ADVANCING BLUEBERRY POSTHARVEST BIOLOGY: THE EFFECT OF SULFUR DIOXIDE FUMIGATION ON BLUEBERRY PHYSIOLOGY AND RNA-SEQ OF THE BLUEBERRY-COLLETOTRICHUM FIORINIAE PATHOSYSTEM IN 'ELLIOTT' FRUIT

By

Patrick Abeli

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Horticulture – Master of Science

2022

ABSTRACT

ADVANCING BLUEBERRY POSTHARVEST BIOLOGY: THE EFFECT OF SULFUR DIOXIDE FUMIGATION ON BLUEBERRY PHYSIOLOGY AND RNA-SEQ OF THE BLUEBERRY-COLLETOTRICHUM FIORINIAE PATHOSYSTEM IN 'ELLIOTT' FRUIT

By

Patrick Abeli

The physiology and biology of blueberry fruit is the most important consideration for understanding postharvest storage. Fruit physiology is responsive to many stimuli including biotic, genetic, and environmental inputs. The extent to which a blueberry fruit are affected by these factors is largely cultivar dependent. The following studies address two aspects of blueberry fruit postharvest biology as it relates to pest control: In Chapter 2, we explore the effect of SO_2 fumigation on five blueberry cultivars: 'Bluecrop', 'Draper', 'Elliott', 'Jersey', and 'Liberty' and the extent to which SO_2 fumigation can be used to disinfest blueberry fruit of blueberry maggot (BBM) *Rhagoletis mendax* Curran. In Chapter 3, we use RNA sequencing (RNA-seq) to reveal genes differentially expressed (DE) in 'Elliott' fruit in response to infection by *Colletotrichum fioriniae* compared to mock- (water-) inoculated fruit. By filtering out DE genes in mock-inoculated fruit postharvest, as well as DE genes in infected 'Jersey' and 'Draper' fruit, we discovered 113 genes unique to 'Elliott' fruit that are inoculation-responsive. This set of genes include canonical plant resistance genes as well as genes associated with secondary metabolite biosynthesis, cell wall metabolism, reactive oxygen species production and scavenging, and the hypersensitive response. These studies provide important groundwork for future blueberry breeding by identifying cultivars with genetics and physiology amenable to different interventions for reducing postharvest fruit losses.

This one’s for me.

ACKNOWLEDGEMENTS

I would like to acknowledge all of the incredible people who helped me throughout the long process of bringing this thesis to completion: to my blueberry industry partners, Ed Wheeler at MBG, and Mike DeGrandchamp who provided insight and fruit without which these projects would never have happened—thank you; to the undergraduate students who helped in staging, inoculating, counting and grinding of blueberries—Amanda Decker, Aubrey Loftus, and Jake McFadden—thank you; to all the members of the Beaudry Lab who came and went through the years—thank you; to Sue Hammar, Jerri Gillette, and Marivi Colle who held my hand through lots and lots of mistakes and showed me the ropes when I was way out of my element—thank you; to my roommates Logan Rowe, Kellie Walters, and Alex Renny who were there for all the little things and all the big things—thank you; to Dan Donovan and Holly Russell-Donovan, Infamoose, and all of the members of the East Lansing ultimate frisbee community who gave me a much bigger purpose for so many years—thank you; to my new community in Kansas City (especially Caitlyn Lisk, and the members of the Pliakoni Lab at K-State Olathe) who helped me keep my head above water during a global pandemic—thank you; to my rock, my best bud, my dog Lenny—thank you; to my family for whom I owe more than I can ever repay—thank you; and finally to my advisor, Dr. Randy Beaudry, with whom I worked for almost a decade, from whom I learned (almost quite literally) everything I know, who nurtured my curiosity and afforded me grace through many troubled times—thank you, thank you, thank you.

What is the most important thing in the world? It is the people, it is the people, it is the people.
– Maori Proverb

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: BLUEBERRIES AND POSTHARVEST PEST CONTROL

Highbush blueberry (*Vaccinium corymbosum L.*) is a native, perennial shrub that is grown commercially throughout the United States for both fresh and processed markets. In the continental United States, growers have planted over 90,000 acres, mainly in Georgia, Michigan, Washington, Oregon and New Jersey, and the crop was worth over $700 million annually in the United States in 2020 (USDA, 2022). Breeding blueberries for a specific trait, such as seasonality, disease resistance, flavor profile, firmness, or plant architecture, is an intensive process that can take up to 15 years (Olmstead and Finn, 2014) and blueberry plantings can be in production for many decades if the variety remains acceptable to consumers.

Blueberry fruit are commonly harvested either by hand or using a mechanical over-the-row harvester. Both systems have their benefits and drawbacks: hand harvesting is more labor-intensive and slower than mechanical harvesting, but is less damaging to both the fruit and the plants, and generally leads to higher quality fruit with longer shelf-life (Retamales and Hancock, 2012). There have been many recent developments in the design and construction of over-the-row harvesters, including the use of soft catching surfaces, that have begun to reduce the quality gap between hand- and machine-harvested fruit (DeVetter et al., 2019). Following harvest, it is important to rapidly cool blueberry fruit to around 1 °C within 4 hours to limit quality loss, reduce softening, and limit the development of decay organisms (Sargent et al., 2006; Beaudry et al., 1992; Ceponis and Cappellini, 1979). Blueberry fruit can be expected to keep two to four weeks in storage at 0.5 °C and above 90% relative humidity, but with the use of controlled atmosphere (CA) storage under an atmosphere of 1-10% O_2 and 10-15% CO_2 the shelf-life can be extended to up to 8 weeks for certain varieties (Hancock et al., 2008; Gross et al., 2016).

Blueberry fruit may be infested by a number of pests that, if not controlled and managed, may cause severe losses postharvest including, insects, fungi, and bacteria. Control of these pests begins pre-harvest with the planting of resistant varieties, regular pesticide sprays, and through proper field sanitation to remove ground-fallen fruit. During harvest, the most important measures for controlling the spread of pests is to reducing fruit damage and the harvest of overripe fruit (Retamales and Hancock, 2012). Blueberry maggot (BBM) (*Rhagoletis mendax* Curran; Diptera; Tephritidae) is one of the most important insect pests of blueberry fruit in the eastern United States, where it is endemic (Retamales and Hancock, 2012). The use of insecticide sprays is the most common preharvest method of control, though planting varieties resistant to infestation, including 'Earliblue' and 'Northland' is also recommended (Liburd et al., 1998). Postharvest control of insects pests like BBM is primarily through the use of cold storage (Armstrong et al., 1995), though many other methods have shown promise including, hot-air and hot water immersion (Mangan and Ingle, 1992), irradiation (Bustos 2004) and fumigation (Armstrong and Couey, 1984). Postharvest fumigation is used to limit the spread of BBM to the western US and abroad; blueberry fruit are commonly fumigated with methyl bromide (Anon 2019; Roth and Richardson, 1970) at a rate of 32 $g \cdot m^{-3}$ for 2 hours at 21 °C (Thang et al., 2016).

Blueberry suffers from diseases caused by a variety of pathogenic organisms, but diseases caused by fungal pathogens rank among the highest priorities for the blueberry industry (Gallardo et al., 2018). Ripe rot, also known as anthracnose, is a fungal disease of blueberry fruit that is caused by fungi in the genus *Colletotrichum* with *C. fioriniae* being the primary causal organism of anthracnose in Michigan (Damm et al., 2012). It is primarily controlled pre-harvest through the planting of resistant varieties such as 'Draper' and 'Liberty' (Miles and Schilder, 2008) though many susceptible varieties are still widely planted, therefore, fungicides sprays

must be performed frequently throughout the growing season at great cost to producers. This high-frequency use can lead to fungicide tolerance or resistance in the pathogen (Hollomon, 2015).

The development of an integrated pest management (IPM) strategy for the control of both insect and fungal pests will include not only the planting of resistant varieties and the use of pesticide sprays, but also proper sanitation and training during harvest and processing, as well as continued research into new control methods. The following studies expand the catalog of knowledge on pest control as it relates to blueberry postharvest biology and physiology through the use of novel control methods for insect pests and development of a deeper understanding of the genetic interactions of blueberry fruit and the anthracnose pathogen.

CHAPTER 2: BLUEBERRY FRUIT QUALITY AND CONTROL OF BLUEBERRY MAGGOT (*RHAGOLETIS MENDAX* CURRAN) LARVAE AFTER FUMIGATION WITH SULFUR DIOXIDE

(Published in Postharvest Biology and Technology, 179 (2021) 111568)

2.1. INTRODUCTION

Postharvest fumigation of fruits and vegetables is an economically important tool for managing pests and diseases that would cause postharvest crop loss, and as a means to disinfest agricultural products and prevent dissemination of pest organisms to regions where they are not present (Mayfield and Norman, 2012). Fumigants used for disinfestation must be highly effective and treated products may be rejected if the fumigant does not properly control the target organism. The effectiveness of fumigants is dependent upon dose, the duration of the treatment, target organism sensitivity, and environmental influences (Davis, 2003). Desirable characteristics of fumigant molecules include high volatility, effective fruit penetration, rapid action, low phytotoxicity, and little residue. To be commercially viable, the duration of the fumigation treatment must be relatively short in order to ensure high product throughput and to minimize negative effects on the condition of treated products. As a fumigant used for postharvest disinfestation in many settings, methyl bromide is applied for 2 to 24 hours, depending on the treatment concentration, with longer times required for lower concentrations (EPA, 2015). The exposure time for most highly perishable commodities is 2 to 3 hours.

Chemical fumigants such as methyl bromide and hydrogen phosphide (phosphine) have been used commercially as a means of eliminating insect and fungal pests in food, feed, and seed (Davis et al., 2003). Research is ongoing to assess the effectiveness of many other postharvest fumigants for controlling disease and insect infestation on various commodities, such as nitric oxide for control of insect pests on lettuce (*Lactuca sativa* L.) (Yang and Liu, 2019), ozone in table grapes for control of Western black widow spiders (*Latrodectus hesperus* Chamberlin &

Ivie, 1935) (Walse et al., 2017) and sour rot (Pinto et al., 2017), and ammonia gas in *Citrus* spp. for control of blue and green mold caused by *Penicillium* spp. (Montesinos-Herrero et al., 2011). Sulfur dioxide (SO_2) is one of the most successful postharvest fumigants in table grapes for reducing grey mold, caused by *Botrytis cinerea* Pers. (1794) (Eckert and Ogawa, 1985). Due to its effectiveness in this application, there is interest in evaluating the efficacy of postharvest SO_2 fumigation in other fruits and vegetables and for other pest species.

While the history of SO_2 fumigation on blueberry dates back nearly 50 years (Brennan et al., 1970), there is recent renewed interest. This is partly due to the global expansion of this crop and the need for long-distance transportation from regions of production to regions of consumption (Retamales and Hancock, 2012). Blueberry fruit destined for fresh markets are often stored in controlled atmospheric (CA) conditions under high CO_2 and low O_2 environments. In laboratory-scale experiments, the addition of SO_2 to chambers under containing either CA (3% O_2 with 3, 6, 12, or 24% CO_2) or air (21% O_2 + 0.04% CO_2) increased the market life of blueberry fruit by reducing decay incidence without reducing the antioxidant activity of berries although differences among cultivars were observed (Cantín et al., 2012). Rivera et al. (2013) determined the optimal dose for control of grey mold (*Botrytis cinerea* Pers.) in blueberry fruit to be around 250-300 ppm·hr at 20 °C. This is almost double the minimum SO_2 dose that is necessary for 99% control of gray mold in table grapes, which is approx. 100 ppm·hr at 0 °C (Smilanick and Henson, 1992). The solubility of SO_2 in water increases with decreasing temperature (Windholz et al., 1983) therefore it is not surprising that an increased dose would be necessary at higher treatment temperatures. Rodriguez and Zoffoli (2016) found that SO_2 fumigation at a dose of 100 ppm·hr at 20 °C was effective in controlling decay during 45 days of storage at 0 °C when used in tandem with modified atmosphere packaging or perforated bags.

While the efficacy of fumigation for fungal disease control has been clearly documented, it is also important to understand whether these treatments affect the survival of insect pest in fruit.

Fruit flies in the family Tephritidae are destructive frugivorous pests whose larvae can contaminate ripe fruit. Post-harvest control of these insects is commonly needed as a quarantine treatment to prevent spread beyond their native range. Approaches to disinfestation of tephritids include cold storage (Armstrong et al., 1995; De Lima et al., 2007; Gould and Sharp, 1990), hot-air and hot water immersion (Armstrong et al., 1995; Mangan and Ingle, 1992; Sharp et al., 1989), irradiation (Bustos 2004; Hallman, and Martinez, 2001) and fumigation (Armstrong and Couey, 1984; Spitler and Couey, 1983; Williamson et al., 1986). Blueberry maggot, *Rhagoletis mendax* Curran (Diptera: Tephritidae), is native to eastern North America and can infest blueberries during the ripening period creating a risk of infestation (Rodriguez-Saona et al., 2015). For export to other regions of North America or to other countries, it is important that this pest is controlled prior to shipment, and so the blueberry industry employs a systems approach that includes frequent pest monitoring and protection of berries with targeted insecticide applications. In addition, many trading partners also expect an effective postharvest treatment to ensure berries are free of this pest. Fumigation against blueberry maggot (BBM) has been used since the 1970s to control this pest (Roth and Richardson, 1970) and use of methyl bromide is an approved fumigant within the certification program to prevent spread of this pest within Canada (Anon., 2019). However, with the expected phase-out of this fumigant (Mayfield and Norman, 2012), there is a need to explore alternatives such as SO_2.

While most fumigations are performed at atmospheric pressure, one method for fumigation of fruits and vegetables that has shown promise in control of post-harvest decay organisms is the use of low pressure (hypobaric) storage (Burg, 2004). It is proposed that by

establishing a hypobaric environment, and evacuating the intracellular gasses within the fruit, the treatment gas has a lower resistance to diffusion into the fruit during treatment. Absent a fumigant, the use of hypobaric storage to control insect pests, including tephritid fruit flies has been explored. Burg (2004) asserts that all life stages of Mediterranean fruit fly (*Ceratitis capitata* Weidemann (Diptera: Tephritidae) infesting papaya (*Carica papaya* L.) fruit stored under hypobaric conditions (2.67 kPa in air at 10 °C) for 3 weeks were killed, while fruit stored under normal atmospheric conditions (101.3 kPa in air at 10 °C) still had adult flies emerge. Caribbean fruit fly (*Anastrepha suspensa* Loew (Diptera: Tephritidae) eggs stored under hypobaric conditions (2.67kPa in air at 13 °C) in Petri dishes supplemented with nutrient media experienced 100% mortality after 11 d storage while eggs treated at atmospheric pressure only exhibited ~60% mortality (Davenport et al., 2006). We aim to expand on previous studies by investigating the use of hypobaric treatments in tandem with SO_2 fumigation for control of blueberry maggot.

It is necessary to find a balance in any fumigation scheme between the risks for damage to the target crop, the degree of control of a disease or insect pest, and commercial requirements for application (e.g., short exposure time). All fumigation methods are phytotoxic past a certain threshold concentration; even components of air such as CO_2 can cause browning and firmness loss of blueberry fruit at high concentrations (Alsmairat et al., 2011; Cantín et al., 2012). Symptoms of SO_2 toxicity in table grapes include discoloration and bleaching that begins at the stem end of the fruit, but in severe cases can envelop the entire fruit (Nelson and Tomlinson 1958; Pentzer et al., 1938).

In this study, our goal was to quantify SO_2 phytotoxicity as it is affected by cultivar and SO_2 concentration. In addition, we evaluated a short-term exposure to SO_2, of similar duration to

that desired for methyl bromide disinfestation, by assessing the impact of treatment duration, barometric pressure, and SO_2 concentration on fruit quality characteristics and on the survival of BBM in infested fruit following packaging and cold storage.

2.2. MATERIALS AND METHODS

2.2.1. Biological material

All fruit were hand harvested from highbush blueberry (*Vaccinium corymbosum* L.) farms in southwest Michigan and transported to Michigan State University in coolers lined with ice packs. Cultivars included in the study were 'Bluecrop,' 'Draper,' 'Elliott,' 'Jersey,' and 'Liberty'. Once the fruit reached MSU, all fruit were placed in a 0 °C cold room until use. Prior to treatment with SO_2 (see below), fruit were warmed to the treatment temperature (10 °C) for at least two hours.

2.2.2. Experiment 1. 16-h exposure to SO_2 at atmospheric pressure

Blueberry fruit were placed into 10-L glass desiccators at 10 °C for fumigation (Fig. 2.1). For each cultivar and SO_2 concentration combination, there were three replicate fruit samples (~130 g) contained in 237-mL, half-pint clamshells. The desiccators were fitted with red rubber septa for administration of the SO_2. To introduce the gas, high purity (>99%) sulfur dioxide gas was removed from a source cylinder (Airgas, Radnor, PA) and transferred to the treatment desiccators on a volumetric basis using small- (1 mL) or large- (100 mL) volume air-tight glass syringes. After introduction of the SO_2, the 100-mL syringe was used as a bellows to mix the chamber atmosphere. Initial SO_2 concentrations were 0, 22, 220, 1100, 2200, 11000, and 22000 $\mu L \cdot L^{-1}$. Treatment duration was 16 h and the pressure in the chambers was maintained at 101 kPa. At the conclusion of the exposure period, chambers were vented and fruit were transferred to a room at 23 °C for evaluation of surface color and appearance after a warming period of 2 h.

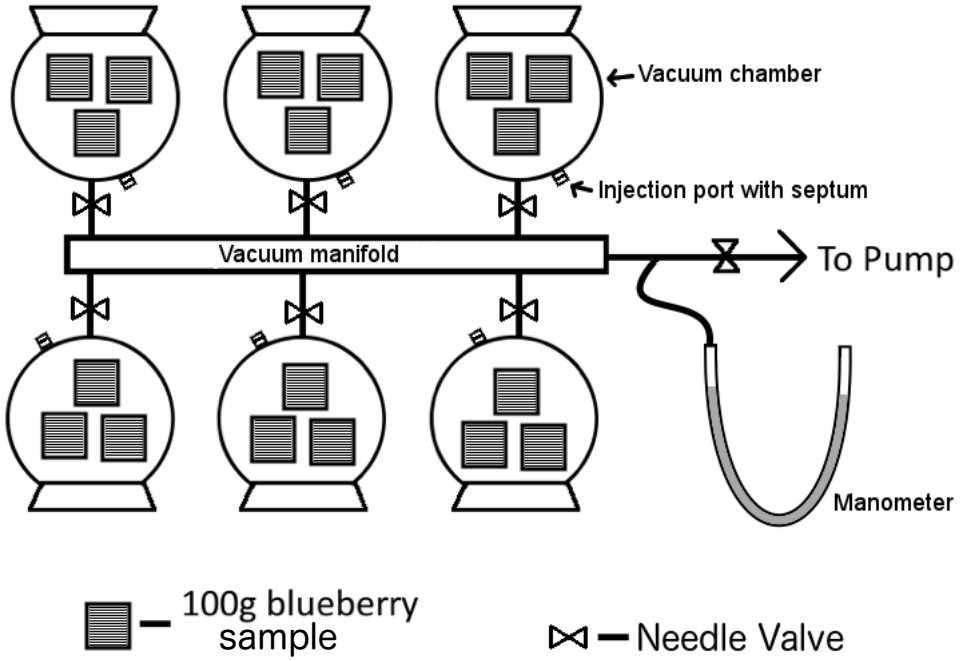

Figure 2.1. Treatment system for SO_2 treatment at ambient or low pressure. Each chamber could be isolated from the manifold using needle valves. Chambers were equipped with a self-sealing septum in the injection port to allow for the introduction of SO_2 gas and air as needed. A mercury manometer was attached in-line between the vacuum manifold and the pump to monitor the pressure prior to treatment. Pressure was regulated using a vacuum regulator. Three replicate samples per treatment level were included in each chamber at the same time. Temperature was regulated by placement of the chambers in an environmental control room.

2.2.2.1. Fruit quality assessment

Fruit color was evaluated for SO_2-treated fruit of four blueberry cultivars (‘Bluecrop,’ ‘Draper,’ ‘Elliott,’ and ‘Jersey'). To determine the extent of color change, we used a colorimeter (CR-400 Chroma Meter, Konica Minolta Inc. Tokyo, Japan) with a 2° observer and C-type illuminant. Prior to fruit measurements, the colorimeter was calibrated using a white color plate (Y: 93.84, x: 0.3132, y: 0.3191). Preliminary experiments showed that fruit color change due to SO_2 exposure occurs first at the stem scar and then moves toward the calyx (data not provided), so we measured color (CIE L*a*b* color space) of the skin adjacent to the stem scar. There were three replicate samples (n=25 berries per sample) per SO_2 concentration/cultivar combination. L* value measures the lightness of the fruit from 0 (black) to 100 (white). The a* value measures the

green (-a*) to red (+a*) component of color, while the b* value measures the blue (-b*) to yellow (+b*) component of color. The combined spatial color change (ΔE) is a representation of the total change in color within the lab color space and is defined by equation 1.

$$\Delta E = \sqrt[2]{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad \text{Eq. 1}$$

ΔE values less than one are considered imperceptible to human eyes, while a ΔE 1-2 is considered perceptible by close observation, and a ΔE greater than 2 is considered perceptible at a glance (Luo, 2016). We considered any ΔE value greater than 2 to be unmarketable due to obvious bleaching by SO_2.

Visual quality analysis was performed on five cultivars (‘Bluecrop,’ ‘Draper,’ ‘Elliott,’ ‘Jersey,’ and ‘Liberty’). To determine if visual assessments, high quality pictures of 25 randomly selected fruit were captured against a white background using a mirrorless digital camera (Sony α6000, Tokyo, Japan). These pictures were then anonymized and assigned a damage index independently by two assessors based on a visual assessment of the severity of the injury symptoms and comparison to a 10-point reference index (Fig. 2.2).

Figure 2.2. Blueberry SO_2 injury index. Injury ratings greater than 2 are considered unmarketable.

2.2.2.2. Statistical Analysis

Statistical analysis (calculation of standard error of the mean) and graphing were performed using commercial software (R version 3.6.1).

2.2.3. Experiment 2. Short-term exposure to SO_2 at atmospheric and hypobaric pressure

'Bluecrop' blueberry fruit were exposed to 24 different short-term SO_2 treatments varying in duration (1 or 2 h), pressure [low pressure (2.76 kPa) or normal pressure (101.3 kPa)], and SO_2 concentration (0, 22, 220, 1100, 2200, 11000, 22000 $\mu L \cdot L^{-1}$) in 10-L desiccators at 10 °C. Three 0.24-L (1/2-pint) clamshells containing 100 g of fruit were treated simultaneously in each of six desiccators differing in target SO_2 concentration. For low-pressure treatments, after loading the desiccators with fruit, a low-pressure environment of 1.38 kPa (10 mm Hg) was established using a vacuum pump (W2V20, LACO Technologies, Salt Lake City, UT) and manifold as depicted (Fig. 2.1). All desiccators were isolated from one another using needle valves (Swagelok, Solon, OH). A vacuum regulator (Model 329 SR, L. J. Engineering, Huntington Beach, CA, USA) and a mercury manometer between the pump and the manifold were used, respectively, to regulate and monitor the pressure in all desiccators. Once the hypobaric environment was established, all desiccators were then sealed by closing the needle valve between the manifold and the desiccator to isolate each desiccator and prevent gas from migrating between desiccators. Target SO_2 concentrations (0, 22, 220, 1100, 2200, 22000 $\mu L \cdot L^{-1}$) were achieved by injecting pure SO_2 gas into the desiccators as previously described. Following this, chamber pressure was brought to 2.76 kPa (20 mm Hg) by injecting air. Desiccators operated at atmospheric pressure were handled as previously described. Exposure duration was either 1 or 2 h. Upon removal from treatment chambers, fruit were stored at 0.5 °C for 0, 7, 14, or 21 d and evaluated as described below.

2.2.3.1. Disinfestation of Blueberry Maggot

Blueberry fruit (cv. 'Bluecrop') were harvested by hand from a planting infested with BBM larvae at an untreated blueberry planting in southwest Michigan. Fruit was harvested

directly into 288 0.24-L (1/2-pint) plastic clamshell containers and transported to Michigan State University in insulated boxes with ice packs. Clamshells with fruit were separated from coming into direct contact with the ice packs. Upon arrival, clamshells were weighed and held at 10 °C until fumigation with SO_2 as described above. After treatment with SO_2, 18 clamshell containers were placed into each of 16, 45 cm x 70 cm pouches composed of 0.0508 mm (2 mil) low-density polyethylene. These were sealed and a portion of the volume removed and replaced with pure CO_2 to generate an initial atmosphere of 10 kPa CO_2 and 18 kPa O_2 to simulate commercial pallet shroud treatments (Macnish et al., 2012). The package was designed to remain aerobic and achieve 10 kPa CO_2 and 3 kPa O_2 at steady state using previously developed models (Beaudry et al., 1992). Fruit were stored for either 0, 7, 14, or 21 days at 0 °C. On each date, CO_2 levels were measured as described by Beaudry et al. (1992) and were found to persist at target concentrations throughout the study (data not shown). On each date, four pouches (containing 3 replicate samples from each of the 24 treatment combinations) were removed from storage for assessment of BBM emergence.

BBM emergence was determined for each clamshell after the allotted storage time. Fruit were removed from the clamshells and placed in rearing chambers consisting of a 473-mL deli cup with a 65-mm hole in the bottom, onto which a round, 80-mm diameter piece of wire mesh with 6-mm x 6-mm holes was secured to allow BMM larva to pass through. These containers were sealed with a ventilated lid to prevent excess moisture build up and suspended in a 946-mL deli cup with a 2-cm deep layer of washed sand in the bottom as a pupation substrate. These rearing chambers were maintained in a walk-in growth chamber (25 °C, 75% RH, and 16:8 light:dark photoperiod) for 21 days.

2.2.3.2. Fruit quality analysis

Blueberry fruit (cv. 'Bluecrop') were harvested by hand from a commercial field in southwest Michigan free of infestation by BBM larvae. Fruit was harvested directly into 288 0.24-L (1/2-pint) plastic clamshell containers and transported to Michigan State University in insulated boxes with ice packs, clamshells with fruit were separated from direct contact with the ice packs. Upon arrival, clamshells were weighed and held at 0 °C until treated. Two hours prior SO_2 exposure, the fruit were transferred to 10 °C to warm to treatment temperature. Fruit were treated with 24 combinations of SO_2 concentration, treatment durations, and pressure, as described previously. There were three replicate clamshells per treatment combination. Immediately after treatment with SO_2, fruit were transferred to room temperature (23 °C) for 2 hours prior to quality analysis. Quality analyses were performed on unstored fruit only.

Firmness was measured on 50-fruit subsamples using a FirmTech 2 fruit firmness tester (Bioworks, Inc. Wamego, KS), which uses a force-deflection method. Units from the FirmTech were in $g \cdot mm^{-1}$ and these were converted to SI units by multiplying by 9.8×10^3 to yield $N.mm^{-1}$. Measurements were made at the equator of each fruit, which reduces fruit-to-fruit variability (Ehlenfeldt and Martin, 2002). The FirmTech 2 was calibrated prior to use using a 250-g weight and a known size standard (3.76 cm dia.).

For total soluble solids (SSC) and titratable acid (TA) analysis, approximately 25 fruit per sample were frozen at -20 °C in sealed 50-mL Falcon™ tubes (BD Biosciences, Franklin Lakes, NJ) to rupture cells and facilitate juice extraction upon thawing. Three replicate samples per treatment combination were used. To extract juice, thawed berries were crushed within the Falcon™ tube with the end of a 15-mL glass culture tube and the macerate was squeezed through cheesecloth into a 15-mL tube for SSC and TA measurements. SSC was determined using a

portable refractometer (Rhino Technology, Oakland, CA) calibrated with a 10% (w/v) sugar solution and is reported as °Brix. TA values, reported as citric acid equivalents, were measured with an automated, digital titrator (Multi-T 2.2, Laboratory Synergy, Inc., Goshen, NY) using 0.1 M NaOH as the titrant. The SSC/TA ratio (a measure of flavor) was calculated based on the SSC and TA measurements of each sample . Fruit color (L*, a*, and b*) analyses were performed as described previously.

