BLUEBERRY AND CRANBERRY FLORAL STIMULATION OF *COLLETOTRICHUM FIORINIAE* AND OTHER FRUIT ROTTING FUNGI

BY

TIMOTHY JAMES WALLER

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ABSTRACT OF THE DISSERTATION

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TIMOTHY JAMES WALLER

Dissertation Director:

Peter V. Oudemans

Colletotrichum fioriniae is an important hemibiotrophic pathogen limiting both highbush blueberry (*Vaccinium corymbosum* L.) and cranberry (*V. macrocarpon* Aiton) production worldwide. Since fungicide applications during bloom are most effective in both crops, the link between host floral signals and pathogen disease cycles were investigated. *C. fioriniae* as well as two other latent infection forming cranberry fruit rot pathogens *C. fructivorum* (*C. gloeosporioides* s.l.) and *Coleophoma cylindrospora* (*C. empetri* s.l.) and a mature fruit infecting fungi *Allantophomopsis lycopodena* were investigated to better describe the temporal dynamics of pathogen stimulation in response to host derived signals produced during bloom.

In order quantify this relationship and visualize pathogen responses, host signals isolated via water or chloroform were utilized in extract-dependent bioassays. The results showed that blueberry and cranberry (as well as multiple other ericaceous species) floral extracts (FEs) affected two important disease cycle stages by stimulating an increased rate (+ 200%) and quantity (+ 500%) of secondary conidiation (inoculum build-up) and appressorial formation (infection structures) of *C. fioriniae* and all other pathogens evaluated, except *A. lycopodena*, linking bloom period infecting fungi to floral signals. Conidia in the presence of FEs also conferred higher levels of disease on detached fruit than conidia alone, suggesting that apparent disease was a function of increased appressorial formation. Bioactivity was readily detected in floral rainwater runoff and became more stimulatory as proximity to flowers or the bloom period increased, thus indicating both mobility of floral signals and the importance of phenology-specific cues. Chloroform-based extractions provided a chemical mirror of the host cuticles first encountered by pathogens. Characterization of multiple tissue types elucidated fatty acid derivative compositional patterns, where specific stimulatory compounds were more abundant in flower cuticular waxes. Multiple fatty acids were identified that stimulated appressorial formation, however, hexadecanoic fatty acid derivatives were concluded to be the most likely source of stimulation due to the paired bioactivity observations and occurrence of this compound within both water- and chloroform-based extraction types.

This research provides strong evidence that flowers contribute substantially to the disease cycle events of replication (sporulation and secondary conidiation) and infection of fruit by *C. fioriniae* and other bloom period infecting fungi, thus providing evidence as to why the bloom period is often referred to as the critical disease control window.

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Chapter I (cited internally as (Waller et al. 2018))

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This work was published as a companion to an open source video protocol as; Waller, T.J., Gager, J.D., Oudemans, P.V. 2019. *Colletotrichum fioriniae* development in water and chloroform-based blueberry and cranberry floral extracts. Journal of Visualized Experiments 146:e58880. The video protocol can be found at the following link: (https://www.jove.com/video/58880/colletotrichum-fioriniae-development-water-chloroform-based-blueberry).

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Chapter IV

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Dedication

To grit and gumption

Without these I may have never made it

I dedicate this effort

To those who can’t be here to read this

…for those that can

I am humbled the faith you have in me

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Introduction

The problem. The large American cranberry (*Vaccinium macrocarpon* Ation) and highbush blueberry (*V. corymbosum* L.) are specialty crops native to the United States (Eck 1990; Oudemans et al. 1998; Polashock 2017). Production of these crops can be severely impacted by insect pests, viruses, climatic/ozone/abiotic factors, and the focus of the current study; fruit rotting fungal pathogens (Polashock 2017). Many of these fruit rotting pathogens infect during bloom but remain dormant until fruit maturation, rendering fungicide applications made outside of the bloom period ineffective (Borve et al. 2010; Damm et al. 2012; DeMarsay 2005; Oudemans et al. 1998; Peres et al. 2005; Polashock 2017; Polashock et al. 2007; Prusky 1996; Prusky et al. 2013; Pszczółkowska et al. 2016; Verma et al. 2006; Wharton 2002; Wharton and Diéguez-Uribeondo 2004; Wharton and Schilder 2008). Fruit rot of both blueberry and cranberry dictates the majority of disease management strategies due to the early season onset of infection coupled to the severe losses that can be experienced if the critical disease control window during bloom is missed (Oudemans et al. 1998; Polashock 2017) (Fig.1). *Colletotrichum fioriniae* is the causal agent of blueberry anthracnose in the northeastern United States as well as an important component of the cranberry fruit rot complex throughout North America (Oudemans et al. 1998; Polashock et al. 2009; Polashock et al. 2005; Waller et al. 2019b; Waller et al. 2018; Wharton and Diéguez-Uribeondo 2004). Taken together, it appeared likely that *C. fioriniae* and other latent fruit rotting fungi were responding to

host signals produced during bloom, thus enabling pathogen synchronization to host plant phenology.

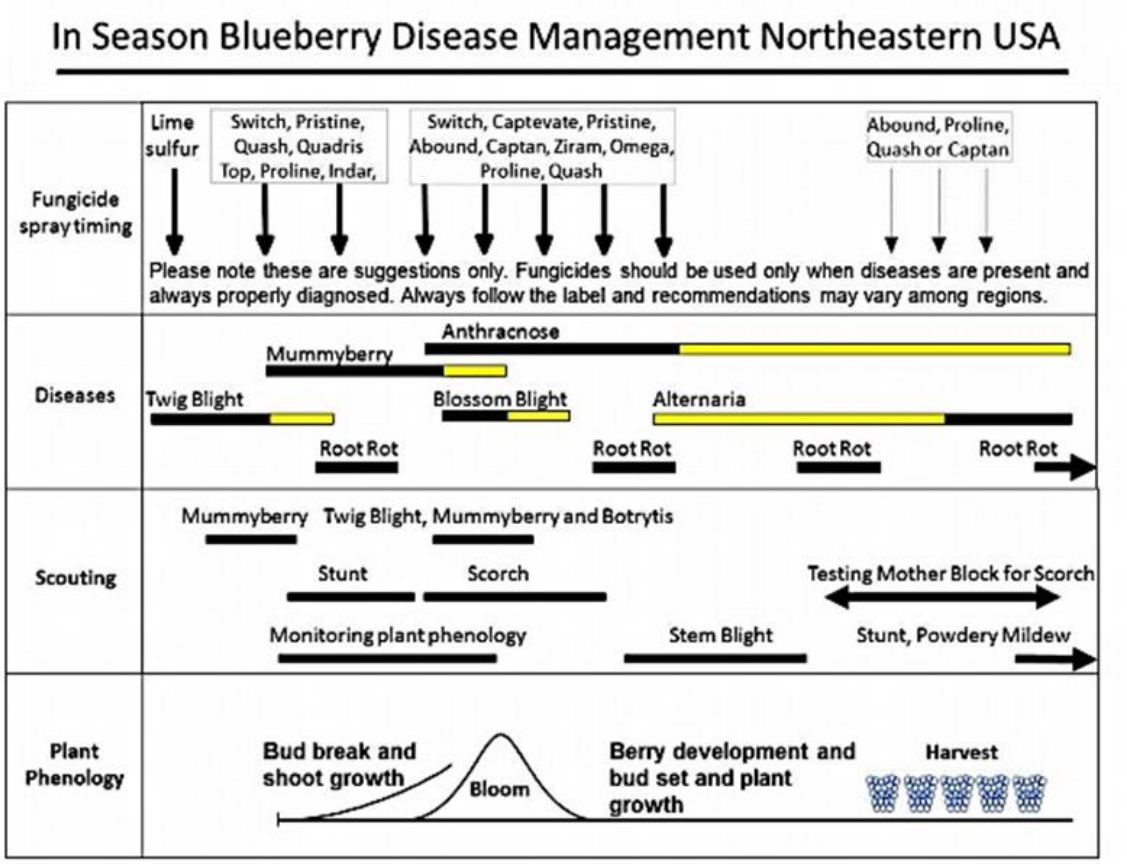

Figure 1. Excerpt from the 2018 Commercial Blueberry Pest Control Recommendations for New Jersey (Oudemans et al. 2018, publication # E265), illustrating the importance of bloom period fungicide applications in the control of *Colletotrichum fioriniae* (blueberry anthracnose).

Hosts: *Vaccinium* species overview and production in North America. The *Vaccinium* genus is subdivided into true blueberries (*Cyanococcus*), cranberries (*Oxycoccus*), lingonberries (*Vitis-idaea*), and billberries (*Myrtillus*), representing the economically important (fleshy-fruit-forming) divisions of the heath family (Ericaceae). These species are characterized as acidophilic woody perennials, growing as either lianas (Oxycoccus) or small to large prostrate bushes (*Vitis-idaea*, *Myrtillus*, *Cyanococcus*), where

Oxycoccus and *Vitis-idaea* are evergreen and *Myrtillus*, *Cyanococcus* are deciduous (Polashock 2017). Although these subgenus groups are phylogenetically similar, their growth habits have a profound effect on the cultural practices implemented. Cranberries, which grow as a carpet of woody vines within a 'bog [bed]' or 'marsh' are often collected via the assistance of water, helping to float the air filled, and thick skinned (exocarp), berries for easier harvest (Eck 1990). Shoulder-height highbush blueberry (*V. corymbosum* L.) bushes are often first hand-harvested due to their stature and the delicate nature of the fruit, as compared to rabbiteye blueberry hybrids (x *V. virgatum* Aiton) grown in the southern United States that are typically planted at higher densities and pruned into compact rows to facilitate machine harvesting (Polashock 2017). Another interesting difference is flower morphology (Fig.2). Blueberry and cranberry flowers are quite distinct from each other; blueberry has fused corollas that form on racemes with many flowers originating from a single floral bud set the season prior, in comparison, cranberry flowers are comprised of four deeply cleft petals that are born individually from a prostrate upright. The upright (inflorescence) is comparable to a raceme though, in the fact that all of the individual flowers are born from a single terminal bud set during the previous season (Polashock 2017).

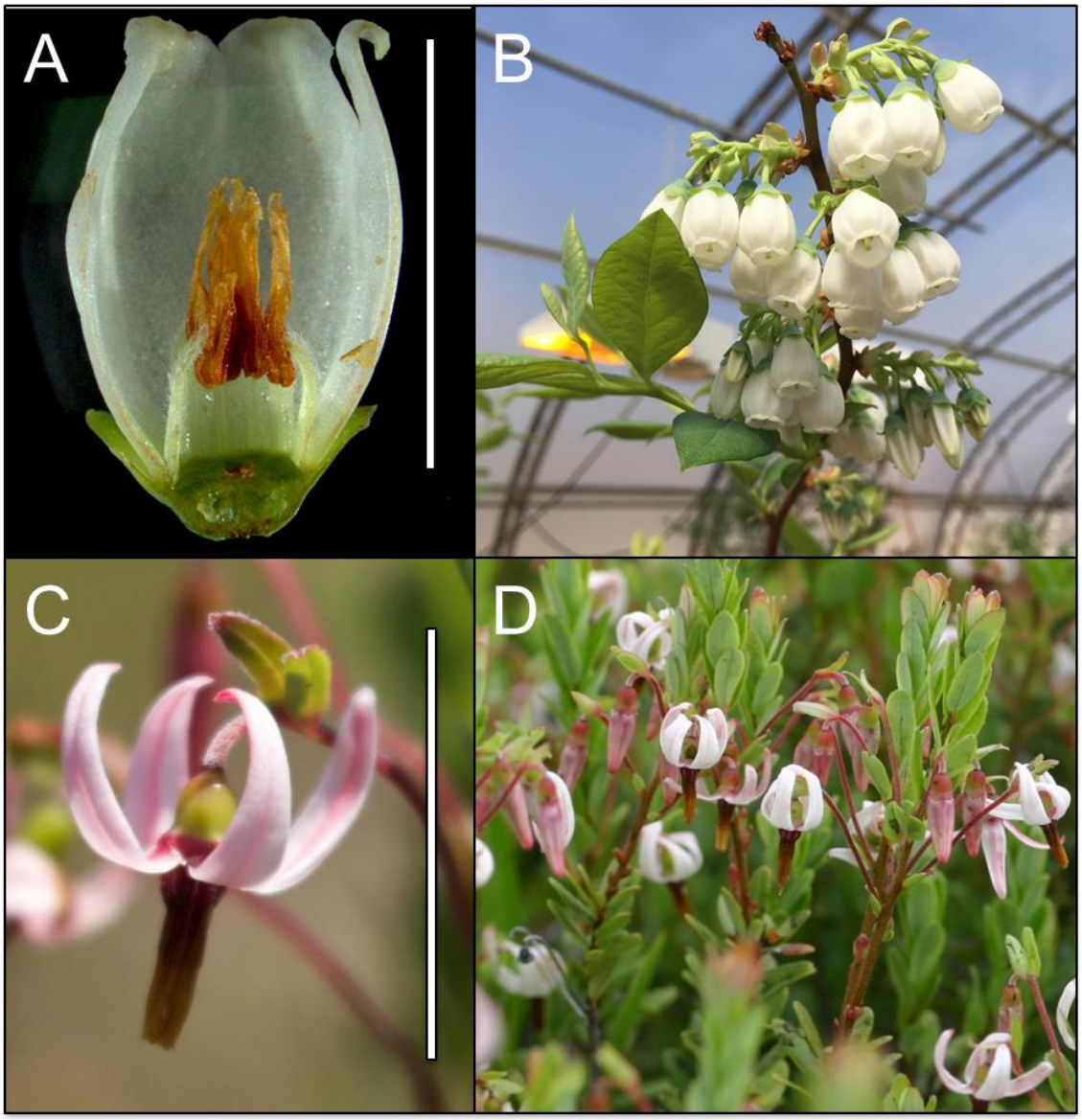

Figure 2. Blueberry and cranberry flowers. **A.** Dissection of blueberry flower showing the fused corolla, inferior ovary, sepals, and characteristic anthers (stigma not present). **B.** Typical inverted blueberry flower inflorescence raceme (early to late spring). Each cluster/raceme is born from a single floral bud, set during the previous season. Note the overlap of adjacent flowers' corollas and ovaries. **C.** Cranberry flower with characteristic 'hooked' / inverted position, owing to its colloquial name 'Crane-berry'. The corolla is comprised of four deeply cleft petals. **D.** A cranberry upright (prostrate inflorescence bearing structure) during bloom (early summer). Note the curved nature of flowers in full bloom compared to the less mature closed and fused blossoms. Each flower is born from an individual bud (1-7 buds can form per upright), however all buds are borne together from a primary terminal bud set during the previous season. Scale bar is approximately 1 cm.

