. This knowledge could contribute to improving our understanding of how wild blueberry innate immunity may be harnessed to improve disease control. Knowledge of the molecular response of defense genes associated with pathogens can be integrated into IDM to induce natural defense through the application of external elicitors.

All the fungicides showed significant effects in controlling Botrytis blossom blight under field conditions, especially under high disease pressure. Among the fungicides tested in this study, Miravis® Prime and Switch® were observed to be consistent and the most effective. All the fungicides tested had a similar mobility pattern within the wild blueberry flowers. The mobility of these fungicides was limited within the flower tissue. The tested fungicides were persistent and residue concentrations of all the fungicides in the flower, and at fruit set were enough to suppress *B. cinerea*. Residues were not detected in harvested berries which is important given that most of the harvested wild blueberries are exported to the international market with strict MRL requirements.

Significant disease suppression was obtained with stand-alone applications of biofungicides, however, stand-alone applications of biofungicides provided intermediate levels of disease control. Although polyoxin D was often as effective as combinations with Switch. Switch® (conventional fungicide) in rotation with biofungicides (biocontrol agents and natural compounds) resulted in improved Botrytis blossom blight control as well as an increased berry yield in two of the four trials. Fungicide rotation programs are necessary to manage Botrytis blight and to safeguard biofungicides.

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APPENDICES

Appendix 1: Future research

This research has generated an extensive amount of new knowledge fundamental to plant responses to microbes, and fungicide activities in wild blueberries, however, there is yet substantial research that can be conducted in the future.

Future research can investigate areas such as transcriptomic analysis of tissue cultured wild blueberry phenotypes under a controlled environment. Transcriptomic research should target the whole genome response of the plant to microbes specifically, *B. cinerea*, *Monilinia vaccinii-corymbosi* and microbe based biofungicides. This will help eliminate the influence of external factors on plant responses as discussed in this study. For integrating the plant's molecular response into wild blueberry disease management, future research can focus on how external elicitors can be used to prime wild blueberry plants and tested some fungal pathogens.

Future research can also focus on investigating fungicides breakdown in flower and leaf tissues over a period. It will be interesting to establish the timeline of dissipation of these fungicides within the flower tissues. The residual efficacy of fungicides in the inhibition of fungal growth on plant tissue should also be tested. Furthermore, other future studies can include the role of pollinators in fungicide distribution within the field. Also, given that this experiment was conducted on the field, it will be important to study the behaviour fungicides within the plant tissue in a controlled environment.

Appendix 2: List of publications and conference presentations

Peer reviewed publication

Abbey, J., Sherin, J. Percival, D., Jaakola, L. and Asiedu, S. (2022). Molecular Responses of Wild Blueberry Phenotypes to *Botrytis cinerea* Infection. Acta Horticulturae (In Press).

Abbey, J. A., Percival, D., Jaakola, L., and Asiedu, S. K. (2021). Potential use of biofungicides and conventional fungicide for the management of Botrytis blossom blight in lowbush blueberries. Canadian Journal of Plant Pathology, 1-10.

Manuscript under prep

Abbey, J., Sherin, J. Percival, D., Jaakola, L. and Asiedu, S. (In Prep). Modulation of defense genes and phenolic compounds in wild blueberry in response to *Botrytis cinerea* under field conditions.

Abbey, J., Percival, D., Jaakola, L. and Asiedu, S. (In Prep). Botrytis blossom blight management and fungicide residues evaluation in wild blueberry.

Conference and seminar presentations

Abbey, J., Percival, D., Jaakola, L. and Asiedu, S. (2021). Potential use of biofungicides for the control of Botrytis blossom blight and Septoria leaf spot in wild blueberry fields. XII ISHS International *Vaccinium* Symposium. Truro, Nova Scotia, Canada. August 30 –Sept. 1, 2021. (Poster Presentation).

Abbey, J., Sherin, J. Percival, D., Jaakola, L. and Asiedu, S. (2021). Molecular Responses of Wild Blueberry Phenotypes to *Botrytis cinerea* Infection. XII ISHS International *Vaccinium* Symposium. Truro, Nova Scotia, Canada. August 30 –Sept. 1, 2021.

Abbey, J., Sherin, J. Percival, D., Jaakola, L. and Asiedu, S. (2019). Comparing the response of pathogenesis gene (*PR genes*) in wild blueberry phenotypes challenged with *Botrytis cinerea*. Nova Scotia Wild Blueberry Conference. Truro, Nova Scotia, Ca. 14-15 November 2019. (Poster presentation).

Abbey, J. and Percival, D. (2019). Management of Botrytis blossom blight in wild blueberry. Maritime Wild Blueberry Annual Field Day. Springhill, NS, Ca. 18 July 2019.