2.2.3.3. Statistical Analysis

To examine the effect of each treatment level (SO_2 concentration, time, and pressure) on the response variables (firmness, SS, TA, SS:TA ratio, and blueberry maggot emergence) we used three-way ANOVA. Tukey's Honestly Significant Difference (HSD) test was used to separate groups within each treatment. Statistical analysis was performed using R version 3.6.1.

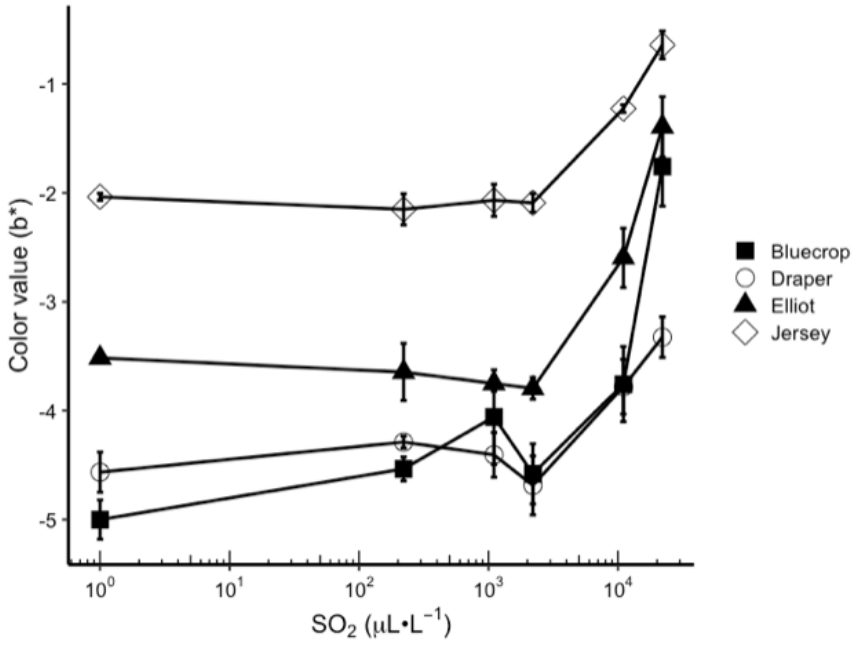

Figure 2.3. Impact of SO_2 concentration on the b* color value of the skin of blueberry fruit sampled near the stem scar. The exposure was performed at atmospheric pressure, the duration was 16 h, and the exposure temperature was 10 °C. Following treatment, fruit were transferred to 23 °C and evaluated within 2 h. Vertical bars represent ±1 standard error.

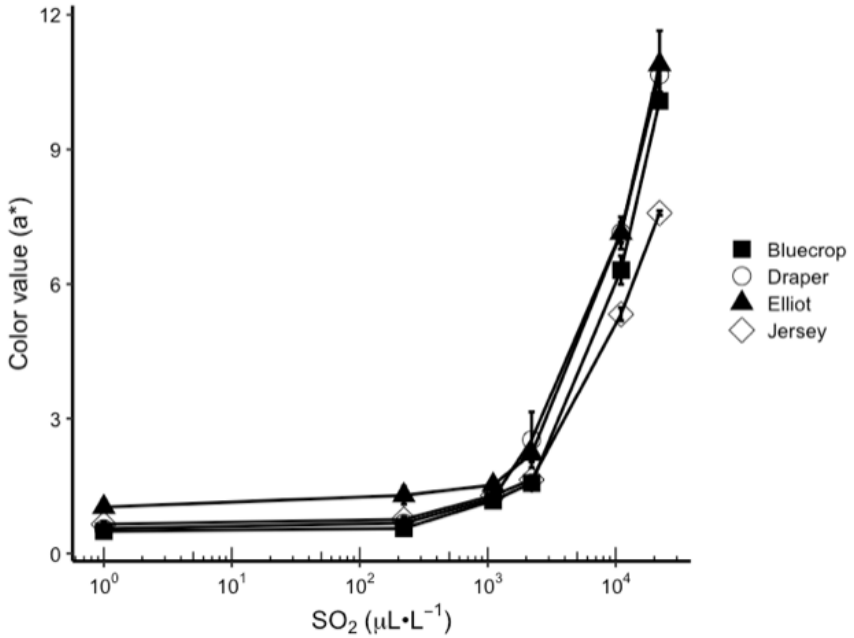

Figure 2.4. Impact of SO_2 concentration on the a* color value of the skin of blueberry fruit sampled near the stem scar. The exposure was performed at atmospheric pressure, the duration was 16 h, and the exposure temperature was 10 °C. Following treatment, fruit were transferred to 23 °C and evaluated within 2 h. Vertical bars represent ±1 standard error.

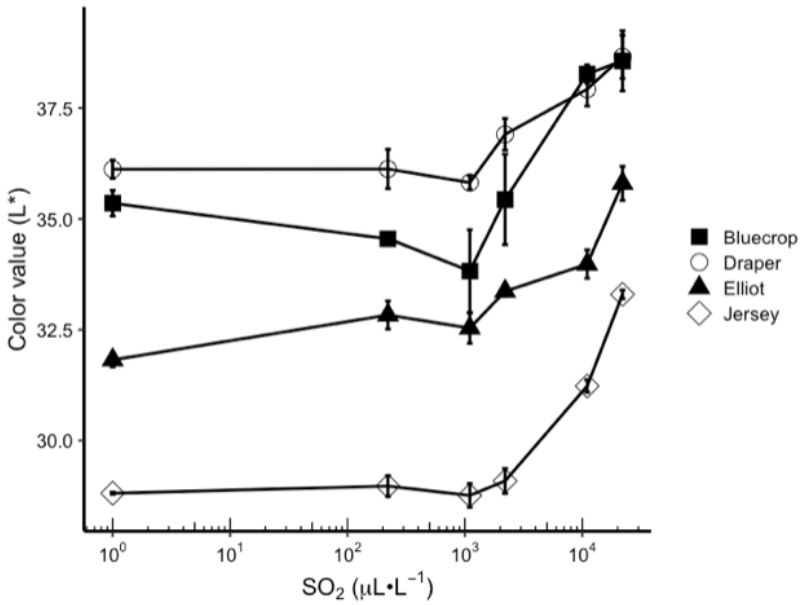

Figure 2.5. Impact of SO_2 concentration on lightness (L*) of the skin of blueberry fruit sampled near the stem scar. The exposure was performed at atmospheric pressure, the duration was 16 h, and the exposure temperature was 10 °C. Following treatment, fruit were transferred to 23 °C and evaluated within 2 h. Vertical bars represent ±1 standard error.

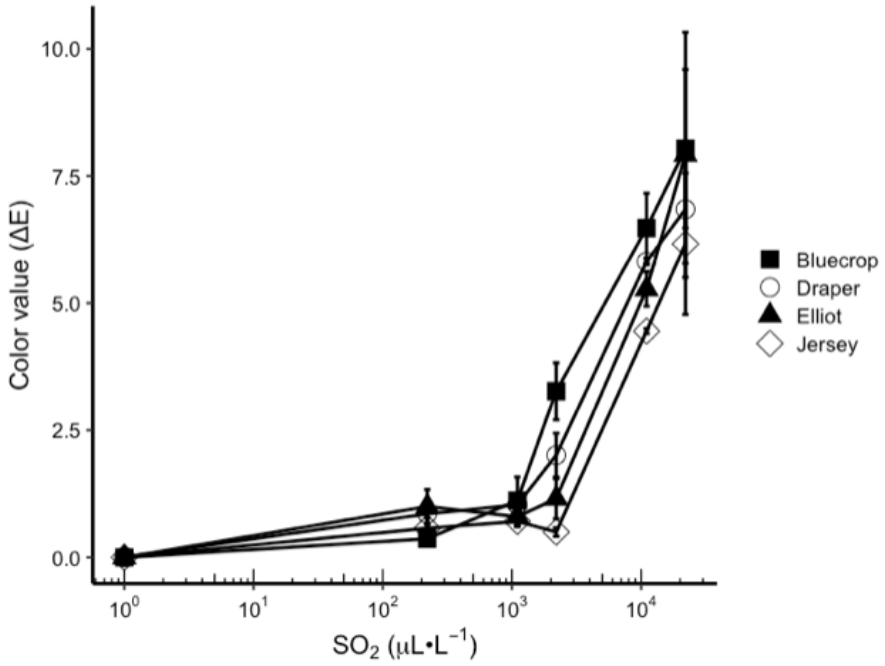

Figure 2.6. Impact of SO_2 concentration on the color difference between control (time 0) fruit and treated fruit for the skin of blueberry fruit sampled near the stem scar. The exposure was performed at atmospheric pressure, the duration was 16 h, and the exposure temperature was 10 °C. Following treatment, fruit were transferred to 23 °C and evaluated within 2 h. Vertical bars represent ±1 standard error.

2.3. RESULTS

2.3.1. Long-term (16-h) SO_2 exposure

The fruit of all cultivars tested were bleached by SO_2 treatment. In general, treated fruit became less blue (increasing b* value; Fig. 2.3), more red (increasing a* value; Fig. 2.4), and lighter in color (increasing L* value; Fig. 2.5) when exposed to SO_2. Total change in fruit color (ΔE) increased non-linearly with increasing SO_2 treatment concentration in all cultivars, and a sharp increase above 10^3 (Fig. 2.6). Average visual damage also increased non-linearly with increasing concentration (Fig. 2.7). The minimum concentration of SO_2 needed to induce a measurable color change from control (0 $\mu L \cdot L^{-1}$ SO_2) fruit depended on the color value being evaluated (Table 2.1).

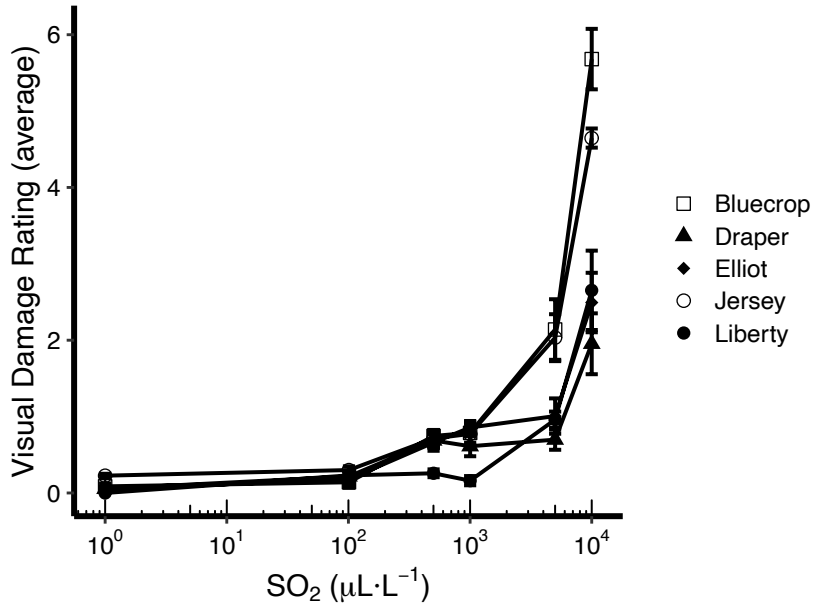

Figure 2.7. Average visual damage rating as a function of SO_2 concentration for five blueberry cultivars. The exposure was performed at atmospheric pressure, the duration was 16 h, and the exposure temperature was 10 °C. Following treatment, fruit were transferred to 23 °C and evaluated within 2 h. Vertical bars represent ±1 standard error.

Table 2.1. The minimum SO_2 level resulting in a change in the color value (L*, a*, b*, and ΔE) of fruit from four cultivars of blueberry following long-term (16 h) exposure to SO_2 at 10 °C at atmospheric pressure. In each case, levels of SO_2 higher than the minimum shown had a significant impact on color value.

	Minimum SO_2 level to change color measure ($p<0.05$)			
Cultivar	L*	a*	b*	ΔE
Bluecrop	11,000	1,100	22,000	1,100
Draper	11,000	2,200	22,000	2,200
Elliot	11,000	11,000	11,000	2,200
Jersey	11,000	1,100	11,000	220

Color difference (ΔE) was most sensitive to SO_2 concentration, with as little as 220 - $\mu L \cdot L^{-1}$ inducing a significant color change for 'Jersey'. 'Bluecrop', 'Draper' and 'Elliot' required, respectively, a minimum of 1100, 2200, and 2200 $\mu L \cdot L^{-1}$ SO_2 to induce a measurable change in color. Blueberry cultivar also affected the subjective assessment of fruit appearance, with concentration needed to cause unacceptable discoloration being lowest for 'Jersey' fruit (Table 2.2). ‘Bluecrop’ and ‘Jersey’ were more susceptible to severe bleaching damage and had as high

as 94% to 77.4% unmarketable fruit, respectively, when treated with 22,000 $\mu L \cdot L^{-1}$ SO_2. In comparison, 'Draper,' 'Elliott,' and 'Liberty' had only 25-35% unmarketable fruit at the same treatment concentration.

2.3.2. Short-term (1- and 2-h) SO_2 exposure

2.3.2.1. Quality parameters

Treatment time, pressure, and concentration all had significant effects on skin color at the stem-end of unstored 'Bluecrop' fruit during short-term treatments of 1 to 2 h (Tables 2.3 and 2.4). Fruit treated at greater than 1100 $\mu L \cdot L^{-1}$ SO_2 underwent significant changes in a*, b* and L* values compared to the control (0 $\mu L \cdot L^{-1}$ SO_2) fruit (Table 2.3). Fruit treated at low pressure for 2 h became significantly lighter, redder, and less blue than any of the 1 h treatments (Table 2.4).

Table 2.2. The effect of long-term (16 h) exposure to SO_2 at 10 °C at atmospheric pressure on the visual damage rating (a) and average percentage unmarketable (b) for five cultivars of blueberry fruit. For the visual damage rating, **bold** indicates that the average damage value exceeds the threshold needed for marketability. For the percentage unmarketable (b), **bold** indicates >4% of the fruit were considered unmarketable.

	SO_2	Cultivar*									
	($\mu L \cdot L^{-1}$)	'Bluecrop'		'Draper'		'Elliott'		'Jersey'		'Liberty'	
a.) Visual damage rating	0	0.09	a	0.05	a	0.05	a	0.23	a	0	a
	220	0.14	a	0.22	a	0.18	ab	0.3	a	0.23	a
	1100	0.75	a	0.69	a	0.66	abc	0.71	a	0.26	a
	2200	0.79	a	0.61	a	0.86	bc	0.77	a	0.16	a
	11000	**2.14**	b	0.7	a	1.01	c	**2.03**	b	0.96	a
	22000	**5.68**	c	1.95	b	**2.49**	d	**4.65**	c	**2.65**	b
b.) Percentage unmarketable	0	1.94		0.67		0.65		3.85		0	
	220	1.28		3.98		3.24		1.99		3.95	
	1100	1.31		3.91		**6.41**		**8.46**		2.67	
	2200	**4.63**		**4.64**		**9.79**		**8.95**		3.85	
	11000	**45.48**		**7.19**		**13.38**		**37.72**		**12.5**	
	22000	**94.02**		**27.83**		**27.36**		**77.27**		**34.33**	

* Values within a column with the same letter are not significantly different from one another (Tukey's HSD; p=0.05).

Table 2.3. Blueberry fruit color (L*, a*, b*) as affected by short-term exposure to different concentrations of SO_2 at 10 °C. Data for high and low pressure (2.67 and 101 kPa) and exposure duration (1 h and 2 h) are pooled.

SO_2 ($\mu L \cdot L^{-1}$)	L*		a*		b*	
0	30.74	ab	0.89	a	-2.26	a
22	30.69	a	0.98	a	-1.95	a
220	31.09	abc	1.03	a	-1.86	ab
1100	31.51	bc	1.64	b	-1.32	bc
2200	31.66	c	1.98	b	-1.25	c
22000	33.37	d	5.45	c	-0.06	d

Values within a column with the same letter are not significantly different from one another (Tukey's HSD; p=0.05).

Short-term (1- or 2-h duration) exposure to SO_2 had variable effects on flavor components (Table 2.5). For the short-term treatments, there was no effect of concentration on

SSC or on the SSC:TA ratio. The SSC:TA ratio for fruit treated at 10 $\mu L \cdot L^{-1}$ SO_2 differed from those given 22,000 $\mu L \cdot L^{-1}$ SO_2 , but these did not differ from all other treatment concentrations. Fruit treated at the highest concentration (22,000 $\mu L \cdot L^{-1}$) had the lowest firmness. The effects of treatment pressure and duration (1 h or 2 h) were relatively minor (Table 2.6). Averaged across all treatment concentrations, fruit treated at low pressure for 1 hour had the highest TA. Fruit treated at low pressure for 2 h had the lowest SSC. Treatment pressure impacted the SSC:TA ratio for those fruit treated with SO_2 for 1 h; however, treatment pressure did not affect the SSC:TA ratio for fruit treated for 2 h with SO_2. Fruit firmness was the lowest in the 1-h, low pressure treatment. No treatment or treatment combination had a significant effect on fruit weight.

Table 2.4. 'Bluecrop' blueberry fruit color value (L*, a*, b*) as affected by the interaction of duration (1 or 2 h) and pressure [low pressure (LP, 2.67 kPa) or high pressure (HP, 101 kPa)] of exposure to SO_2 at 10 °C. Data for all SO_2 concentrations (0, 22, 220, 1100, 2200, 11000, 22000 $\mu L \cdot L^{-1}$) are pooled.

Treatment		L*		a*		b*	
1 hr	LP	30.78	a	1.65	a	-1.79	a
	NP	31.42	a	1.60	a	-2.04	a
2 hr	LP	32.79	b	2.64	c	-0.39	b
	NP	31.04	a	2.09	b	-1.58	a

Values within a column with the same letter are not significantly different from one another (Tukey's HSD; p=0.05).

Table 2.5. 'Bluecrop' blueberry fruit quality characteristics as affected by short-term exposure to different concentrations of SO_2 at 10 °C. Data for high and low pressure (2.67 and 101 kPa) and exposure duration (1 h and 2 h) are pooled.

SO_2 ($\mu L \cdot L^{-1}$)	TA (mg mL^{-1})		SSC (°Brix)		SSC:TA		Firmness (N mm^{-1})	
0	6.71	ab	12.7	a	1.93	a	1.335	a
22	6.58	b	12.1	a	1.86	a	1.344	a
220	7.11	ab	12.4	a	1.80	a	1.346	a
1100	6.73	ab	12.5	a	1.88	a	1.247	a
2200	7.23	ab	13.0	a	1.87	a	1.252	a
22000	7.79	a	12.9	a	1.70	a	1.102	b

Values within a column with the same letter are not significantly different from one another (Tukey's HSD; p=0.05).

Table 2.6. 'Bluecrop' blueberry fruit quality characteristics as affected by the interaction of duration (1 or 2 h) and pressure [low pressure (LP, 2.67 kPa) or high pressure (HP, 101 kPa)] of exposure to SO_2 at 10 °C. Data for all SO_2 concentrations (0, 22, 220, 1100, 2200, 11000, 22000 $\mu L \cdot L^{-1}$) are pooled.

Treatment Duration	Pressure	TA (mg/mL)		SSC (ºBrix)		SSC:TA		Firmness (N mm^{-1})	
1 hr	LP	8.22	a	12.8	a	1.59	c	1.171	b
	NP	6.38	b	12.9	a	2.05	a	1.322	a
2 hr	LP	6.48	b	11.5	b	1.81	bc	1.301	a
	NP	7.02	b	13.2	a	1.90	ab	1.278	a

Values within a column with the same letter are not significantly different from one another (Tukey's HSD; p=0.05).

2.3.2.2. BBM disinfestations

Longer treatment duration, higher fumigant concentration, and higher chamber pressure all had significant negative effects on the emergence of BBM from infested fruit (Table 2.7). Treatment pressure and the interaction between treatment concentration and treatment time (Conc * Time) had significant effects ($p<0.05$) on BBM emergence in unstored (0 d) fruit. Higher fumigant concentrations significantly reduced BBM emergence when combined with 7 and 14 d of storage at 0 °C in modified atmosphere packages.

Table 2.7. ANOVA p-values for blueberry maggot emergence from infested 'Bluecrop' blueberry for treatments of SO_2 concentration [Concentration (0, 22, 220, 1100, 2200, 11000, and 22000 $\mu L \cdot L^{-1}$)], exposure duration [Duration (1 and 2 h)], and pressure [Pressure (2.67 and 101 kPa)] and the interaction between concentration and treatment duration (Concentration×Duration) after 1, 7, 14, and 21 d storage at 0.5 °C in modified atmosphere packages.

	Storage Duration			
	0 days	7 days	14 days	21 days
Concentration	0.1822	**0.0009**	**0.0052**	0.984
Duration	0.2924	0.1464	0.1277	0.947
Pressure	**0.0045**	0.2679	0.2742	0.168
Concentration×Duration*	**0.0033**	0.7171	0.0952	0.313

Bold indicates significance $p<0.05$; ANOVA).
*No other interactions were significant.

Despite the effectiveness of the highest concentration of SO_2, the data indicate that cold storage was still necessary to eliminate BBM entirely. Fruit stored for 0 days (Fig. 2.8; squares and solid line) had BBM emergence even when previously treated with 2.2% SO_2 for two hours. However, after 21 days of storage, BBM mortality was >99% even in the control (0 $\mu L \cdot L^{-1}$) treatment. Averaged across all treatment conditions, BBM emergence was negatively correlated with storage duration (Fig. 2.9). Averaged across the six treatment concentrations and the four storage times, the 1-h low-pressure treatment had the lowest BBM emergence and 2-h normal pressure treatments had the highest emergence (Fig. 2.10). Only the 22,000 $\mu L \cdot L^{-1}$ SO_2 treatment yielded significantly lower BBM emergence than the control (0 $\mu L \cdot L^{-1}$) (Fig. 2.11).

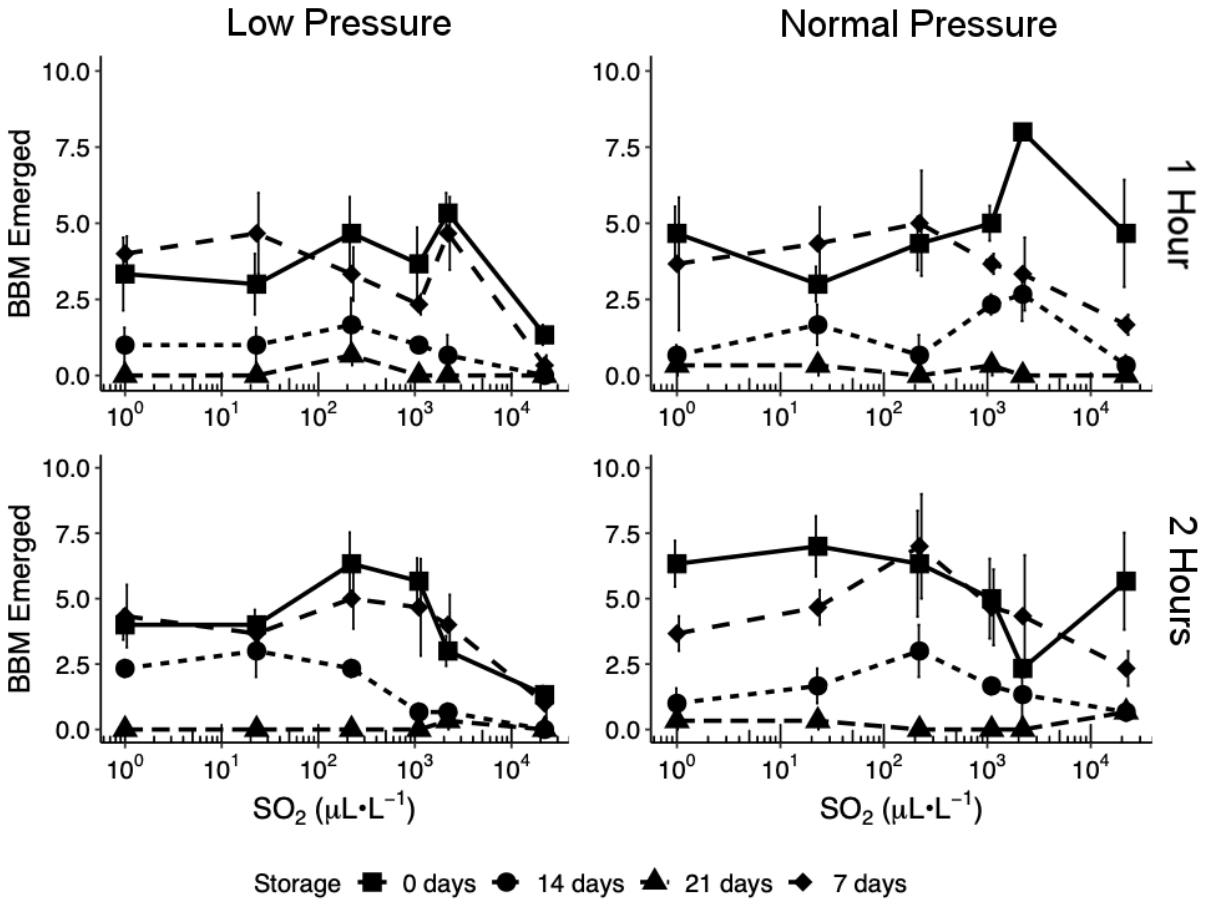

Figure 2.8. Emergence of blueberry maggot (*R. mendax*, BBM) from 130-g samples of infested 'Bluecrop' blueberry fruit following SO_2 fumigation and storage at 0 °C for 0, 7, 14, and 21 d at ~99% RH in modified atmosphere packages. Fumigation was for 1 h (top row) or 2 h (bottom row) at low pressure (2.67 kPa, left column) or normal pressure (101 kPa, right column). The temperature at the time of fumigation was 10 °C and fumigant concentrations were 0, 22, 220, 1100, 2200, 22000 $\mu L \cdot L^{-1}$ SO_2. BBM emergence was determined by incubating treated fruit at 25°C, 75% RH and 16:8 light:dark photoperiod for 21 d. Errors bars represent ± 1 SE.

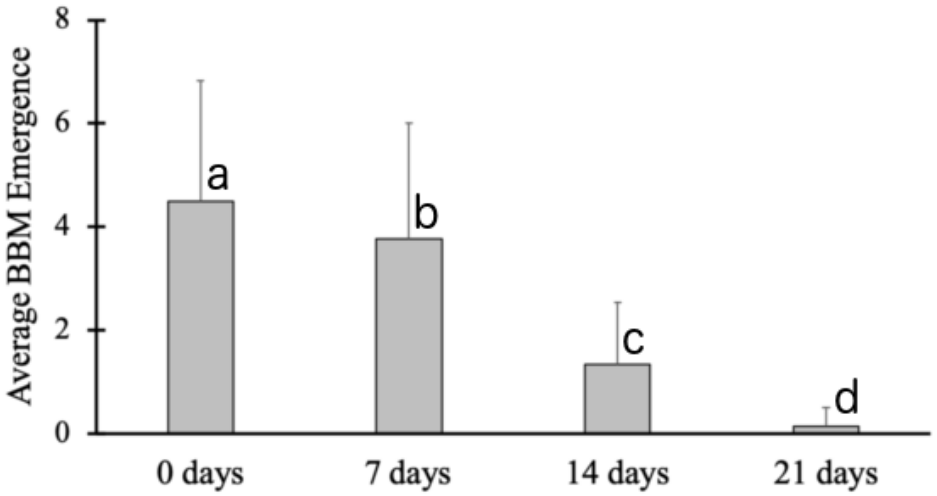

Figure 2.9. Blueberry maggot (*R. mendax*, BBM) emergence per 130 g sample of 'Bluecrop' blueberry fruit after fumigation with SO_2 and 0, 7, 14, and 21 d of storage in modified atmosphere packages at 0 °C. Data for all SO_2 concentrations (0, 22, 220, 1100, 2200, 11000, 22000 $\mu L \cdot L^{-1}$), pressures (2.67 and 101 kPa), and exposure durations (1 and 2 h) are pooled. The incubation for BBM emergence was performed at 25 °C, 75% RH and 16:8 light:dark photoperiod for 21 d. Values with the same letter are not significantly different (Tukey's HSD; $p<0.05$). Errors bars represent ± 1 SD.