The fruit of highbush blueberry, the large American cranberry (*V. macrocarpon* Aiton) and lowbush blueberry (*V. angustifolium* Aiton) are high-value specialty crops native to North America that have a rich tradition in holiday meals and celebratory dishes, including sauces for Thanksgiving and pie fillings/toppings for summer holidays. These fruits are also consumed daily as fresh fruit or processed into juices, sauces, jams, jellies, candies, and sweetened dried fruits, either consumed alone or as a health conscious ingredient. Blueberries and cranberries are acclaimed 'Super Foods', due to their reported health benefits, often citing the rich pigment producing proanthocyanidin A / anthocyanidins, and other flavonoids as beneficial for inhibiting the adherence of *E. coli* bacteria to the urinary tract and increased cardiovascular health as well as numerous anti-cancer properties and increased memory function in Alzheimer's patients among many other anecdotal remedies (Howell 2002; Krikorian et al. 2010; Neto et al. 2003). However, due to the prevalence of pests and pathogens, these crops have made their way onto the notorious Environmental Working Group's (a consumer advocacy group) "Dirty Dozen List" due to the detection of pesticide residues on fresh fruit.

North American production of blueberry and cranberry is centralized around both coasts, the Great Lakes region, and the Pacific Northwest of the United States, as well as corresponding regions of Canada (Polashock 2017). Lowbush "wild" blueberry production, is restricted to the northeastern regions of North America with the state of Maine harvesting 17-thousand acres producing 67-million pounds of fruit valued at $17.5-million, with less than 1% entering the fresh market in 2017 (USDA 2018). In 2016, over 92-thousand acres of blueberry (here consisting of northern and southern highbush and rabbiteye varieties) were harvested nationally producing over 580-million

pounds of fresh and processed fruit valued at approximately $720-million. In New Jersey, the crop was valued at $59-million, dwarfing apple and peach production in the state valued at $35-million and $28-million, respectively. In the case of cranberry, approximately 40-thousand acres produced 8.4-million barrels (the standard unit of measure for cranberry equaling 100-lbs) of fruit valued at $260-million dollars in 2016, with only ~3% of this fruit sold into the fresh market (USDA 2017). Blueberry anthracnose and cranberry fruit rot are among the main factors limiting production of these economically important crops, with 20% of the multi-hundred-million dollar industry attributed to the use of fungicides nationwide (Gianessi and Reigner 2005).

Early cranberry pathology. The large American cranberry has had a complex history, providing both insight into the history of fruit rot and the future trajectory of disease management approaches. This crop, originally harvested by indigenous peoples and early colonialist of northeastern North America, came into commercial cultivation in the early nineteenth century to meet increased demand for the fruit, both domestically and in Europe (Oudemans et al. 1998). Entrepreneurs of Massachusetts and New Jersey established some of the first production areas in the United States but were almost immediately plagued by "the rot" (Hall 1950). Prevailing knowledge surfaced that plantings could only be productive for five to ten years before losses were too great, often leading to bog abandonment. However by the 1890s there was nationwide appreciation of fungal plant pathology, especially in New Jersey where Byron Halsted, a botanist based out of the New Jersey State Experiment Station, implicated fungi as the cause of two important cranberry diseases (gall, caused by *Synchytrium vaccinii* and scald (a fruit and

foliage rot) caused by *Guignardia vaccinii* (*Phyllosticta vaccinii*) (Griffith et al. 1997; Halsted 1889; Oudemans et al. 1998). Halsted also popularized the use of spray applications of fungicides (a term he coined at the American Pomologicial Society meeting in 1883), primarily consisting of Bordeaux-mixture (copper sulfate + lime) (Griffith et al. 1997). Forty years later, researchers Cornelius Shear, Neil Stevens, Henry Bain, and associates published "Fungous Diseases of the Cultivated Cranberry" (USDA Technical Bulletin No. 258) that described additional fungal pathogens as well as the chemical (Bordeaux-mixture, now with soap added) and cultural control measures thought to combat them (Shear et al. 1931). This publication revolutionized the study of fungal pathology in cranberry production, yet moving to current day, fruit rot is *still* the main limiting factor in North America even with a greatly increased chemical armory and understanding of pathogen biology (Polashock 2017).

Fruit rot and pathogen infection strategies: Typically, once small seeded fleshy-fruit have reached maturity (i.e. seed maturation) they decay and become more attractive to birds and mammals, who aid in seed dispersal through consumption and defecation (Cipollini and W. Stiles 1992; Cipollini and Stiles 1993; Janzen 1977). In addition to physiological breakdown, mature fruits undergo many dramatic changes in secondary metabolites that ultimately provide more palatable fruits with higher sugar contents and lowered phytoalexins or other preformed toxic compounds (Janzen 1977; Prusky 1996; Prusky et al. 2013; Prusky and Lichter 2007). It is also during this stage of fruit ontogeny that mature fruit often begin to show signs or symptoms of microbial colonization (Janzen 1977). Microbial colonization of blueberry and cranberry by certain fungal

pathogens may present a selective evolutionary advantage to the host by aiding in seed dispersal. In blueberry, *C. fioriniae* is thought to speed the conversion of acids to sugars thereby making the fruit more appealing to animals (Cipollini and W. Stiles 1992). Conversely, cranberries have thick leathery skins (exocarp) that must be compromised before the air-filled/floating fruits (locules) can disseminate the seeds via water, as this fruit is not commonly consumed by birds or mammals (Cipollini and W. Stiles 1992; Cipollini and Stiles 1993). Many of the fruit rotting pathogens such as *C. fioriniae*, *C. fructivorum,* and *Coleophoma cylindrospora* infect during the bloom period and remain latent (dormant) until seed maturation potentially aiding in this dispersal pattern (Oudemans et al. 1998). Following on this, latent pathogen synchronization to host signals produced during bloom would benefit both organisms and potentially guard against additional infections from non-beneficial fungi, as plant defenses increase with fruit maturation (Ficke et al. 2002; Prusky 1996). Additionally, if an evolutionary relationship does exist it could explain why controlling blueberry and cranberry latent pathogens is so difficult in practice. It is important to note that the term 'pathogen' in this case refers to an organism that does not benefit the humans cultivating the crop, yet does not necessary mean the organism is detrimental to the host plant.

A generalized lifecycle of fruit rotting fungal pathogen entails inoculum dispersal, recognition of susceptible tissues, breaching the plant cuticle, colonization, and ultimately replication (Agrios 2005). However, this process is highly nuanced and adoption of any given lifestyle by a pathogen depends on the host or tissue type being colonized. For example the gray mold fungus *Botrytis cinerea* is considered to be necrotrophic on mature strawberries and senescing tissues yet is biotrophic on actively

growing leaves and immature fruit (Petrasch et al. 2019b; Petrasch et al. 2019; Veloso and Van Kan 2018). During a necrotrophic lifecycle or phase, fungi kill host tissues very quickly prior to colonization by secreting toxins and cell wall degrading enzymes or eliciting programmed cell death (PCD), gathering the resources needed for rapid inoculum buildup (Van Kan 2006). In biotrophic colonization, fungi secrete effector proteins that suppress host immune responses, enabling the pathogen to feed on living tissues (Oliver and Ipcho 2004; Petrasch et al. 2019b; Petrasch et al. 2019). However, recent studies have demonstrated that many fruit rotting pathogens are not strictly necrotrophs or biotrophs but a combination of both phases, and are termed hemibiotrophic pathogens (Alkan and Fortes 2015; Blanco-Ulate et al. 2016; Oliver and Ipcho 2004; Petrasch et al. 2019; Prusky and Lichter 2007). In blueberry and cranberry, *C. fioriniae* adopts this hemibiotrophic lifestyle, which is characterized by a variable lag-time (a function of fruit maturity) between initial biotrophic infection and subsequent necrotrophic colonization (known as latent or quiescent infections) (Peres et al. 2005; Prusky 1996; Prusky et al. 2013; Prusky and Lichter 2007; Prusky et al. 1991; Wharton and Diéguez-Uribeondo 2004; Wharton and Schilder 2008). Thus, preemptive and protective fungicides applications are recommended, and absolutely required in the northeastern growing regions, to optimally control pathogens with this lifestyle, as latent infections can be initiated days (blueberry anthracnose) to months (cranberry fruit rot) prior to symptom development (Oudemans et al. 2005; Oudemans et al. 1998; Polashock 2017; Prusky 1996). The applications are aimed at preventing the rain-splash dispersed conidia (asexual reproductive spores) from germinating, further sporulating (conidiation), or forming infection structures (appressoria) by protecting susceptible tissues such as

flowers, ovaries and developing fruit of both blueberry and cranberry (Madden et al. 1996; Miles et al. 2013; Peres et al. 2005; Polashock 2017; Wharton 2002; Yang et al. 1990).

Pathogens can gain entrance into the host in a variety of ways. Numerous devastating plant pathogens, such as multiple *Colletotrichum* spp., the rice blast fungus *Magnaporthe grisea,* and multiple *Phyllosticta* spp. infect via an appressorium (Mendgen and Deising 1993). This is a highly specialized, melanized structure that generates tremendous turgor pressure to puncture the plant cuticle (wax layer covering the surface of all aerial plant parts) (Parbery and Blakeman 1978; Ryder and Talbot 2015; Serrano et al. 2014). In association with extracellular enzymes such as cutinase and pectinase, a penetration peg (hypha) is forced directly into the epidermal layer of cells, where the pathogen can remain quiescent until changes in host physiology triggers necrotrophic colonization (Dickman et al. 2003; Prusky 1996; Ryder and Talbot 2015; Wheeler 1975). The appressorium also functions as a survival structure to both desiccation as well as fungicide applications (Peres et al. 2005; Wharton and Diéguez-Uribeondo 2004). Some pathogens use less forceful means, instead searching for natural openings in the cuticle. The bean rust pathogen, *Uromyces viciae-fabae* also differentiates a specialized cell similar to an appressorium, however this structure is used to locate and gain entry through stomatal cells, where a feeding haustorium is then formed within a mesophyll cell (Mendgen and Deising 1993). Still other pathogens simply grow through the stomata, or enter through wounds in the epidermal tissues, utilizing combinations of cellular degrading enzymes to colonize and feed on the plant host (Petrasch et al. 2019). Regardless of how the pathogens gain entry into a host, many pathogens embark on a

hemibiotrophic lifestyle, relying on host derived signals to trigger or allow the next phase of colonization (Oliver and Ipcho 2004; Prusky 1996).

Host stimulation of pathogens. How host signals produced during bloom alter the initial patterns of colonization, sporulation, or infection of *C. fioriniae* on blueberry and cranberry remains unclear. *Colletotrichum* spp. have been shown to preferentially overwinter in dormant floral buds of numerous host species (Bernardi et al. 1983; Borve and Stensvand 2006; DeMarsay 2005), as well as the synchrony between *C. fioriniae* conidial production and the bloom period (DeMarsay 2005; Miles et al. 2013; Wharton 2002), suggest an early, possibly evolutionary, relationship of primary inoculum and floral tissues / signals. Floral signals have been implicated in at least two important pathosystems involving members of the *C. acutatum* s.l. disease complex (Leandro et al. 2003; MacKenzie et al. 2010). *C. acutatum* s.l. has been shown to form latent infections on persistent calyces (buttons) as dormant appressoria in citrus. During bloom, these appressoria germinate and then sporulate in response to floral signals (nectar sugars, particularly sucrose) washed throughout the canopy (MacKenzie et al. 2010). Subsequently, the newly formed conidia infect citrus flowers and developing ovaries that later trigger the abscission of green fruit (Citrus post-bloom fruit drop), leaving infected persistent calyces, thus completing the disease cycle (Agostini et al. 1993; Agostini and Timmer 1994; MacKenzie et al. 2010; Peres et al. 2005). Similarly, water-based extractions of strawberry leaf and floral tissue stimulated secondary conidiation (production of conidia without the development of traditional conidiogenous structures such as acervuli or pycnidia) (Leandro et al. 2001, 2003). Leandro et al. (2003) went on

to suggest that secondary conidiation was epiphytic and asymptomatic, thus greatly increasing the potential for inoculum buildup (Eastburn and Gubler 1990).