Abbey, J., Anku, K. and Percival, D. (2019). Disease Management and Remote Sensing 2019/2020. PEI Wild Blueberry Growers Annual Conference. Charlottetown, PEI, Ca. 5 April 2019.

Appendix 3: Phenolic compounds used in HPLC-DAD and primers used in the qRT-PCR.

Table A 1. List of phenolic compounds, their retention times and wavelength of determination

Compound	Retention time (min)	Wavelength λ (nm)
Catechin	25.30	280
Procyanidin B2	25.84	280
m-coumaric acid	28.93	280
p-coumaric acid	28.22	302
Chlorogenic acid	26.17	302
Caffeic acid	26.72	302
Neochlorogenic acid	22.69	302
Quercetin-3- galactoside	29.20	355
Quercetrin (Quercetin 3-rhamnoside)	30.00	355
Kaempferol -3- glucoside	30.10	355

Table A 2. List of primer pairs used for gene expression studies.

Gene name	Accession number	Primer	Sequence details (5'-3')	Amplicon size
PR3	MK292726	Forward	AGACTTGGTAGCAACTGAC	82
		Reverse	GGAAGGTTTCGGGGATTG	
PR4	MK292724	Forward	TAACTACAACCCGGAGCAGG	164
		Reverse	GCAAGCACTTCCCACAAGAA	
PR5	This study	Forward	TAGACGGGTTCAATGTGCCA	91
		Reverse	CACTGCCCGTTTATATCAGCA	
CHS	MK333527	Forward	TCCCAGATCAAGAAGAGGTACA	119
		Reverse	ATTTCCACAACCACCATATCCT	
FLS	MK333531	Forward	CTCCTTCTTACAGGGAAGCTAATG	79
		Reverse	GACAGCCACTTGAACAACTTG	
LOX	MT006245	Forward	CGTGCTTCACCCGATTCATA	78
		Reverse	GTCTGTCTAGCGAGTGCATTTA	
ANS	MK333528	Forward	GAATCACCTGAGAGCCCTAAC	75
		Reverse	AGCCTGTCTTCTTCCAATCC	
ANR	MH321471	Forward	CAAAGACCCTAGCGGAGAAAG	98
		Reverse	GGAGAAACACCAGCCATAAGA	
DFR	MK333524	Forward	CTGCTGGAACCGTCAATGT	139
		Reverse	GCTGCTTTCTCTGCTAGTGTT	
CCR	This study	Forward	CCTGTTACTGATGACCCAGAAG	115
		Reverse	CGATCGATGAGGTGAACACTAC	
LOX	MT006245	Forward	CGTGCTTCACCCGATTCATA	78
		Reverse	GTCTGTCTAGCGAGTGCATTTA	
GAPDH	AY123769	Forward	CAAACTGTCTTGCCCCACTT	207
		Reverse	CAGGCAACACCTTACCAACA	

Appendix 4 Botrytis infection periods and Environmental conditions

Table A 3. Infection periods for Botrytis blight observed at Murray Siding, NS in June 2018