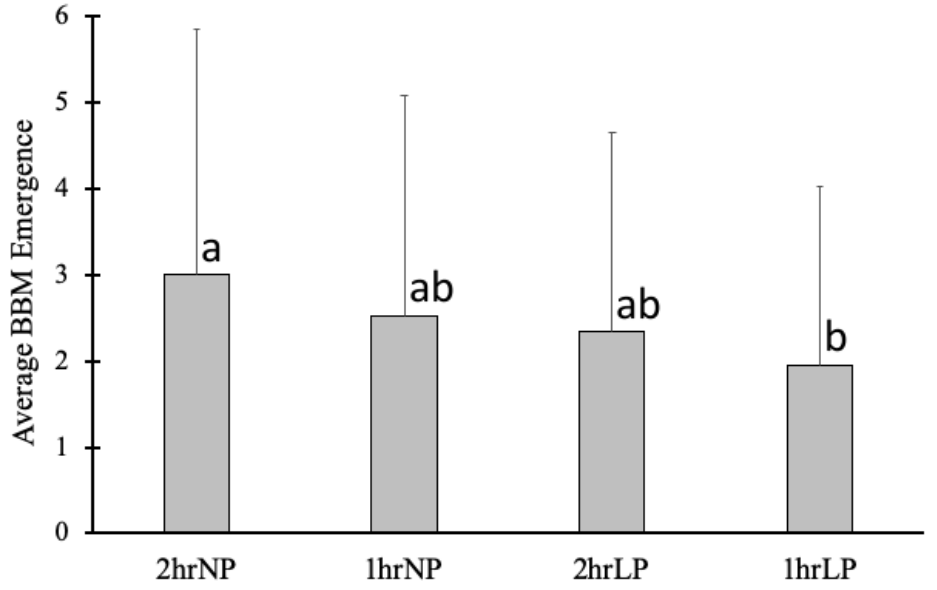

Figure 2.10. Blueberry maggot (*R. mendax*, BBM) emergence per 130-g sample of 'Bluecrop' blueberry fruit after fumigation with SO_2 and 0, 7, 14, and 21 d of storage in modified atmosphere packages at 0 °C as affected by the interaction of exposure duration (1 or 2 h) and exposure pressure (2.72 and 101 kPa). Data for all SO_2 concentrations (0, 22, 220, 1100, 2200, 11000, 22000 $\mu L \cdot L^{-1}$) are pooled. The incubation for BBM emergence was performed at 25 °C, 75% RH and 16:8 light:dark photoperiod for 21 d. Values with the same letter are not significantly different (Tukey's HSD; $p<0.05$). Errors bars represent ± 1 SD.

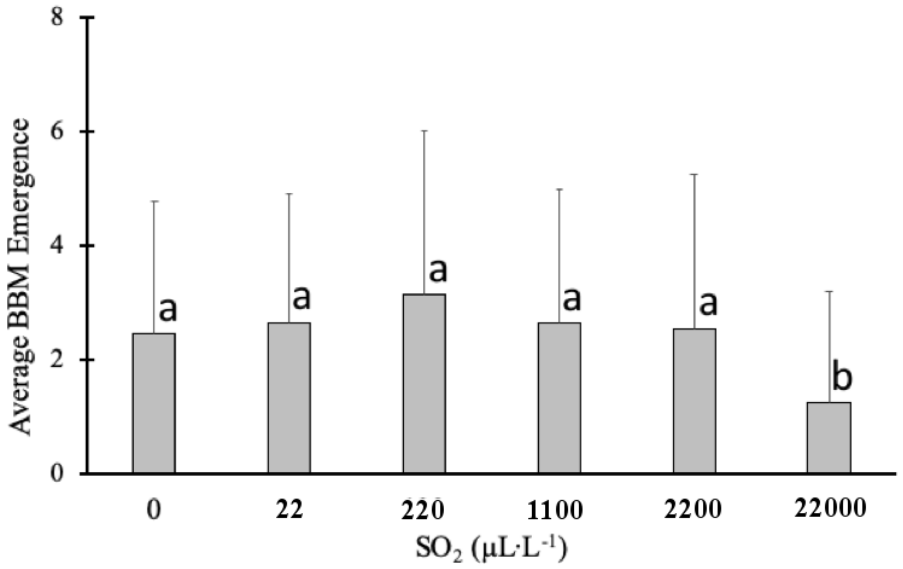

Figure 2.11. Blueberry maggot (*R. mendax*, BBM) emergence from 'Bluecrop' blueberry fruit given short-term SO_2 treatments at 10 °C. Data are the average for fruit exposed to 16 duration/pressure/storage duration combinations (1 and 2 h storage duration, high and low pressure and storage at 0 °C in MAP for 0, 7, 14 and 21 d). The incubation for BBM emergence was performed at 25°C, 75% RH and 16:8 light:dark photoperiod. Values with the same letter are not significantly different (Tukey's HSD; $p<0.05$). Errors bars represent ± 1 SD.

2.4. DISCUSSION

In the present study, we show that SO_2 treatment can lead to bleaching of fruit and this damage increases with increasing SO_2 concentration as measured both objectively (Fig. 2.6) and subjectively (Fig. 2.7). We found that this bleaching is driven by a decrease in 'blueness' (Figure 2.3), an increase in 'redness' (Fig. 2.4), and a lightening (Fig. 2.5) of the fruit which combine to produce light-pink colored fruit at the most severe treatment levels (Fig. 2.2). Sulfur dioxide injury to grape berries has been noted since the late 1930s (Pentzer et al., 1938) and is tolerated to some extent in commercial grape production to this day. Increasing bleaching around the stem-end of the fruit with increasing SO_2 concentrations (Gao et al., 2003; Lichter et al., 2008; Nelson and Tomlinson, 1958), exposure time and temperature (Gao et al., 2003) has also been demonstrated in grape. It is important to note that blueberries are often slightly red or pink when under ripe, so a moderate level of bleaching in some fruit may be tolerated by consumers,

however, no more than 4% of the fruit in any blueberry lot is permitted to be permanently damaged according to USDA guidelines (USDA 1995), hence the selection of this threshold for determinations of cultivar sensitivity.

The cultivar-dependence of the bleaching response found for blueberry was also found in table grapes (Gao et al., 2003). Various table grape cultivars were found to have a range of susceptibility to bleaching by SO_2 delivered using either SO_2-releasing pads or using gaseous SO_2 injections in a fumigation chamber with ‘Ribier’, ‘Hongbaoshi’, and ‘Red Globe’ found to be very susceptible and ‘Longyan’ and ‘Black Autumn’ very resistant. In some blueberry cultivars, fruit of approximately the same size had large differences in the bleaching response at the same treatment concentration (Fig. 2.12).

Figure 2.12. Selected fruit taken from within the same replicate samples for each cultivar demonstrating the range in color for fruit exposed to long-term SO_2 vapors (16 h) at 10 °C and atmospheric pressure. The SO_2 treatment concentrations from which the fruit were selected were 11000, 22000, 22000, 11000, and 22000 $\mu L \cdot L^{-1}$, respectively, for 'Bluecrop', 'Draper', ‘Elliott’, 'Jersey', and 'Liberty'.

This may be a result of differences in the rate at which SO_2 diffuses and/or dissolves into the tissues due to microscopic injuries and abrasions or inherent differences in the sensitivity of the fruit to SO_2 damage. In table grapes, SO_2 diffuses into ‘Emperor’ and ‘Tokay’ fruit through lenticels and at the stem scar, as well as through injuries such as scratches or skin tears (Nelson

and Tomlinson, 1958). While we expect similar mechanisms are at play in blueberry fruit, the factors affecting SO_2 diffusion have not been described for blueberry and the mechanism responsible for the bleaching response in any fruit is not well understood. SO_2 has been shown to reprogram the transcriptome of grapes (cv 'Crimson Seedless') by up-regulating genes for sulphate transport and down-regulating genes associated with sulfite oxidation, including the grape ortholog of *Arabidopsis* sulfite oxidase (*SiO*), and has been shown to affect redox/ROS signaling genes (Giraud et al., 2011). In *Arabidopsis,* sulfite oxidation by sulfite oxidase has been shown to consume molecular oxygen and form hydrogen peroxide, H_2O_2, and it is proposed that H_2O_2 is subsequently used in a non-enzymatic step to oxidize another molecule of sulfite (Hänsch et al., 2006). While the mechanisms for bleaching by SO_2 are not well understood, future research in blueberry could help elucidate both the genetic and chemical factors involved in this response to better understand cultivar-dependent differences in bleaching sensitivity.

Softening of blueberry by SO_2 has not been previously reported. Our results show that a modest softening occurred during or soon after treatment as the measurements were made within 2 h of fumigation. Blueberry fruit firmness can be affected by cell wall dynamics (Chen et al., 2015), postharvest handling (Beaudry, 1992), and water loss (Paniagua et al., 2013). Given that we found a reduction in fruit firmness at the highest treatment concentration without a concurrent reduction in fruit weight (data not shown), firmness loss due to SO_2 treatment is likely not due to water loss. Furthermore, bruising is not likely the cause of softening because the fruits were hand-picked and specifically handled to reduce the incidence of bruising during treatment, and there was no difference in handling between treatments. Loss of membrane and/or cell wall integrity, or changes in composition of either, can affect fruit firmness. In cucumber (*Cucumis sativus* L.) fruit, chilling injury is characterized by soft, sunken tissue at the injury site and is

correlated with damaged and leaking plasma membranes as well as cell wall deterioration (Fernández-Trujillo and Martínez 2006). In blueberry, firmness generally decreases with increasing storage time as well as storage temperature and is associated with changes in cell wall composition as well as cell wall-degrading enzyme activity (Chen et al., 2015). High CO_2 treatments during postharvest controlled atmosphere storage of blueberry fruit are often used to help control decay. Decreasing firmness and increasing internal injury of the fruit of some highbush blueberry cultivars was associated with increasing CO_2 treatment concentration during storage (Alsmairat et al., 2011). While the mechanism for changes in firmness due to SO_2 exposure are not explicitly known in blueberry, it warrants further investigation if SO_2 is to become a more widely used postharvest fumigant.

The increase in titratable acidity with increasing SO_2 concentration (Table 2.4) is similar to the gain in TA found for SO_2-treated table grapes (Pretel et al., 2005). Pretel et al. (2005) found that 'Aledo' table grapes treated with SO_2 and subject to long-term storage had a nearly 50% (w/w) increase in citric acid over the control after 60-70 d storage, however, malic acid decreased over time in the SO_2 treated fruit, albeit to a much lesser extent, and tartaric acid was not affected by SO_2 treatment. The primary organic acid component in blueberry fruit is citric acid, with additional malic, quinic, and succinic acids (Forney et al., 2012). While we did not measure each acid individually, it would be interesting to see if blueberry acids behave similarly to those in grape during treatment with SO_2. This response may be a result of sulfur dioxide reacting to form sulfurous and/or sulfuric acid when dissolved in water (Windholz et al., 1983). In grapes, the majority of applied SO_2 is retained as inorganic sulfur and 10% (w/w) of applied SO_2 is accumulated as sulfites that are then oxidized to sulfates (Considine et al., 2015).

The exposures to SO_2 used in these experiments showed that short-term (1 or 2 h) fumigation concentrations necessary to reduce BBM exceed 10^4 $\mu L \cdot L^{-1}$ to impact the survival of BBM. However, at such high concentrations there would be considerable reduction in marketability of the fruit due to bleaching. We found that in all blueberry varieties tested, treatment concentrations exceeding 10^4 $\mu L \cdot L^{-1}$ caused the percentage of unmarketable fruit to exceed 7% in the most tolerant variety (cv. 'Draper') and 45% in the most sensitive variety (cv. 'Bluecrop') (Table 2.2). This would be an unacceptable level of damage unless these fruit could be sorted out prior to distribution, leading to increased production costs. These results suggest that short-term fumigation with SO_2 could not likely be considered as a standalone control method for BBM. Further evaluations of low dose and long-term fumigation strategies using SO_2 for controlling BBM are currently underway to identify combinations that provide the pest control without fruit damage.

Nevertheless, we found that BBM control was possible with a combination of SO_2 exposure and cold treatment (0 °C). Cold treatment has previously been shown to be effective for the control of other tephritid flies without the addition of SO_2, though some species are more cold tolerant than others. De Lima et al. (2011) demonstrated complete disinfestation of table grapes infested with Mediterranean fruit fly (*Ceratitis capitata* Weidemann [Diptera: Tephritidae]) in 16, 18, and 20 days at 1, 2, and 3 °C, respectively, and Queensland fruit fly (*Bactrocera tryoni Froggatt* (Diptera: Tephritidae)) in 12 days and 14 days at 1 and 2-3 °C, respectively. A similar result was found for Queensland fruit fly infesting rabbiteye blueberry fruit; 100% mortality was achieved after 12 days at 1 °C (Jessup et al., 1998). We show that BBM were able to survive even after 21 days of cold storage in untreated control fruit, albeit at low levels (Fig. 2.8), so the combination is needed for control.

Few studies have focused on short-term treatments of high-concentration SO_2 exposure, instead, most studies have addressed the use of SO_2 as an additive during long-term treatment storage. In this context, SO_2 is most commonly used as a control for post-harvest diseases such as grey mold of grape (Eckert and Ogawa, 1985; Palou et al., 1992) and blueberry (Rodriguez and Zoffoli 2016; Rivera et al., 2013). In these studies, the minimum SO_2 treatment rate for effective control was around 100-1000 ppm·hrs. Our studies show that these rates do not cause injury to blueberry fruit, but are ineffective for the control of BBM. The use of SO_2 releasing pads during long-term cold storage (up to 8 weeks at ~1 °C) has been shown to be effective for the control of some insect pests such as western flower thrips (*Frankliniella occidentalis* Pergande), grape mealybug (*Pseudococcus maritimus* Ehrhorn), and various species of spider mite (*Tetranychus* spp.) at concentrations around 1.0 ppm in laboratory- and commercial-scale systems (Yokoyama et al., 2001). Lower treatment concentrations may be more effective in these instances because these pests generally live on the outside of commodities where SO_2 vapor can more readily interact with the pest, whereas BBM lives most of its life cycle within the fruit where SO_2 vapor has more barriers to diffuse through before interacting with the pest. Treatment concentrations as low as 0.4 $\mu L \cdot L^{-1}$ for 4 d has been shown to decrease survival and increase development time in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) larvae and pupae with significant differences among various experimental lines, however no significant effect on the fecundity of adults was found (Ginevan and Lane 1978). To our knowledge, this study is the first to evaluate control of a tephritid using SO_2. Use of SO_2 as a fumigant has not been established in any fruit crops.

2.5. CONCLUSIONS

This study builds on previous research in blueberry by quantifying the effects of sulfur dioxide fumigation treatment on blueberry fruit quality traits including flavor components and fruit color. We show that blueberry fruit are damaged beyond marketability at high SO_2 concentrations, and under long-duration treatments, and that these effects are variable according to the cultivar. In this study and others, it has been shown that long-term cold storage of blueberry fruit is sufficient to control BBM infestation. However, there is an interest in alternative strategies for disinfestation when long-term storage is not an option such as in shipment from the United States to Canada where fruit may only be a few weeks old at most. We found that the most effective combination of treatments to control BBM emergence was a combination of 22,000 $\mu L \cdot L^{-1}$ SO_2 fumigation for 1-h under low pressure (2.67 kPa) followed by 21-days of storage at 0 °C, which eliminated greater than 99% of blueberry maggot from infested highbush blueberry fruit. However, the impact of this high concentration of SO_2 on fruit quality characteristics, especially fruit color, was too severe to consider this treatment combination for commercial use. If longer treatment durations would be commercially acceptable, it may be useful to evaluate continuous SO_2 concentrations below 1000 $\mu L \cdot L^{-1}$ for their utility in disinfestation of blueberry maggot.

CHAPTER 3: EXPLORING DIFFERENTIALLY EXPRESSED GENES IN A RESISTANT BLUEBERRY CULTIVAR IN RESPONSE TO INFECTION BY *COLLETOTRICHUM FIORINIAE*

3.1. INTRODUCTION

Blueberry suffers from diseases caused by a variety of pathogenic organisms, but diseases caused by fungal pathogens rank among the highest priorities for the blueberry industry (Gallardo et al., 2018). Anthracnose fruit rot, also known as ripe rot, is caused by *Colletotrichum spp.* and is one of the most important fungal pathogens of blueberry in Michigan, causing 10-20% preharvest loss and up to 100% loss postharvest if not properly controlled (Miles and Schilder, 2008; Milholland, 1995). *C. fioriniae* is the causal agent of anthracnose fruit rot of blueberry, due to a re-classification of many members of *Colletotrichum acutatum* (Damm et al., 2012). Fungal pathogens like *Colletotrichum acutatum* sensu lato are primarily controlled with the use of fungicides and by planting resistant varieties (Miles and Schilder, 2008). Fungicides sprays must be performed frequently throughout the growing season at great cost to producers. This high-frequency use can lead to fungicide tolerance or resistance (Hollomon, 2015). In fact, populations of a related species, *Colletotrichum siamense,* causing anthracnose leaf spot on blueberry in South Carolina have shown resistance to azoxystrobin (FRAC 11) and thiophanate-methyl (FRAC 1) (Hu et al., 2015). In strawberries FRAC 11 resistance in *C. fioriniae* has been linked to two single nucleotide polymorphisms and seems to be increasing in Florida (Forcelini et al. 2016). Planting anthracnose resistant blueberry varieties reduces the need to spray as frequently and may decrease the development of fungicide resistance at the same time reducing costs for growers. While susceptible varieties made up over 10% of new plantings between 2015-2018 (Fig. 3.1A), there is a long-term trend of increased planting of ‘resistant’ and ‘moderately resistant’ varieties since 1985 (Fig. 3.1B).

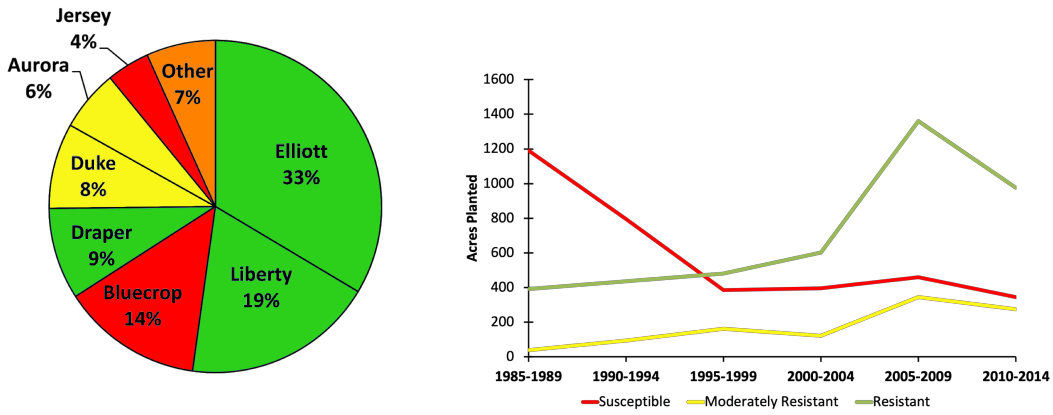

Figure 3.1. A.) New blueberry plantings from 2007-2011 by variety and susceptibility to ripe rot. Adapted from Longstroth and Hanson (2012) B.) Number of acres planted of the seven most popular varieties since 1985 (90% of total new plantings) by susceptibility class. Red = susceptible, yellow = moderately resistant, green = resistant. Adapted from USDA (2015, 2012, 2007, 2004, 2001).

C. fioriniae is known to develop quiescent infections in immature blueberry fruit, infecting fruit as early as fruit-set and remaining dormant until the fruit matures (Wharton et al., 2004, Peres et al., 2005). The disease presents first as sunken lesions on the fruit surface followed by orange-colored spore masses known as acervuli appearing to leak from the fruit surface (Wharton and Schilder, 2008). *C. fioriniae* spores are spread through rain- or irrigation-splash or by mechanical means to neighboring plants where they attach to the fruit surface and produce new infections (Milholland, 1995). Around 50% of blueberry fields in Michigan are irrigated through overhead sprinklers (USDA, 2018) which contributes to the spread of spores during the growing season.

Understanding the genetic and biochemical mechanisms used *Colletotrichum* by these pathogens to infect fruit and other plant tissues, as well as the mechanisms of resistance employed by the hosts is important to mitigating loss and preserving food security in the future. One important first step in this process is the release and accessibility of genetic and genomic

datasets from a variety of *Colletotrichum*-host pathosystems that can be leveraged in future breeding studies toward creating more resilient blueberry cultivars.

It is unlikely that resistance to *C. fioriniae* by blueberry fruit is controlled by a single gene. Resistance in cv. 'Elliott' is characterized by high ROS production (Miles et al., 2011) as well as the accumulation of phenolic (Miles et al., 2009) and antifungal compounds (Miles et al., 2013). No variety is completely resistant; while 'Elliott' is considered one of the most resistant cultivars, it only slows the rate of disease development rather than completely inhibiting growth of *C. fioriniae* in the fruit. This virulence may be due, in part, to the diverse infection strategies employed by *C. fioriniae* in various cultivars of highbush blueberry fruit. In the susceptible cultivar 'Jersey', *C. fioriniae* utilizes an infection strategy known as 'intracellular hemibiotrophy', characterized by large primary hyphae that develop within the initially invaded, living host cell followed by smaller secondary hyphae that invade neighboring cells (Wharton and Schilder, 2008). Infection in the resistant cultivar 'Elliott' is described as 'intramural necrotrophy,' where the first cell is similarly invaded by a primary hypha and subsequently dies before secondary hyphae are produced (Wharton and Schilder, 2008). Hemibiotrophic pathogens go through a lifestyle transition during infection, and it is possible that we may capture this transition happening at different times during infection in a resistant or susceptible cultivar by assaying gene expression of the host over time. Miles et al. (2011) showed that many of the same genes are activated later in 'Jersey' than in 'Elliott'.

It has been shown that the resistant cultivar 'Elliott' produces higher level of anthocyanins than the susceptible cultivar 'Jersey' (Miles et al., 2011). Additionally, anthocyanin production is known to contribute to the active disease response to *C. fioriniae* infection (Miles et al., 2009). Recent developmental time series of antioxidant and phytonutrient production by

Colle et al. (2019), coupled with previous analyses of the genes involved in antioxidant production based on RNAseq (Li et al., 2012) and EST datasets (Zifkin et al., 2012), are crucial to the understanding of disease resistance mechanisms in blueberry fruit. The complete suite of molecular and genetic mechanisms leading to a disease response, or a lack thereof, in both susceptible and resistant cultivars largely remains a mystery in blueberry, however due to the broad host range of *Colletotrichum* species, we can draw insight from other host-pathogen interactions for better understanding.

In other *Colletotrichum*-host pathosystems, resistance has been linked to a single gene or genetic locus. Resistance within a single host species can be specific to particular strains or pathogenicity groups of a particular pathogen depending on growing region and host variety (Padder et al., 2018). In octoploid strawberry fruit (*Fragaria x ananassa*), high resistance to European isolates of *C. acutatum* from pathogenicity group 2 was mapped to a single dominant locus (*FaRca2*) while intermediate resistance was shown to be quantitative and controlled by various minor genes (Denoyes-Rothan et al., 2005). A second distinct locus (*FaRCa1)* was identified in an analysis of 33 full-sib families of strawberry and shown to confer resistance to isolates from pathogenicity group 1 (Salinas et al., 2019). Resistance to *Colletotrichum* crown rot (CCR), caused by *C. gloeosporioides*, was mapped to a quantitative trait locus (QTL), *FaRCg1,* on linkage group 6B and is consistent with a single, partially dominant allele, though other minor alleles may contribute to resistance (Anciro et al., 2018). Multiple single-gene resistance mechanisms have been found in the chili pepper (*Capsicum annum)*-*C. acutatum* pathosystem. These include a single dominant gene in ripe fruit and a single recessive gene in unripe fruit (Mahasuk et al., 2009) in one pepper line. In the interspecific hybrid *Capsicum annum x chinense*, resistance to *Colletotrichum capsici* was linked to a single recessive gene

(Pakdeevaraporn et al., 2005). Kim et al. (2007) also found resistance to *C. acutatum* in a different cross of the interspecific hybrid *C. annum x chinense* to be consistent with a single recessive gene based on segregation ratios of the progeny. In the common bean, *Phaseolus vulgaris*, resistance to two races of *Colletotrichum lindemuthianum* was controlled by two separate genes (Geffroy et al., 2008) and seventeen resistance loci have been mapped to 8 chromosome regions using data from twelve *P. vulgaris* cultivars (Padder et al., (2018).

Here, we aim to increase our understanding of the complex host-pathogen interaction between blueberry fruit and *Colletotrichum fioriniae* to enhance the ongoing goal of breeding more resistant blueberry cultivars to reduce pre- and postharvest loss. By revealing genes associated exclusively with the resistance phenotype of 'Elliott', one of the most resistant highbush blueberry cultivars currently grown widely in Michigan, using RNAseq, this work will provide deeper understanding of the genetic underpinnings of high-level resistance and elucidate resistance mechanisms more completely than they were known previously.

3.2. MATERIALS AND METHODS

3.2.1. Isolation of inoculum and single-spore culture isolation

Blueberry fruit (cv. 'Draper') infected with *Colletotrichum acutatum sensu lato* were collected from the field, placed in a half-pint clamshell container and transported back to MSU uncooled. When we returned to the lab, the fruit were placed in a reusable aluminum baking pan along with a petri-dish filled with water to maintain a high relative humidity. The whole pan was then enclosed within a 2.0-mil thick LDPE bag and sealed tightly with a binder clip. The fruit were removed when acervuli were noticeable and significant sporulation had occurred, indicated by orange-colored masses on the surface of the fruit. The fruit were then placed in a 50-mL Falcon™ tube along with 10 mL sterile deionized water and rinsed for one minute to dislodge

the spores by gently inverting the tube. After rinsing, the berries were removed, and the suspension was allowed to settle for at least 30 min at room temperature. Approximately 8 mL of the supernatant was removed, and the spores were resuspended in the remaining 2 mL by vortexing. The resulting spore suspension was separated equally into two 1.5-mL microcentrifuge tubes and briefly centrifuged for 15 s at 1000×g to collect the spores. The supernatant was removed, and the spores were washed twice to remove contaminants by resuspending in 1.0 mL sterile deionized water and briefly centrifuging (15 s at 1000×g) to collect the spores and removing the supernatant. After the final wash, one tube containing 1 mL of the spore suspension was placed at -80 °C for future use. The spores in the second tube were resuspended in 200 µL sterile deionized water. The spore suspension was then transferred to a petri plate containing potato dextrose agar (PDA) under sterile conditions and incubated on the bench top under ambient conditions for 24 hr. After incubation, contaminating colonies were removed using a sterile loop of wire in the fume hood. To ensure that all contaminating colonies were removed successfully, the plate was incubated on the bench top for 24 hr and checked for contamination the following day. When no contamination was found, the plate was flooded with 1.0 mL sterile deionized water and the spores were dislodged with a sterile L-shaped rod. 200 µL of the spore suspension was transferred to a new PDA plate and incubated on the bench top under ambient conditions for at least 72 hr until the entire plate was colonized with a lawn of orange- or salmon-colored microconidia and no contamination was found.

The microconidia-covered plate was flooded with 2 mL sterile deionized water and microconidia were dislodged with a sterile L-shaped glass rod. A single-spore isolate was derived from this spore suspension through serial dilution using sterile deionized water. 10 µL of the primary spore suspension stock was diluted in 10 mL sterile deionized water to produce the

secondary dilution then 10 µL of the secondary dilution was diluted with 10 mL of sterile deionized water to produce the tertiary dilution. This process was repeated a third time to produce the final dilution. 1.0 mL of the final dilution was plated on ½ strength PDA and allowed to grow at room temperature under ambient conditions. When colonies had formed, a single colony was selected and transferred to a full-strength PDA plate using a sterilized cork borer and allowed to colonize the plate. After the plate had been fully colonized and the presence of microconidia was apparent by the light orange color, the plate was flooded with 2 mL of sterile DI water and were dislodged with a sterile L-shaped glass rod. This spore suspension was allowed to settle at room temperature for one hour, the supernatant was removed, and the spores were resuspended with 100 µL of sterile DI water. This concentrated spore suspension was plated onto full-strength PDA and incubated at room temperature for 24 hr. After incubation, the plate was flooded with 2 mL of sterile DI water, spores dislodged as above, and 1 mL was transferred to a new plate while 1 mL was saved at -80 ºC for further use.