In both of these examples, floral signals have only been linked to the spread of disease via sporulation / secondary conidiation. However, appressorial formation of numerous pathogens has been linked to host epi- and intracuticular waxes, citing pathogen preference of specific compounds over the influences of hydrophobicity or hard surface recognition (Barthlott et al. 2017; Dickman et al. 2003; Kolattukudy et al. 1995; Koller et al. 1991; Woloshuk and Kolattukudy 1986). These waxes cover the outer surface of all terrestrial plants and are primarily composed of fatty acid derivatives, wax esters, alcohols, aldehydes, and alkanes (Gulz 1994). The intracuticular waxes are formed primarily from the biopolymer cutin, a polyester comprised of scaffolding C_{16} and C_{18} hydroxylated and epoxy-hydroxylated fatty acid derivatives that function as both the transpiration barrier and frontline in pathogen defense (Barthlott et al. 2017). Thus, it appears logical that *C. fioriniae* would respond to host cuticular waxes, as appressoria are necessary to breach this barrier (Parbery and Blakeman 1978; Serrano et al. 2014).

Importance. As stated prior, many fruit rotting pathogens are only controlled with bloom time fungicide applications, leading to question if plant signals produced during bloom play a critical role in the disease cycle of these pathogens. If host derived signals produced during bloom are more stimulatory than those produced during fruit maturation, this would present a counterpoint or alternate view of ontogenic resistance (Ficke et al. 2002). This research set out to describe how floral signals alter the initial stages of pathogen development as this knowledge could ultimately lead to more robust disease management approaches aimed at disrupting pathogen-host phenology synchronization.

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Chapter I

Evidence that blueberry floral extracts influence secondary conidiation and appressorial formation of *Colletotrichum fioriniae*

Introduction:

Highbush blueberry (*Vaccinium corymbosum* L.) is an important specialty crop in the United States with more than 89,000 acres grown nationwide and producing upwards of 560-million pounds of fresh or processed fruit with a value of over $811-million annually according to the 2015 USDA Noncitrus Fruits and Nuts Summary (USDA 2013). Blueberry anthracnose, caused by the plant pathogen *Colletotrichum fioriniae* (Damm et al. 2012) can cause significant pre-harvest and post-harvest crop losses and is a primary target for disease management in many growing regions.

C. fioriniae was recently delineated from the *Colletotrichum acutatum* species complex (Damm et al. 2012; Shivas and Tan 2009; Sreenivasaprasad and Talhinhas 2005) that affects many valuable horticultural species (Adaskaveg and Hartin 1997; Dieguez-Uribeondo et al. 2004; Eastburn and Gubler 1990; Freeman et al. 2001; Ivey et al. 2004; Jones et al. 1996; MacKenzie et al. 2010; Moral et al. 2009; Oudemans et al. 1998; Polashock et al. 2009; Rodriguez-Salamanca 2012; Smith and Black 1990; Wharton and Diéguez-Uribeondo 2004). In the original description of *Colletotrichum acutatum* var. *fioriniae* (teleomorph: *Glomerella acutata* var. *fioriniae* var. nov.), isolates collected from scale insects (*Fiorinia externa)* shared 100% sequence similarity to isolates collected from New Jersey blueberry fruit in the D1/D2 region of the 28S

ribosomal DNA (28S), partial beta tubulin (TUB2), and mating-type gene MAT1–2 (Marcelino et al. 2008). Isolates now reclassified as *C. fioriniae* are important pathogens of almond, apple, avocado, mango, nectarine, peach, and strawberry, in addition to several *Vaccinium* species including highbush blueberry (Freeman and Shabi 1996; Guerber et al. 2003; Kou et al. 2014; Polashock et al. 2009).

C. fioriniae has a latent, hemibiotrophic lifestyle with a variable time lag between infection and symptom development depending on timing of infection and ripeness of the fruit (Milholland 1995; Peres et al. 2005; Prusky et al. 2013; Wharton and Diéguez-Uribeondo 2004; Wharton and Schilder 2008). The pathogen is known to also infect tissues other than berries causing symptoms such as tip dieback and blossom blight, in addition to symptomless infections on leaves, inflorescence buds and stems (Milholland 1995; Yoshida et al. 2007). On blueberry, the pathogen overwinters in blighted tissues such as dead twig tips and previous seasons fruit pedicels (Milholland 1995; Stretch 1967) as well as dormant inflorescence buds (DeMarsay 2005; Wharton and Diéguez-Uribeondo 2004). Inflorescence buds have also been implicated as inoculum reservoirs in other horticultural species including apple (Bernardi et al. 1983), sweet cherry, and sour cherry (Borve and Stensvand 2006), suggesting a close relationship of primary inoculum with floral tissues. Most *Colletotrichum* species, including *C. fioriniae*, are well adapted to rain-splash dispersal, with conidia arising from infected tissues and spread in water droplets throughout the canopy during rain events (Boudreau and Madden 1995; Madden et al. 1996; Milholland 1995). Rain-splash dispersed conidia may either germinate to form an appressorium that can lead to infection or increase inoculum via secondary

conidiation on the surface of plant tissues (Leandro et al. 2001; MacKenzie et al. 2010; Zulfiqar et al. 1996).

Typical fungicide applications on blueberry and other hosts are aimed at preventing *C. fioriniae* from sporulating, germinating and infecting host tissues (Boudreau and Madden 1995; Madden et al. 1996; Miles et al. 2013; Milholland 1995; Peres et al. 2005; Wharton 2002; Yang et al. 1990). For successful management of blueberry anthracnose, fungicide applications initiated during open bloom are most effective for preventing the formation of season long latent infections (Milholland 1995; Verma et al. 2006). Interestingly, several studies have shown a synchronization of conidial production and the bloom period (DeMarsay 2005; Miles et al. 2013; Wharton 2002); however, it is unknown if flowers signal the initiation of conidial production and disease development. Since the critical disease control period begins during bloom, the biological importance of floral components on the disease cycle of *C. fioriniae* needs to be explored more fully.

Flowers have been implicated in at least two pathosystems involving members of the *C. acutatum* species complex (Agostini et al. 1993; Leandro et al. 2001, 2003; MacKenzie et al. 2010; Peres et al. 2005; Wharton and Diéguez-Uribeondo 2004). *C. acutatum* (*sensu lato*) can also cause floral infections on almond, citrus, mango, and strawberry leading to significant crop losses (Adaskaveg and Hartin 1997; Jeffries et al. 1990; Maas 1984; Zulfiqar et al. 1996). This research was conducted to investigate the role of water-dispersible blueberry floral components on secondary conidiation and appressorial formation of *C. fioriniae* during the initial stages of pathogen development.

Materials and Methods:

Fungal isolates. The primary isolate of *C. fioriniae* used in this research was BB#10, isolated in 2005 from an infected blueberry fruit at the P. E. Marucci Center for Blueberry and Cranberry Research and Extension, Rutgers University in Chatsworth, NJ (PEM). BB#10 matched the description of *C. fioriniae* and shared 100% sequence similarity to the diagnostic regions (ITS), TUB2, and partial actin (ACT) (Gen Bank JQ040070, JQ040075 and JQ040050, respectively) of *C. fioriniae* type material, CBS 128517 (Gen Bank NR_111747, JQ949943 and JQ949613). The TUB2 region (Gen Bank EF593325) of the original *Colletotrichum acutatum* var. *fioriniae* isolate (Marcelino et al. 2008), was compared as well and showed 99% sequence similarity. In addition to BB#10, four other *C. fioriniae* isolates were used in one assay. BB-89 and CB-KPCF16 were isolated from naturally infected blueberry and cranberry fruit, respectively, collected at PEM. CB-PMAP182 and CB-PMAP184 were collected from symptomatic cranberry fruit at local growers' farms in Chatsworth, NJ and Shamong, NJ, respectively. All isolates matched *C. fioriniae* conidial and morphological descriptions as well as 99% sequence similarity with the accessions previously described (P. Oudemans, *in press*).

Single spore isolates were stored on corn-meal agar (CMA) (Becton, Dickinson and Company, Sparks, MD). High-density sporulation was induced by streaking conidia onto clarified V8 juice agar (cV8A) (Miller 1955) with no calcium carbonate and higher concentration of agar (28 g/L). Cultures were incubated at 25˚C in the dark for seven days and spore suspensions were prepared by washing the conidia with sterile deionized water (SDW) from the high-density plates. Spore concentrations were estimated using a

hemocytometer and adjusted to either 1.0 X 10^5 or 5.0 X 10^5 conidia per ml of SDW, depending on assay.

Preparation of Floral Extracts (FE). Blueberry flowers (*Vaccinium corymbosum* L.) were collected from two fungicide free blueberry plantings at PEM during peak bloom over three growing seasons, 2013-2016. Flowers were processed using a protocol modified from Leandro et al. (2003). Prior to destructive extraction, the ovaries, sepals and peduncles were discarded and the remaining corolla, stigma, style and stamen were then divided into 10 g samples and stored up to 3 d at 4˚C until processed. FEs were prepared by grinding 10 g samples with a mortar and pestle in 90 ml SDW, a 1:9 weight to volume ratio, for 30 seconds (care was taken to not pulverize the samples). The resulting pulp sample was strained through 4 layers of sterile cheesecloth and further clarified by centrifugation for 10 minutes at 8055 x g. The supernatant was vacuum filtered through Whatman No.1 filter paper (Maidenstone, UK) and filter sterilized (CAMEO 25 GAS 0.22-µm, Osmonics Penang, Malaysia). Extracts were stored at -20˚C in 15 ml aliquots until experimental use. Blueberry FE prepared using this method included anthracnose susceptible *V. corymbosum* L. cultivars Bluecrop and Coville, moderately resistant ‘Duke’, and resistant ‘Elliot’. Flowers of four ericaceous species, were also collected in the forest surrounding PEM as well as a greenhouse grown, sterile triploid mutant of Lingonberry (*Vaccinium vitis-idaea* L.) collected from the pathology research greenhouse at PEM. Flowers from various ericaceous species were collected during the peak bloom period (early spring through early summer) for each species

examined and FE was made using methods and floral tissues described. A passive extraction was utilized in one assay. 'Bluecrop' flowers (50 g whole flowers with only peduncles removed) were placed into plastic mesh containers and sprayed with 250 ml of SDW using a standard laboratory spray/mist bottle and the collected runoff, referred to as 'Bluecrop' passive FE, was divided into 15 ml aliquots and frozen at -20˚C until use.

Bioactivity of FEs. A protocol modified from Leandro et al. (2003) was utilized to estimate biological activity of numerous FEs. *C. fioriniae* isolates, described above, were assayed for growth responses (secondary conidiation and appressorial formation) in the presence or absence of blueberry FE. Assays were conducted in 40 µl droplets placed on 22 x 22 mm glass coverslips (triple rinsed with 95% EtOH and dried prior to use). Each 40 µl droplet contained equal volumes conidial suspension (1.0×10^5 for all assays except 24 h time-course where the concentration was 5.0×10^5 conidia per ml SDW), SDW and FE, aliquoted from a previously combined solution (500 µl portions). 'Bluecrop' FE was used in this assay and for untreated controls SDW replaced FE so that conidial concentrations were consistent. Glass coverslips with droplets were incubated in plastic cell culture dishes at high humidity at 25˚C in the dark. Growth responses in the droplets were evaluated at 0 h and 24 h by first adding 15 µl of lactophenol cotton-blue (LCB) (20.0 g phenol crystals, 20.0 ml 2.5% lactic acid, 40.0 ml glycerol, 0.05 g cotton blue) to the droplet and then carefully inverting coverslips and placing them droplet side down on a glass microscope slide to facilitate microscopic examination. Observations were made across 16 microscope fields at 400x magnification, totaling an area of 3.808

mm^2. Two treatments were replicated three times in a completely randomized design and the experiment was conducted twice. A 24 h time-course assay was also conducted as previously described using one *C. fioriniae* isolate, BB#10, with evaluations every 2 h over a 24 h period and consisted of three replicates per time point per treatment in a completely randomized design. The experiment was conducted twice. In order to determine if any cultivar effects were present two susceptible cultivars, Coville and Bluecrop and two resistant cultivars, Duke and Elliot were selected (Polashock et al. 2005) and the bioactivity of the FEs on *C. fioriniae* (BB#10) were compared among cultivars following the methods described above. Evaluation of appressorial formation was made at 24 h post-inoculation and the experiment consisted of three replicates per treatment in a completely randomized design. The experiment was conducted three times. In another experiment, the FEs of four ericaceous species were evaluated for bioactivity on *C. fioriniae* (BB#10) following methods described above. Treatments included the FEs of the four ericaceous species, a SDW control, and 'Bluecrop' FE as a positive control. Evaluation of growth responses were made at 24 h post-inoculation and the experiment consisted of three replicates per treatment in a completely randomized design. The experiment was conducted twice.

Blueberry fruit inoculation. An inoculation trial was conducted to determine if FE from 'Bluecrop' (susceptible) and 'Elliot' (resistant) increased the occurrence of infection on fruit. Visually healthy 'Elliot' fruit were collected from a field at PEM that received no fungicides. This cultivar was utilized as substrate due to its low frequency of anthracnose compared to 'Bluecrop', where high background levels of *C. fioriniae* are persistent.

Plastic sealable containers (12”x5”x3”), lined with sterile, moistened paper towels and sterile plastic netting placed above towels, were used as inoculation chambers. Forty fruit placed into each container represented a single replicate. A total of five replicates were used for each of the six treatments: ‘Elliot’ fruit treated with FE alone (either ‘Bluecrop’ or ‘Elliot’), ‘Bluecrop’ or ‘Elliot’ FE plus conidia (BB#10), conidia alone, and a SDW only control. Containers were organized as a randomized complete block design and each were treated individually by misting approximately 1 ml of inoculum over the fruit using a standard laboratory pump sprayer (30 ml capacity). Containers were then sealed to promote a high relative humidity and held at room temperature (approximately 22˚C) with 12 h photoperiods. Fruit were evaluated for disease development 1 week post inoculation and the entire experiment was conducted twice.