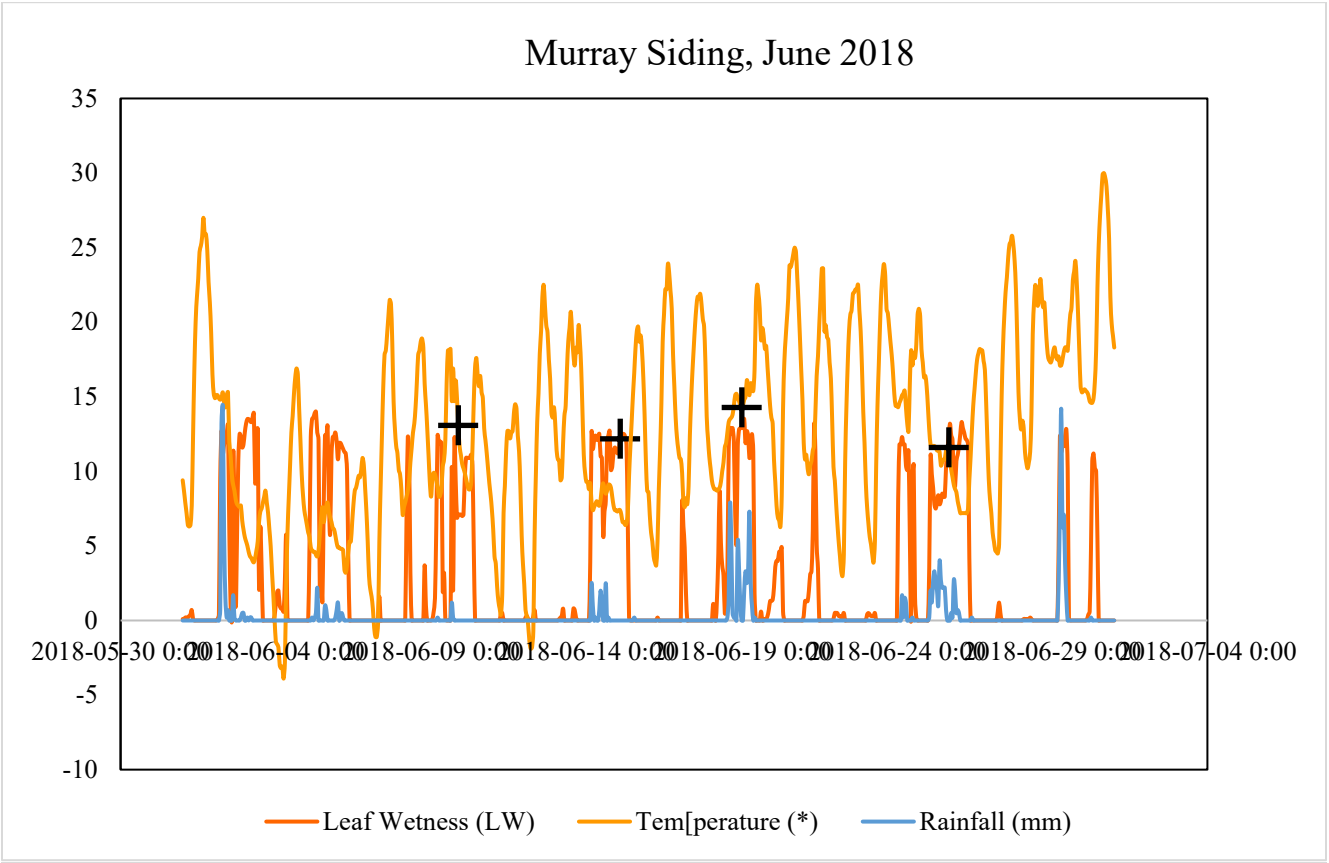
Start and end of infection period (Date, time)			Wetness duration (Hours)	Mean Temperature (°C)	Infection Period Rating
2018-06-09 16:00	-	2018-06-10 9:00	18.00	11.9	Moderate
2018-06-14 3:00	-	2018-06-15 8:00	30.00	7.8	Moderate
2018-06-18 13:00	-	2018-06-19 9:00	20.00	14.6	Moderate
2018-06-25 1:00	-	2018-06-26 8:00	32.00	9.8	Moderate

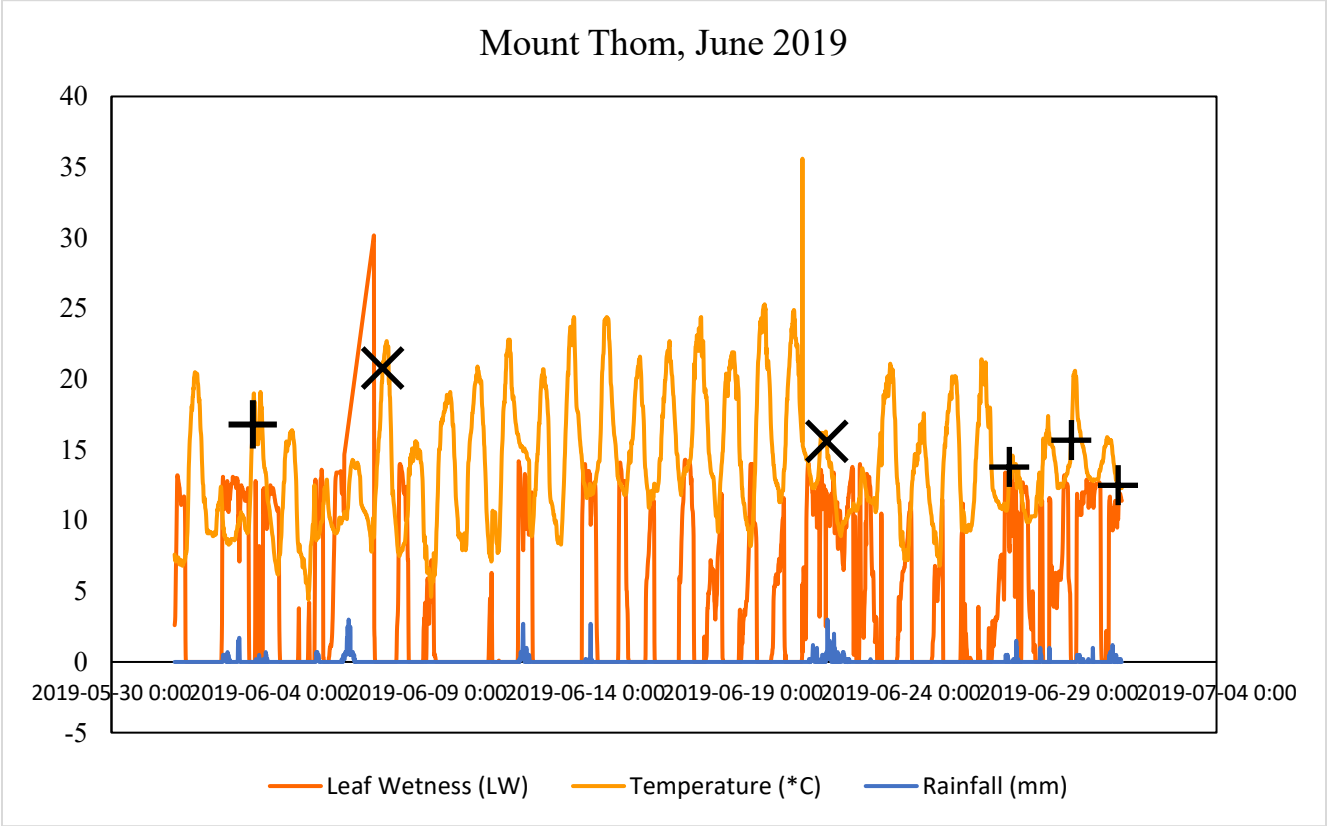
Table A 4. Infection periods for Botrytis blight observed at Mount Thom, NS in June 2019