3.2.2. Inoculum production and inoculation procedure

C. fioriniae microconidia were grown on a full-strength PDA plate at room temperature for at least three days. The plate was flooded with 2.0 mL sterile deionized water and microconidia were dislodged with a sterile L-shaped glass rod. 10 µL of this spore suspension was diluted into 10 mL of sterile deionized water and the number of spores was counted using a hemocytometer. The spore suspension was adjusted to a concentration of $1x10^6$ spores per milliliter with sterile distilled water and subsequently used for inoculation.

We staged the fruit one day prior to inoculation by placing 100 fruit in a reusable aluminum baking pan along with a petri-dish filled with water to maintain a high relative humidity. The fruit were situated calyx-end up and distributed so no fruit were touching the walls

of the pan or any other fruit. The whole pan was then enclosed within a 2.0-mil LDPE bag and sealed tightly with a binder clip and left on the benchtop under ambient conditions for approximately 16 hours. Fifteen baking trays were made per variety. The following day, fifty fruit per tray were inoculated with a microconidial spore suspension by pipetting 50 µL of the inoculum onto the calyx side of the fruit. Fifty fruit within each tray were mock inoculated with 50 µL of sterile deionized water on the calyx end. After inoculation, the whole tray was enclosed in a 2.0-mil LDPE bag and sealed tightly with a binder clip. The trays were incubated on the bench top under ambient conditions (21 °C 12h:12h light:dark) until they were sampled.

3.2.3. Blueberry harvest and fruit sampling

Blueberry fruit (cv. 'Elliott,' 'Draper,' and 'Jersey') were hand harvested in Southwest Michigan on July 30, 2018 and shipped to Michigan Blueberry Growers, Inc. (MBG) where they were stored at 0 °C in 1-pint plastic clamshells. We picked the fruit up from MBG on the following day and transported it back to MSU in ice-lined coolers. When the fruit arrived at MSU, it was placed at 0 °C overnight until use.

Fruit tissue sampling took place at five time points: one hour after inoculation (0 days) and 1, 2, 3, 4 days after inoculation for each variety and treatment (inoculated or mock-inoculated) with three replications for a total of 90 individual samples. Fruit tissue was sampled by using a single-edged razor blade to excise all tissue within 2-3 mm of the calyx. This piece of tissue was immediately dropped in liquid nitrogen until all 50 fruit for a given treatment were sliced then the frozen tissue was collected in a 50-mL tube (Denville Scientific, Inc.) and stored at -80 °C. The frozen fruit slices from each sample were subsequently ground in liquid nitrogen using a clean mortar and pestle and then stored at -80 °C.

3.2.4. Total RNA extraction and RNA sequencing

For each of the 90 samples, approximately 50 mg of frozen ground tissue was transferred to an individual 2-mL microfuge tube for RNA extraction. RNA was extracted using the MagMax™ plant RNA Isolation Kit (Applied Biosystems/ThermoFisher Scientific) according to the manufacturers recommended protocol for use with the KingFisher™ Flex Magnetic Particle Processor 96DW (ThermoFisher Scientific). RNase-free PVP40 (2% w/v) was added to the lysis buffer according to the manufacturers recommendation to deal with polyphenol- and polysaccharide-rich samples. Following tissue lysis, the clarified lysate was stored at -20 °C for a period not longer than five days until all samples could be processed according to the manufacturer's recommendation. When all samples were processed, the clarified lysate was allowed to thaw on ice before adding the RNA binding beads and ethanol and were mixed thoroughly to ensure proper binding. All plates for the KingFisher™ portions of the protocol were prepared at room temperature immediately prior to use. RNA was eluted in 100 µL of RNase-free water at the end of the protocol. A 1-µL aliquot of a random subset of 40 samples (out of a total of 90) was used to check RNA quality in a 1% TAE gel with GelRed dye as a marker. The remainder of the RNA was immediately placed on ice then stored at -80 °C until further use.

Preparation of RNAseq libraries was performed using the mRNA HyperPrep Kit for Illumina® Platforms along with the Dual-Indexed Adapter Kit for Illumina platforms (KAPA Biosystems/Roche). Adapters were diluted to 7 uM according to the manufacturers recommendation for use with 500-1000 ng RNA prior to use and stored at -80 °C. Total isolated RNA was quantified using a Qubit 3 fluorometer (ThermoFisher Scientific). When sufficient quantities were available, RNA eluted from the previous kit were diluted to 20 ng/µL in 25 µL

RNase-free water. If there was not at least 500 ng of RNA, 25 µL of the original eluate was used for library preparation. The library preparation protocol was followed according to the manufacturers recommendation with the following changes: all reaction volumes were halved to extend the number of samples a single kit can accommodate, fragmentation (step 3.3 from manufacturers protocol) was performed at 94 °C for 6 min based on the manufacturers recommendation for achieving a mean library insert size between 200-300 bp, the library amplification (step 9.3 from manufacturers protocol) was run for 9 cycles according to the manufacturers recommendation for working with 501-1000 ng starting material, and the DNA was eluted in 11 µL of Tris-HCl (pH 8.0) at the end of the protocol. The DNA concentration of the final eluate was determined from a 1 µL aliquot using the Qubit 3 fluorometer (ThermoFisher Scientific) with the Qubit™ dsDNA Broad-range (BR) assay kit.

In order to minimize the cost-per-sample, 12 libraries were run in each lane of the Illumina flow cell by appending library-specific primers to each individual sample. Library sizes were standardized to 40 ug μL^{-1} and pooled in a total of 8 lanes, then submitted to the MSU Research Technology Support Facility (RTSF) Genomics Core and sequenced using 150-bp paired-end reads on an Illumina HiSeq4000 system. Following sequencing, all data was downloaded from the RTSF database for processing and downstream analysis.

3.2.5. Data pre-processing, trimming, and quality filtering

Read quality was confirmed using FastQC (Andrews, 2010). All reads were processed to remove adapters, reads that were too short, and low-quality leading or trailing bases using Trimmomatic v0.38 (Bolger et al., 2014) with the following options: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:8:TRUE LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. This also paired the first and second strand read files into a single paired-end read file.

3.2.6. V. corymbosum read alignment and expression analysis

To separate RNAseq reads from the host and pathogen, we first aligned the reads to the 'Draper' reference genome (Colle et al., 2019) using STAR v2.7.2b (Dobin et al., 2013). The genetic background of commercial blueberry is not very wide, with the majority of the genome derived from fewer than ten wild relatives, however, in order to capture the most representative picture of all transcripts expressed in the full dataset, even those that may not be in the 'Draper' reference genome, and without being too resource or time intensive, we used a subset of samples that only included the inoculated, day 4 alignments from STAR to create a genome-guided transcriptome assembly using Trinity v2.8.5 (Grabherr et al., 2011). An overview of the process can be found at (https://github.com/trinityrnaseq/trinityrnaseq/wiki). Paired transcripts from the forward (R1.fastq.gz) and reverse (R2.fastq.gz) reads were run through Trinity to align using Bowtie2 (Langmead and Salzberg, 2012) and estimate abundances using the "RNA-seq by Expectation Maximum" (RSEM) method (Li and Dewey; 2011).

3.2.7. Functional annotation and differential expression analysis

The Trinotate pipeline was utilized to generate de novo RNAseq assemblies, quantify transcript levels, and provide functional annotation (Bryant et al., 2017). The Trinity-derived transcripts were compared to the translated amino acid annotations contained in the UniProtKB/Swiss-Prot 2019_10 database using the blastx tool within the NCBI BLAST+ (Camacho et al., 2008) with an e-value cutoff of 0.001. Predicted protein-coding regions of the of the Trinity-derived transcripts were translated into amino acid sequences using TransDecoder v5.5.0 (https://github.com/TransDecoder/TransDecoder) and compared to the UniProtKB/Swiss-Prot 2019_10 database using the blastp tool within Blast+ with an e-value cutoff of 0.001. The top hit from each version of BLAST was added to an SQLite annotation database for each

Trinity-derived transcript. Protein family (Pfam) domains were predicted for each TransDecoder-derived amino acid sequence using HMMER v3.3 (Eddy 2011) and the top hit was added to the SQLite annotation database. The TransDecoder-derived amino acid sequences were searched for predicted signal peptides using SignalP v4.1 (Petersen et al., 2011) and presence or absence of a predicted signal peptide was added to the SQLite database. The presences of trans-membrane helices were predicted using TMHMM v2 (Krogh et al., 2001; Sonnhammer et al., 1998) and these annotations were added to the SQLite database.

Differential expression was performed using the R (version 3.5.1 "Feather Spray") package DESeq2 (Love et al., 2014) within the Trinity pipeline. We broke our differential expression analysis into nine different contrasts in two broad categories; 'within-cultivar' and 'between-cultivar.' Differential expression within-cultivar was considered as genes differentially expressed (DE) in the comparison between inoculated (T) and mock-inoculated (N) treatments of each cultivar ('Jersey' – 'J', 'Elliott' – 'E', 'Draper' – 'D'). DE genes between cultivars were further considered in two categories: between cultivars of mock-inoculated fruit (ENvsDN, JNvsDN, JNvsEN), and between cultivars of inoculated fruit (JTvsET, JTvsDT, and ETvsDT). Genes were considered DE if the $\log_2$-fold change > 2 when comparing inoculated and mock-inoculated samples within each cultivar and for mock-inoculated samples between cultivars. For treated fruit, genes were considered DE if the $\log_2$-fold change ($|LFC|$) > 4 . For all analyses, $p > 0.001$ was used as a cutoff to reduce false-discovery rate. Genes in the between-cultivar comparisons were further filtered according to the method of Padder et al. (2016) by subtracting the NvN DE genes from the TvT DE genes to eliminate background differential expression. Strong candidates for genes responsible for the resistant phenotype of 'Elliott' were found by isolating genes present in both the ENvET dataset and the JTvET after removing the JNvEN DE

genes and then eliminating any genes that were common between these two datasets and genes in the DE dataset of DNvDT (Figure 8). For these analyses, only genes with good functional annotation based on the Trinotate pipeline will be considered.

3.3. RESULTS

3.3.1. Laboratory infection varies by cultivar

Three varieties of blueberry with different levels of resistance to *C. fioriniae* were inoculated and any fruit that appeared to have infection from the field, based on the presence of acervuli, were removed prior to sampling. After seven days, the final disease incidence was measured for three replicate samples of inoculated and mock-inoculated fruit (Fig. 3.2). 'Elliott' fruit had the lowest incidence of disease (29.3%) while 'Draper' and 'Jersey' had approximately equal incidence of disease (89.3% and 87.3%, respectively).

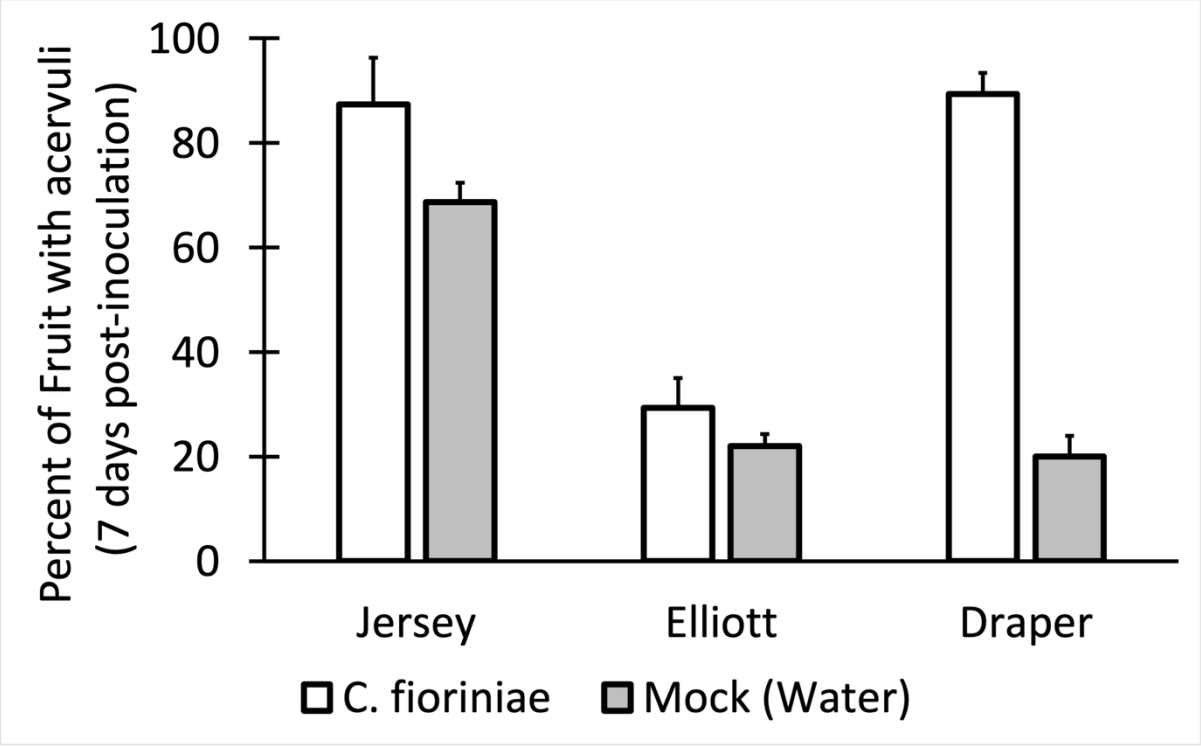

Figure 3.2. Proportion of blueberry fruit with visible acervuli after 7 days of incubation. Error bars represent ± 1 SE.

3.3.2. RNA extraction resulted in high-quality RNAseq reads

The RNA extraction protocol yielded an average of 3.04 ± 0.86 ug of RNA. RNA quality was assessed using 1 uL each of a subset of 40 samples by gel electrophoresis using a 1X TAE gel with GelRed® nucleic acid gel stain (Biotium, Fremont, CA) to visualize (Fig. 3.3). Around 507 ng of RNA was diluted to 25 uL in RNase-free water for library preparation. Library preparation yielded an average of 17.96 ± 7.23 ng uL^{-1} of cDNA for sequencing.

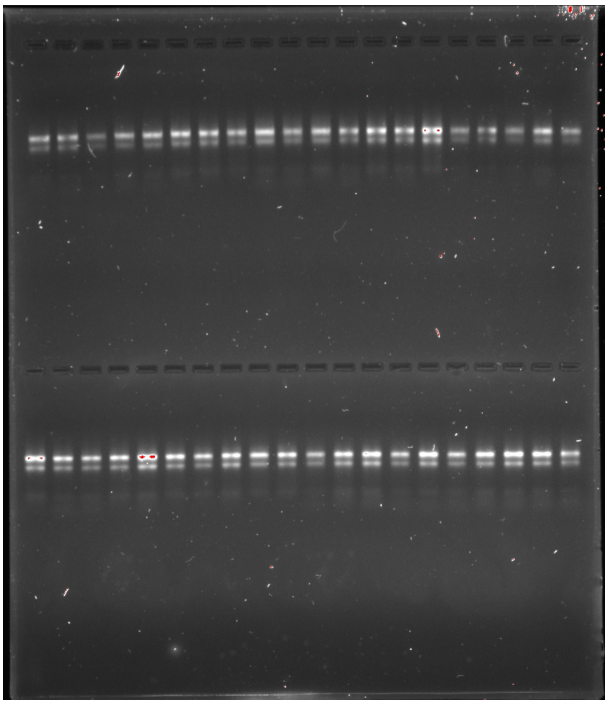

Figure 3.3. RNA gel (1% agarose) of a random sample of total RNA extracts for visual analysis of RNA quality.

RNA sequencing produced between 20.60 million and 42.30 million paired-end reads per sample an average of 26.42 ± 3.41 million. The average GC content of each sample was 46.06%. An average of 24.26 ± 3.23 million paired-end reads (92.03 ± 1.12%) had both pairs survive trimming and quality filtering. Of unpaired reads, an average of 1.88 ± 0.32 million of the first strand was the only read that survived trimming and an average of 92.92 ± 15.16 thousand of the second strand was the only surviving read. An average of 116.98 ± 89.88 thousand reads had

both the first and second strand dropped due to quality and/or length parameter settings of Trimmomatic.

3.3.3. Differential expression and gene clustering shows dynamic expression patterns

Trinity v2.8.5 was used to assemble 479,502 ‘genes’ based on 584.98 M assembled bases from 213.87 M input reads. These ‘genes’ were based on 654,817 predicted isoforms with 41.32% GC content from 337.52 Mbp. The contig N50 length was 1513 bp, with a median contig length of 509 bp and an average contig length of 893.36 bp. Based on only the longest isoform per gene, the contig N50 was 1064 bp with a median contig length of 410 bp and an average contig length of 703.9 bp. The within-cultivar differential expression resulted in a lower number of DE features (‘gene’ assembled by Trinity) than the between-cultivar differential expression (Table 3.1).

Table 3.1. Differentially expressed features (up- or down-regulated genes per day; $p>0.001$, $|Log_2FC| >2$ or 4) in each comparison in this study.

Comparison	DE features	
	|LFC|>2	|LFC|>4
DNvDT	886	-
JNvJT	4761	-
ENvET	1417	-
JTvDT	-	22667
JTvET	-	18477
ETvDT	-	20560

Differentially expressed genes were divided into sub-clusters with similar expression patterns using a tree-cutting algorithm based on hierarchical clustering generated by the R package hclust using the ‘complete’ agglomeration method (https://www.rdocumentation.org/packages/stats/versions/3.6.1/topics/hclust). In general, the expression patterns of interest fell into two categories, those with dynamic expression patterns in

one treatment (or cultivar) (Fig. 3.4), and those that were constitutively expressed in one treatment (or cultivar) (Fig. 3.5). Different cut-off percentages were used for each comparison based on visual analysis of the resulting cluster expression patterns. The ENvET was clustered at 20% of the total hierarchical cluster tree height producing 20 sub-clusters with the largest sub-cluster containing 687 transcripts and the smallest only containing a single transcript.

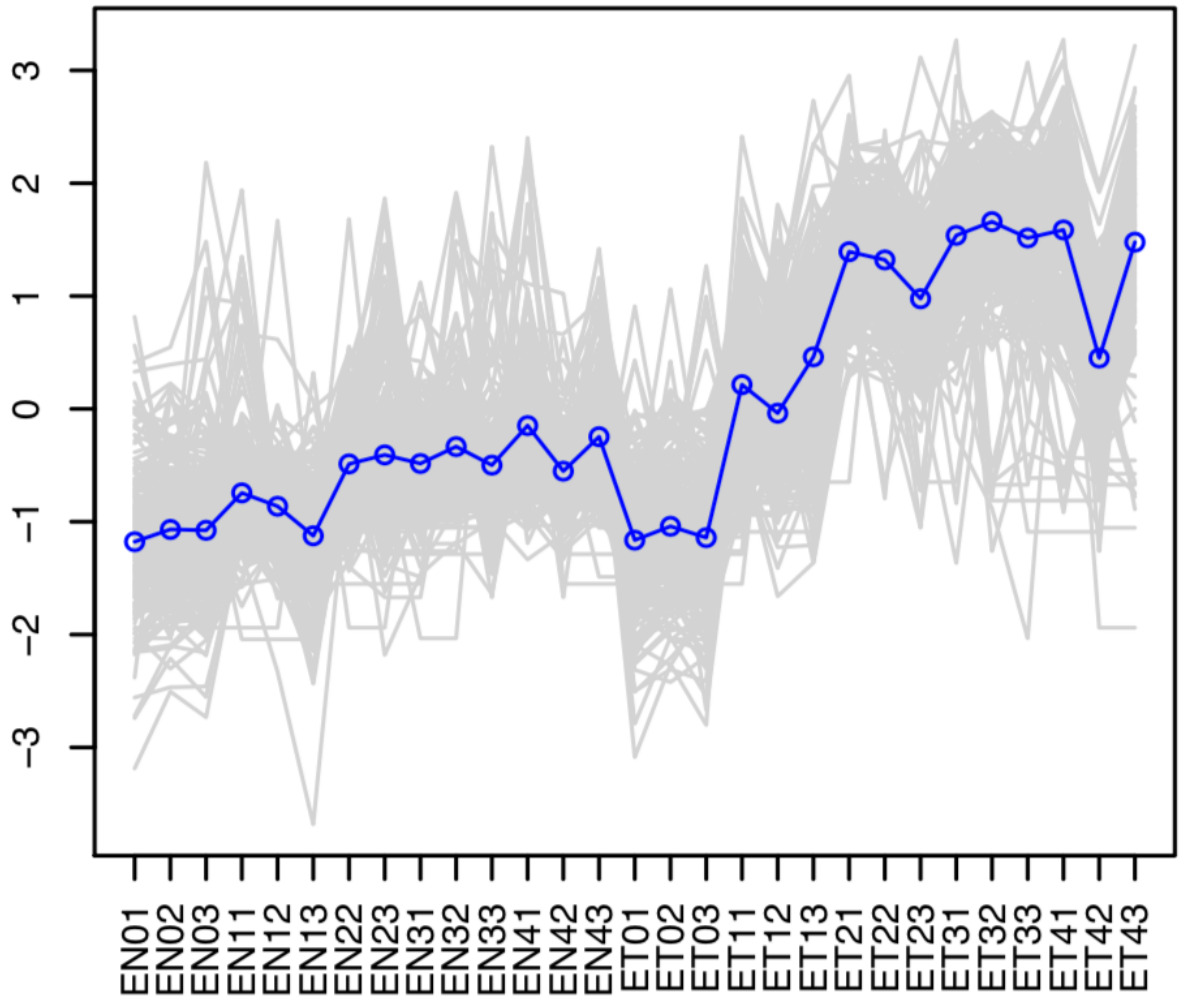

Figure 3.4. Transcript sub-cluster 2 (P=20%) with 74 members from the ENvET comparison demonstrating 'dynamic differential expression.' The grey lines are individual genes and the blue line is the average expression pattern for this cluster. Samples coded with an EN are 'Elliott' fruit that were mock-inoculated with water. Samples coded with ET are 'Elliott' fruit inoculated with a spore suspension of *C. fioriniae.* Sample numbers are in the form of 'sampling day' then 'replicate number'.

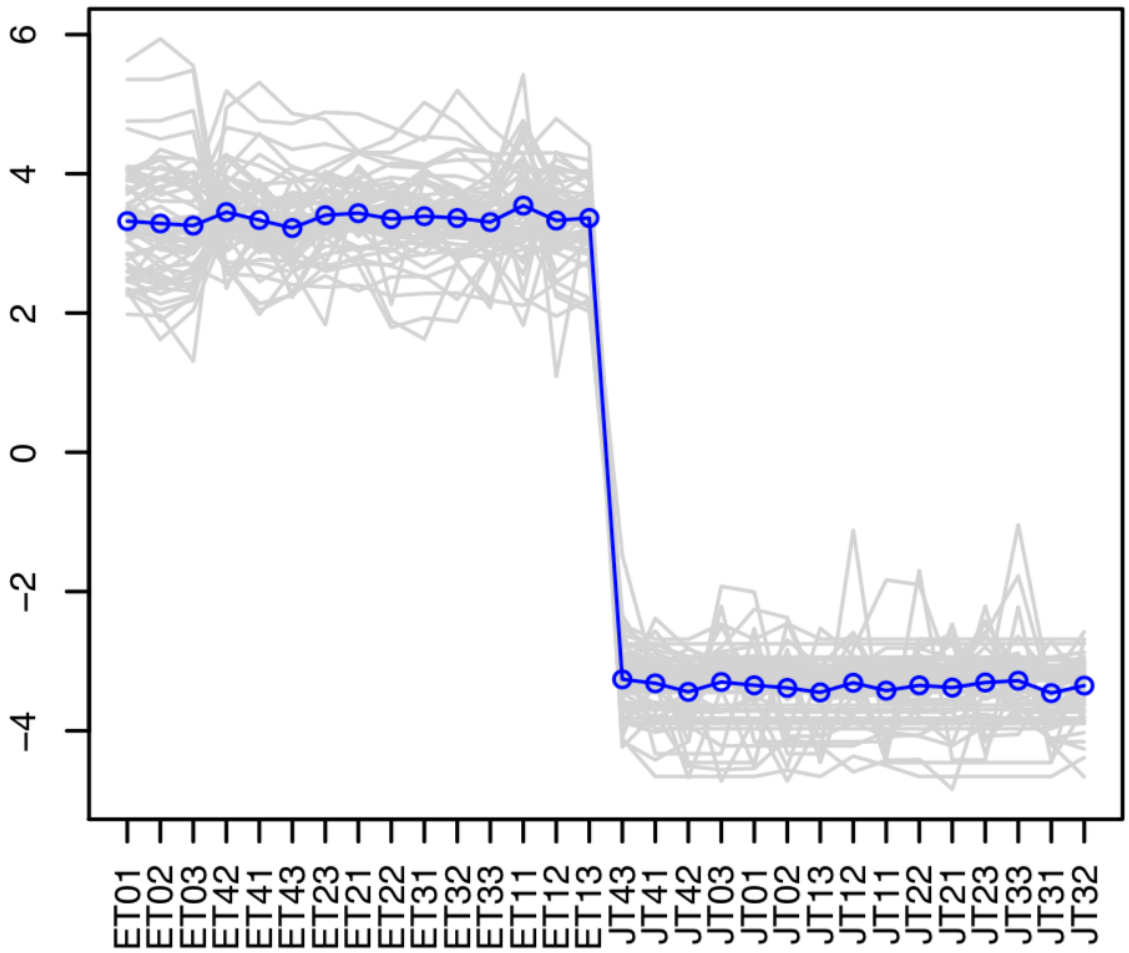

Figure 3.5. Transcript sub-cluster 18 (P=20%) with 60 transcripts from the ETvJT comparison demonstrating 'constitutive differential expression.' The grey lines are individual genes and the blue line is the average expression pattern for this cluster. Samples coded with an EN are 'Elliott' fruit that were mock-inoculated with water. Samples coded with ET are 'Elliott' fruit inoculated with a spore suspension of *C. fioriniae.* Sample numbers are in the form of 'sampling day' then 'replicate number'.

3.3.4. 'Jersey' had the highest number of DE genes of the within-cultivar comparisons

There was a total of 906, 1590 and 4955 DE features in 'Draper,' 'Elliott,' and 'Jersey,' respectively, when inoculated with *C. fioriniae* compared to fruit that were mock inoculated with sterile water (|LFC|>2, p<0.001). Of these DE features, 194, 639, and 1914 were annotated through the Trinotate pipeline for 'Draper,' 'Elliott,' and 'Jersey,' respectively. The number of up-regulated genes generally increased over the course of the sampling period with a peak on

day 4 (158 DE genes) in ‘Draper,’ a peak on day 3 (400 DE genes) in ‘Elliott,’ and a peak on day 4 (3281 DE genes) in ‘Jersey.’ The number of down-regulated genes was the highest on day 0 in ‘Draper’ (103 DE genes) and ‘Elliott’ (242 DE genes) and decreased thereafter. In ‘Jersey,’ the number of down-regulated genes peaked on day 1 (533 DE genes) (Fig. 3.6).

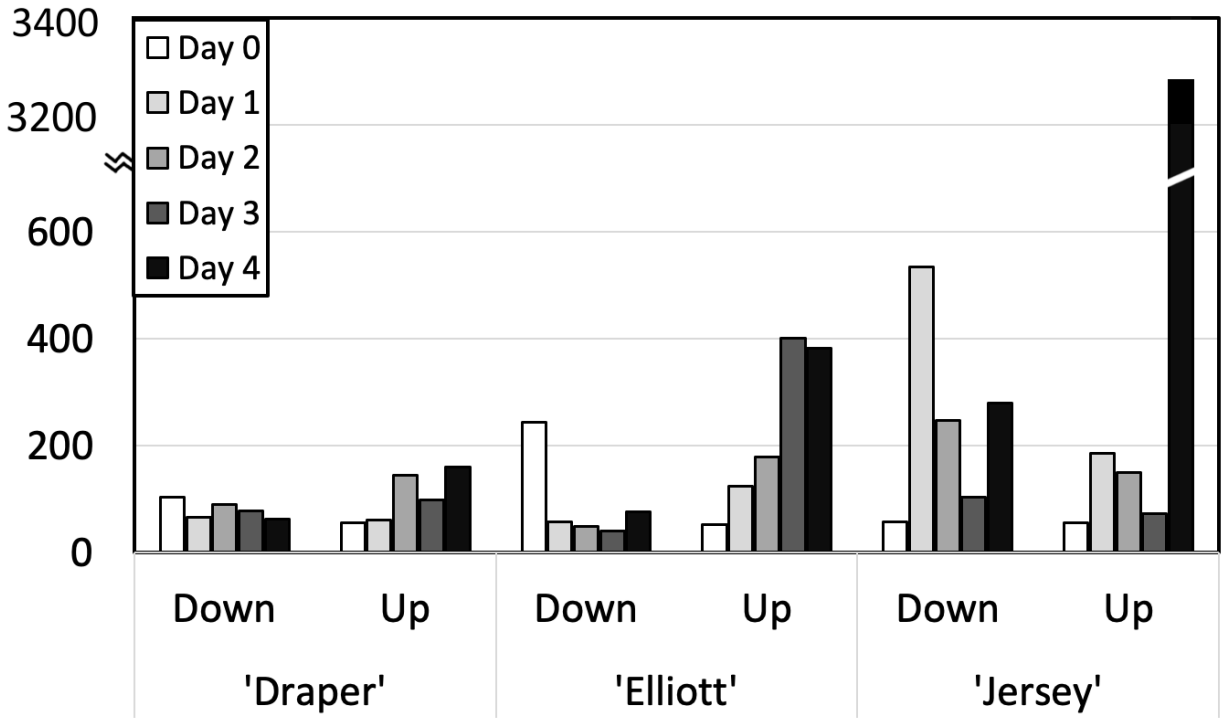

Figure 3.6. Differentially expressed blueberry genes for inoculated fruit in each cultivar compared to the mock-inoculated fruit ($p<0.001$; LFC>2). ‘Down’ represents genes that are down-regulated each day, and ‘Up’ represents up-regulated genes each day.