Comparison of destructive and passive ‘Bluecrop’ FE, and rainwater runoff collection of FEs. Destructive (mortar/pestle) FE and passive (SDW spray) FEs were compared for bioactivity on *C. fioriniae* (BB#10) following methods described. Evaluation of growth responses (secondary conidiation and appressorial formation) were made at 24 h post-inoculation and the experiment consisted of three replicates per treatment in a completely randomized design. The experiment was conducted twice. Rainwater was collected from 3 ‘Bluecrop’ bushes by placing a funnel connected to a 50 ml centrifuge tube either directly beneath an inflorescence (denoted as *flower*), halfway between top of the plant and crown (*stem*), at the base of the bush (*crown*), as well as rainwater that had not contacted the bush (*ground*). The assay was performed using the same methods as previously described with evaluations at 24 h post-inoculation using *C.*

fioriniae isolate BB#10. Experiment consisted of three replicates per treatment in a completely randomized design. The experiment was conducted twice.

Sporulation on developing inflorescence buds. A total of 100 'Bluecrop' stems each containing at least 5 inflorescence buds were collected in November following leaf-drop. Five non-surface sterilized 'Bluecrop' stems were placed in plastic sealable containers (12"x5"x3") lined with sterilized wetted paper towels and sterile plastic netting. Containers were inoculated individually by misting ~1 ml of either 1:1, BC FE to SDW or SDW alone over the fruit using a standard laboratory pump sprayer (30 ml capacity). Containers were then sealed to promote a high relative humidity and held at room temperature (approximately 22˚C) with 12 h photoperiods. Data were collected on first 5 inflorescence buds from the apical end of each stem per treatment per block. Inflorescence buds were visually assessed for active sporulation at 2, 3, 4 weeks post-inoculation. Experiment consisted of three replicates per treatment, and the assay was conducted twice.

Data analyses. All analyses were conducted using CoStat version 4.0 (CoHort Software, Monterey, CA) (Cardinali 2013). For the coverslip bioactivity experiments, data from 16 microscope fields were summed for each replicate. Data were checked for normality using Bartlett's Test for homogeneity of variances and if necessary, normalized using a $\log_{10}$ transformation with a constant of 0.5 added to compensate for 0 count data. All experiments were conducted at least twice and a completely randomized 2-way ANOVA was applied using experiment and treatment as independent variables and either total

conidia or appressoria as the dependent variable. The results were expressed as the back transformed means per mm^2 of total conidia (comprised of primary and secondary conidia) or appressoria per mm^2. In cases where a significant interaction between experiment and treatment occurred, the experiments were analyzed independently using a completely randomized 1-way ANOVA. Fruit inoculation and sporulation on developing inflorescence buds were analyzed using a randomized complete block 1-way ANOVA. The results were reported as percent infected fruit and percent sporulating buds, respectively. A post hoc Fishers Least Significant Difference (LSD) test was performed for means separation.

Results:

'Bluecrop' FE stimulates an increased rate and magnitude of secondary conidiation and appressorial formation of *C. fioriniae.* Two assays were implemented to first determine the effects of 'Bluecrop' FE on multiple *C. fioriniae* isolates and a second, detailed 24 h time-course, to estimate a growth response timeline. SDW control (Fig. 1a) compared with 'Bluecrop' FE (BC FE) (Fig. 1b), where BC FE had pronounced effects on secondary conidiation and appressorial formation of all *C. fioriniae* isolates at 24 h (Table 1).

The FE was found to have three visible effects on *C. fioriniae* 1) increased secondary conidiation similar to that reported previously (Leandro et al. 2001, 2003) (Fig. 1c), 2) increased appressorial formation (Fig. 1d) and 3) increased hyphal density although not quantified in this study (Fig. 1e). In a detailed time-course experiment, the rate of secondary conidiation and appressorial formation was compared with or without

'Bluecrop' FE over 24 h using isolate BB#10. Secondary conidiation began after 6 h in the presence of FE and was significantly greater ($P = 0.0391$) than SDW treatments (Fig. 2a). Appressorial formation was also significantly greater in the presence of 'Bluecrop' FE after 6 h ($P \leq 0.0001$) (Fig. 2b). Conidia visibly produced more germ tubes leading to an overall greater hyphal density in the presence of FE as compared with conidia in SDW where conidia typically produced a single germ tube, infrequently producing appressoria sessile to conidia. In many cases, conidia in the presence of FE formed appressoria at early time points, which germinated to form additional appressoria. FE-treated conidia would ultimately form aggregates resulting in appressoria stacked upon each other by 24 h post-inoculation.

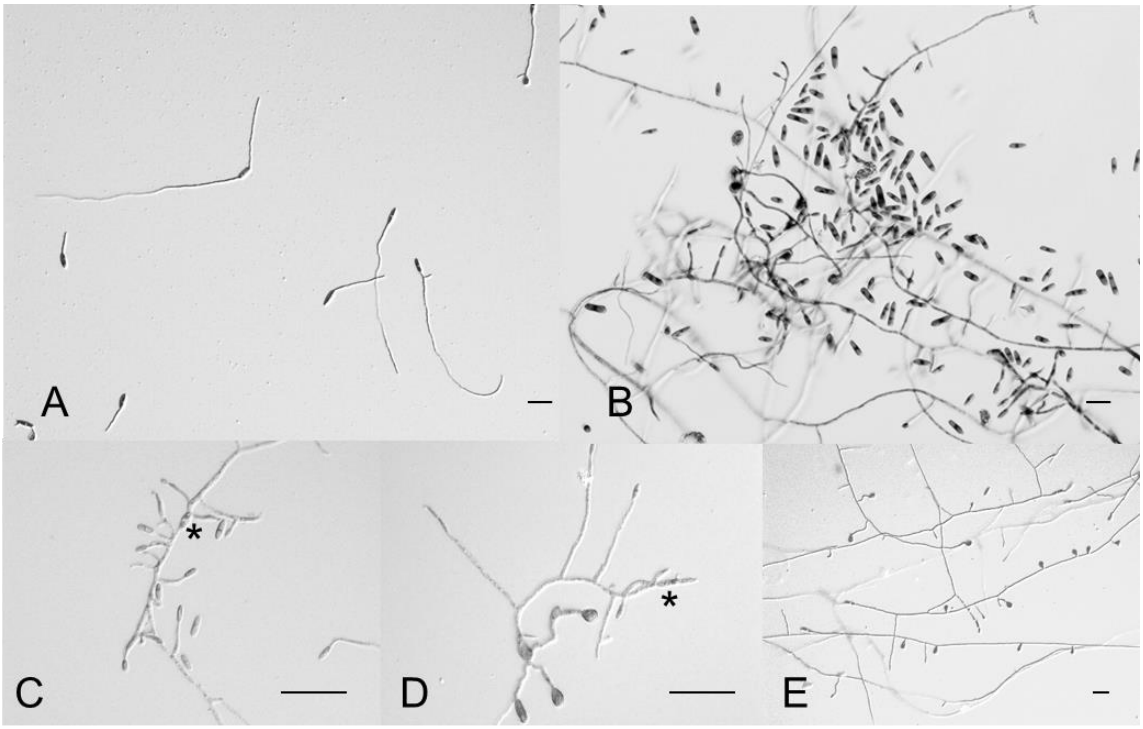

Figure 1. *Colletotrichum fioriniae* on glass coverslips with and without floral extracts at 24 h post-inoculation. **(A)** Primary conidia in sterile deionized water (SDW). **(B)** Primary and secondary conidia, with appressoria in the presence of 'Bluecrop' floral extract (BC FE). **(C)** Primary conidium (*) forming secondary conidia and **(D)** in the process of appressorial formation in the presence of BC FE. **(E)** Mycelial growth and abundant appressorial formation in the presence of rainwater collected from under a 'Bluecrop' inflorescence in the field. Scale bars: 40 µm.

Table 1. Comparison of secondary conidiation and appressorial formation with and without 'Bluecrop' floral extract (BC FE) using five *Colletotrichum fioriniae* isolates.

Isolate	Treatment	0 h Conidia [z]	24 h Conidia [z]	24 h APP [z]
BB#10	BC FE	2.9 a	37.0 a	10.7 a
	SDW	2.7 a	4.7 b	0.5 b
BB-89	BC FE	2.9 a	56.1 a	24.2 a
	SDW	2.7 a	5.4 b	0.9 b
CB-PMAP182	BC FE	3.1 a	70.3 a	23.2 a
	SDW	2.6 a	9.9 b	0.6 b
CB-PMAP184	BC FE	2.8 a	42.1 a	20.6 a
	SDW	2.7 a	4.6 b	0.4 b
CB-KPCF16	BC FE	2.5 a	45.7 a	31.0 a
	SDW	2.6 a	4.9 b	0.4 b

[z] Analysis performed on $\log_{10}$ transformed means of total conidia (denoted as Conidia, comprised of primary and secondary conidia) and appressoria coverslip count data at 0, 24 h per isolate. Values are presented as back-transformed means per mm^2, experiment was conducted twice. Letters indicate significant differences between treatments per isolate at $P < 0.05$ according to Fischer's Least Significant Difference test (LSD). Abbreviations: Sterile deionized water, SDW. Appressoria, APP.

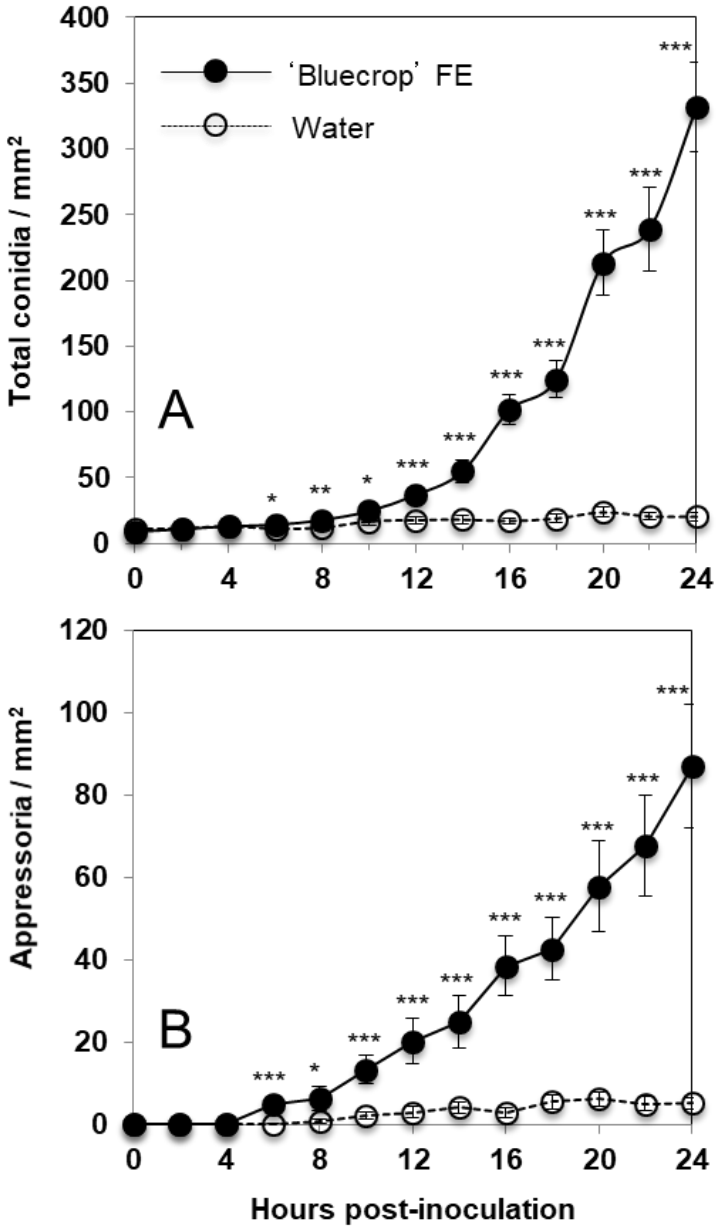

Figure 2. Time-course study of secondary conidiation and appressorial formation of *Colletotrichum fioriniae* (isolate BB#10) in the presence (●) or absence (○) of 'Bluecrop' floral extract (BC FE). **(A)** Secondary conidiation was evaluated as total conidia (primary conidia at 0 h were 9.0 and 10.8 conidia per mm^2, BC FE and water respectively, $P = 0.773$). **(B)** Appressorial formation. Asterisks indicate level of significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. Error bars indicate standard deviation of count data.

FEs of susceptible cultivars stimulated the greatest *C. fioriniae* appressorial response in vitro. Given that there is a known range of susceptibility in blueberries to anthracnose, a test was conducted to determine if FEs of susceptible and resistant cultivars affected *C. fioriniae* appressorial formation differently. FEs from susceptible 'Coville' and 'Bluecrop' induced significantly more appressoria than those of moderately resistant 'Duke', resistant 'Elliot' and the SDW control at 24 h post-inoculation (Table 2). Observations clearly demonstrated active secondary conidiation of *C. fioriniae* in the presence of all blueberry FEs evaluated. Results for each experiment are presented separately due to a significant interaction between experiment and treatment. Results from the three replicated trials all show significantly greater appressorial formation for the susceptible cultivars FEs as compared to the resistant and moderately resistant cultivars, and SDW control in vitro.

Table 2. Comparison of floral extracts from susceptible and resistant blueberry cultivars on appressorial formation of *Colletotrichum fioriniae*[x].