Start and end of infection period (Date, time)			Wetness duration (Hours)	Mean Temperature (°C)	Infection Period Rating
2019-06-03 19:15	-	2019-06-04 7:30	12.5	10.1	Moderate
2019-06-06 0:00	-	2019-06-07 7:30	31	11	High
2019-06-21 0:00	-	2019-06-22 13:45	38	12.3	High
2019-06-27 0:00	-	2019-06-27 17:00	17	12	Moderate
2019-06-28 17:00	-	2019-06-29 8:45	16	13.6	Moderate
2019-06-29 13:45	-	2019-06-30 8:00	18.5	14.2	Moderate

Table A5. Infection periods for Botrytis blight observed at Debert, NS in June 2019

Start and end of infection period (Date, time)			Wetness duration (Hours)	Mean Temperature (°C)	Infection Period Rating
2019-06-02 12:30	-	2019-06-03 7:30	19	10.5	Moderate
2019-06-06 3:15	-	2019-06-06 18:15	15	11.8	Moderate
2019-06-17 17:30	-	2019-06-18 7:45	14.5	14.5	Moderate
2019-06-21 0:30	-	2019-06-22 16:30	40	13.6	High
2019-06-28 18:15	-	2019-06-29 9:30	15	15	Moderate
2019-06-29 12:30	-	2019-06-30 9:15	21	15.9	High


Murray Siding, June 2018
35
30
25
20
15
10
5
0
-5
-10
2018-05-30 0:00
2018-06-04 0:00
2018-06-09 0:00
2018-06-14 0:00
2018-06-19 0:00
2018-06-24 0:00
2018-06-29 0:00
2018-07-04 0:00
Leaf Wetness (LW)
Tem[perature (*)
Rainfall (mm)


Mount Thom, June 2019
40
35
30
25
20
15
10
5
0
-5
2019-05-30 0:00
2019-06-04 0:00
2019-06-09 0:00
2019-06-14 0:00
2019-06-19 0:00
2019-06-24 0:00
2019-06-29 0:00
2019-07-04 0:00
Leaf Wetness (LW)
Temperature (*C)
Rainfall (mm)