3.3.5. The ‘Jersey’ vs ‘Draper’ comparison had the most DE genes in the between-cultivar comparisons

To account for genome differences and discover differences in gene expression between cultivars, we compared gene expression with slightly more strict parameters ($|LFC|>4$; $p<0.001$). These parameters resulted in a total of 46446, 44730, and 29625 differentially expressed features over the course of the sampling period for the ‘Jersey’ vs ‘Draper’, ‘Elliott’ vs ‘Draper’, and the ‘Jersey’ vs ‘Elliott’ comparisons, respectively (Fig. 3.7). No clear trends were found in the total

number of DE genes per day. The ‘Jersey’ vs ‘Draper’ comparison was highest on day 3 (13,181 DE genes). The ‘Elliott’ vs ‘Draper’ comparison was highest on day 2 (11,809 DE genes) and day 4 (11,624 DE genes). The ‘Jersey’ vs ‘Elliott’ comparison had the lowest number of overall DE genes but was highest on day 4 (11,914 DE genes).

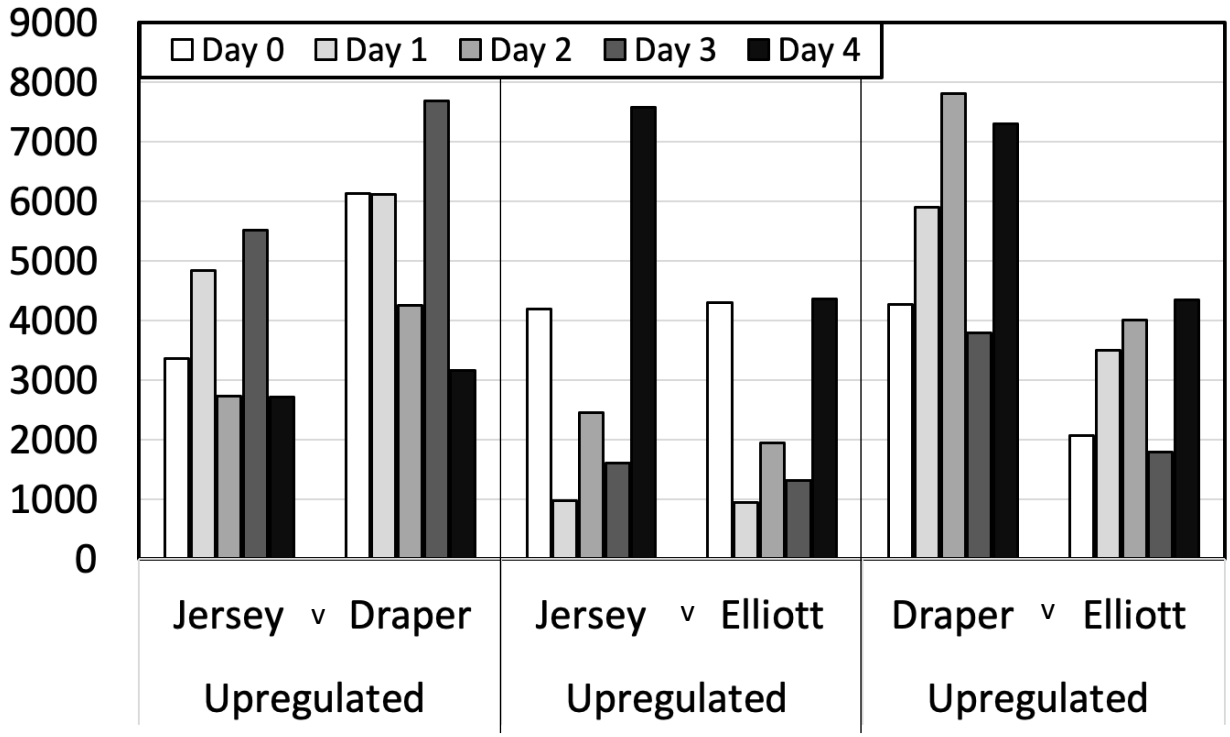

Figure 3.7. Up-regulated genes ($p<0.001$; LFC>4) in each between-cultivar comparison by day, separated by comparison. In all comparisons individually, any gene that was down-regulated in the first listed cultivar is considered to be up-regulated in the second listed cultivar.

3.3.6. DE genes unique to the C. fioriniae response of ‘Elliott’ fruit were found

To find genes that are strong candidates for defense-related genes responsible for the strong resistance phenotype of ‘Elliott’, we used a strict filtering procedure (Fig. 3.8). We first identified differentially expressed genes ($p<0.001$, $|LFC|>2$) in the mock (H_2O) inoculated fruit between ‘Jersey’ and ‘Elliott.’ This revealed 8029 unique DE genes over time, but because some genes were differentially expressed on more than one day, a total of 12951 features (combinations of genes x days).

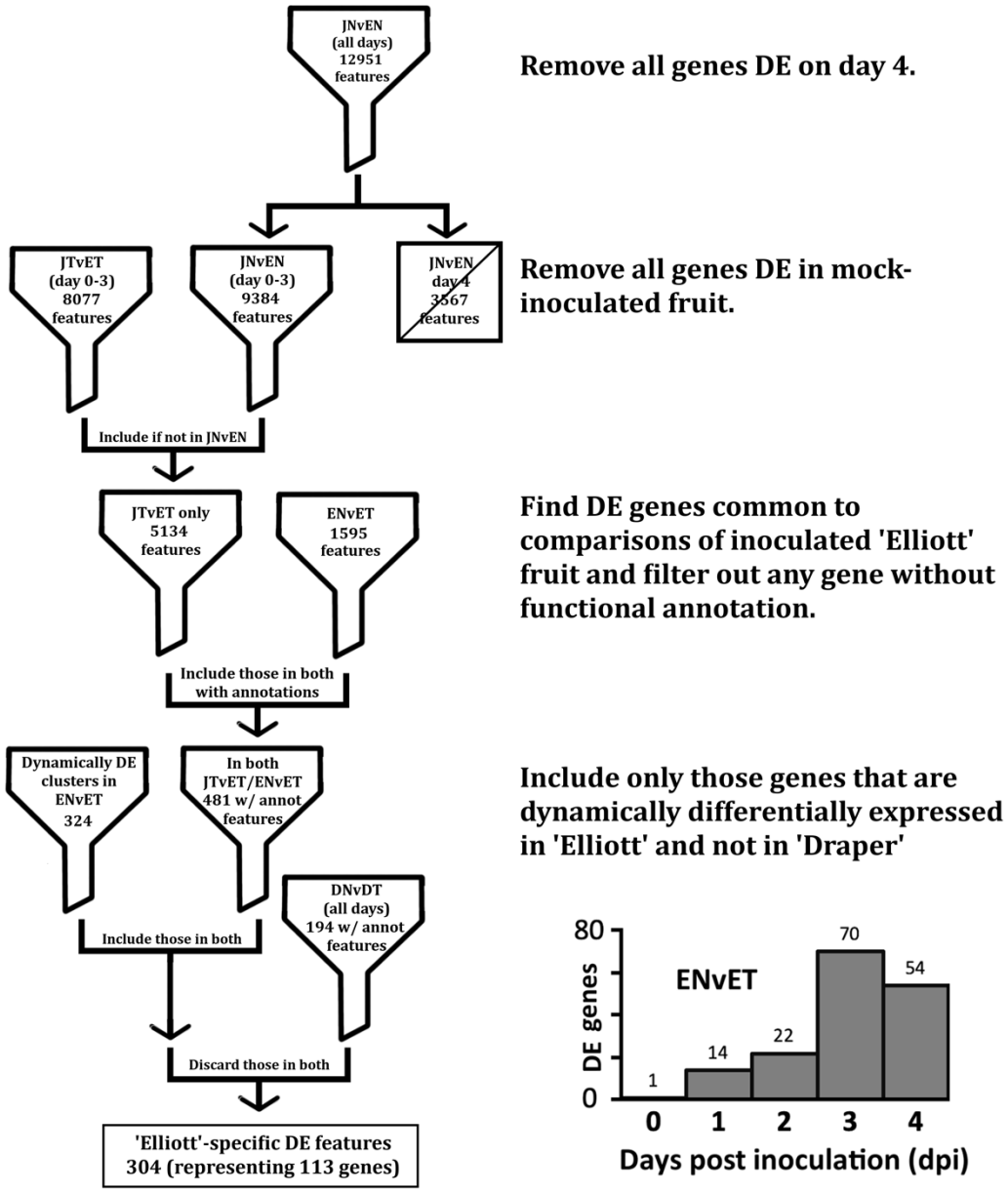

Figure 3.8. Filtering procedures for isolating 'Elliott'-specific DE genes.

We then analyzed DE genes in the JTvET comparison ($p>0.001$, $|LFC|>2$) and found 8077 features and 5280 unique annotated genes. After subtracting genes common in both sets, we were left with 4152 DE genes unique to the inoculated comparison, hereafter referred to as the subtracted JTvET dataset. Following this gene filtering, we analyzed the DE genes ($p<0.001$, $|LFC|>2$) between mock inoculated and *C. fioriniae* inoculated 'Elliott' fruit and found 443

annotated DE gene features comprised of 393 unique genes. The genes common to the ENvET and the subtracted JTvET datasets, but not in the DNvDT DE dataset, were removed. DE genes in the ENvET comparison that had a dynamic expression pattern over time (subclusters 2 and 5; P=20%; Fig. 3.9) were finally isolated resulting in 113 'Elliott'-specific, inoculation responsive resistance-related DE genes.

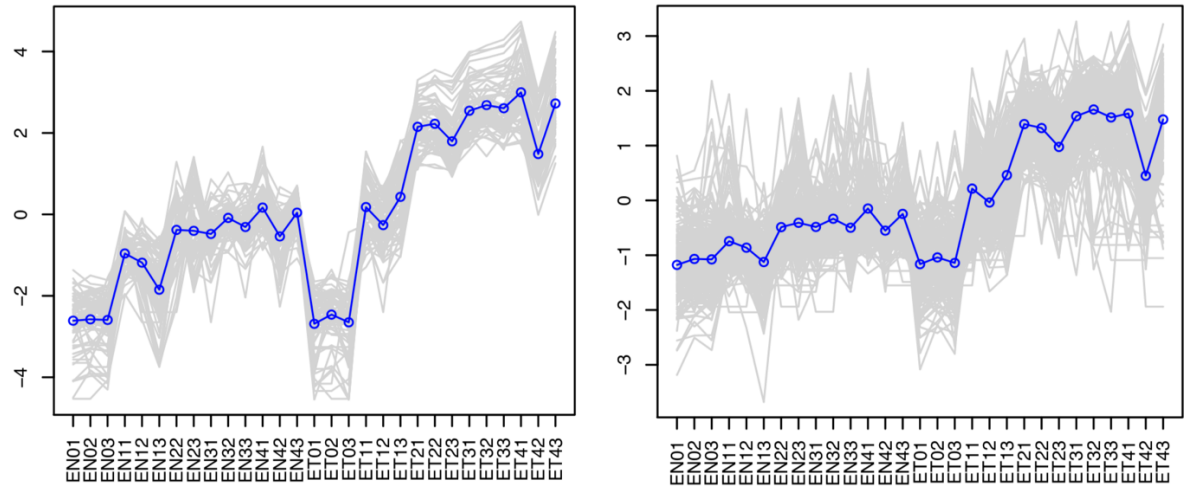

Figure 3.9. Sub-clusters showing patterns of dynamic differential expression formed by the hierarchical clustering program hclust at 20% of the total tree height of all differentially expressed genes in the ENvET comparison. A) Sub-cluster 2. B) sub-cluster 5. Samples are grouped by treatment and replicate where E = 'Elliott', N = mock-inoculated fruit, T = fruit inoculated with *C. fioriniae*, and the two number code is sampling day followed by sample replicate. X-axis represents the Log_2-fold change in expression.

3.3.7. Genes implicated in pathogen-triggered immunity (PTI) were common

Of the 113 genes DE in 'Elliott' fruit that passed our filtering protocol (Fig. 3.8) many were homologous to genes involved in pathogen-triggered immunity in other host-pathogen systems. Three genes had homology to resistance genes (R genes) based on BLAST and UniProt best hits (Table 3.2). One predicted R gene (Vcor_4198C309g1), one of the most highly up-regulated (|LFC|=9.68) genes in the dataset, was upregulated on day 2. Nine genes showed strong evidence for homology to known secondary metabolite biosynthesis genes (Table 3.3), including genes related to phytoalexin and saponin biosynthesis. Sixteen genes were related to fungal or

plant cell wall metabolic genes (Table 3.4), including six with peptide sequences that mark them for secretion into the apopolast.

Table 3.2. Canonical resistance (R) genes up-regulated (|LFC|>2, p-value<0.001) in 'Elliott' fruit inoculated with *Colletotrichum fioriniae* compared to mock-inoculated fruit. ^{S}: indicates a predicted secreted protein (according to SignalP version 4.0).

Gene name	UniProtKB Best Hit	Protein Name	References	Log$_2$-fold Change				
				Day 0	Day 1	Day 2	Day 3	Day 4
Vcor_2547c19g1^{S}	LRR1_CAPAN	**Leucine-rich repeat protein 1**	Jung et al., (2004) Jung and Hwang, (2007) Choi et al., (2012)	-	-	-	+3.20	+2.75
Vcor_4198c309g1	RLP13_ARATH	**Receptor-like protein 13**	-	-	-	+9.68	-	-
Vcor_1132c178g1^{S}	9DC3_SOLPI	**Receptor-like protein 9DC3**	Kruijt et al., (2004)	-	-	-	+3.07	-

Table 3.3. Secondary metabolite-related genes up-regulated (|LFC|>2, p-value<0.001) in 'Elliott' fruit inoculated with *Colletotrichum fioriniae* compared to mock-inoculated fruit.

Gene Name	UniProtKB Best Hit	Protein name	Publication	Log$_2$-fold change				
				Day 0	Day 1	Day 2	Day 3	Day 4
Vcor_1620c10g1	C93A1_SOYBN	3,9-dihydroxypterocarpan 6A-monooxygenase	Schopfer et al., (1998)	-	-	-	+2.18	-
Vcor_4132c3g1	C7A17_VITVI	Beta-amyrin 28-monooxygenase	Fukushima et al., (2011)	-	-	-	-	+2.03
Vcor_1595c3g1				-	-	-	+2.02	-
Vcor_1065c184g1	DXS2_ORYSJ	Probable 1-deoxy-D-xylulose-5-phosphate synthase 2, chloroplastic	-	-	-	-	+2.25	-
Vcor_1276c82g1	SQE1_PANGI	Squalene monooxygenase SE1	Li et al., (2016)	-	-	+3.08	+3.05	+2.19
Vcor_1839c13g1				-	-	-	+3.57	-
Vcor_2125c13g1				-	+2.15	-	-	-
Vcor_5631c44g1				-	-	+3.04	-	+2.49
Vcor_1703c120g1	VESTR_MEDSA	Vestitone reductase	Guo et al., (1995) Kessmann et al., (1990)	-	-	-	+2.36	+2.44

Of the predicted secreted proteins and those genes homologous to them, four were class V chitinases (Vcor_1653c5g1, Vcor_5264c11g1, Vcor_7534c36g1, and Vcor_2181c20g1) which are implicated in the breakdown of fungal cell walls. Cell wall-related genes were mostly up-regulated on days 3 and 4, though some (Vcor_2317c486g1 and Vcor_1617c20g1) were up-

regulated as early as day 1. Genes related to extracellular sugar scavenging and agglomeration, such as homologs of lectins, were primarily up-regulated on day 4, though two predicted secreted invertase genes (Vcor_6357c2080g1 and Vcor_3209c164g1) were up-regulated only on day 2 and 3, respectively (Table 3.5). Other genes related to PTI, such as homologs of pathogen-related protein PRPX (Vcor_21c0g1 and Vcor_2317c66g1) and homologs of genes in the NRT1/PTR family (Vcor_4269c2g1, Vcor_1578c2g1, and Vcor_7266c8g1) were primarily up-regulated on day 3 (Table 3.6).

Table 3.4. Plant and fungal cell wall-related genes up-regulated (|LFC|>2, p-value<0.001) in 'Elliott' fruit inoculated with *Colletotrichum fioriniae* compared to mock-inoculated fruit. ^{S}: indicates a predicted secreted protein (according to SignalP version 4.0).

Gene Name	UniProtKB Best Hit	Protein name	Publication	Log_2-fold change				
				Day 0	Day 1	Day 2	Day 3	Day 4
Vcor_1664c1g1	AB11G_ARATH	ABC transporter G family member 11	Takeuchi et al., (2018)	-	-	-	+3.19	+2.74
Vcor_2202c9g1				-	-	-	+3.24	+2.52
Vcor_7534c67g1				-	-	+3.30	+3.86	-
Vcor_1617c20g1^{S}	AT3g10190	Probable calcium-binding protein CML36	Ha Le et al., (2014)	-	+2.14	-	-	-
Vcor_4218c3g1	CERK1_ORYSJ	Chitin elicitor receptor kinase 1	Shimizu et al., (2010) Yamaguchi et al., (2013)	-	-	-	-	+2.24
Vcor_1653c5g1^{S}	CHT5A_MEDTR	Class V chitinase CHIT5a	Zhang et al., (2016)	-	-	-	+2.64	+2.28
Vcor_5264c11g1^{S}				-	-	+2.41	-	+2.26
Vcor_7534c36g1				-	-	+2.24	+2.84	-
Vcor_2181c20g1^{S}				-	-	-	+2.86	+2.19
Vcor_3592c63g1^{S}	E13B_VITVI	Glucan endo-1,3-beta-glucosidase	-	-	-	-	+3.99	-
Vcor_1132c16g1	GIP2_NICBE	Probable aspartic proteinase GIP2	Ma et al., (2017)	-	-	-	+4.51	+3.12
Vcor_1132c2g1^{S}				-	-	-	+2.61	-
Vcor_1132c2g2				-	-	-	+3.36	+2.78
Vcor_1132c10g1				-	-	-	+4.15	-
Vcor_2317c486g1	LYM2_ARATH	LysM domain-containing GPI-anchored protein 2	Shinya et al., (2012)	-	+3.40	-	-	-
Vcor_1489c56g1	PME41_ARATH	Probable pectin esterase/pectinesterase inhibitor 41	Bethke et al., (2014)	-	-	-	+3.95	-
Vcor_1373c22g1	XAT3_ORYSJ	Alpha-1,3-arabinosyltransferase XAT3	Anders et al., (2012)	-	-	+2.64	+3.09	-
Vcor_1718c148g1				-	-	-	+2.30	-
Vcor_1373c6g1				-	-	+2.30	+2.79	+2.34

Table 3.5. Lectin and sugar-related genes up-regulated (|LFC|>2, p-value<0.001) in 'Elliott' fruit inoculated with *Colletotrichum fioriniae* compared to mock-inoculated fruit. [S]: indicates a predicted secreted protein (according to SignalP version 4.0).

Gene Name	UniProtKB Best Hit	Protein name	Reference	Log$_2$-fold change				
				Day 0	Day 1	Day 2	Day 3	Day 4
Vcor_2272c0g1	LEC2_CLAKE	Agglutinin-2	Barre et al., (2001) Lannoo and Van Damme, (2014)	-	-	-	+4.57	+4.29
Vcor_6988c1g1				-	-	-	-	+4.20
Vcor_5658c1g1	LEC_LENCO	Lectin	Barre et al., (2001) Lannoo and Van Damme, (2014)	-	-	-	-	+4.30
Vcor_329c15g1[S]	P4H7_ARATH	Probable prolyl 4-hydroxylase 7	Vlad et al., (2007)	-	-	-	-	+2.28
Vcor_7025c42g1[S]				-	-	-	-	+2.23
Vcor_6357c2080g1[S]	INV1_ARATH	Beta-fructofuranosidase, insoluble isoenzyme CWINV1	Veillet et al., (2016)	-	-	+2.99	-	-
Vcor_3209c164g1[S]				-	-	-	+2.46	-

Table 3.6. Miscellaneous PTI-related genes up-regulated (|LFC|>2, p-value<0.001) in 'Elliott' fruit inoculated with *Colletotrichum fioriniae* compared to mock-inoculated fruit. [S]: indicates a predicted secreted protein (according to SignalP version 4.0).

Gene name	UniProtKB Best Hit	Protein Name	References	Log$_2$-fold change				
				Day 0	Day 1	Day 2	Day 3	Day 4
Vcor_2317c436g1	BAD1_ARATH	**Ankyrin repeat-containing protein BDA1**	Yang et al., (2012)	-	-	-	+2.67	-
Vcor_1718c27g1[S]	CRR38_ARATH	**Cysteine-rich repeat secretory protein 38**	-	-	-	-	+2.51	+2.62
Vcor_7494c135g1			-	-	-	-	+2.20	-
Vcor_147c5g1	PUB24_ARATH	**E3 ubiquitin-protein ligase PUB24**	Trujillo et al., (2008)	-	-	-	+2.42	-
Vcor_2544c35g1	PIP1_ARATH	**PAMP-induced secreted peptide 1**	Hou et al., (2014)	-	-	-	+2.65	-
Vcor_21c0g1	PRPX_HORVU	**Pathogen-related protein**	-	-	-	-	+2.46	-
Vcor_2317c66g1			-	-	-	-	+2.45	+2.06
Vcor_4269c2g1	PTR3_ARATH	**Protein NRT1/ PTR FAMILY 5.2**	Karim et al., (2007)	-	-	-	-	+2.09
Vcor_1578c2g1				-	-	-	+2.11	+2.20
Vcor_7266c8g1	PTR4_ARATH		-	-	-	-	+2.13	-
Vcor_2544c1565g1	U74F2_ARATH	**UDP-glycosyltransferase 74F2**	Song et al., (2006)	-	-	-	+9.28	-

3.3.8. Hypersensitive response (HR)-related genes were represented

We expect genes related to the hypersensitive response in other hosts to be up-regulated late in the infection process in 'Elliott' fruit. We found at least 21 genes with strong similarity to HR-related genes (Table 3.7). These genes include fatty acid metabolic genes (Vcor_4217c965g1, Vcor_4217c962g1, Vcor_4119c7g1, Vcor_4217c18g1, Vcor_1620c276g1, and Vcor_1110c28g1), ROS metabolism including hydrogen peroxide metabolism-related genes

(Vcor_2884c19g1, Vcor_2884c2g1, and Vcor_2406c125g1), as well as genes related to programmed cell death (Table 3.7). Most of the hypersensitive response-related genes were up-regulated on day 4.

Table 3.7. Hypersensitive response (HR)-related genes up-regulated (|LFC|>2, p-value<0.001) in 'Elliott' fruit inoculated with *Colletotrichum fioriniae* compared to mock-inoculated fruit. ^{S}: indicates a predicted secreted protein (according to SignalP version 4.0).

Gene Name	UniProtKB Best Hit	Protein name	Reference	Log$_2$-fold change				
				Day 0	Day 1	Day 2	Day 3	Day 4
Vcor_1675c16g1^{S}	ASO_CUCMA	L-ascorbate oxidase	García-Pineda et al., (2004)	-	-	-	+3.04	+2.50
Vcor_1148c4g1	CYT1_ACTDE	Cysteine proteinase inhibitor 1	Solomon et al., (1999)	-	-	-	+3.44	+2.67
Vcor_7091c5g1				-	-	-	+3.90	+3.06
Vcor_7025c101g1	DOX1_ARATH	Alpha-dioxygenase 1	De Leon et al., (2002) Hamberg et al., (1999) Vicente et al., (2012)	-	-	-	-	+2.86
Vcor_329c0g1				-	-	+2.72	-	+2.46
Vcor_4217c965g1	FAD2_VERFO	Delta(12)-fatty-acid desaturase FAD2	Nguyen et al., (2019)	-	-	-	-	+2.30
Vcor_4217c962g1				-	-	-	-	+2.63
Vcor_4119c7g1	FD6E2_SOYBN	Omega-6 fatty acid desaturase, endoplasmic reticulum isozyme 2	-	-	-	-	-	+2.17
Vcor_4217c18g1				-	-	-	-	+2.91
Vcor_1620c276g1				-	-	-	+2.03	-
Vcor_403c175g1	GLOX_VITPS	Aldehyde oxidase GLOX	Guan et al., (2011) Zhao et al., (2013)	-	-	-	-	+2.88
Vcor_5724c144g1				-	-	-	-	+2.72
Vcor_7213c9g1	GSTU7_ARATH	Glutathione S-transferase U7	Gullner et al., (2018)	-	-	-	-	+2.42
Vcor_6577c28g1	LAC14_ARATH	Laccase-14	-	-	-	-	-	+4.09
Vcor_2317c96g1				-	-	-	+3.57	+4.44
Vcor_1110c28g1	LOX31_SOLTU	Linoleate 13S-lipoxygenase 3-1, chloroplastic	Royo et al., (1996,1999)	-	-	-	+2.22	-
Vcor_2498c26g1	PCR2_ARATH	PLANT CADMIUM RESISTANCE 2	Luhua et al., (2008)	-	-	-	+2.17	-
Vcor_2406c125g1^{S}	PER51_ARATH	Peroxidase 51	Almagro et al., (2009)	-	-	-	-	+2.95
Vcor_2884c19g1	PERX_NICSY	Lignin-forming anionic peroxidase	Almagro et al., (2009)	-	-	-	+2.34	-
Vcor_2884c2g1^{S}				-	-	-	+2.91	-
Vcor_2406c103g1^{S}	PLP2_ARATH	Patatin-like protein 2	La Camera et al., (2005, 2009) Yang et al., (2012)	-	-	-	-	+3.02

3.3.9. Genes related to a general abiotic and/or biotic 'stress-response' are differentially expressed

Genes homologous to known 'stress-responsive' genes, including those related to both biotic and abiotic stress response were up-regulated throughout the infection process (Table 3.8). We found evidence for homologs of ethylene-biosynthesis genes (Vcor_1718c98g1, 1929c33g1,

and 3261c18g1), a predicted wound-induced protein (WUN1) (Vcor_5543c28g1), as well as multiple homologs of a gene necessary for cuticle development (Vcor_1164c1g1, Vcor2202c9g1, and Vcor7534c67g1). The WUN1 homolog was the earliest gene in this group to be up-regulated, though most were up-regulated on days 2-4.

Table 3.8. General stress response-related genes up-regulated (|LFC|>2, p-value<0.001) in 'Elliott' fruit inoculated with *Colletotrichum fioriniae* compared to mock-inoculated fruit. ^{S}: indicates a predicted secreted protein (according to SignalP version 4.0).