Cultivar	Fruit rot susceptibility [y]	Appressoria per mm^2 [z]		
		Trial 1	Trial 2	Trial 3
Coville	Susceptible	11.9 b	20.4 a	11.5 a
Bluecrop	Susceptible	20.8 a	11.8 b	11.1 a
Duke	Moderately resistant	6.5 c	3.4 c	2.1 b
Elliot	Resistant	4.8 c	3.9 c	0.3 c
SDW	…	1.7 d	0.3 d	0 c

[x] *C. fioriniae* isolate BB#10 was used in this experiment.
[y] Blueberry cultivar anthracnose susceptibility ranking based on fruit inoculations by Polashock et al.. 2005.
[z] Analysis performed on $\log_{10}$ transformed means of appressoria coverslip count data at 24 h, values are presented as back-transformed means per mm^2. Letters indicate significant differences at $P < 0.05$ according to Fischer's Least Significant Difference test (LSD).

Blueberry FE enhances *C. fioriniae* infectivity on 'Elliot' fruit. Prior assays demonstrated an increase in *C. fioriniae* appressorial formation in the presence of some FEs, leading to question if FEs could enhance *C. fioriniae* infection on sound, fungicide free, 'Elliot' blueberry fruit. *C. fioriniae* (BB#10) conidia in the presence of either susceptible 'Bluecrop' or resistant 'Elliot' FE induced significantly higher levels of disease as compared with treatments lacking FE (Table 3). FE without conidia and conidia without FE produced similar levels of disease according to a LSD test with an alpha of 0.05. Data were collected at 1 week post-inoculation and were analyzed separately due to interaction effects between experiment and treatment, however, both trials followed the same general trend of increased infection with conidia in the presence of FEs.

Table 3. Impact of floral extracts (FE) on percent infection of 'Elliot' blueberry fruit by *Colletotrichum fioriniae*[y].

FE	*C. fioriniae*	% Infection at 1 week [z]	
		Trial 1	Trial 2
Bluecrop	no conidia	29.5 b	38.5 b
Elliot	no conidia	35 b	38.5 b
Bluecrop	conidia	51.5 a	64 a
Elliot	conidia	45.5 a	69.5 a
none	conidia	32 b	42 b
none	none	3.5 c	22 c

[y] *C. fioriniae* isolate BB#10 was used in this experiment.
[z] Analysis performed on percent infected fruit showing signs of sporulation at 1 week post-inoculation. Letters indicate significant differences at $P < 0.05$ according to Fischer's Least Significant Difference test (LSD). Trial 1; block $P = 0.2556$. Trial 2 (later in the season); block $P = 0.3373$.

Bioactive stimulants can be captured through passive extraction and blueberry flower rainwater runoff. Two extraction types, destructive and passive, were compared to determine whether or not FEs could be moved passively into water without the need for tissue maceration. ‘Bluecrop’ passive FE, prepared by mimicking a wetting event such as rainfall, stimulated greater secondary conidiation than the SDW control, although not to the same extent observed with destructive ‘Bluecrop’ FE obtained with mortar and pestle (Fig. 3a). This same trend was also observed for appressorial formation where destructive ‘Bluecrop’ FE stimulated 42.2 appressoria per mm^2, followed by ‘Bluecrop’ passive FE (15.4 appressoria per mm^2) and SDW control (5.1 appressoria per mm^2), demonstrating the movement of stimulatory compounds by non-destructive means. Rainwater collected from ‘Bluecrop’ bushes directly beneath inflorescence (*flower*), halfway between inflorescence and crown (*stem*), base of bush (*crown*) and virgin rainwater collected away from the bush (*ground*) were compared with SDW and showed different levels of stimulatory effects on conidia (Fig. 3b). Secondary conidial production and appressorial formation decreased as rainwater collections were further removed from flowers. Comparing the rainwater (*ground*) and SDW treatments there was no significant increase of appressorial formation and only a slight increase of secondary conidiation in the virgin rainwater.

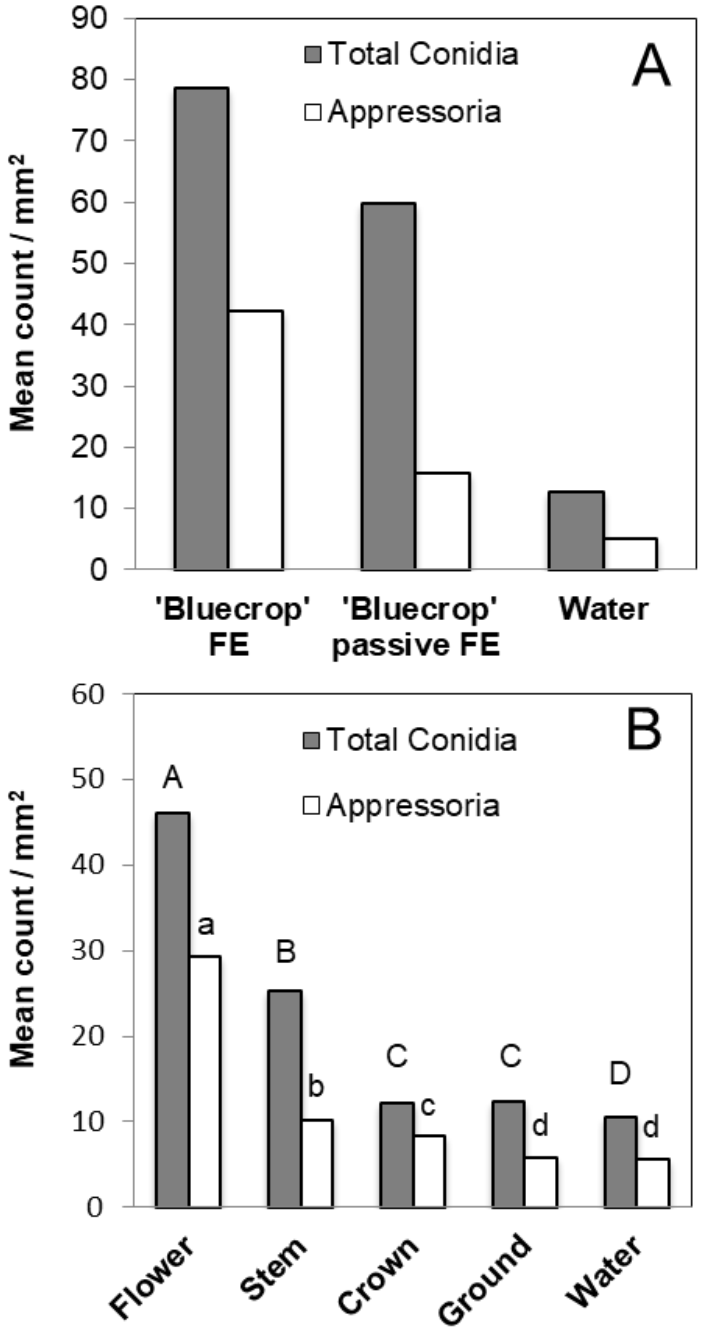

Figure 3. Results of bioassays to detect activity of floral extracts (FE) collected from different sources on *Colletotrichum fioriniae* (isolate BB#10) at 24 h post-inoculation. Secondary conidiation and appressorial formation increased in the presence of passive FEs. **(A) '**Bluecrop' passive FE performed similarly to standard 'Bluecrop' FE although with a lower response. **(B)** Activity of rainwater collected at increasing distances from 'Bluecrop' inflorescences. Letters indicate significant differences at $P < 0.05$ according to Fischer's Least Significant Difference test (LSD), uppercase, total conidia; lowercase, appressoria.

Multiple ericaceous species FEs stimulate *C. fioriniae* secondary conidiation and appressorial formation. FEs from five ericaceous species including *Kalmia latifolia* L. (mountain laurel, native), *Lyonia mariana* (L.) D. Don (staggerbush, native), *Rhododendron maximum* L. (rhododendron a.k.a. great laurel, native), *Vaccinium macrocarpon* Aiton (cranberry, native) and *V. vitis-idaea* L. (lingonberry, non-native) were compared with *V. corymbosum* L. ('Bluecrop') FE for their effects on secondary conidiation and appressorial formation. FE from four of the five non-blueberry sources stimulated significantly more secondary conidiation than the SDW control. The sole exception was FE from a greenhouse grown, sterile triploid mutant of *V. vitis-idaea* L. (Table 4). FE from blueberry, 'Bluecrop', and the related species *V. macrocarpon* Aiton (cranberry), consistently had the highest level of secondary conidiation compared with extracts from the other plant species. Likewise, with the exception of *V. vitis-idaea* L.*,* FEs from the different plant species significantly increased appressorial formation when compared with the SDW control (Table 4). Data from each experiment were analyzed independently due to considerable variation in secondary conidiation between trials.

Table 4. Comparison of floral extracts (FE) from select species in *Ericaceae* on secondary conidiation and appressorial formation of *Colletotrichum fioriniae*[x].

FE [y]	24 h Conidia [z]		24 h Appressoria [z]	
	Trial 1	Trial 2	Trial 1	Trial 2
Vaccinium corymbosum	89.4 a	350 a	11.9 ab	13.4 a
V. macrocarpon	72.3 a	174 b	4.7 abc	2.5 b
Kalmia latifolia	59.7 a	78.1 d	15.1 a	8.6 a
V. vitis-idaea	42.4 ab	95.1 cd	4 bc	1.6 bc
Lyonia mariana	54.2 a	76.2 d	10.4 ab	8.9 a
Rhododendron maximum	72.9 a	126 c	10.5 ab	10.6 a
SDW	13.7 b	11.8 e	2.9 c	0.6 c

[x] *C. fioriniae* isolate BB#10 was used in this experiment. [y] Flowers collected in Chatsworth, NJ over two growing seasons (2014, 2015). 'Bluecrop' FE was utilized for *V. corymbosum*. [z] Analysis performed on log-10 transformed means of total conidia (denoted as Conidia, comprised of primary and secondary conidia) and appressoria coverslip count data at 24 h. Values are presented as back-transformed means per square mm, experiment was conducted twice. Letters indicate significant differences at $P < 0.05$ according to Fischer's Least Significant Difference test (LSD).

FE increases *C. fioriniae* sporulation on developing blueberry inflorescence buds. To determine if flowers could influence sporulation of overwintered appressoria, stems with dormant inflorescence buds were treated with 'Bluecrop' FE and were evaluated at 2, 3, and 4 week post-inoculation for active sporulation. Stems examined at 2 week post-treatment in the first trial showed a significant increase in sporulation in the presence of FE compared with SDW ($P = 0.016$), but as time further progressed, floral buds were beginning to expand and sporulation was abundant in both treatments (Table 5). In a second experiment, inflorescences had begun to emerge and sporulation was not significantly different. Results were analyzed independently due to interaction effects between experiment and treatment.

Table 5. Effect of 'Bluecrop' floral extract (BC FE) on conidiation of native *Colletotrichum fioriniae* from dormant 'Bluecrop' inflorescence buds.

Trial	Time (weeks)	Treatment [y]	% Sporulation [z]	*P*-value
1	2	BC FE	2.8	0.016*
		SDW	0	
1	3	BC FE	9.6	0.120
		SDW	4.8	
1	4	BC FE	14.0	0.909
		SDW	12.8	
2	2	BC FE	3.2	0.609
		SDW	4.4	
2	3	BC FE	3.6	0.319
		SDW	4.8	
2	4	BC FE	3.6	0.609
		SDW	5.6	

[y] Note; no conidia were added for this experiment.
[z] Analysis performed on mean percent sporulating inflorescence buds at designated time points, for both trials separately. Asterisk indicates significance at $P < 0.05$. Inflorescence buds were from non-fungicide treated plots over two growing seasons. Time indicates duration of floral extract on inflorescence buds. Abbreviations: Sterile deionized water, SDW.

Discussion:

Extracts from blueberry flowers were found to affect all *Colletotrichum fioriniae* isolates evaluated in this study similarly. The primary effects were increased rate and magnitude of both secondary conidiation and, unique to this study, appressorial formation. Furthermore, an increase of fruit infection was observed with *C. fioriniae* conidia in the presence of FEs. Interestingly, appressorial formation was influenced by cultivar, with FEs from susceptible varieties inducing more appressoria in vitro than those of resistant varieties. The activity of FE was detected in both simulated rainfall and natural rainwater collected from flowers in the field. The observations collectively provide strong evidence that FEs likely affect the disease cycle of *C. fioriniae*.

Previous studies have demonstrated increased secondary conidiation of *C. acutatum* (*sensu lato*) in response to floral components in the strawberry anthracnose pathosystem (Leandro et al. 2003) as well as sporulation of persistent calyces (buttons) in citrus post-bloom fruit drop (MacKenzie et al. 2010), both resulting in greater disease. In this study, blueberry FEs not only increased inoculum but also had decided effects on appressorial formation.

FEs affected secondary conidiation and appressorial formation differently, suggesting that their induction may be due to different floral compounds. All blueberry cultivars stimulated secondary conidiation equally, suggesting a nutritional role of the FEs. MacKenzie et al. (2010) demonstrated increased propagule formation in response to 2.5% sucrose solutions, a common floral componet. However, differences in host genotype were found here to influence the pattern of appressorial formation and this may be related to other components such as long chain fatty acids (Gager 2015; Podila et al.