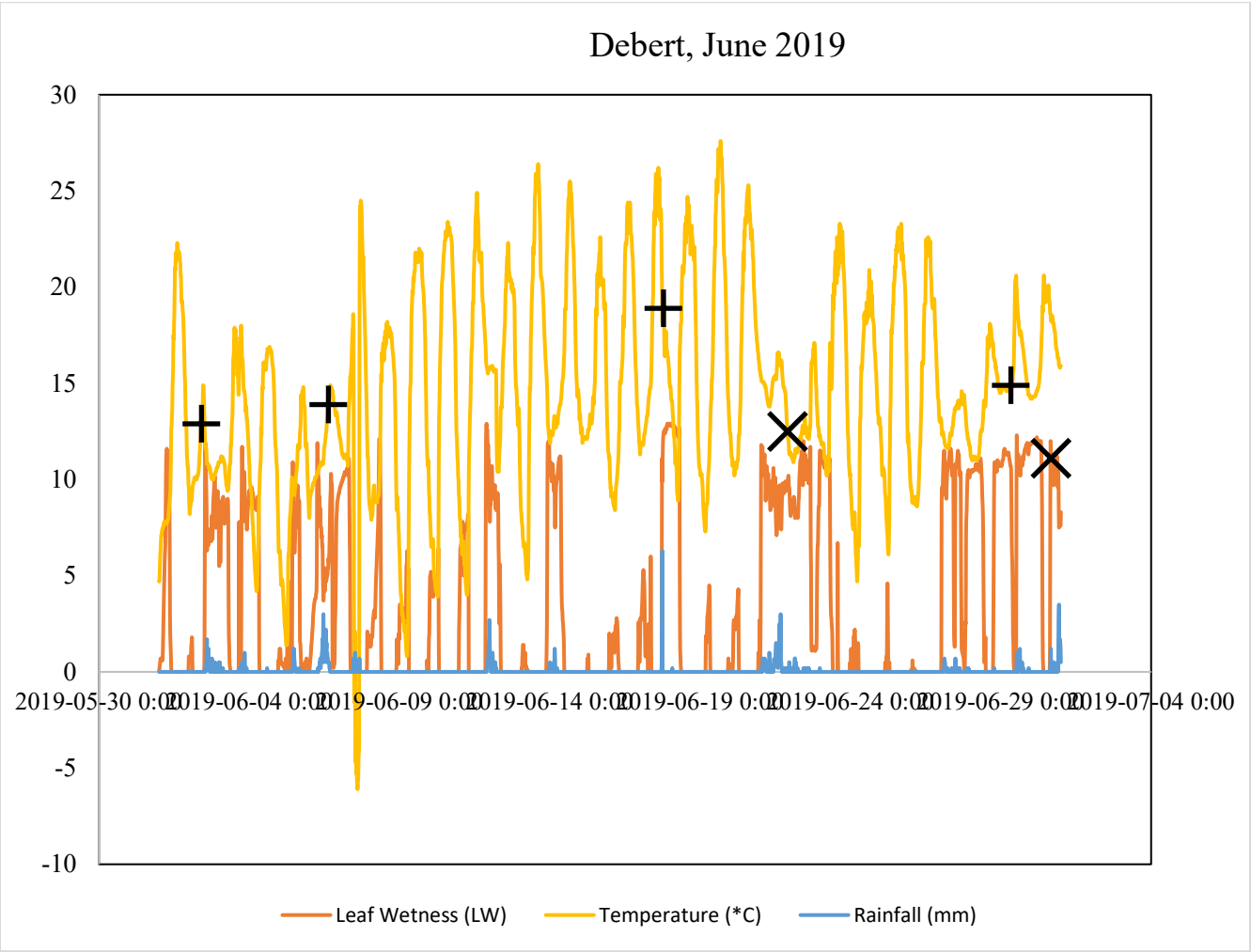


Figure A1. Environmental conditions (leaf wetness, temperature, and rainfall) observed in Murray Siding, Mount Thom and Debert, NS in 2018 and 2019. X: High risk Botrytis infection period, +: Moderate risk Botrytis infection period.

Table A6. Infection periods for Botrytis Blight observed at Pigeon Hill, NS in June 2018

Start and end of infection period (Date, time)	Wetness duration (hours)	Mean Temperature (^{0}C)	Infection period Rating
2018-06-02 3:00 - 2018-06-03 1:00	34	8.08	High
2018-06-05 3:00 - 2018-06-06 2:00	35	4.38	Moderate
2018-06-14 3:00 - 2018-06-15 8:00	30	6.8	Moderate
2018-06-18 4:00 - 2018-06-19 9:00	30	13.5	High
2018-06-24 9:00 - 2018-06-26 6:00	33	8.97	High
2018-06-29 1:00 - 2018-06-29 12:00	11	17	Moderate
2018-06-29 9:00 - 2018-06-30 7:00	10	17.2	Moderate

Table A7. Infection periods for Botrytis Blight observed at Blue Mountain, NS in June 2018

Start and end of infection period (Date, time)	Wetness duration (Hours)	Mean Temperature (°C)	Infection Period Rating
2018-06-06 6:45 - 2018-06-06 20:40	14	15.7	High
2018-06-06 20:45 - 2018-06-07 19:55	23	12.4	High
2018-06-07 20:00 - 2018-06-08 18:05	22	12.3	Medium
2018-06-08 18:10 - 2018-06-09 11:15	17	11.2	Medium
2018-06-09 20:40 - 2018-06-10 22:20	25.5	10.6	Medium
2018-06-10 4:25 - 2018-06-12 6:25	31	3.8	Low