Gene	UniProtKB Best Hit	Protein name	Reference	Log$_2$-fold change				
				Day 0	Day 1	Day 2	Day 3	Day 4
Vcor_1718c98g1	ACCO3_PETHY	1-aminocyclopropane-1-carboxylate oxidase 3	-	-	-	-	+2.09	-
Vcor_1929c16g1	ACCH1_ARATH	1-aminocyclopropane-1-carboxylate oxidase homolog 1	-	-	-	-	+2.08	+2.62
Vcor_3261c18g1			-	-	-	-	-	+2.09
Vcor_1065c23g1	HHP2_ARATH	Heptahelical transmembrane protein 2	-	-	-	+2.00	+2.16	-
Vcor_3668c5g1	IQM4_ARATH	IQ domain-containing protein IQM4	-	-	-	+2.89	-	-
Vcor_1923c33g1^{S}	OS35_SOLCO	Osmotin-like protein OSML15	-	-	-	-	+2.83	+3.32
Vcor_7092c102g1	SRO2_ARATH	Probable inactive poly [ADP-ribose] polymerase SRO2	-	-	-	-	-	+2.82
Vcor_1578c18g1	BSK5_ARATH	Serine/threonine-protein kinase BSK5	Tang et al., (2008) Li et al., (2012)	-	-	-	+2.35	-
Vcor_5543c28g1	WUN1_SOLTU	Wound-induced protein 1	-	-	+2.79	-	-	-

3.3.10. Many genes could not be well classified into any of the above categories

Many of the DE genes did not have strong enough evidence for classification into any of the above categories (Table 3.9), including those with predicted transmembrane domains (Vcor_356c2g1, Vcor_1293c3g1, Vcor_1798c0g1, Vcor_2097c0g1, Vcor_2181c723g1, Vcor_2498c42g1, Vcor_5646c0g1), a predicted gibberellin-regulated protein (Vcor_6966c12g1), and two homologs of a probable cysteine desulfurase (Vcor_2206c3g1 and Vcor_4493c0g1).

Table 3.9. Genes with low-evidence for disease resistance up-regulated (|LFC|>2, p-value<0.001) in 'Elliott' fruit inoculated with *Colletotrichum fioriniae* compared to mock-inoculated fruit.

Gene	UniProtKB Best Hit	Protein name	Log$_2$fold change				
			Day 0	Day 1	Day 2	Day 3	Day 4
Vcor_1107c46g1	AB2A_ARATH	ABC transporter A family member 2	-	-	-	+2.27	-
Vcor_7495c139g1	AIR3_ARATH	Subtilisin-like protease SBT5.3	-	-	-	+3.02	-
Vcor_1623c2g1	AOX1_TOBAC	Ubiquinol oxidase 1, mitochondrial	-	+2.56	-	-	-
Vcor_5494c1747g1	ATPAM_ARATH	ATP synthase subunit alpha, mitochondrial	-	-	+14.40	-	-
Vcor_146c15g2	BBE2_ARATH	Berberine bridge enzyme-like 2	-	-	-	+2.06	-
Vcor_5438c7g1	CPC_CUCSA	Cucumber peeling cupredoxin	-	-	+2.38	-	+2.70
Vcor_2206c3g1	CSD_STAAN	Probable cysteine desulfurase	-	-	-	+3.17	-
Vcor_4493c0g1			-	-	-	-	+2.51
Vcor_2514c8g1	DCE1_ARATH	Glutamate decarboxylase 1	-	+4.22	-	+4.74	+3.71
Vcor_6576c119g1	DIOX3_PAPSO	Codeine O-demethylase	-	-	-		+2.47
Vcor_6966c12g1	GASA1_ARATH	Gibberellin-regulated protein 1	-	-	-	+2.33	-
Vcor_1937c8g1	GATA5_ARATH	GATA transcription factor 5	-	-	+2.42	+2.63	-
Vcor_1319c194g1	HQGT_RAUSE	Hydroquinone glucosyltransferase	-	+2.06	-	+2.56	-
Vcor_5785c33g1	IST1L_DICDI	IST1-like protein	-	-	+2.77	-	+2.59
Vcor_406c28g1	ITH5_CUCMA	Inhibitor of trypsin and hageman factor	-	-	-	-	+2.43
Vcor_1609c5g1	KITH_ORYSJ	Thymidine kinase	-	-	-	+2.08	+2.07
Vcor_2317c55g1	LHTL8_ARATH	Lysine histidine transporter-like 8	-	+2.34	+2.62	+2.51	-
Vcor_5608c11g1			-	+2.43	+2.58	-	-
Vcor_7092c71g1	PHO13_ARATH	Phosphate transporter PHO1 homolog 3	-	-	-	-	+3.12
Vcor_1929c798g1	POLR2_ARATH	Retrovirus-related Pol polyprotein from transposon RE2	-6.89	-	-	-	-
Vcor_1798c5g1	PR1F2_ARATH	PRA1 family protein F2	-	-	-	+2.56	
Vcor_5646c12g1			-	+2.93	-	-	-
Vcor_3629c1026g1	RS272_ARATH	40S ribosomal protein S27-2	-	-	+3.49	+3.91	-
Vcor_1107c151g1	SRO2_ARATH	Probable inactive poly [ADP-ribose] polymerase SRO2	-	-	-	+2.71	-
Vcor_1624c160g1	VQ1_ARATH	VQ motif-containing protein 1	-	+4.97	-	+3.52	+3.10
Vcor_2915c16g1	Y1465_ARATH	Late embryogenesis abundant protein At1g64065	-	-	-	+2.22	-
Vcor_2317c682g1		Ankyrin repeat-containing protein	-	-	+3.80	-	-
Vcor_356c2g1		Predicted transmembrane domain-containing protein	-	-	+2.18	-	-
Vcor_1293c3g1				+2.86		+2.22	+2.16
Vcor_1798c0g1			-	-	-	+2.57	-
Vcor_2097c0g1			-	-	-	+2.44	-
Vcor_2181c723g1			-	-	-	+2.60	+2.54
Vcor_2498c42g1			-	+2.10	-	-	-
Vcor_5646c0g1			-	+3.17	-	-	-

3.4. DISCUSSION

No blueberry cultivars grown in Michigan are completely resistant to infection by *C. fioriniae*, though many widely grown cultivars are considered to be 'highly resistant'. Using

RNA-seq, we show that discovering differentially expressed genes in field-grown highbush blueberry fruit can be achieved even in the presence of background levels of infection, however, our results challenge the common designations of field resistance in 'Draper' fruit grown in western Michigan. We found that 'Draper' fruit, which is generally considered 'resistant' (Miles and Schilder, 2008; Polashock et al., 2005) in Michigan had a similar level of background infection from the field as 'Elliott' (Fig. 3.2), a cultivar generally considered one of the most resistant cultivars in both field and laboratory studies. When inoculated with *C. fioriniae* in a laboratory setting, 'Draper' displayed similar resistance to 'Jersey', which is considered 'moderately susceptible' in the same context. A similar disparity between field- and lab-infected levels of resistance has been reported for 'Duke,' 'Croatan,' and 'Murphy' fruit (Miles and Schilder, 2008). Likely these differences are a consequence of environmental factors experienced in the field compared to the relatively stable and highly favorable conditions for pathogen development created in a laboratory-scale experiment.

To maintain favorable traits from older, potentially more susceptible cultivars, many of the newly released cultivars contain minority introgressions from other *Vaccinium* species. 'Draper' for instance, is primarily *V. corymbosum* with small genetic contributions from *V. tenellum*, *V. ashei*, and *V. darrowi* (Hancock, 2004). 'Draper' is more resistant than many earlier released cultivars (Miles and Schilder, 2008) and it is possible these minority contributions may be the genetic basis for resistance even though they make up a small portion of the genome. Introgression of resistance genes from wild relatives or interspecific crosses is common in plant breeding. A gene for resistance to *Meloidogyne exigua* was introgressed into the widely cultivated *Coffea arabica* from a wild interspecific hybrid from Timor (*C. arabica* x *C. canephora*) and breeding programs are underway to use this same wild cross to introgress

resistance to *Colletotrichum kahawae* as well as other genetic resistance mechanisms (Bertrand et al., 2008). Similar efforts to use introgression from wild relatives or interspecific crosses for anthracnose resistance are underway in many other crops, including pepper (Yoon et al., 2006) and common bean (Pereira et al., 2013; Mongi et al., 2009). Future studies aimed at discovering the genomic location and source (*V. corymbosum* or a wild species) of the genes identified herein may direct further studies focusing on incorporating more of those genetic sources into breeding programs to increase resistance to *C. fioriniae* even further.

We used a strict filtering procedure for identifying strong candidate genes responsible for the resistant phenotype of 'Elliott' fruit by starting with genes that are up-regulated in both the ENvET comparison and the JTvET comparison and then excluding any genes that are also up-regulated in inoculated 'Draper' fruit. Using this approach, we identified 113 DE genes unique to 'Elliott' fruit that show a dynamic response over time in the context of cell wall and cell membrane dynamics, ROS metabolism and scavenging, and the production of secondary metabolites.

The cell surface is the first interface for host-pathogen interactions. In this study, we show differential expression in a number of genes with homology to known cell wall and cell membrane-related genes in other plants, including some known to contribute to resistance to other plant pathogens. We found evidence for genes associated with response to microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) up-regulated in 'Elliott' fruit. PAMPs are molecules such as chitin in fungi or flagellin in bacteria that are characteristic of each class of pathogen. PAMPs and/or MAMPs that are recognized through interaction with constitutively expressed pattern recognition receptors (PRRs) may trigger what is known as PAMP-triggered immunity, or PTI (Jones and Dangl, 2006).

Canonical resistance genes (R genes) are a part of the gene-for-gene resistance mechanism in plants where one host gene confers resistance in a plant to a pathogen strain containing a specific avirulence (Avr) gene. Avirulence genes often produce secreted effectors while R genes are often membrane bound receptors or lucine-rich-repeat (LRR) containing receptor-like proteins (RLPs). We found evidence for two genes that code for RLPs including one with homology to the RLP 9DC3 gene from *Solanum pimpinellifollium* which confers resistance to *Cladosporium fulvum* through recognition of the *Avr9* effector protein (Kruijt et al., 2004) as well as a homolog of a LRR gene from *C. annum* (LRR1) that can act both as a negative or positive regulator of cell death through interaction with HIR1 (Jung and Hwang, 2007) or PR10 (Choi et al., 2012), respectively (Table 3.2). Finding three canonical R gene homologs after strict filtering for 'Elliott'-specific infection responsive genes indicates that there are potentially more R gene homologs involved in other the other cultivars that we may have filtered out. *Colletotrichum* spp. are notoriously promiscuous with respect to their host, so it is possible that studies aimed at probing our data for other canonical R genes could provide valuable insight into not only blueberry anthracnose, but resistance in other systems as well.

Saponins are a diverse class of steroidal triterpene glycosides produced in a number of plant species including oat (Crombie et al., 1984), soybean (Yoshiki et al., 1998), and tomato (Friedman, 2002) that are associated with competitive interaction between plants as well as anti-herbivory and defense against fungal pathogens (Mugford and Osbourn, 2012). It is generally recognized that the mode of action for saponin-induced fungal defense is through pore formation when saponins complex with fungal membrane sterols (Osbourn, 1998; Price et al., 1987). In saponin-deficient oat plants produced through mutagenesis *via* sodium azide treatment showed increased susceptibility to infection by *Gauemannomyces graminis* var. *tritici* (Papadopoulou et

al., 1999). To our knowledge, no studies have specifically identified saponins in blueberry, though saponins isolated from shallot, *Allium sepa* L. aggregatum group, have been shown to strongly inhibit the growth of *Colletotrichum* species, including *C. acutatum, C. gloeosporioides* and *C. destructivum* (Teshima et al., 2013).

The evidence from our study points to an increase in the expression of saponin biosynthesis-like genes (Table 3.3). It is possible that these genes are utilized in blueberry fruit toward other ends. Saponin biosynthesis shares similarities with the synthesis of terpenes and other steroidal compounds and we found evidence of at least one other terpenoid biosynthesis-like gene, a probable 1-deoxy-D-xylulose-5-phosphate (DXP) synthase which was up-regulated (|LFC| = 3.60) on day 3. Saponins are generally constitutively expressed *in planta* as opposed to phytoalexins, which are induced in response to infection by fungal pathogens (Osbourn, 1998). We found evidence of two putative homologs of phytoalexin biosynthesis genes, vestitone reductase and a cytochrome P450, 3,9-dihydroxypterocarpan 6A-monoxygenase (CYP93A1), up-regulated in inoculated fruit (Table 3.3). Vestitone reductase catalyzes the second to last step in the production of the phytoalexin medicarpin in alfalfa, *Medicago sativa* L. (Guo et al., 1994), which accumulates in response to elicitation from cell wall preparations of *Colletotrichum lindemuthianum* (Kessmann et al., 1990). 3,9-dihydroxypterocarpan 6A-monoxygenase is involved in the biosynthesis of the phytoalexin glyceollin in soybean, *Glycine max* L., which has been shown to accumulate in response to treatment with a ß-1,3-glucan elicitor from *Phytophthora sojae* produced by acid hydrolysis of purified mycelial cell walls (Schopfer et al., 1998).

Blueberry is known for producing many putative anti-fungal compounds (Miles et al., 2013; Miles et al., 2009). This study provides evidence for the role of both saponins and

phytoalexins in the resistance mechanisms uniquely employed by 'Elliott' fruit in response to inoculation with *C. fioriniae,* though future research into whether blueberry saponins and phytoalexins are not only produced in appreciable amounts but also that these compounds attenuate the growth or virulence of *Colletotrichum* spp. is warranted.

Both plants and fungi incorporate polysaccharides chains into their cell walls as structural components: plant cell walls contain many polysaccharides mostly consisting of ß-1,4-glucans like cellulose and hemicelluloses (glucomannan, xylan and xyloglucan) and lignin, while fungal cell walls contain mostly ß-1,3- and ß-1,6-glucans and chitin (Ruiz-Herrera and Ortiz-Castellanos, 2019). These polysaccharide chains are major targets for both host and pathogen attack during infection, and many fungal polysaccharides are considered PAMPs. Fungi express and excrete glucanases during infection to disrupt the host cell wall, such as the xyloglucan-specific endo-ß-1,4-glucanase 1 (XEG1) gene of *Phytophthora spp.,* the causal organisms of stem and root rots in a number of plant species, and *Peronospora parasitica,* the causal organism of downy mildew in both *Arabidopsis* and soybean (*Glycine max* L.) (Ma et al., 2017). We found evidence for up-regulation of four homologs of the PRR glucanase-inhibitor protein (GIP2) gene from tobacco, *Nicotiana benthamiana,* on days 3 and 4 (Table 3.4). The glucanase-inhibitor protein produces an enzyme that recognizes and inhibits the xyloglucanase activity of XEG1 and contributes to increased resistance to *Phytophthora spp.* (Ma et al., 2017).

We found three homologs (Vcor_1664c1g1, Vcor_2202c9g1, Vcor_7534c67g1) of the ABC transporter G family member 11 (ABCG11) gene from *A. thaliana*, which is required for cutin biosynthesis (Table 3.4). Cutin plays an important role in the interaction between plants and their potential bacterial and fungal pathogen (Aragón et al., 2017). Cutin monomers have been shown to induce the production of cutinase, an enzyme that breaks down cutin polymers, as

well as a lipid induced protein kinase that is necessary for appressorium formation in *Colletotrichum trifolii*, the causal agent of alfalfa anthracnose (Ahmed et al., 2003; Dickman et al., 2003). Loss of cutin and, therefore, increased leaf permeability has been linked to increased resistance to *Botrytis cinerea* in *Arabidopsis* (Bessire et al., 2007). There is also evidence that the perception of cutin monomers may induce a plant defense response (Serrano et al., 2014). While the specific mechanism is unknown, there is evidence that necrotrophic, and not biotrophic, pathogenesis is attenuated as a result of cutin and cutinase-related processes (Chassot et al., 2007). In the case of the ABCG11-homologs we found in blueberry fruit, this would indicate a transition to necrotrophic growth around 3 days post-infection (Table 3.4). Given that *Colletotrichum* species exhibit both biotrophic and necrotrophic lifestyles, it is possible that the DE of cutin-related genes in blueberry fruit reacting to *C. fioriniae* infection is a signal of the transition to necrotrophic growth, though this warrants further research.

Our data shows evidence for active cell wall modification in 'Elliott' fruit in response to inoculation with *C. fioriniae* in the form of up-regulation of three homologs of the alpha-1,3-arabinosyltransferase (XAT3) gene from *O. sativa*. In blueberry fruit cell walls, the main non-cellulosic neutral sugars are xylose and arabinose and while xylans (a polysaccharide primarily composed of xylose subunits) are usually a minor hemicellulose in primary cell walls compared to xyloglucose, it is proposed that xylans are abundant in blueberry fruit cell walls (Vicente et al, 2007). The XAT3 gene modifies xylan through arabinosylation (Scheller and Ulvskov, 2010). Arabinose units within the xylan backbone may cross-link with lignin to strengthen the cell wall (McNeil et al., 1984), therefore it is possible that an increase in arabinosylation of xylan could be a mechanism used by 'Elliott' to defend against *C. fioriniae* invasion. Alkan et al., (2015) detected up-regulation of hemicellulose-related genes during the necrotrophic stage in the *C.*

gloeosporioides-tomato pathosystem as well as an increase in gene ontology terms related to cell wall modification.

Another major component of the plant cell wall is pectin, which is produced in the golgi and modified by pectin methylesterases (PME) before being incorporated into the plant cell wall. We found a PME up-regulated (|LFC| = 3.95) on day 3 in inoculated 'Elliott' fruit (Table 3.4). It has been shown that PMEs are induced in *Arabidopsis* when challenged with both necrotrophic and hemibiotrophic pathogens after 24 to 48 h, and that PMEs contribute to resistance to the hemibiotrophic pathogen *Pseudomonas syringae* (Bethke et al., 2014).

The *A. thaliana* chitin elicitor-binding protein LYM2 (CEBiP), is an example of a PRR-encoding gene which codes for a lysin motif (LysM) domain-containing protein that binds chitin oligosaccharides and triggers an immune response (Shinya et al., 2012). A homolog of LYM2 was up-regulated on day 1 in 'Elliott' fruit in response to *C. fioriniae* inoculation along with 13 other genes. Another LysM domain-containing protein that was up-regulated in 'Elliott' fruit was a homolog of the chitin elicitor receptor kinase 1 (CERK1) gene from rice, *Oryza sativa,* that encodes for a receptor kinase-domain containing protein and is known to interact with CEBiP to signal downstream components of the PAMP-triggered immune (PTI) response (Shimizu et al., 2010). We also found evidence for a homolog of a gene, calmodulin-like protein 36 (CML36), which encodes a protein shown to interact with CERK1 in a yeast two-hybrid system (Le et al., 2014).

In response to fungal pathogen invasion, PTI activates defense genes to mitigate damage and control the invading organism. Components of the fungal cell wall, such as chitin and ß-glucans, appear to be a major target for plant cell defensive response to fungal infection. One family of genes activated during PTI in plant-fungi pathosystems are chitinases which are

released by the plant cell to break down chitin, a major component of the fungal cell wall (Tariq et al., 2018; Casado-Diaz et al., 2006). We found evidence for the activation of four homologs of class V chitinase genes and at least one glucosidase gene (Table 3.4), which break down ß-1,3-glucans, up-regulated in 'Elliott' in response to inoculation by *C. fioriniae*, in addition, many of these genes were computationally predicted by SignalP to contain protein domains that mark them for transport into the apoplast (Petersen et al., 2011). We also found a homolog of a predicted glucan endo-1,3-beta-glucosidase from *Vitis vinifera* L. (VIT_06s0051g00120) up-regulated on day 3, providing more evidence for active regulation of genes that break down fungal cell wall components.

ß-1,3-glucans have been found in high proportion in the appressoria and necrotrophic hyphae of *C. graminicola* and ß-1,3-glucan synthase has been shown to be necessary for pathogenicity (Oliveira-Garcia and Deising, 2013). Transgenic sugar cane, *Saccharam spp.*, that is normally susceptible to red rot caused by *Colletotrichum falcatum* Went. has been shown to develop high levels of resistance when transformed with a class II chitinase from barley, *Hordeum vulgare* (Tariq et al., 2018) or a ß-1,3-glucanase from the fungus *Trichoderma viridae* (Nayyar et al., 2017) demonstrating that these enzymes alone are sufficient for resistance in a unique host-pathogen system, however, downregulation of chitinases and ß-1,3-glucanases have been demonstrated in highly susceptible strawberry cultivars infected by *C. acutatum* (Casado-Diaz et al., 2006) demonstrating that these enzymes are not only sufficient, but may be necessary for high-level resistance.

Plant-pathogen interactions are often referred to as an 'arms race' where both develop defensive and offensive capabilities against the other over the course of their co-evolutionary struggle. In these transgenic systems where a single non-host gene can confer resistance to a

common pathogen, the co-evolutionary 'arms race' is, in a sense, circumvented through the introduction of a novel host defense mechanism where the pathogen has no evolutionary time to develop a counterattack. In the *C. fioriniae-V. corymbosum* interaction, we provide evidence that the expression of glucanases and chitinases alone are not sufficient for complete resistance (Fig. 3.2) in blueberry fruit though they are part of the immune response mechanism that produces the high resistance phenotype of 'Elliott'.

The breakdown of glucans and chitin releases mono- and oligosaccharides, respectively. Lectins are receptor proteins that bind and agglomerate free carbohydrates and are most well-studied in mammalian systems (Brown and Gordon, 2005; Fesel and Zuccaro, 2015). Lectins in plants are less well-studied than those in mammals, however, growing evidence suggests a role in plant defense response through recognition of glycans from non-host organisms (Barre et al., 2001; Lannoo and Van Damme, 2014; Wang et al., 2014).

We found evidence for at least three homologs of mannose-specific lectin proteins up-regulated on day 4 in inoculated 'Elliott' fruit when compared to mock-inoculated fruit (Table 3.5). Additionally, we found evidence for multiple homologs of the *A. thaliana* gene P4H7 which post-translationally modifies proline-rich plant glycoproteins, including lectins, at proline rich sites to produce 4-hydroxyproline in response to hypoxia and mechanical wounding, which are then further modified at the hydroxyproline residues through glycosylation (Vlad et al., 2007). This suggests an additional mechanism for scavenging of exogenous sugars in addition to the inherent receptor activity of lectin proteins. We also found two homologs of the *A. thaliana* beta-fructofuranosidase, insoluble isoenzyme (CWINV1) gene, a secreted cell wall invertase, up-regulated on days 2 and 3. Other cell wall invertase genes have been shown to be responsible for cleavage of apoplastic sucrose into glucose and fructose during *B. cinerea* infection (Veillet et

al., 2016), so it is possible CWINV1 plays a similar role during *C. fioriniae* infection of 'Elliott' fruit. Further studies specifically evaluating the effect of *C. fioriniae* infection on cell wall biochemistry of blueberry fruit could provide valuable targets for targeted breeding approaches at increasing resistance.

Pathogen-triggered immunity and effector-triggered immunity (ETI) are complex processes with many genes functioning downstream of the receptors immediately responsible for binding PAMPs and effectors (Table 3.6). We found evidence for homologs of *A. thaliana* peptide transporters that are necessary for resistance to *P. syringae* (Karim et al., 2007) as well as up-regulation of the PAMP-induced secreted peptide 1 (PIP1) gene from *A. thaliana* that acts as a positive regulator of immune response (Hou et al., 2014). We also found a homolog of E3 ubiquitin-protein ligase (PUB24) from *A. thaliana* that acts as a negative regulator of the immune response to PAMPs (Trujillo et al., 2008) and a gene with homology to a UDP-glycosyltransferase (UGT74F2) that glycosylates benzoic acid derivatives, over-expression of which increases susceptibility to *P. syringae* (Song, 2006).

Some of the genes we found that responded to *C. fioriniae* inoculation have been identified experimentally in other pathosystems upon pathogen infection, though their precise function is unknown. For instance, we identified two homologs of a cysteine-rich repeat (CRR) secretory protein (CRR38) from *A. thaliana* up-regulated in response to *C. fioriniae* infection. Recent research into the CRR superfamily shows that some, CRR56 for example, might be better classified as plasmodesmata-located proteins (PDLPs) and may be involved in cell-to-cell communication during infection to regulated callose deposition (Caillaud et al., 2014) or the induction of systemic acquired resistance, or SAR (Lim et al., 2016). As the study of plant-

pathogen interactions matures in the next few decades, it is possible the function of these genes will be elucidated.

Plants challenged by certain pathogens will sometimes employ what is known as the hypersensitive response (HR) in which the cells immediately surrounding the site of infection undergo programmed cell death (PCD) in order to starve the pathogen of the necessary nutrients. It is characterized by the influx and efflux of ions across the cell membrane, fatty acid metabolism, ROS production, and lignin biosynthesis (Morel and Dangl, 1997). HR can be induced within hours of host-pathogen contact, but in the context of hemibiotrophic pathogens, which undergo a lifestyle transition from biotrophic growth to necrotrophic growth during infection, the signals that induce HR in the host may be delayed until this transition takes place. In 'Elliott' fruit challenged with *C. fioriniae*, we found a number of HR-related genes up-regulated during the latter half of our sampling period (Table 3.7).

We found evidence for a number of genes related to cell membrane and fatty acid metabolism up-regulated on days 3 and 4 in 'Elliott' fruit, including three homologs of the soybean omega-6 fatty acid desaturase 2 (FAD2-2) gene, and two homologs of the delta-12 fatty acid desaturase (FAD2) gene from tung (*Vernicia fordii),* which converts oleic acid into linoleic acid (Dyer et al., 2002). FAD2 genes are known to be involved in endoplasmic reticulum (ER) membrane metabolism, and an *A. thaliana FAD2* deficient mutant produced a HR upon treatment with tunicamycin which is known to induce ER stress (Nguyen et al., 2019). While it is not clear if *Colletotrichum spp.* target the ER as part of their infection strategy, the HopD1 effector protein from *Pseudomonas syringae* pv. *Tomato* DC3000 has been shown to target the ER localized protein NTM1-like 9 (NTL9) to suppress effector-triggered immunity (ETI) in *A.*

thaliana (Block et al., 2013) and there is a body of evidence that suggests a complex interplay between ER stress signaling and plant defense response (Kørner et al., 2015).

We also found homologs of the alpha-dioxygenase 1 (DOX1) gene from *A. thaliana* up-regulated in 'Elliott' fruit. DOX1 is involved in oxygenation of fatty acids to form oxylipins and has been shown to be associated with both compatible and incompatible interactions in bacterial infections (Ponce de León et al., 2002). In interactions where DOX1 is more highly expressed, it is associated with induction of a HR and *dox1* knockout mutants show a reduction in the systemic acquired resistance (SAR) response (Vicente et al., 2012).

Within the complex interaction between pathogen and host, it is beneficial to the pathogen to attempt to induce the expression of genes that will contribute to increased host susceptibility. We found evidence for up-regulation of a patatin-like protein 2 (PLP2) which hydrolyzes galactolipids present in the host cell membrane and has been shown to increase the susceptibility of *A. thaliana* to both necrotrophic fungal pathogens and avirulent bacterial pathogens (La Camera et al., 2005), not Cucumber mosaic virus (La Camera et al., 2009), by reducing the efficiency of the HR and promoting cell death (La Camera et al., 2005). Given that *C. fioriniae* is a fungal pathogen it is likely induction of PLP2-like genes in blueberry fruit is detrimental to resistance, however we did not investigate the biochemical responses commonly associated with HR in this study, so cannot predict how the induction of this gene may have affected the extent of the HR in inoculated fruit.

Reactive oxygen species generation is a common response to pathogens and a key component of the HR (Torres et al., 2006) and many of the genes we found up-regulated in 'Elliott' fruit were ROS-related (Table 3.7). We found evidence for the up-regulation of homologs of aldehyde oxidase (GLOX) genes from chinese wild grapevine (*Vitis*

pseudoreticulata), which produce enzymes responsible for creating hydrogen peroxide (H_2O_2) from aldehyde-containing molecules. Transformation of *V. vinifera* with a GLOX-gene containing plasmid was sufficient to produce resistance to powdery mildew caused by *Erysiphe necator* in a susceptible cultivar (Zhao et al., 2013; Guan et al., 2011). While we did not examine H_2O_2 concentrations in this study, blueberry fruit infected with *C. fioriniae* have been shown to produce an oxidative burst as early as 18-24 hours post infection in both 'Jersey' and 'Elliott' (Miles et al., 2011).