1993). FE from susceptible ‘Bluecrop’ increased the rate of appressorial formation, suggesting some floral components strongly influence the infection period and may shorten the time needed for a successful infection to occur. Previous research concluded that a minimum of 10 to 12 h of continual wetness is required for blueberry fruit infections at 11 to 27˚C (Hartung et al. 1981; Verma et al. 2007). Since those studies were conducted in the absence of floral tissues, the estimated minimum wetness period may be too conservative during the blueberry bloom period. Dieguez-Uribeondo et al. (2011) reported a dramatically shorter wetness period required to establish infection on almond blossoms (3 h at 15-20˚C) compared to almond leaves (24 h at 15-20˚C) when inoculated with *C. acutatum,* suggesting that floral infections occur more rapidly. In light of the results presented here, we hypothesize that floral stimulation likely reduces infection period time requirements.

The rainwater collections provide evidence that the stimulatory compound(s) originate from blueberry flowers and can be mobilized in rainwater. The mobility of FEs in water under field conditions suggest that stimulatory effects may extend to infection courts located on the fruit pedicel, on fertilized ovaries where petals have already dropped as well as other susceptible tissues. This is similar to floral compounds being “washed” or mobilized from citrus flowers, over some distance, onto the persistent calyces and stimulating the production of conidia (MacKenzie et al. 2010). Thus, FEs carried in rainwater could affect multiple inoculum reservoirs and synchronize *C. fioriniae* sporulation on dead twig tips as well as on developing blueberry inflorescences (DeMarsay 2005; Miles et al. 2013; Milholland 1995; Wharton 2002).

Resistance to anthracnose in highbush blueberry has been examined by comparing infection and colonization on resistant ‘Elliot’ and susceptible ‘Jersey’. Wharton and Schilder (2008) demonstrated significant differences in the time needed to cause infection in the resistant cultivar and Miles et al. (2011) supported these findings. In this current study all blueberry FEs stimulated secondary conidial production, however, the FEs from susceptible ‘Coville’ and ‘Bluecrop’ stimulated significantly more appressorial production at 24 h post-inoculation as compared with the more resistant cultivars ‘Duke’ and ‘Elliot’. Equal levels of disease were recorded on ‘Elliot’ fruit in the inoculation assay, indicating that *C. fioriniae* conidia in the presence of either FE over long infection periods (greater than 24 h) are able to produce sufficient appressoria and secondary conidia for successful infections to occur. Cultivar susceptibility may be related to altered infection period duration in response to the FEs of susceptible and resistant cultivars. If FE from susceptible varieties promote shorter infection periods, then there would be a greater number of overall infection periods, ultimately leading to higher levels of disease. *C. acutatum* (*sensu lato*) has been shown to overwinter in the inflorescence buds of blueberry (DeMarsay 2005) as well as several other plant species including apple (along with *Colletotrichum gloeosporioides)* in Brazil (Bernardi et al. 1983), mulberry in Japan (Yoshida and Shirata 1999), sour cherry and sweet cherry in Norway (Borve and Stensvand 2006). This overwintering reservoir is not unique to *Colletotrichum* species as other plant pathogens such as *Xanthomonas arboricola* pv. *julandis* also overwinter in flower buds, where there is a strong correlation of bacterial population size in dormant floral buds with disease incidence. (Lindow et al. 2014). This close proximity of primary inoculum with the developing floral tissues provides an opportunity for movement of

chemical signals from flowers to the dormant pathogen, highlighting a possible evolutionary relationship.

C. fioriniae is also responsible for severe losses in *V. macrocarpon* and preliminary data (not presented) suggest that cranberry FE also stimulates important members of the cranberry fruit rot pathosystem (*C. fioriniae, C. fructicola,* and *Coleophoma empetri*) (Waller 2014). In agreement with this, all of the cranberry isolates evaluated in this study were dramatically affected by blueberry 'Bluecrop' FE, indicating similar disease cycle components in the cranberry *C. fioriniae* pathosystem. FEs from *Kalmia latifolia* L., *Lyonia mariana* (L.) D. Don, *Rhododendron maximum* L.*, V. corymbosum* L.*,* and *V. macrocarpon* Ation*,* stimulated both secondary conidiation and appressorial formation of *C. fioriniae*, suggesting some commonality amongst these FEs across members of Ericaceae. In addition to these findings, floral infections by *C. acutatum* (*sensu lato*) have been documented on numerous other host genera where flowers have been implicated in other aspects of the disease cycle beyond blossom necrosis (Adaskaveg and Hartin 1997; Jeffries et al. 1990; Maas 1984; Wharton and Diéguez-Uribeondo 2004; Zulfiqar et al. 1996).

This research raises several questions regarding the role of floral components in both secondary conidiation and appressorial formation. For example ontogenic resistance, the process of changing susceptibility over time during development, may in part be influenced by the presence of flowers (Cooley et al. 1996; Ficke et al. 2002). In agreement with this, our results suggest stimulatory floral compounds alter the infectivity of the pathogen by providing less stringent conditions for successful infection during bloom. This research identifies the importance of water-mobilized floral components

during the initial stages of conidiation and appressorial formation of *C. fioriniae* and indicates the need for floral information in the development of effective IPM strategies.

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Chapter II

Colletotrichum fioriniae development in water and chloroform-based blueberry and cranberry floral extracts

Introduction:

Note: this chapter was written and formatted to accompany an open source video protocol, found at: (https://www.jove.com/video/58880/colletotrichum-fioriniae-development-water-chloroform-based-blueberry).

Colletotrichum fioriniae causes a fruit rot of both Highbush Blueberry (*Vaccinium corymbosum* L.) and the large American Cranberry (*V. macrocarpon* Aiton) (Oudemans et al. 1998; Pszczółkowska et al. 2016). This pathogen was recently delineated from the *C. acutatum* species complex (Damm et al. 2012; Marcelino et al. 2008; Pennycook 2017; Shivas and Tan 2009) and is a causal agent of blueberry anthracnose and a member of the cranberry fruit rot complex, in addition to causing numerous other plant diseases worldwide (Wharton and Diéguez-Uribeondo 2004). *C. fioriniae* has a latent, hemibiotrophic lifestyle, with infections occurring during bloom and symptom development not becoming apparent until the fruit are in final stages of maturation (Polashock 2017; Prusky et al. 2013). In blueberry and cranberry, fruit rot is only adequately controlled with fungicide applications made during the bloom period. The pathogen overwinters in dormant blueberry floral bud scales (DeMarsay 2005) and sporulates during bloom. Conidia are moved throughout the canopy via rain-splash dispersal (Madden et al. 1996; Yang et al. 1990) and inoculum buildup has been strongly

correlated to the bloom period (Wharton 2002). Response of *Colletotrichum* species to host flowers is not unique to *Vaccinium* spp., as flowers are important components of citrus post bloom fruit drop (PFD) (MacKenzie et al. 2010) as well as strawberry anthracnose (Leandro et al. 2003), in both cases causing the pathogen to sporulate. All of these cases highlight the need for effective methods to evaluate the temporal dynamics of floral chemical cues on *C. fioriniae* and other pathogens that infect during bloom. The insights provided by the methods described here are becoming increasingly more valuable.

This protocol details methods of floral extract (FE) procurement, and guides the evaluation of *C. fioriniae* responses to FE via glass coverslip bioassays (Gager 2015; Waller et al. 2018). The floral extraction techniques are broken into two main types; water-based extractions (*active*-FE, *passive*- (*pass*-FE), field rainwater-based (rw-FE)), and chloroform-based (ch-FE) extractions. The water-based extractions allow for inspection of water mobilized floral chemical cues. These mobilized cues are likely important components of the infection court since FE greatly increases the speed of infection (Waller et al. 2018), in addition to providing the moisture required for the infection to occur. Additionally, they represent a more natural condition as floral stimulation can be washed throughout the canopy during wetting-events as previously observed in blueberry and other crop systems (MacKenzie et al. 2010; Waller et al. 2018). Chloroform-based floral extractions (ch-FE) also provide valuable information pertaining to pathogen response to host surface waxes (Gager 2015; Podila et al. 1993), elucidating the early growth stages of conidia once deposited onto susceptible host organs (i.e. flowers, ovaries and developing fruit). Pathogen response to seasonal changes in host

surface waxes can also be monitored using this protocol. Accordingly, the bioassays are tailored to working with either water-based FE or chloroform-based FE to mitigate the inherent differences between these two materials.

The data generated from the bioassays revealed that water-based extractions stimulate higher levels of secondary conidiation than chloroform-based extractions where there was a definitive appressorial response, therefore implicating multiple compounds present in the FE. Interestingly, both of these growth responses were observed when using rainwater that had run off of blueberry and cranberry flowers, indicating multiple stimulatory compounds can be washed from the surface of flowers. Thus, monitoring for floral stimulation will provide insight into the probability of pathogen success in an agricultural system.

The ultimate goal of this protocol is to provide a methodology for generating baseline biological information on fungal plant pathogens in response to floral chemical cues, as well as initiating methodologies that can utilize this floral information to aid in site-specific disease management and decision making processes.

Protocol:

1) Fungal isolates and spore suspensions

1.1) Isolate *Colletotrichum fioriniae* from a naturally infected host (Polashock et al. 2009). Then store the clean cultures on corn meal agar (CMA) slants. Place plug of culture (from CMA slant) onto a standard plastic cell culture dish (9 cm diameter) containing either clarified V8 juice agar (cV8A) (modified from Miller 1955), or non-clarified V8 juice agar (V8A) (Miller 1955). When colonies begin to sporulate, streak

conidia (orange conidial masses) onto another cV8A or V8A containing cell culture dish (with a standard sterile loop) to produce a high-density sporulating culture.

1.1.1) Any protocol steps with fungi should be performed in a Laminar flow hood to reduce the possibility of contaminating fungal cultures and/or bioassays.

1.2) After 7 days of growth, using a standard sterile loop gather a small amount of conidia from the high-density culture (by lightly touching the loop to the conidial mass) and stir this into a 15 ml centrifuge tube containing 10 ml of sterile deionized water (SDW). Vortex this sample for 10 s, then using a standard pipette plunge up and down numerous times to further mix the sample.

1.3) Then, place a drop of the vortexed sample onto the hemocytometer and estimate spore concentrations. Count 5-10 fields on the hemocytometer, obtain the average, and multiply average by the appropriate dilution factor (i.e. 10,000), thus obtaining the conidial concentration per ml SDW.

1.4) Then using a volume (V)/concentration (C) equation (V1•[C1]=V2•[C2]) adjust (with SDW) to 1.0 X 105 conidia per ml of SDW with a 5 ml final volume. This is referred to as the spore suspension and is only made immediately prior to use in either bioassay.

2) Active, water-based floral extracts (active-FE) (Leandro et al. 2003; Waller et al. 2018). *(Supplemental Figures 1-3, Supplemental Movie 1)*

2.1) Carefully hand-collect blueberry (April-May) and cranberry (June-July) flowers during their respective peak blooms in the field (supplemental Figure 1). Wear and change nitrile gloves between sampling locations (cultivars/varieties/physical locations)

to inhibit human skin oil contamination as well as cross contamination between floral varieties. Place either blueberry or cranberry flowers (from a single source) into plastic bags (fill 100 mm X 150 mm bag) and immediately refrigerate at 4˚ C once flowers are collected.

2.2) The active- extraction is modified from Leandro et al. (2003). Prior to extraction, remove any deteriorated, damaged, diseased flowers, then using healthy flowers remove the ovaries, sepals and peduncles with curved forceps (45˚ tweezers) and discard. Use only the remaining organs (corolla, stigma, style and stamen) for active- extractions. Cut cheesecloth (150 mm X 150 mm) and place within a 7 x 7 mm funnel, which is then placed into a clean 50ml centrifuge tube, set aside.

2.3) Combine 1 part processed flowers to 9 parts SDW (1 wt : 9 vol ratio) in a mortar. Gently grind with a pestle for 30 s (take care to not completely pulverize the samples). Strain resulting pulp through prepared cheesecloth/funnel and collect in centrifuge tube (50 ml). Clean or use new mortar/pestle between each extraction with 95% EtOH and warm running water.

2.4) Prepare a vacuum filtration apparatus; gather Buchner funnel, vacuum filter Erlenmeyer flask (up to 1000 ml capacity), Watman No1 Qualitative 55 mm circle filter paper, vacuum hose/source. Add filter paper to an appropriate sized Buchner funnel and place atop the flask, then connect apparatus to a water / suction source via vacuum hose, set aside.

2.5) Further clarify the blueberry floral extracts by centrifuging for 10 min at 8055 x g. Pour off supernatant into prepared vacuum filter apparatus, turn on vacuum source, filter supernatant then pour flask contents into a new centrifuge tube (50 ml). Clean all

apparatus components between each filtration with 95% EtOH and warm running water. Further filter through a syringe adapted with a 0.22 µm pore size, acetate sterilizing filter into a new centrifuge tube (50 ml).

2.5.1) For the cranberry floral extracts, only vacuum filter through No1 55 mm circle filter paper and pour flask contents into a new centrifuge tube (50 ml).

2.6) Resulting preparation is referred to as active- floral extract (active-FE). Store all water-based floral extracts (active-, passive-, rainwater collections) at -20˚ C in 5-50 ml aliquots until experimental use. Repeat extractions with multiple samples (at least 3 extractions per sample type) to provide replicates.

3) Passive, water-based floral extracts (pass-FE) (Waller et al. 2018)

(Supplemental Movie 2)

3.1) Hand collect whole blueberry flowers (50 g) as above, refrigerate after collecting. Prior to extraction, remove any deteriorated, damaged, diseased flowers and remove only the peduncles from intact flower. Refrigerate prepared samples until the passive-extraction system, described below, is in place.