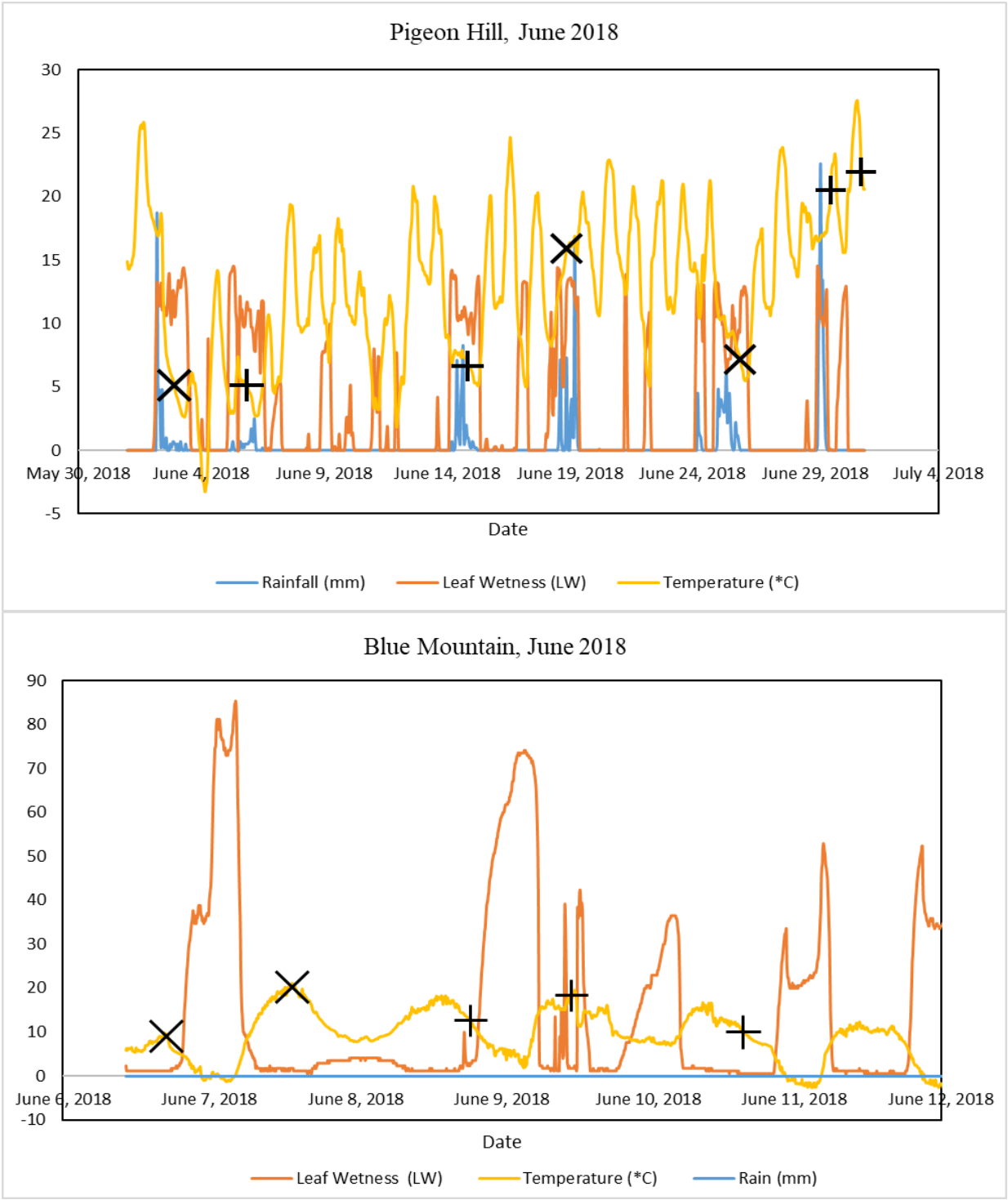


Figure A2. Environmental conditions (leaf wetness, temperature, and rainfall) observed in Pigeon Hill and Blue Mountain, NS in 2018. X: High risk Botrytis infection period, +: Moderate risk Botrytis infection period. Weather data for Blue Mountain were obtained from Data Garrison Satellite Weather Station, Blue Sky Station, NS

Table A8. Infection periods for Botrytis Blight observed at Earltown, NS in June 2019

Start and end of infection period (Date, time)	Wetness duration (hours)	Mean Temperature (^{0}C)	Infection period Rating
2019-06-02 12:15 - 2019-06-03 8:15	20	9.20	Moderate
2019-06-06 12:30 - 2019-06-06 6:30	18	11.25	Moderate
2019-06-16 11:30- 2019-06-17 10:30	11	13.9	Moderate
2019-06-20 10:00 - 2019-06-22 11:45	38	12.6	High
2019-06-27 2:00 - 2019-06-27 5:00	15	12.15	Moderate
2019-06-28 5:00 - 2019-06-29 8:45	16	13.62	Moderate
2019-06-29 1:45 - 2019-06-30 8:00	18	14.20	Moderate

Table A9. Infection periods for Botrytis Blight observed at Farmington, NS in June 2019

Start and end of infection period (Date, time)	Wetness duration (hours)	Mean Temperature (^{0}C)	Infection period Rating
2019-06-02 2:00 - 2019-06-03 7:15	18	9.3	Moderate
2019-06-03 3:15 - 2019-06-04 6:15	15	10.3	Moderate
2019-06-11 18:45- 2019-06-12 6:45	12	14	High
2019-06-13 21:30 - 2019-06-14 9:15	12	12.4	High
2019-06-20 22:15 - 2019-06-22 15:00	41	12.5	High
2019-06-29 12:15 - 2019-06-30 9:00	21	14.6	High