When high levels of oxidative damage occur and intracellular concentrations of ROS increase beyond a certain threshold, PCD is induced (Levine et al., 1994). Induction of PCD is an important step of the HR, however, PCD is a one-way street so host cells may benefit from tight control over PCD when faced with hemibiotrophic pathogens. Many proteins are involved in limiting oxidative stress, including those in the glutathione S-transferase (GST) family (Gullner et al., 2018). A homolog of the *A. thaliana* GSTU7 gene was up-regulated on day 4 in 'Elliott' fruit (Table 3.7). GST-family genes were also shown to be induced in *N. benthamiana* by both *C. destructivum* and *C. orbiculare* infection and expression knock-down through virus-induced gene silencing (VIGS) of NbGSTU1 showed increased susceptibility to *C. orbiculare* (Dean et al., 2005). Genes related to glutathione biosynthesis are necessary for resistance to *C. gloeosporioides* in *Arabidopsis* (Hiruma et al., 2013).

Cysteine proteases are important for the induction of PCD in soybean as a result of oxidative stress. Modulating cysteine protease activity by regulating the expression of cysteine protease inhibitor genes is another mechanism to control plant response to oxidative stress (Solomon et al., 1999). We found two homologs of cysteine proteinase inhibitor 1 from kiwi, *Actinidia deliciosa,* up-regulated concurrently with the majority of the other HR-related genes

(Table 3.7) indicating that resistance in 'Elliott' fruit may be related to tight control of PCD as a result of oxidative stress.

A later step in the HR is the production of lignin, which is closely tied to ROS metabolism and is thought of as a mechanism to wall off the pathogen and delineate dead cells from healthy tissue (Morel and Dangl, 1997). We found evidence for up-regulation of a number of ROS- and lignin-metabolism related genes (Table 3.7). This includes homologs of *A. thaliana* laccase (LAC14) as and peroxidase (PER51) genes and homologs of lignin-forming peroxidases from *Nicotiana sylvestris* (PERX), genes that are responsible for H_2O_2 scavenging and oxidation of phenolic compounds such as lignin, producing oxygen radicals and leading to cross-linking of cell wall components (Almagro et al., 2009), which can produce stronger cell wells and serve as early resistance mechanism to pathogens (Brisson et al., 1994).

Plant stress such as salinity, drought, wounding, and pathogen attack induce the expression of a wide variety of genes and many of these stresses involve hormone signaling to propagate the stress signal through the plant. We found a gene with homology to the brassinosteroid signaling transduction gene BSK1 from *A. thaliana* (Tang et al., 2008), as well as homologs of ethylene-producing 1-aminocyclopropane-1-carboxylate oxidase (ACCO3) genes which are known to be induced following infection of many pathogens including the rice blast fungus *Magnaporthe grisea* (Iwai et al., 2006). RNAi-mediated suppression of ACCO genes has been shown to increase susceptibility of rice, *Oryza sativa,* to *M. oryzae* as well as reduces expression of many defense-related genes (Helliwell et al., 2016). In addition, we found many other genes with homology to known or predicted 'stress related' genes (Table 3.8). Stress response to *C. fioriniae* infection in 'Elliott' fruit likely involves many more genes than those in

this report, though the expression of the genes presented herein represent those unique to 'Elliott' compared to the moderately resistant cultivar 'Draper' and the susceptible cultivar 'Jersey'.

Of the total 113 inoculation-response resistance associated genes we found in 'Elliott' fruit, 34 genes did not have strong enough homology to known genes through functional annotation to assign them to one of the above groups (Table 3.9). Eight of these have predicted transmembrane domains, similar to other membrane-associated proteins such as RLPs, though in some cases that is the only homology we were able to detect. It is possible that some of these genes predicted *in silico* are gene fragments derived from incomplete computational reconstruction by the Trinity pipeline, though they are present in many samples and demonstrate differential expression in inoculated vs. mock-inoculated fruit so more in-depth study may reveal more conclusive information.

In this study, we used RNA-seq followed by a strict filtering protocol to identify over one hundred genes expected to play a part in the resistance phenotype of 'Elliott' fruit in response to infection by *C. fioriniae* over the background of known field-derived infection. We found this host-pathogen interaction to yield a complex multi-day response characterized by DE of genes responsible for host cell wall rearrangement, the secretion of fungal cell wall degrading enzymes, secondary metabolite biosynthesis, reactive oxygen species metabolism, and programmed cell death. We found genes known in other pathosystems to be both positive and negative regulators of resistance. It is difficult to determine which genes are induced by pathogen-derived effectors and which genes are induced by host-derived factors, though it is possible both are at play. In order to better understand how this study fits in with the bigger picture of biochemistry and genetics within blueberry fruit in response to *C. fioriniae,* We have suggested further studies into a number of processes, such as R gene mediated resistance, secondary metabolite production,

cell wall dynamics, and ROS production. Additional genetics studies utilizing these data along with mutagenesis, knockout and/or overexpression studies, could also provide important tools for current and future blueberry breeders toward the goal of developing more resistant varieties for many years to come.

CHAPTER 4: FUTURE DIRECTIONS FOR POSTHARVEST BLUEBERRY RESEARCH

The results from the previous studies demonstrate recent advances in the science of blueberry postharvest physiology as it relates to fumigation and the genetics of anthracnose disease responses in blueberry fruit. With the decreasing costs of sequencing and increasing sophistication of computing algorithms and data processing resources, the pace of our understanding of blueberry genetics and physiology will only continue to accelerate. However, moving forward many challenges will continue to be practical, experimental ones, so looking to other, more mature plant-pathogen systems will give us a roadmap for future research in blueberry.

In the study described in chapter 2, we used a dose-response analysis to assess the effect of SO_2 fumigation on blueberry fruit and the survival of BBM. One consideration not well addressed in this study was the definition of dose. Generally, dose is considered as the product of time and treatment concentration (e.g. ppm·hr), where a 'dose' of 100 ppm SO_2 for 10 hr would be theoretically equivalent to a 'dose' of 10 ppm SO_2 for 100 hr. In our study, we calculated the dose of SO_2 volumetrically by determining the headspace of the treatment containers and injecting a single aliquot of the required SO_2 to achieve a desired headspace concentration. The concentration was verified using an external SO_2 sensor, however it was not continuously monitored throughout the duration of treatment. It is possible that the SO_2 was rapidly taken up by the fruit as SO_2 dissolves rapidly in water, and in that case may have been metabolized by the fruit into sulfur-containing compounds. The rate at which fruit uptakes SO_2 from the atmosphere may be cultivar dependent and, therefore, the headspace concentration may have been different for each cultivar. To address this, future studies continual monitoring and maintaining a target SO_2 concentration are warranted to determine if 'dose' is truly equivalent across a range of

concentration x time treatments. Pads that slowly release SO_2 during storage are used in table grape to maintain a target concentration for the control of grey mold caused by *B. cinerea* (Lichter et al., 2008). It is possible that these studies may find that lower treatment concentrations for longer durations may control BBM more effectively than the high concentration, short duration treatments we performed while achieving lower fruit damage.

In the study described in chapter 3, we utilized field-collected fruit that had a presence of background infection (Figure 3.1). One challenge this presents is a dilution of the signal-to-noise ratio in our RNAseq data. The presence of potential prior infections may prime the plant to express a systemically acquired resistance (SAR) response that can increase the HR and/or attenuate pathogen growth through the production of secondary metabolites and produce long-term resistance in distal tissues (Durrant and Dong, 2004). In order to eliminate the potential for this effect to confound the true disease response caused by a targeted inoculation, it would be necessary to grow a large number of plants in a greenhouse or growth chamber free from *C. fioriniae* spores. This is a practical approach for many plants, but could be difficult in the short term for blueberry research due to the large number of fruit needed for a good sample size (ca. 50 fruit) and the 2-3 year delay from planting/propagation to the time when blueberry bush fruit set achieves significant production. However, with good phytosanitary practices, a *Colletotrichum*-free plot could provide decades of valuable research once established.

C. fioriniae can infect all the above ground tissues of a blueberry plant. One of the most important goals of understanding disease resistance mechanisms in fruit is to develop molecular and genetic markers for resistance that can be deployed in a breeding program to allow breeders high-throughput screening of progeny for resistance before they produce fruit. Attempts to use a leaf-disc assay for resistance showed that foliar resistance to *C. fiorinae* is not well correlated

with fruit rot resistance (Ehlenfeldt et al., 2006). However, the same genomic DNA is present in both tissues, so molecular markers are a promising approach to speed up the screen process. Gichuru et al. (2008) used 31 amplified fragment length polymorphism (AFLP) and 57 microsatellite markers to screen for resistance to *C. kahawae* (the causal organism of coffee berry disease (CBD) in *Coffea arabica*) in two F_2 populations of a cross between a resistant and susceptible cultivar and mapped the first resistance locus (*Ck-1*) for CBD in *C. arabica*. In the future, it would be interesting to develop populations in this way and screen the progeny for some or all of the 113 genes identified from 'Elliott' fruit in our study. This screening along with inoculations of the leaves, stems, or fruit of the progeny in these populations has the potential to reveal a robust marker for resistance. It is also possible a resistance gene was filtered out, so a broader genome-wide association study (GWAS) may be necessary to narrow down the genomic regions of interest as has been performed for phenology related traits (Nagasaka et al., 2022).

LITERATURE CITED

LITERATURE CITED

Alkan, N., Friedlander, G., Ment, D., Prusky, D., and Fluhr, R. 2015. Simultaneous transcriptome analysis of Colletotrichum gloeosporioides and tomato fruit pathosystem reveals novel fungal pathogenicity and fruit defense strategies. New Phytol. 801–815.

Almagro, L., Gómez Ros, L., Belchi-Navarro, S., Bru, R., Ros Barceló, A., and Pedreño, M. 2009. Class III peroxidases in plant defence reactions. J. Exp. Bot. 60: 377–390.

Alsmairat, N., Contreras, C., Hancock, J., Callow, P., and Beaudry, R., 2011. Use of combinations of commercially-relevant O_2 and CO_2 partial pressures to evaluate the sensitivity of nine highbush blueberry fruit cultivars to controlled atmospheres. HortScience. 46, 74-79.

Anciro, A., Mangandi, J., Verma, S., Peres, N., Whitaker, V.M., and Lee, S. 2018. FaRCg1: a quantitative trait locus conferring resistance to Colletotrichum crown rot caused by Colletotrichum gloeosporioides in octoploid strawberry. Theor. Appl. Genet. doi: 10.1007/s00122-018-3145-z.

Anders, N., Wilkinson, M.D., Lovegrove, A., Freeman, J., Tryfona, T., Pellny, T.K., Weimar, T., Mortimer, J.C., Stott, K., and Baker JM 2012. Glycosyl transferases in family 61 mediate arabinofuranosyl transfer onto xylan in grasses. PNAS. 109: 989–993.

Andrews, S. 2010. FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

Angeletti, P., Castagnasso, H., Miceli, E., Terminiello, L. 2010. Effect of preharvest calcium applications on postharvest quality , softening and cell wall degradation of two blueberry (Vaccinium corymbosum) varieties. Postharvest Biol. Technol. 58: 98–103.

Anon., 2019. D-02-04: The Blueberry Certification Program and domestic phytosanitary requirements to prevent the spread of blueberry maggot (*Rhagoletis mendax*) within Canada. http://www.inspection.gc.ca/plants/plant-pests-invasive-species/directives/horticulture/d-02-04/eng/1320046578973/1320046655958. (accessed 12 Jan, 2021).

Aragón, W., JJ Reina-Pinto, and M. Serrano. 2017. The intimate talk between plants and microorganisms at the leaf surface. J. Exp. Bot. 68(19)5339-5350.

Armstrong, J.W., Couey, M.H., 1984. Methyl bromide fumigation treatments at 30 C for California stone fruits infested with the Mediterranean fruit fly (Diptera: Tephritidae). J. Econ. Entomol. 77, 1229-1232.

Armstrong, J.W., Hu, B.K.S., Brown, S.A., 1995a. Single-temperature forced hot-air quarantine treatment to control fruit flies (Diptera: Tephritidae) in papaya. J. Econ. Entomol. 88, 678-682.

Armstrong, J.W., Silva, S.T., Shishido, V.M., 1995b. Quarantine cold treatment for Hawaiian carambola fruit infested with Mediterranean fruit fly, melon fly, or oriental fruit fly (Diptera: Tephritidae) eggs and larvae. J. Econ. Entomol. 88, 683-687.

Baroncelli R, Sreenivasaprasad S, Sukno S a, Thon MR, Holub E. 2014. Draft genome sequence of *Colletotrichum acutatum sensu lato* (*Colletotrichum fioriniae*). Genome Announc. 2:2.

Barre A, Bourne Y, Van Damme EJM, Peumans WJ, Rougé P. 2001. Mannose-binding plant lectins: Different structural scaffolds for a common sugar-recognition process. Biochimie 83: 645–651.

Beaudry, R., 1992. Blueberry quality characteristics and how they can be optimized. Annu. Rpt. of the Michigan State Hort. Soc. (122nd). 140-145.

Beaudry, R.M., A.C. Cameron, A. Shirazi and D.L. Dostal-Lange. 1992. Modified atmosphere packaging of blueberry fruit: Effect of temperature on package oxygen and carbon dioxide. J. Amer. Soc. Hortic. Sci. 117:436-441.

Bell, J., 1980. Response of plants to sulphur dioxide. Nature. 284:399-400.

Bertrand B, Anthony F, Lashermes P, Rica IC. 2001. Breeding for resistance to *Meloidogyne exigua* in *Coffea arabica* by introgression of resistance genes of *Coffea canephora*. Plant Pathol. 50: 637–643.

Bessire M, Chassot C, Jacquat AC, Humphry M, Borel S, Petétot JM, Métraux JP, Nawrath C. 2007. A permeable cuticle in *Arabidopsis* leads to a strong resistance to *Botrytis cinerea*. The EMBO Journal 26, 2158–2168.

Bethke G, Grundman RE, Sreekanta S, Truman W, Katagiri F, Glazebrook J. 2014. Arabidopsis PECTIN METHYLESTERASEs contribute to immunity against *Pseudomonas syringae*. Plant Physiol 164: 1093–1107.

Bian Y, Ballington J, Raja A, Brouwer C, Reid R, Burke M, Wang X, Rowland LJ, Bassil N, Brown A. 2014. Patterns of simple sequence repeats in cultivated blueberries (*Vaccinium* section of *Cyanococcus* spp.) and their use in revealing genetic diversity and population structure. Mol. Breed. 34(2):675-689.

Block A, Toru TY, Elowsky CG, Zhang C, Steinbrenner J, Beynon J, Alfano JR. 2014. The *Pseudomonas syringae* type III effector HopD1 suppresses effector-triggered immunity, localizes to the endoplasmic reticulum, and targets the Arabidopsis transcription factor NTL9. New Phytol. 201: 1358–1370.

Bolger AM, Lohse M, and Usadel B. 2014. Trimmomatic: A flexible trimmer for Illumina Sequence Data. Bioinformatics, btu170.

Brennan, E., Leone, I.A., Daines, R.H., 1964. Investigation of SO_2 effects on rubber trees as a means of forestalling injury to Malayan plantations from refinery emissions. J. Air Pollut. Control Assoc. 14, 229-233.

Brennan, E., Leone, I.A., Daines, R.H., 1970. Toxicity of sulfur dioxide to highbush blueberry. Plant Dis. Rpt. 54, 704-706.

Brisson LF, Tenhaken R, Lamb C, Brisson LF, Tenhaken R, Lamb C. 1994. Function of Oxidative Cross-Linking of Cell Wall Structural Proteins in Plant Disease Resistance. Plant Cell 6: 1703–1712.

Brown GD, Gordon S. 2005. Immune recognition of fungal ß-glucans. Cell Microbiol. 7: 471–479.

Bryant DM, Johnson K, DiTommaso T, Tickle T, Couger MB, Payzin-Dogru D, Lee TJ, Leigh ND, Kuo TH, Davis FG, Bateman J, Bryant S, Guzikowski AR, Tsai SL, Coyne S, Ye WW, Freeman RM Jr, Peshkin L, Tabin CJ, Regev A, Haas BJ, Whited JL. 2017. A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. Cell Rep. 18(3):762-776.

Burg, S.P., 2004. Postharvest Physiology and Hypobaric Storage of Fresh Produce. CABI Publishing. Cambridge, MA, USA.

Bustos, M.E., Enkerlin, W., Reyes, J., Toledo J., 2004. Irradiation of mangoes as a postharvest quarantine treatment for fruit flies (Diptera: Tephritidae). J. Econ. Entomol. 97, 286-292.

Caillaud M, Wirthmueller L, Sklenar J, Findlay K, Piquerez SJ. 2014. The plasmodesmal protein PDLP1 localises to haustoria-associated membranes during downy mildew infection and regulates callose deposition. PLOS Pathog. 10: 1–13.

Camacho C., Coulouris G., Avagyan V., Ma N., Papadopoulos J., Bealer K., TL Madden T.L. 2008. BLAST+: architecture and applications. BMC Bioinformatics 10:421.

Cantín, C.M., Minas, I.S., Goulas, V., Jiménez, M., Manganaris, G.A., Michailides, T.J., Crisosto, C.H., 2012. Sulfur dioxide fumigation alone or in combination with CO_2-enriched atmosphere extends the market life of highbush blueberry fruit. Postharvest Biol. Technol. 67, 84-91.

Casado-Diaz A, Encinas-Villarejo S, de los Santos B, Schiliro E, Yubero-Serrano E-M, Amil-Ruiz F, Pocovi MI, Pliego-Alfaro F, Dorado G, Rey M. 2006. Analysis of strawberry genes differentially expressed in response to Colletotrichum infection. Physiol. Plant 128: 633–650.

Chassot, C., Nawrath, C., and Metraux, J. 2007. Cuticular defects lead to full immunity to a major plant pathogen. *Plant J.* 49, 972–980. doi: 10.1111/j.1365-313X.2006.03017.x

Chen, H., Cao, S., Fang, X., Mu, H., Yang, H., Wang, X., Xu, Q., Gao, H., 2015. Changes in fruit firmness, cell wall composition and cell wall degrading enzymes in postharvest blueberries during storage. Sci. Hort. 188, 44-48.

Choi DS, Hwang IS, Hwang BK. 2012. Requirement of the Cytosolic Interaction between REPEAT PROTEIN1 for Cell Death and Defense Signaling in Pepper. Plant Cell. 24: 1675–1690.

Colle M, CP Leisner, CM Wai, Shujun Ou, Kevin A Bird, Jie Wang, Jennifer H Wisecaver, Alan E Yocca, Elizabeth I Alger, Haibao Tang, Zhiyong Xiong, Pete Callow, Gil Ben-Zvi, Avital Brodt, Kobi Baruch, Thomas Swale, Lily Shiue, Guo-qing Song, Kevin L Childs, Anthony Schilmiller, Nicholi Vorsa, C Robin Buell, Robert VanBuren, Ning Jiang, and Edger, P.P. 2019. Haplotype-phased genome and evolution of phytonutrient pathways of tetraploid blueberry, *GigaScience*, 8(3)giz012, doi: 10.1093/gigascience/giz012.

Considine, M.J., Foyer, C.H., 2015. Metabolic responses to sulfur dioxide in grapevine (*Vitis vinifera* L.): photosynthetic tissues and berries. Front. Plant Sci. 6, 1–10.

Crombie L., W. Crombie & D. Whiting, 1984. Structures of the four avenacins, oat root resistance factors to 'take-all' disease. Chemical Communications, 246-248.

Damm U, Cannon PF, Woudenberg JHC, Crous PW. 2012. The *Colletotrichum acutatum* species complex. Stud Mycol 73: 37–113.

Davenport, T.L., Burg, S.P., White, T.L., 2006. Optimal low-pressure conditions for long-term storage of fresh commodities kill Caribbean fruit fly eggs and larvae. HortTechnology. 16, 98-104.

Davis, R., 2003. Fumigants, in Trugo, L., Finglas P.M. (Eds). Encycl. Food Sci. Nutr., 2nd ed. Academic Press, Cambridge, pp. 2821-2826.

de León IP, A S, Hamberg M, Castresana C. 2002. Involvement of the Arabidopsis a-DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. Plant J. 29: 61–72.

De Lima, C., Jessup, A., Mansfield, E., Daniels, D., 2011. Cold treatment of table grapes infested with Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) and Queensland fruit fly *Bactrocera tryoni* (Froggatt) Diptera: Tephritidae. N. Z. J. Crop Hort. Sci. 39, 95–105.

Dean JD, Goodwin PH, Hsiang T. 2005. Induction of glutathione S-transferase genes of Nicotiana benthamiana following infection by Colletotrichum destructivum and C. orbiculare and involvement of one in resistance. J. Exp. Bot. 56: 1525–1533.

Denoyes-Rothan B, Guérin G, Lerceteau-köhler E, Risser G, Cedex M. 2005. Inheritance of Resistance to Colletotrichum acutatum in Fragaria × ananassa. Phytopathology 95: 405–412.

DeVetter LW, Yang W, Takeda F, Korthuis S, Li C. 2019. Modified Over-the-Row Machine Harvesters to Improve Northern Highbush Blueberry Fresh Fruit Quality. Agriculture. doi: 10.3390/agriculture9010013.

Dickman M, Ha Y, Yang Z, Adams B, Huang C. 2003. A Protein Kinase from Colletotrichum trifolii Is Induced by Plant Cutin and Is Required for Appressorium Formation. Mol. Plant-Microbe Interact. 16:411–421.

Die JV, Rowland LJ. 2013. Advent of genomics in Blueberry. Mol. Breeding 32:493-504.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29: 15–21.

Dyer JM, Chapital DC, Kuan JW, Mullen RT, Turner C, Mckeon TA, Pepperman AB. 2002. Molecular Analysis of a Bifunctional Fatty Acid Conjugase/Desaturase from Tung. Implications for the Evolution of Plant Fatty Acid Diversity 1. Plant Physiol. 130: 2027–2038.

Eckert, J.W., Ogawa, J.M., 1985. The Chemical Control of Postharvest Diseases: Subtropical and Tropical Fruits. Ann. Rev. Phytopathol. 23, 421-454.

Eddy SR. 2011. Accelerated Profile HMM Searches. PLOS Comput. Biol. 7(10): e1002195.

Ehlenfeldt MK, Polashock JJ, Stretch AW, Kramer M (2006) Leaf disk infection by *Colletotrichum acutatum* and its relation to fruit rot in diverse blueberry germplasm. HortScience. 41:270-271.

Ehlenfeldt, M.K., Martin, R.B. 2002. A survey of fruit firmness in highbush blueberry and species-introgressed blueberry cultivars. HortScience. 37, 386-389.

EPA, 2015. Methyl Bromide Quarantine Fumigant, EPA Reg. No. 8536-29.

Fernández-Trujillo, J.P. Martínez, J.A., 2006. Ultrastructure of the onset of chilling injury in cucumber fruit. J. Appl. Bot. Food Qual. 80, 100-110.

Fesel PH, Zuccaro A. 2016. ß-glucan: Crucial component of the fungal cell wall and elusive MAMP in plants. Fungal. Genet. Biol. 90: 53–60.

Forcelini BB, Seijo TE, Amiri A. 2003. Resistance in Strawberry Isolates of *Colletotrichum acutatum* from Florida to Quinone-Outside Inhibitor Fungicides. Plant Dis. 100: 2050–2056.

Forney, C.F., Kalt, W., Jordan, M.A., Vinqvist-Tymchuk, M.R., Fillmore, S.A.E., 2012. Blueberry and cranberry fruit composition during development. J. Berry Res. 2, 169-177.

Friedman M. 2002. Tomato Glycoalkaloids: Role in the Plant and in the Diet. J. Agr. Food Chem. 50: 5751–5780.

Fukushima EO, Seki H, Ohyama K, Ono E, Umemoto N, Mizutani M, Saito K, Muranaka T. 2011. CYP716A Subfamily Members are Multifunctional Oxidases in Triterpenoid Biosynthesis. Plant Cell Physiol. 52: 2050–2061.

Gallardo K, Zhang Q, Dossett M, Polashock J, Rodriguez-Saona C, Vorsa N, Edger P, Ashrafi H, Babiker E, Finn C. 2018. Breeding Trait Priorities of the Blueberry Industry in the United States and Canada. HortScience. 53: 1021–1028.

Gao, H., Hu, X., Zhang, H., Wang, S., Liu, L., 2003. Study on sensitivity of table grapes to SO_2. Acta Hort. 628, 541–548.

García-Pineda E, Castro-Mercado E, Lozoya-Gloria E. 2004. Gene expression and enzyme activity of pepper (Capsicum annuum L.) ascorbate oxidase during elicitor and wounding stress. Plant Sci. 166: 237–243.

Gasic K, Hernandez A, Korban SS. 2004. RNA Extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA. Plant Mol. Biol. Report. 22: 437a–437g.

Geffroy V, Sévignac M, Billant P, Dron M, Langin T. 2008. Resistance to Colletotrichum lindemuthianum in Phaseolus vulgaris: a case study for mapping two independent genes. Theor. Appl. Genet. 116:407–415.

Giraud, E., Ivanova, A., Gordon, C.S., Whelan, J., Considine, M.J., 2011. Sulphur dioxide evokes a large scale reprogramming of the grape berry transcriptome associated with oxidative signaling and biotic defense response. Plant Cell Environ. 35, 405-417.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A. 2011. Full-length transcriptome assembly from RNA-seq data without a reference genome. Nat. Biotechnol. 29(7):644-52.

Gross K.C., Wang C.Y, and M. Saltveit, eds. 2016. The Commercial Storage of Fruits, Vegetables, and Florist and Nursery Stocks. Agriculture Handbook Number 66.

Guan X, Zhao H, Xu Y, Wang Y. 2011. Transient expression of glyoxal oxidase from the Chinese wild grape *Vitis pseudoreticulata* can suppress powdery mildew in a susceptible genotype. Protoplasma. 248: 415–423.

Gullner G, Komives T, Király L, Schröder P. 2018. Glutathione S-Transferase Enzymes in Plant-Pathogen Interactions. Front. Plant Sci. 9: 1–19.

Guo L, Dixon RA, Paival NL. 1994. Conversion of Vestitone to Medicarpin in Alfalfa (*Medicago sativa* L.) Is Catalyzed by Two Independent Enzymes. J. Biol. Chem. 269: 22372–22378.

Hallman, G.J., Martinez, L.R., 2001. Ionizing irradiation quarantine treatment against Mexican fruit fly (Diptera: Tephritidae) in citrus fruits. Postharvest Biol. Technol. 23, 71-77.

Hamberg M, Sanz A, Castresana C. 1999. α-oxidation of fatty acids in higher plants: identification of a pathogen-inducible oxygenase (piox) as an α-dioxygenase and biosynthesis of 2-hydroperoxylinolenic acid. J. Biol. Chem. 274: 24503–24513.

Han J-H, Chon J-K, Ahn J-H, Choi I-Y, Lee Y-H, Kim KS. 2016. Whole genome sequence and genome annotation of *Colletotrichum acutatum*, causal agent of anthracnose in pepper plants in South Korea. Genomics Data. 8: 45–46.

Hancock, J. 2004. Blueberry plant denominated 'Draper'. US. Patent No. USPP15103P3.

Hänsch, R., Lang, C., Riebeseel, E., Lindigkeit, R., Gessler, A., Rennenberg, H., Mendel, R.R., 2006. Plant sulfite oxidase as a novel producer of H_2O_2: combination of enzyme catalysis with a subsequent non-enzymatic reaction step. J. Biol. Chem. 281, 6884-6888.

Helliwell EE, Wang Q, Yang Y. 2016. Ethylene Biosynthesis and Signaling Is Required for Rice Immune Response and Basal Resistance Against *Magnaporthe oryzae* Infection. Mol. Plant-Microbe Interact. 29: 831–84.

Hiruma K, Fukunaga S, Pi M. 2013. Glutathione and tryptophan metabolism are required for Arabidopsis immunity during the hypersensitive response to hemibiotrophs. PNAS. 1: 2–7.

Hollomon DK. 2015. Fungicide Resistance: Facing the Challenge. Plant Protection Science 51(4):170-176.

Hou H, Atlihan N, Lu Z-X. 2014. New biotechnology enhances the application of cisgenesis in plant breeding. Front. Plant Sci. 5: 1–5.

Hu M, Grabke A, Dowling ME, Holstein HJ, Schnabel G. 2015. Resistance in Colletotrichum siamense From Peach and Blueberry to Thiophanate-Methyl and Azoxystrobin. Plant Dis. 99: 806–814.

Iwai T, Miyasaka A, Seo S, Ohashi Y. 2006. Contribution of Ethylene Biosynthesis for Resistance to Blast Fungus Infection in Young Rice Plants. Plant Physiol. 142: 1202–1215

Jacob, H. 1929. The Use of Sulfur Dioxide in Shipping Grapes. Univ. California Agr. Expt. Sta. Bul.