3.2) Rinse all components prior to use with 95% ethanol (EtOH) and warm running water to prevent contamination (plastic mesh sheet, two plastic mesh baskets, glass bread pan, and pump mist bottle). Also prepare a 4 layer cheesecloth/funnel/50 ml centrifuge tube described above, for each extraction, as described above (in step 2.2), set aside.

3.3) Prepare the sieve; place a plastic mesh sheet (aka clear bar matting) into one plastic mesh basket (114 mm X 102 mm). Place a second, identical, mesh basket upside down (inverted) into a glass bread-pan (127 mm X 229 mm) (to avoid flowers sitting in SDW)

and place the mesh sheet containing basket atop. Add 50 g of prepared flowers into the sieve.

3.3.1) Alternatively, small test tube [cleansing] baskets can be used in place of the originally used plastic mesh baskets (old, green, strawberry mesh pint baskets are often difficult to source).

3.4) Evenly mist flowers with 250 ml using a pump mist bottle and capture runoff in the glass bread-pan. Then strain filtrate through the prepared cheesecloth/funnel into a clean centrifuge tube. Resulting preparation is referred to as passive- floral extract (pass-FE), store as per above (step 2.6).

3.5) Clean all components between each extraction with 95% EtOH and warm running water. Repeat extractions with multiple samples to provide replicates (at least 3 per sample type).

4) Chloroform-based floral extracts (ch-FE) (Gager 2015)

4.1) Collect blueberry and cranberry flowers as per above (step 2.1), and prepare flowers for extraction (step 2.2, without cheesecloth/funnel preparation). Keep prepared flowers refrigerated until prior to use.

4.1.1) Any work performed with chloroform must be performed in a fume hood for safety reasons. This includes preparation of materials / glassware, extraction procedures, and bioassay conductance (steps using ch-FE).

4.2) Clean all components described below with 95% EtOH twice, then twice with chloroform to prevent contamination for each extraction; threaded glass culture tubes, 2 glass beakers, small stainless steel screen, and a [glass] graduate cylinder, set aside to dry

(upside-down). Rinse polytetrafluoroethylene (PTFE) lined caps twice with 95% EtOH only (chloroform will damage the outer materials of the cap) and set aside to dry.

4.3) Combine 1 part processed flowers to 9 parts chloroform (1 wt : 9 vol ratio) in a beaker (flowers then chloroform), gently swirl for 30 s, and strain through a stainless steel screen into the second beaker. Pour ch-FE from the second beaker into the glass culture tube (10 - 15 ml) and affix the PTFE cap. Wrap the cap with parafilm to prevent evaporation.

4.4) This preparation is the chloroform-based floral extract (ch-FE). Store sample in darkness (to reduce light degradation) at 4˚ C until experimental use. Repeat extractions with multiple samples to provide replicates (at least 3 per sample type).

5) Collection of rainwater from blueberry flowers (BB rw-FE) (Waller et al. 2018)

5.1) The blueberry floral rainwater collection device consists of an air spray gun disposable paint spray cup with connection adapter (cup: female thread, adapter: male to male thread), 50 ml centrifuge tubes (polypropylene), parafilm and plastic coated wires (standard telephone wire, individual internal wire strand contents).

5.2) Select multiple locations within a blueberry bush to capture rainwater run off of flowers prior to creating collection devices. These include directly under inflorescences (flower) to the very base of the bush (crown). Record diameter of the stems (ranging from 1-5 cm) at selected locations, as this will dictate the size of holes used for device attachment, described below.

5.3) For each selected location create a collection device. First drill a hole at the bottom of a spray cup (where the cup curves towards the threaded opening) to the corresponding

stem diameter, with a step-bit attached to a drill press. Then, cut a straight line from the top of the hole to the mouth of the spray cup. Drill 4 equidistant holes (large enough to thread the plastic coated wire), at the mouth of the spray cup, attach one end of the plastic coated wires leaving one end free.

5.4) Drill a hole large enough to thread the paint sprayer cup adapter into a 50 ml centrifuge cap lid. Seal adapter threads with parafilm to prevent leaking. Attach this to both the centrifuge cap and threaded portion of the sprayer cup. Attach the mated 50 ml centrifuge tube.

5.5) Repeat steps 5.3-5.4 to create multiple devices as per selected locations, a minimum of 4 collection devices per sampling location.

5.6) Deploy devices at selected locations by flexing sprayer cups to fit onto stems. Orient the cut-side of the spray cup upwards using the plastic coated wires attached to other stems (to insure water passing over flowers is captured). Affix parafilm any openings that could leak rainwater. (Supplemental Figures 3-6).

5.7) Label collection tubes with deployment date / time. After a rain-event remove and replace the bottom (tube) portion of the centrifuge tube (label date / time of collection). Bring runoff collections (referred to as blueberry rainwater floral extract (BB rw-FE)) inside and vacuum filter (filtration described in steps 2.4-2.5). Store as above (step 2.6), until used in a water-based bioassay.

6) Collection of rainwater from cranberry flowers (CB rw-FE)

6.1) The floral rainwater collection device in cranberry consists of a 7 X 7 cm polypropylene funnel, 50 ml centrifuge tubes (polypropylene), parafilm, and 4 standard, plastic coated twist ties (per device).

6.2) First, heat puncture 8 equidistant holes (diameter of twist ties) around the mouth of the funnel using a metal probe. Insert twist ties into 4 holes. Attach the other ends to that holes' opposite location, forming a neat crossing pattern. Wrap funnel down-stem with parafilm and set aside.

6.3) Drill a hole large enough to insert the funnel down-stem into a 50 ml centrifuge cap with a step-bit. Insert prepared funnel into the centrifuge cap. Repeat steps 6.2-6.3 to create multiple devices, a minimum of 4 collection devices per sampling location.

6.4) Deploy labeled (date/time) devices into selected cranberry bogs. Neatly tuck two flower bearing inflorescences (known as uprights) under the crossed plastic twist ties. Then vertically orient the device by piercing the centrifuge tube into the cranberry canopy *(Supplemental Figures 7-8, Supplemental Movie 3).*

6.5) After rainfall or overhead irrigation remove and replace the bottom (tube) portion of the centrifuge tube (label date / time of collection). Bring runoff (referred to as cranberry rainwater floral extract (CB rw-FE)) inside and vacuum filter (filtration described in steps 2.4-2.5). Store as above (step 2.6), until used in a water-based bioassay.

7) Bioassay using water-based floral extracts (Leandro et al. 2003; Waller et al. 2018) (active-FE, pass-FE, rw-FE) (Figure 1)

7.1) this bioassay is prepared in a Laminar flow hood. Prepare materials; triple rinse glass coverslips with 95% EtOH and air dry, put aside. Cut paper towel disks to the internal diameter of a standard plastic cell culture dishes (9 cm). Place 2 layers of paper disks within culture dishes and soak with 2 ml SDW.

7.2) Prepare at least 5 ml of a 1.0 X 105 conidia per ml of SDW spore suspension (*C. fioriniae*) as per above (step 1.2-1.4), set aside. Next, mix equal volumes of SDW and water-based floral extract in 2 ml microcentrifuge tubes. Then, add an equal volume of the spore suspension to the prepared 2 ml microcentrifuge tubes (SDW plus FE), resulting preparation is referred to as an aqueous treatment mixture. For the control, omit FE portion and replaced with SDW, to keep conidial concentrations consistent.

7.2.1) Note portion size is dependent on number of replicates and time-points. Typically portions do not exceed 500 µl. Once conidia and FE are combined the bioassay has begun; 0 h post-inoculation.

7.3) Place the pre-cleaned glass coverslips on top of the soaked paper towels within the cell culture dish. Place a 40 µl droplet of aqueous treatment mixture onto the center of a coverslip. Repeat for desired treatments, including the control, close the cell culture dish. Repeat in separate cell culture dishes for replications (at least 3) and time points (each dish is for 1 time point). Once all treatments and replicates have been dispensed, place all replicated cell culture dishes into a sealed plastic container (30 mm X 13 mm X 7 mm) and incubated at 25˚ C in the dark.

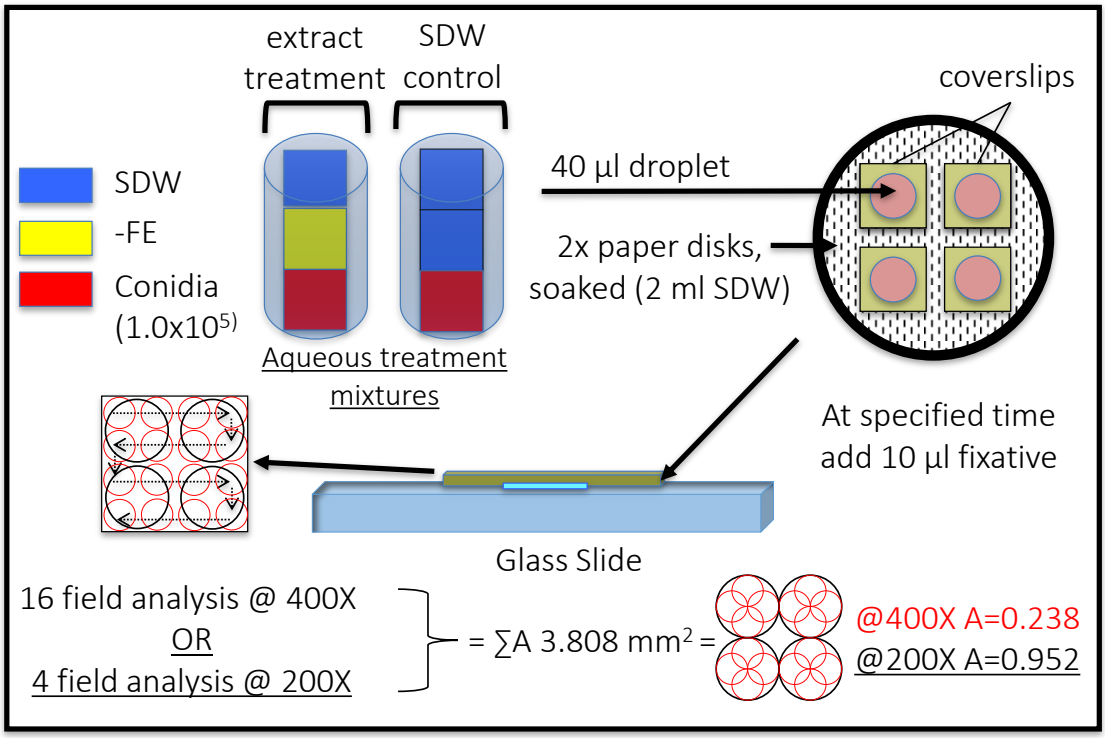
7.4) At predetermined time-points, add 10 µl of a fixative like lactophenol cotton-blue (20.0 g phenol crystals, 20.0 ml 2.5% lactic acid, 40.0 ml glycerol, 0.05 g cotton blue) to the droplets, stopping growth and semi-preserving the mount.

7.4.1) Wait 2 h to collect the 0 h time point as this allows conidial adhesion to the glass surface but is not long enough for conidial germination. For all other time points add fixative at corresponding time.

7.5) Once a fixative has been added, carefully invert the coverslips, placing them droplet side down on a glass microscope slide to facilitate microscopic examination (1 coverslip per slide). When all coverslips are on the glass microscope slides, leave them to settle and partially dry in the flow hood for 20 min.

7.6) Count all conidia (total conidia) present on the coverslip (primary conidia, germinated conidia, and newly formed secondary conidia) as well as appressoria at either 400x magnification (counting 16 fields) or 200x (counting 4 fields), totaling an area of 3.808 mm^2. Replicate entire bioassay for statistical analysis. For assays using water-based FE, analyze data as described in Waller et al. (2018), typically displaying as mean count / mm^2 (total conidia or appressoria).

(Figure 1)



General overview of the water-based floral extract (FE) bioassay (Figure 1). This assay was utilized for water-based floral extracts with both blueberry and cranberry flowers; *active*- floral extracts (*active*-FE), *passive*- floral extracts (*pass*-FE) and floral rainwater runoff (rw-FE). The -FE portion typically constitutes the experimental / variable factor. Conversely the -FE portion can remain constant and time points/hours post-inoculation can be evaluated. Preference has been made towards 4 field analysis at 200x magnification. Abbreviations. Sterile deionized water, SDW. Area per field of view, A.

8) Bioassay using chloroform-based floral extracts (ch-FE) (Gager 2015)

(Figure 2)

8.1) Prepare materials and cell culture dishes as per above (steps 7.1). Additionally, rinse an equal number of Van Tieghem cells (VanT. cells) (8 mm OD, 6 mm ID) to coverslips, as well as a glass pipette (1 ml with 1 µl increments) within a fume hood, (twice with 95% EtOH then twice with chloroform) set aside.

8.2) In laminar flow hood, using a 2 ml microcentrifuge tube add two equal volumes (at least 500 µl portions) of SDW and 1 volume of spore suspension, set aside. This is the aqueous treatment mixture for ch-FE bioassays.

8.3) In a fume hood, place a VanT. cell onto a glass coverslip within the prepared plastic cell culture dish. Dispense (with glass pipette) 33 µl of desired ch-FE into the center of the Van T. cell (do not touch the walls of the VanT. cell), for the control treatment, add virgin chloroform, allow to dry. Repeat in separate cell culture dishes for replications (at least 3).