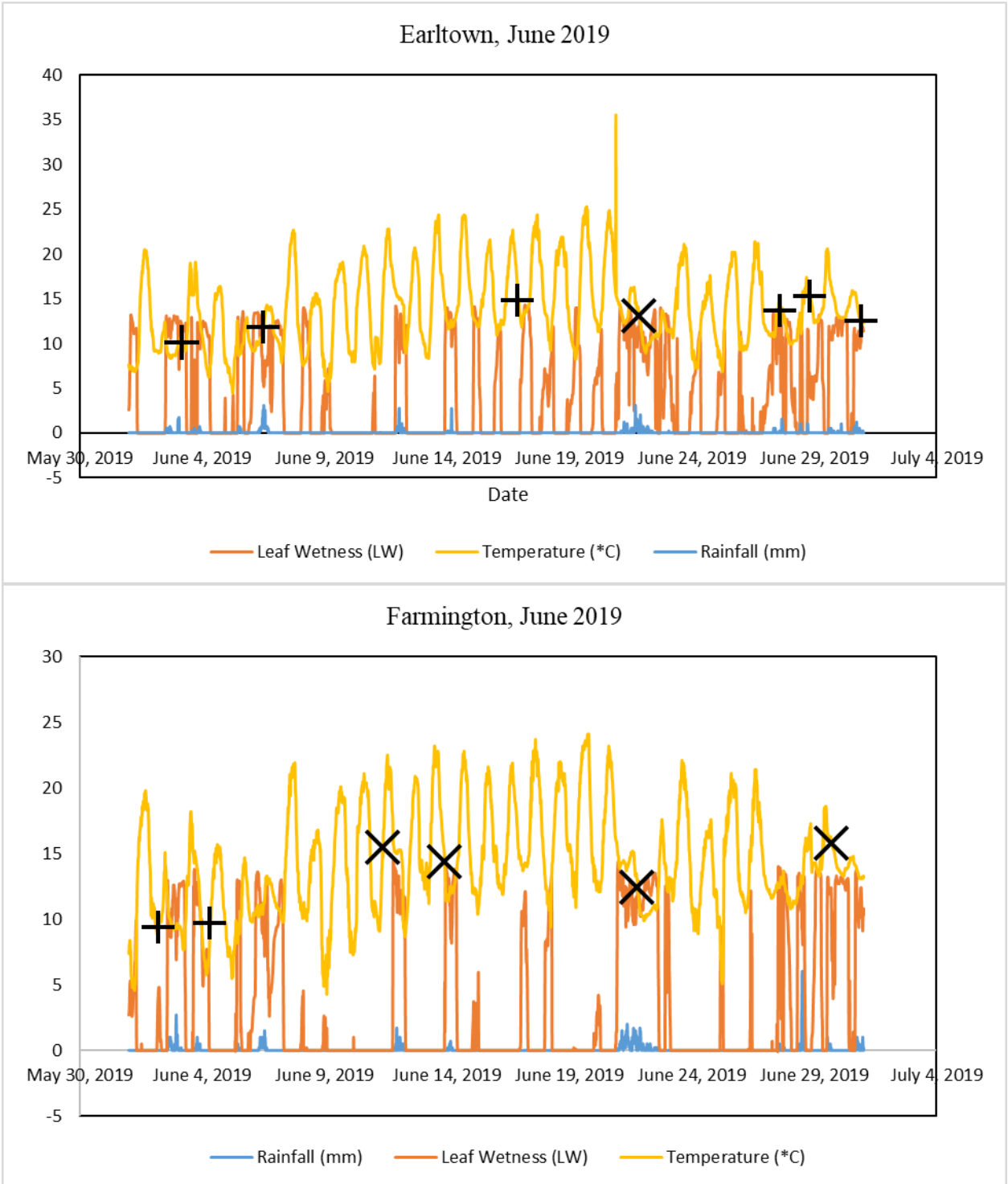


Figure A3. Environmental conditions (leaf wetness, temperature, and rainfall) observed in Earltown and Farmington, NS in 2019. X: High risk Botrytis infection period, +: Moderate risk Botrytis infection period

Table A10. *Botrytis cinerea* infection periods predictive model based on environmental conditions (leaf wetness and temperature). (Delbridge and Hildebrand, 2007)

	Mean Temperature (°C) during Infection Period				
Wetness Duration (Hrs)	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

Delbridge, R and Hildebrand, P. (2007). Botrytis blight control for wild blueberries. Fact sheet No. 212, UMaine Extension No. 2027. https://extension.umaine.edu/blueberries/ factsheets/ disease/212-botrytis-blight-control-for-wild-blueberries. Accessed December 22, 2021.