Jessup, A., Sloggeti, R., Quinn, N., 1998. Quarantine disinfestation of blueberries against *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) by cold storage. J. Econ. Entomol. 91, 964-967.

Jones J, Dangl J. 2006. The plant immune system. Nature 444: 323–329.

Jung EH, Won H, Chul S, Wook S. 2004. Identification of a novel pathogen-induced gene encoding a leucine-rich repeat protein expressed in phloem cells of *Capsicum annuum*. Biochem. Biophys. Acta 1676: 211–222.

Jung H, Hwang B. 2007. The leucine-rich repeat (LRR) protein, CaLRR1, interacts with the hypersensitive induced reaction (HIR) protein, CaHIR1, and suppresses cell death induced by the CaHIR1 protein. Mol. Plant Pathol. 8: 503–514.

Karim S, Abul KH, Dahl P, Hohmann S, Brader G, Palva ET, Pirhonen M. 2007. AtPTR3, a wound-induced peptide transporter needed for defence against virulent bacterial pathogens in Arabidopsis. Planta. 225: 1431–1445.

Kessmann H, Edwards R, Geno PW, Dixon RA. 1990. Stress Responses in Alfalfa (Medicago sativa L.) V. Constitutive and elicitor-induced accumulation of isofavonoid conjugates in cell suspension cultures. Plant Physiol. 94: 227–232.

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14: 1–13.

Kim SH, Yoon JB, Do JW, Park HG. 2007. Resistance to Anthracnose Caused by Colletotrichum acutatum in Chili Pepper (Capsicum annuum L .). J Crop Sci. Biotech. 10: 277–280.

Kim YS, Ko MK, Oh B. 2001. A cytochrome P450 gene highly expressed in the incompatible interaction. European Patent Application No. EP20000301010.

Kørner C, Du X, Vollmer M, Pajerowska-Mukhtar K. 2015. Endoplasmic Reticulum Stress Signaling in Plant Immunity — At the Crossroad of Life and Death. Int. J. Mol. Sci. 16: 26582–26598.

Krogh A, B Larsson, G von Heijne, ELL Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J. Mol. Biol. 305(3):567-580.

Kruijt M, Brandwagt BF, De Wit PJGM. 2004. Rearrangements in the Cf-9 disease resistance gene cluster of wild tomato have resulted in three genes that mediate Avr9 responsiveness. Genetics. 168: 1655–1663.

La Camera S, Balagué C, Göbel C, Geoffroy P, Legrand M, Feussner I, Roby D, Heitz T. 2009. The Arabidopsis Patatin-Like Protein 2 (PLP2) Plays an Essential Role in Cell Death Execution and Differentially Affects Biosynthesis of Oxylipins and Resistance to Pathogens. Mol. Plant-Microbe Interact. 22: 469–481.

La Camera S, Geoffroy P, Samaha H, Ndiaye A, Rahim G, Legrand M, Heitz T. 2005. A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in Arabidopsis. Plant J. 810–825.

Langfelder P, Horvath S. 2008. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. doi: 10.1186/1471-2105-9-559.

Langmead B, Salzberg S. 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods. 9:357-359.

Lannoo N and EJM Van Damme. 2014. Lectin domains at the frontiers of plant defense. Front. Plant Sci. 5: 1–16.

Le MH, Cao Y, Zhang X, Stacey G. 2014. LIK1, A CERK1-Interacting Kinase, Regulates Plant Immune Responses in Arabidopsis. PLoS One. 9: 1–10.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell. 79, 583–593.

Li, B., Dewey, C.N. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 12(323). doi:10.1186/1471-2105-12-323.

Li J, Liu S, Wang J, Li J, Liu D, Li J, Gao W. 2016. Fungal elicitors enhance ginsenosides biosynthesis, expression of functional genes as well as signal molecules accumulation in adventitious roots of Panax ginseng C. A. Mey. J. Biotechnol. 239: 106–114.

Li X, Sun H, Pei J, Dong Y, Wang F, Chen H, Sun Y, Wang N, Li H, Li Y. 2012. De novo sequencing and comparative analysis of the blueberry transcriptome to discover putative genes related to antioxidants. Gene. 511:54-61.

Liburd, O.E., Alm, S.R., and Casagrande, R.A. 2006. Susceptibility of highbush blueberry cultivars to larval infestation by *Rhagoletis mendax* (Diptera: Tephritidae). Environ. Ent. 27, 817-821.

Lichter, A., Zutahy, Y., Kaplunov, T., Lurie, S., 2008. Evaluation of table grape storage in boxes with sulfur dioxide-releasing pads with either an internal plastic liner or external wrap. HortTechnology. 18, 206-214.

Lim G, Shine MB, Lorenzo L De, Lee J, Lim G, Shine MB, Lorenzo L De, Yu K, Cui W, Navarre D. 2016. Plasmodesmata Localizing Proteins Regulate Transport and Signaling during Systemic Acquired Immunity in Plants. Cell Host Microbe. 19: 541–549.

Longstroth M, Hanson E. 2012. The Michigan blueberry industry. MSU Extension Online. 1-5.

Lorenzini, G., Panattoni, A., Guidi, L., Schenone, G., 1992. On the effects of exposure to realistic sulfur dioxide levels on six host/pathogen combinations. J. Environ. Sci. Health Part A. 27, 1863-1873.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. (15)550. doi: 10.1186/s13059-014-0550-8.

Lozano R, Hamblin MT, Prochnik S, Jannink J. 2015. Identification and distribution of the NBS-LRR gene family in the Cassava genome. BMC Genomics. 16: 1–14.

Luhua S, Ciftci-Yilmaz S, Harper J, Cushman J, Mittler R. 2008. Enhanced tolerance to oxidative stress in transgenic Arabidopsis plants expressing proteins of unknown function. Plant Physiol. 148: 280–292.

Luo, M.R., 2016. CIE L*a*b*. Encyclopedia of Color Science and Technology, Springer, New York.

Ma Z, Zhu L, Song T, Wang Y, Zhang Q, Xia Y, Qiu M, Lin Y, Li H, Kong L. 2017. A paralogous decoy protects *Phytophthora sojae* apoplastic effector PsXEG1 from a host inhibitor. Science. (80)355: 710–714.

Macnish, A.J., M.S. Padda, F. Pupin, P.I. Tsouvaltzis, A.I. Deltsidis, C.A. Sims, J.K. Brecht, and E.J. Mitcham. 2012. Comparison of pallet cover systems to maintain strawberry fruit quality during transport. HortTechnology. 22:493-501.

Mahasuk, P., Taylor, P.W.J., and Mongkolporn, O. 2009. Identification of two new genes conferring resistance to *Colletotrichum acutatum* in *Capsicum baccatum*. Phytopathology. 99:1100-1104.

Mayfield, E.N., Norman, C.S., 2012. Moving away from methyl bromide: Political economy of pesticide transition for California strawberries since 2004. J. Environ. Mgt. 106, 93-101.

McNeil M, Darvill A, Fry S, Albersheim P. 1984. Structure and function of the primary cell walls of plants. Annu. Rev. Biochem. 53: 625–663.

Michelmore R. 2000. Genomic approaches to plant disease resistance. Curr. Opin. in Plant Biol. 3:125-131.

Miles TD, Day B, Schilder AC. 2011. Identification of differentially expressed genes in a resistant versus a susceptible blueberry cultivar after infection by *Colletotrichum acutatum*. Mol. Plant. Path. 12(5):463-477.

Miles TD, Schilder AC. 2008. Michigan Blueberry Facts: Anthracnose Fruit Rot (Ripe Rot). Michigan State University Extension Bulletin E-3039.

Miles TD, Vandervoort C, Nair MG, Schilder AC. 2013. Characterization and biological activity of flavonoids from ripe fruit of an anthracnose-resistant blueberry cultivar. Physiol. Mol. Plant Pathol. 83: 8–16.

Miles TD, Wharton PS, Schilder AC. 2009. Cytological and chemical evidence for an active resistance response to infection by *Colletotrichum acutatum* in 'Elliott' blueberries. Acta Hort. 810: 361–368.

Milholland RD. 1995. Anthracnose fruit rot (ripe rot). In: Compendium of Blueberry and Cranberry Diseases, p. 17. Eds. F. L. Caruso and D. C. Ramsdell. APS Press, The American Phytopathological Society, St. Paul, MN, USA.

Mongi R, Mwalyego F, Elanga A, Kiwovele B. 2009. Introgressing genes conferring resistance to angular leaf spot and anthracnose in common bean landraces southern highlands of Tanzania. African Crop Sci. Conf. Proc. pp 517–520.

Montesinos-Herrero, C., Smilanick, J.L., Tebbets, J.S., Walse, S., Palou, L., 2011. Control of citrus postharvest decay by ammonia gas fumigation and its influence on the efficacy of the fungicide Imazalil. Postharvest Biol. Technol. 59, 85-93.

Morel JB, Dangl JL. 1997. The hypersensitive response and the induction of cell death in plants. Cell Death Differ. 4: 671–83.

Mugford S.T., Osbourn A. 2012. Saponin Synthesis and Function. In: Bach T., Rohmer M. (eds) Isoprenoid Synthesis in Plants and Microorganisms. Springer, New York, NY.

Nagasaka K, Nishiyama S, Fujikawa M, Yamane H, Rowland LJ. 2022. Genome-Wide Identification of Loci Associated With Phenology-Related Traits and Their Adaptive Variations in a Highbush Blueberry Collection. Front. Plant Sci. **12**: 1–18.

Narusaka Y, Narusaka M, Park P, Kubo Y, Hirayama T, Seki M, Shiraishi T, Ishida J, Nakashima M, Enju A. 2004. RCH1, a locus in Arabidopsis that confers resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*. Mol. Plant Microbe Interact. 17: 749–762.

Nayyar S, Sharma BK, Kaur A, Kalia A, Sanghera S, Thind KS, Yadav IS, Sandhu JS. 2017. Red rot resistant transgenic sugarcane developed through expression of β-1,3-glucanase gene. PLoS One 12: 1–16.

Nelson, K.E., Tomlinson, F.E., 1958. Some factors influencing bleaching and wetness of Emperor and Tokay grapes. Proc. Amer. Soc. for Hort. Sci. 71, 190-198.

Nepusz T, Yu H, Paccanaro A. 2012. Detecting overlapping protein complexes from protein-protein interaction networks. Nature Methods. doi:10.1038/nmeth.1938.

Nguyen VC, Nakamura Y, Kanehara K. 2019. Membrane lipid polyunsaturation mediated by FATTY ACID DESATURASE 2 (FAD2) is involved in endoplasmic reticulum stress tolerance in Arabidopsis thaliana. Plant J. 99: 478–493.

O'Connell RJ, Thon MR, Hacquard S, Amyotte SG, Kleemann J, Torres MF, Damm U, Buiate EA, Epstein L, Alkan N. 2012. Lifestyle transitions in plant pathogenic Colletotrichum fungi deciphered by genome and transcriptome analyses. Nat. Genet. 44: 1060–1065.

Oliveira-Garcia E, Deising HB. 2013. Infection structure–specific expression of b-1,3-glucan synthase is essential for pathogenicity of Colletotrichum graminicola and evasion of b-glucan–triggered immunity in maize. Plant Cell. 25: 2356–2378.

Olmstead JW and CE Finn. 2014. Breeding highbush blueberry cultivars adapted to machine harvest for the fresh market. HortTechnology. 24(3):290-294.

Osbourn AE. 1996. Preformed Antimicrobial Compounds and Plant Defense against Fungal Attack. Plant Cell. 8: 1821–1831.

Padder BA, Kamfwa K, Awale HE, Kelly JD. 2016. Transcriptome Profiling of the Phaseolus vulgaris - Colletotrichum lindemuthianum Pathosystem. PLoS ONE. 11(11): e0165823. doi:10.1371/journal.pone.0165823.

Pakdeevaraporn, P., Wasee, S., Taylor, P.W.J., and Mongkolporn, O. 2005. Inheritance of resistance to anthracnose caused by *Colletotrichum capsici* in *Capsicum*. Plant Breeding. 124:206-208.

Palou, L., Crisosto, C.H., Garner, D., Basinal, L.M., Smilanick, J.L., Zoffoli, J.P., 2002. Minimum constant sulphur dioxide emission rates to control gray mold of cold stored table grapes. Amer. J. Enol. Viticult. 53, 110-115.

Paniagua, A.C., East, A.R., Hindmarsh, J.P., Heyes, J.A., 2013. Moisture loss is the major cause of firmness change during postharvest storage of blueberry. Postharvest Biol. Technol. 79, 13-19.

Papadopoulou K, Melton RE, Leggett M, Daniels MJ, Osbourn AE. 1999. Compromised disease resistance in saponin-deficient plants. Proc. Natl. Acad. Sci. 96: 12923–12928.

Pentzer, W.T., Asbury, C.E., Hamner, K.C., 1938. Effects of fumigation of different varieties of *Vinifera* grapes with sulphur dioxide gas. Proc. Amer. Soc. for Hort. Sci. 29, 339-344.

Pereira AC, Poersch NL, Crescêncio P, Carneiro S, José T, Júnior P. 2013. Introgression of resistance to pathogens in common bean lines with the aid of molecular markers. Crop Breed. Appl. Biotechnol. 13: 120–126.

Peres NA, Timmer LW, Adaskaveg JE, Correll JC. 2005. Lifestyles of *Colletotrichum acutatum*. Plant Dis. 89: 784–796.

Perfect SE, Hughes HB, O'Connell RJ, Green JR. 1999. Colletotrichum: A model genus for studies on pathology and fungal-plant interactions. Fungal Genet. Biol. 27: 186–198.

Petersen TN, S Brunak, G von Heijne and H Nielsen. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods. 8:785-786.

Pinto, L., Caputo, L., Quintieri, L., de Candia, S., Baruzzi, F., 2017. Efficacy of gaseous ozone to counteract postharvest table grape sour rot. Food Microbiol. 66, 190-198.

Polashock J, Ehlenfeldt M, Stretch A, Kramer M. 2005. Anthracnose Fruit Rot Resistance in Blueberry Cultivars. Plant Dis. 89: 33–38.

Ponce de León I, Sanz A, Hamberg M, Castresana C. 2002. Involvement of the Arabidopsis alpha-DOX1 fatty acid dioxygenase in protection against oxidative stress and cell death. Plant J. 29(1):61-62.

Pretel, M.T., Martínez-Madrid, M.C., Martínez, J.R., Carreño, J.C., Romojaro. F., 2005. Prolonged storage of 'Aledo' table grapes in a slightly CO_2 enriched atmosphere in combination with generators of SO_2. LWT - Food Sci. Technol. 39, 1109-1116.

Price KR, I. T. Johnson, G. R. Fenwick & M. R. Malinow. 1987. The chemistry and biological significance of saponins in foods and feedingstuffs, CRC Critical Reviews in Food Science and Nutrition, 26:1, 27-135 .

Retamales, J.B., Hancock, J.F., 2012. Blueberries. CABI, Wallingford, UK.

Rivera, S.A., Zoffoli, J.P., Latorre, B.A., 2013. Determination of optimal sulfur dioxide time and concentration product for postharvest control of gray mold of blueberry fruit. Postharvest Biol. Technol. 83, 40-46.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26:139–140.

Rodriguez, J., Zoffoli, J.P., 2016. Effect of sulfur dioxide and modified atmosphere packaging on blueberry postharvest quality. Postharvest Biol. Technol. 117, 230-238.

Rowland LJ, Alkarouf N, Darwish O, Ogden EL, Polashock JJ, Bassil NV, Main D. 2012. Generation and analysis of blueberry transcriptome sequences from leaves, developing fruit, and flower buds from cold acclimation through deacclimation. BMC Plant Biol. 12:46.

Royo J, León J, Vancanneyt G, Albar JP, Rosahl S, Ortego F, Castañera P, Sánchez-Serrano JJ. 1999. Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight. Proc. Natl. Acad. Sci. 96: 1146–1151.

Royo J, Vancanneyt G, Pe AG, Sanz C, Sto K, Rosahl S, Sa JJ. 1996. Characterization of Three Potato Lipoxygenases with Distinct Enzymatic Activities and Different Organ-specific and Wound-regulated Expression Patterns. J. Biol. Chem. 271: 21012–21019.

Ruiz-Herrera J, Ortiz-Castellanos L. 2019. Cell wall glucans of fungi. A review. Cell Surf. 5:100022. doi: 10.1016/j.tcsw.2019.100022.

Salinas N, Verma S, Peres N, Whitaker VM. 2019. FaRCa1: a major subgenome-specific locus conferring resistance to Colletotrichum acutatum in strawberry. Theor. Appl. Genet. 132: 1109–1120.

Scheller HV, Ulvskov P. 2010. Hemicelluloses. Annu. Rev. Plant Biol. 61: 263–289.

Schopfer CR, Kochs G, Lottspeich F, Ebel Y. 1998. Molecular characterization and functional expression of dihydroxypterocarpan 6a-hydroxylase , an enzyme specific for pterocarpanoid phytoalexin biosynthesis in soybean (Glycine max L.). FEBS Lett. 432: 182–186.

Serrano M, Coluccia F, Torres M, Haridon FL, Métraux J. 2014. The cuticle and plant defense to pathogens. Front. Plant. Sci. 5: 1–8.

Shimizu T, Nakano T, Takamizawa D, Desaki Y, Ishii-Minami N, Nishizawa Y, Minami E, Okada K, Yamane H, Kaku H. 2010. Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. Plant J. 64: 204–214.

Shin S, Mackintosh CA, Lewis J, Heinen SJ, Radmer L. 2008. Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum*. J. Exp. Bot. 59: 2371–2378.

Shinya T, Motoyama N, Ikeda A, Wada M, Kamiya K, Hayafune M, Kaku H, Shibuya N. 2012. Functional characterization of CEBiP and CERK1 homologs in Arabidopsis and rice reveals the presence of different chitin receptor systems in plants. Plant Cell Physiol. 53: 1696–1706.

Smilanick, J.L., Hartsell, P., Henson, D., Fouse, D., Assemi, M., Harris, C., 1990. Inhibitory activity of sulfur dioxide on the germination of spores of *Botrytis cinerea*. Phytopathol. 80, 217-220.

Smilanick, J.L., Henson, D.J., 1992. Minimum gaseous sulphur dioxide concentrations and exposure periods to control *Botrytis cinerea*. Crop Prot. 11, 535-540.

Solomon M, Belenghi B, Delledonne M, Menachem E, Levine A. 1999. The Involvement of Cysteine Proteases and Protease Inhibitor Genes in the Regulation of Programmed Cell Death in Plants. Plant Cell. 11: 431–443.

Song JT. 2006. Molecules and Induction of a Salicylic Acid Glucosyltransferase, AtSGT1 , Is an Early Disease Response in Arabidopsis thaliana. Mol. Cells. 22: 233–238.

Sonnhammer ELL, G. von Heijne, and A. Krogh. 1998. A hidden Markov model for predicting transmembrane helices in protein sequences.In J. Glasgow, T. Littlejohn, F. Major, R. Lathrop, D. Sankoff, and C. Sensen, editors, Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology, pages 175-182, Menlo Park, CA.

Spitler, G.H., Couey, H.M., 1983. Methyl bromide fumigation treatments of fruits infested by the Mediterranean fruit fly (Diptera: Tephritidae). J. Econ. Ent. 76, 547-550.

Takeuchi M, Kegasa T, Watanabe A, Tamura M. 2018. Expression analysis of transporter genes for screening candidate monolignol transporters using Arabidopsis thaliana cell suspensions during tracheary element differentiation. J. Plant. Res. 131: 297–305.

Tang W, Kim T, Oses-prieto JA, Sun Y, Deng Z, Wang R, Burlingame AL, Wang Z. 2009. Brassinosteroid-Signaling Kinases (BSKs) mediate signal transduction from the receptor kinase BRI1 in Arabidopsis. Science. (80)321: 557–560.

Tariq M, Khan A, Tabassum B, Toufiq N. 2018. Antifungal activity of chitinase II against *Colletotrichum falcatum* Went. causing red rot disease in transgenic sugarcane. Turkish J. Biol. 42: 45–53.

Teshima Y, Ikeda T, Imada K, Sasaki K, El-sayed MA, Shigyo M, Tanaka S, Ito S. 2013. Identification and Biological Activity of Antifungal Saponins from shallot (Allium cepa L. aggregatum group). J. Agric. Food Chem. 61:7440–7445.

Thang K, Au K, Prakash A. 2016. Effect of phytosanitary irradiation and methyl bromide fumigation on the physical, sensory, and microbiological quality of blueberries and sweet cherries. J. Sci. Food Agric. 4382–4389.

Torres M, Jones J, Dangl J. 2006. Reactive Oxygen Species Signaling in Response to Pathogens. Plant Physiol. 141: 373–378.

Trujillo M, Ichimura K, Casais C. 2008. Report Negative Regulation of PAMP-Triggered Immunity by an E3 Ubiquitin Ligase Triplet in Arabidopsis. Curr. Biol. 18: 1396–1401.

USDA. 1995. United States Standards for Grades of Blueberries. United States Department of Agriculture, Agricultural Marketing Service, Fruit and Vegetable Programs, Fresh Products Branch. Reprinted: January 1997. https://www.ams.usda.gov/grades-standards/blueberries-grade-and-standards (accessed 12 Jan, 2021).

USDA. 2001. Michigan Rotational Survey Fruit Inventory. National Agricultural Statistics Service, Washington, DC.

USDA. 2004. Michigan Rotational Survey Fruit Inventory. National Agricultural Statistics Service, Washington, DC.

USDA. 2007. Michigan Rotational Survey Fruit Inventory. National Agricultural Statistics Service, Washington, DC.

USDA. 2012. Michigan Rotational Survey Fruit Inventory. National Agricultural Statistics Service, Washington, DC.

USDA. 2015. Michigan Rotational Survey Fruit Inventory. National Agricultural Statistics Service, Washington, DC.

USDA 2018. Small Fruits and Hops Inventory: Michigan Rotational Survey Fruit Inventory. National Agricultural Statistics Service, Washington, DC.

USDA. 2022. National Agricultural Statistics Service – Blueberries, Tame, Fresh Market Production, Measured in US Dollars. nass.usda.gov. (accessed 24 Feb., 2022).

Vanburen R, Mockler TC, Wai CM, Ou S, Pardo J, Edger P, Michael TP. 2018. Extreme haplotype variation in the desiccation-tolerant clubmoss *Selaginella lepidophylla*. Nat Commun. doi: 10.1038/s41467-017-02546-5.

Veillet F, Gaillard C, Coutos-Thévenot P, La Camera S. 2016. Targeting the AtCWIN1 Gene to Explore the Role of Invertases in Sucrose Transport in Roots and during Botrytis cinerea Infection. Front. Plant Sci. 7: 1–20.

Vicente A, Ortugno C, Rosli H, Powell A, Greve L, Labavitch J. 2007. Temporal Sequence of Cell Wall Disassembly Events in Developing Fruits . 2 . Analysis of Blueberry (Vaccinium Species). J. Agric. Food Chem. 55: 4125–4130.

Vicente J, Cascón T, Vicedo B, García-Agustín P, Hamberg M, Castresana C. 2012. Role of 9-Lipoxygenase and α-Dioxygenase Oxylipin Pathways as Modulators of Local and Systemic Defense. Mol. Plant. 5: 914–928.

Vlad F, Spano T, Vlad D, Daher FB, Ouelhadj A, Kalaitzis P. 2007. Arabidopsis prolyl 4-hydroxylases are differentially expressed in response to hypoxia, anoxia and mechanical wounding. Physiol. Plant 130: 471–483.

Walse, S.S., Tebbets, J.S., Leesch, J.G., 2017. Postharvest fumigation of California table grapes with ozone to control Western black widow spider (Araneae: Theridiidae). Postharvest Biol. Technol. 134, 1–4.

Wang G, Ellendorff U, Kemp B, Mansfield JW, Forsyth A, Mitchell K, Zipfel C, Wit PJGM De, Bastas K, Liu C. 2008. A Genome-Wide Functional Investigation into the Roles of Receptor-Like Proteins in Arabidopsis. Plant. Physiol. 147: 503–517.

Wang Y, Bouwmeester K, Beseh P, Shan W, Govers F. 2014. Phenotypic Analyses of Arabidopsis T-DNA Insertion Lines and Expression Profiling Reveal That Multiple L-Type Lectin Receptor Kinases Are Involved in Plant Immunity. Mol. Plant-Microbe Interact. 27: 1390–1402.

Wharton P, Diéguez-Uribeondo J. 2004. The biology of Colletotrichum acutatum. An. del Jard bontanico Madrid 61: 3–22.

Wharton PS, Schilder AC. 2008. Novel infection strategies of Colletotrichum acutatum on ripe blueberry fruit. Plant Pathol. 57: 122–134.

Williamson, D.L., Summy, K.R., Hart, W.G., Sanchez-r, M., Wolfenbarger, D.A., Bruton, B.D., 1986. Efficacy and phytotoxicity of methyl bromide as a fumigant for the Mexican fruit fly (Diptera: Tephritidae) in grapefruit. J. Econ. Entomol. 79, 172-175.

Windholz, M., Budavari, S., Blumetti, R.F., Otterbein E.S., eds. 1983. The Merck Index, Tenth Edition. Merck & Co., Inc. Rahway, New Jersey.

Winkler, A., Jacob, H., 1925. The utilization of sulfur dioxide in the marketing of grapes. Hilgardia. 1, 107-131.

Wisecaver JH, Borowsky AT, Tzin V, Jander G, Kliebenstein DJ. 2017. A Global Coexpression Network Approach for Connecting Genes to Specialized Metabolic Pathways in Plants. Plant Cell. 29: 944–959.

Yamaguchi K, Yamada K, Ishikawa K, Yoshimura S, Hayashi N, Uchihashi K, Ishihama N, Kishi-kaboshi M, Takahashi A, Tsuge S. 2013. A Receptor-like Cytoplasmic Kinase Targeted by a Plant Pathogen Effector Is Directly Phosphorylated by the Chitin Receptor and Mediates Rice Immunity. Cell Host Microbe. 13: 347–357.

Yang W, Zheng Y, Chul S, Pan X, Li M, Sy H. 2012. The Patatin-Containing Phospholipase A pPLAII a Modulates Oxylipin Formation and Water Loss in Arabidopsis thaliana. Mol. Plant 5: 452–460.

Yang, X., Liu, Y.B., 2019. Nitric oxide fumigation for postharvest pest control on lettuce. Pest Mgt. Sci. 75, 390-395.

Ye W, Wang Y, Dong S, Zheng X. 2017. A paralogous decoy protects Phytophthora sojae apoplastic effector PsXEG1 from a host inhibitor. Science. 80(714):710–714.

Yokoyama, V., Miller, G., 2001. Pest response in packed table grapes to low temperature storage combined with slow-release sulfur dioxide pads in basic and large-scale tests. J. Econ. Entomol. 94, 984-988.

Yoon JB, Yang DC, Do JW, and HG Park. 2006. Overcoming two post-fertilization genetic barriers in interspecific hybridization between capsicum annuum and C. baccatum for introgression of anthracnose resistance. Breeding Science. 56:31-38.

Yoshiki Y, Kudou S, Okubo K. 1998. Relationship between Chemical Structures and Biological Activities of Triterpenoid Saponins from Soybean. Biosci. Biotechnol. Biochem. 62: 2291–2299.

Zhang L, Cai J, Li R, Liu W, Wagner C, Wong K, Xie Z, Staehelin C. 2016. A single amino acid substitution in a chitinase of the legume *Medicago truncatula* is sufficient to gain Nod-factor hydrolase activity. Open Biol. 6(7):160061. doi: 10.1098/rsob.160061.

Zhao H, Guan X, Xu Y, Wang Y. 2013. Characterization of novel gene expression related to glyoxal oxidase by agro-infiltration of the leaves of accession Baihe-35-1 of Vitis pseudoreticulata involved in production of H_2O_2 for resistance to *Erysiphe necator*. Protoplasma 250: 765–777.

Zifkin M, Jin A, Ozga JA, Zaharia LI, Schernthaner JP, Gesell A, Abrams SR, Kennedy JA, Constabel CP. 2012. Gene expression and metabolite profiling of developing highbush blueberry fruit indicates transcriptional regulation of flavonoid metabolism and activation of abscisic acid metabolism. Plant Physiol. 158(1), 200–224.