Appendix 5. ANOVA output for gene expression analysis gene expression analysis in 2019

Table 1. Pathogenesis-related gene 3 *(PR3)*

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
phenotype	5	46	3.33	0.0119
time	3	46	3.19	0.0321
phenotype*time	15	46	2.29	0.0159

Table 2. Pathogenesis-related gene 5 (*PR5*)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
phenotype	5	47	6.44	0.0001
time	3	47	14.52	<.0001
phenotype*time	15	47	1.53	0.1332

Table 3. Chalcone synthase (*CHS*)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
phenotype	5	44	4.06	0.0041
time	3	44	16.66	<.0001
phenotype*time	15	44	2.88	0.0033

Table 4. Flavonol synthase (*FLS*)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
phenotype	5	45	4.82	0.0013
time	3	45	14.55	<.0001
phenotype*time	15	45	1.18	0.3225

Appendix 6. ANOVA output for gene expression analysis gene expression analysis

Table 1. Pathogenesis-related gene 3 *(PR3)*

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Phenotype	2	256	17.15	<.0001
time	3	256	2.00	0.1138
Phenotype*time	6	256	0.80	0.5737
trt	1	256	3.94	0.0482
Phenotype*trt	2	256	3.06	0.0484
time*trt	3	256	2.01	0.1129
Phenotype*time*trt	6	256	0.79	0.5796

T Grouping for Phenotype Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
Phenotype	**Estimate**	
A	1.9081	A
N	1.3905	B
M	0.8155	C

Table 2. Pathogenesis-related gene 5 (*PR5*)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Phenotype	2	258	37.91	<.0001
time	3	258	7.12	0.0001
Phenotype*time	6	258	4.34	0.0003
trt	1	258	0.03	0.8674
Phenotype*trt	2	258	4.78	0.0092
time*trt	3	258	5.18	0.0017
Phenotype*time*trt	6	258	3.07	0.0064

T Grouping for Phenotype Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
Phenotype	**Estimate**	
N	4.8902	A
A	4.3676	B
M	3.2788	C

Table 3. Chalcone synthase (CHS)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Phenotype	2	257	43.44	<.0001
time	3	257	14.31	<.0001
Phenotype*time	6	257	1.22	0.2952
trt	1	257	3.36	0.0679
Phenotype*trt	2	257	2.03	0.1332
time*trt	3	257	3.73	0.0119
Phenotype*time*trt	6	257	1.08	0.3731

T Grouping for Phenotype Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
Phenotype	**Estimate**	
A	0.8164	A
N	0.6467	A
M	-0.07418	B

Table 4. Flavonol synthase (FLS)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Phenotype	2	253	84.61	<.0001
time	3	253	30.58	<.0001
Phenotype*time	6	253	1.34	0.2402
trt	1	253	5.01	0.0261
Phenotype*trt	2	253	2.39	0.0935
time*trt	3	253	2.55	0.0560
Phenotype*time*trt	6	253	1.05	0.3960

T Grouping for Phenotype Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
Phenotype	**Estimate**	
N	4.4995	A
A	3.5439	B
M	3.0086	C

Table 5. Anthocyanin synthase (*ANS*)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Phenotype	2	257	5.28	0.0057
time	3	257	12.63	<.0001
Phenotype*time	6	257	1.57	0.1559
trt	1	257	2.30	0.1305
Phenotype*trt	2	257	2.98	0.0525
time*trt	3	257	1.28	0.2828
Phenotype*time*trt	6	257	0.73	0.6254

T Grouping for Phenotype Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
Phenotype	**Estimate**	
A	2.1115	A
M	1.8521	B
N	1.7284	B

Table 6. Cinnamoyl CoA reductase (*CCR*)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Phenotype	2	257	17.95	<.0001
time	3	257	6.04	0.0005
Phenotype*time	6	257	2.56	0.0201
trt	1	257	3.25	0.0724
Phenotype*trt	2	257	0.49	0.6107
time*trt	3	257	2.47	0.0622
Phenotype*time*trt	6	257	1.96	0.0718

T Grouping for Phenotype Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
Phenotype	**Estimate**	
M	2.3298	A
A	2.2056	A
N	1.8080	B

Table 7. Lipoxygenases (*LOX*)

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Phenotype	2	254	16.12	<.0001
time	3	254	6.52	0.0003
Phenotype*time	6	254	3.19	0.0049
trt	1	254	0.14	0.7054
Phenotype*trt	2	254	2.36	0.0962
time*trt	3	254	1.29	0.2780
Phenotype*time*trt	6	254	0.32	0.9276

T Grouping for Phenotype Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
Phenotype	**Estimate**	
A	5.7932	A
M	5.6526	A
N	5.1679	B