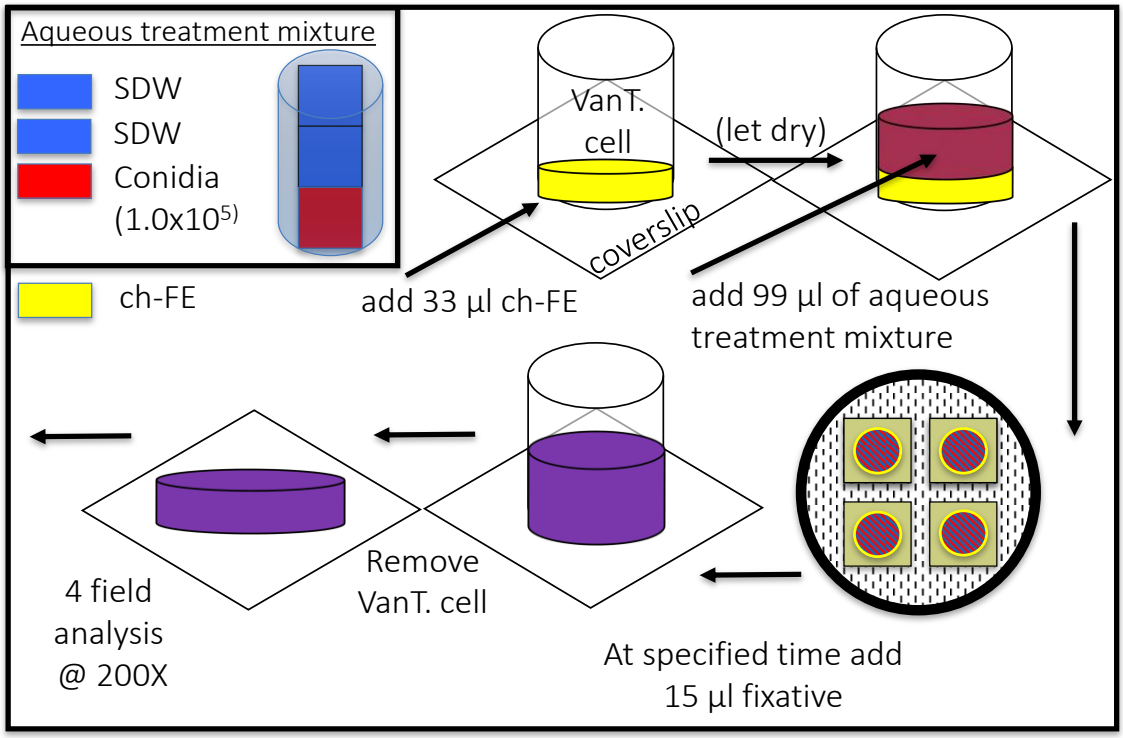
8.4) Once ch-FE has dried, dispense 99µl of the prepared aqueous treatment mixture with a standard pipette into the center of the VanT. cell, then close all cell culture dishes. Once the aqueous treatment mixture has come in contact with the dried ch-FE treatments, the bioassay has begun; 0h post-inoculation.

8.5) Once all treatments and replicates have been dispensed, place all (closed) cell culture dishes into a sealed plastic container (30 mm X 13 mm X 7 mm) and incubated at 25˚ C in the dark.

8.6) At predetermined time-points, add 15 µl of a fixative (lactophenol cotton-blue) to the VanT. cell and let sit for at least 5 min to insure adequate fungal staining. After that time,

carefully remove the VanT. cell and follow coverslip inversion and data acquisition steps above (steps 7.5-7.6). For data analysis, follow methods described in Gager 2015, typically displaying data as appressorium formation, which is the ratio of total conidia to appressoria counted in the area observed (3.808 mm2).

(Figure 2)



General overview of the chloroform-based floral extract (ch-FE) bioassay (Figure 2). This assay was utilized for both blueberry and cranberry (flowers, ovaries and fruit). Only one type of aqueous treatment mixture was used in this assay; 1 part spore suspension, 2 parts SDW (to keep conidial concentration consistent due to ch-FE evaporation). This assay can be used to compare multiple ch-FE (waxes from various plant surfaces), or multiple time points/hours post-inoculation using a single ch-FE. Abbreviations: Sterile deionized water, SDW. Van Tieghem [glass] cells, VanT. cell.

9) Cranberry phenology-based extractions (Gager 2015)

9.1) Hand collect cranberry flowers (in June) (100 g), immature fruit (twice; July and August) (200 g), and mature cranberry fruit at harvest (October) (200 g), place into appropriately sized plastic bags and refrigerate at 4˚ C immediately after collection. Use the contamination precautions outlined above (step 2.1).

9.1.1) There will be extra plant material collected at each time but these amounts guard against extracting obviously fungal infected ovaries and fruit (showing symptoms and signs of disease).

9.2) Prior to extraction, remove any deteriorated, damaged, diseased flowers, then using only healthy flowers, remove the sepals, peduncles, corollas, stigmas, styles and stamens with curved forceps (45˚ tweezers) and discard all but the ovaries. Once ovaries are collected, perform the chloroform-based extraction at a 1 wt : 9 vol ratio (detailed in steps 4.2-4.4, using only ovaries). Store samples until all other extractions are complete, i.e. until bioassay conductance.

9.3) Once fruit are collected, perform a chloroform-based extraction (steps 4.2-4.4, using collected fruit instead of flowers), but add 10 g of fruit to 90 ml of chloroform and once extracted, allow the solution to evaporate to 9 ml (resulting in a 10 g : 9 ml, wt : vol solution).

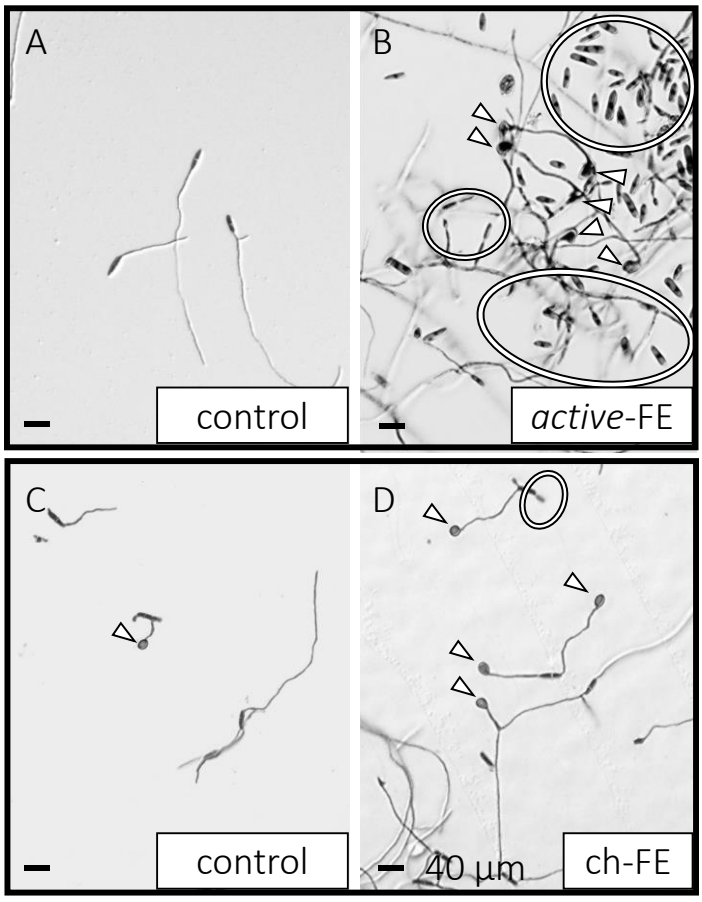
9.4) Make fruit extractions immediately after collecting in July, August, and October. Store as per above (step 4.4) until all extractions are collected. After the last chloroform-based fruit extraction, subject all penology-based chloroform extracts collected for this assay to the chloroform-based bioassay and analyze accordingly (steps 8.1-8.6).

Representative results:

Results overview. The results presented here are a few examples of the many assays that can be performed using this methodology. Figure 1 is an illustrated guide to the water-based FE bioassay, and is supplemented by Figure 2, which follows on to the chloroform-based FE bioassay. Figure 3 provides a visual guide to what can be expected upon microscopic evaluation of *C. fioriniae* at 24 h, in both water- and chloroform-based bioassays (compared SDW controls). Figure 4 details a 24 h time-course study with *C. fioriniae* in the presence of the cranberry variety Stevens ch-FE, and gives visual reference to an import result of this research; FE decreased the time needed to form infection structures compared to SDW. Figure 5 provides an example of data collected from a coverslip bioassay using cranberry floral rainwater runoff (CB "Flower" rw-FE). Figure 6 represents another important result; floral ovary ch-FE was much more stimulatory than fruit ch-FE, indicating the importance of bloom in the lifecycle of *C. fioriniae.* The supplemental photos and movies provide important visuals of the flowers used in the extractions and floral rainwater collection devices / deployment, in addition to movies that visualize the *active*- and *passive*- extraction (water-based) processes.

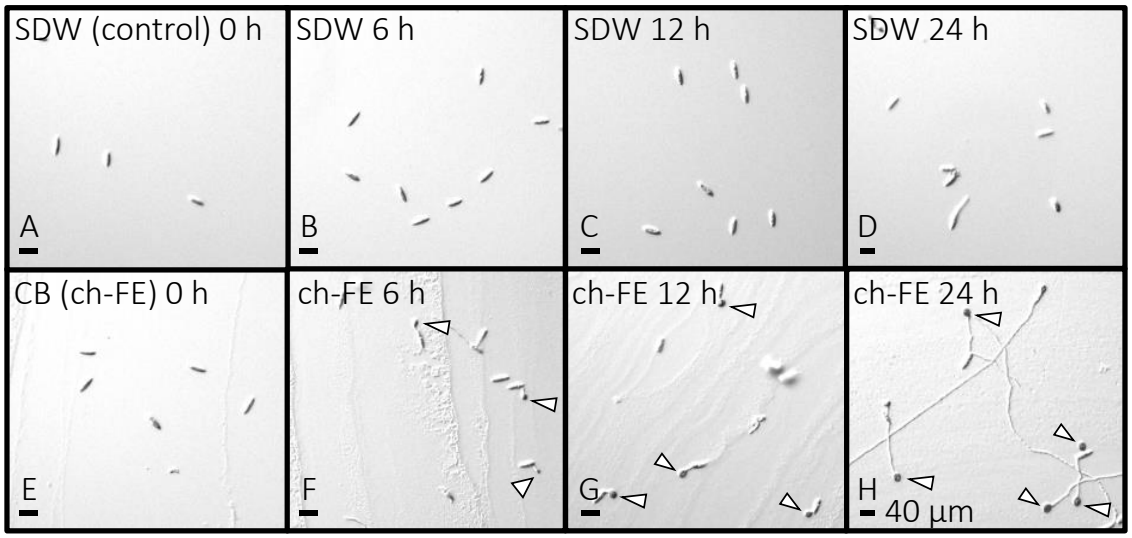
Visual comparison of *Colletotrichum fioriniae* in the presence of water-based FE and ch-FE (Figure 3). In this assay blueberry 'Bluecrop' (*active*-FE, water-based) (fungal isolate: BB#10) and cranberry 'Stevens' chloroform-based (ch-FE) (fungal isolate: CB-PMAP182) floral extracts were compared to SDW controls. A dramatic increase in secondary conidiation (rings) and appressorium formation (arrowheads) were observed when comparing conidia in the presence of SDW (control) **(A)** to *active*-FE **(B)** at 24h post-inoculation. However, secondary conidiation was not as apparent when comparing the chloroform bioassay SDW control **(C)** to ch-FE **(D),** rather *C. fioriniae* growth shifted towards appressorial formation. Shown is a common response to each extraction type, water-based and chloroform-based, regardless of host/floral species described.

(Figure 3)

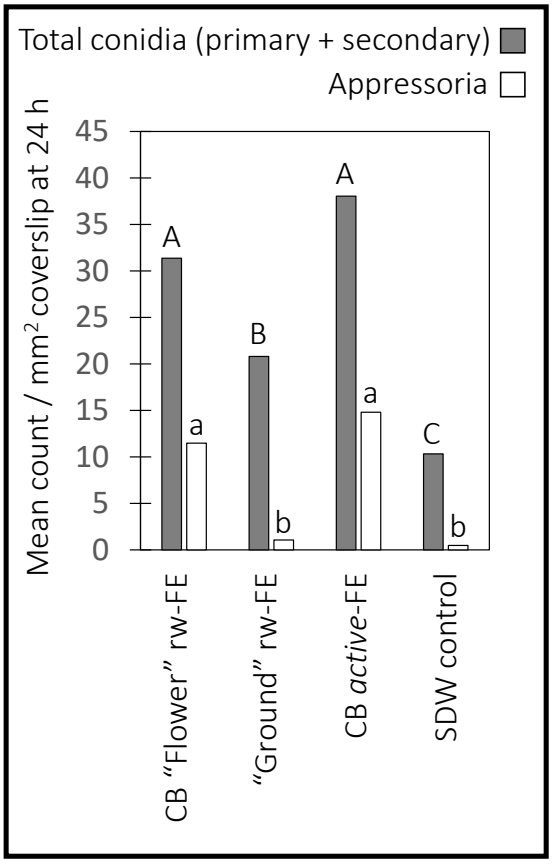


Time-course study (24h) with *Colletotrichum fioriniae* in the presence of ch-FE (Figure 4). In this assay a SDW control **(A-D)** and cranberry 'Stevens' ch-FE **(E-F)** were visually inspected at 0, 6, 12, 24 h post-inoculation (an example of variable time points instead of comparing multiple FE). Appressorium formation (arrowheads) began at 6 h in the ch-FE and steadily increased throughout subsequent time points. This result eludes to an important factor of pathogen biology during the bloom period; flowers reduce the time needed to form infection structures.

(Figure 4)

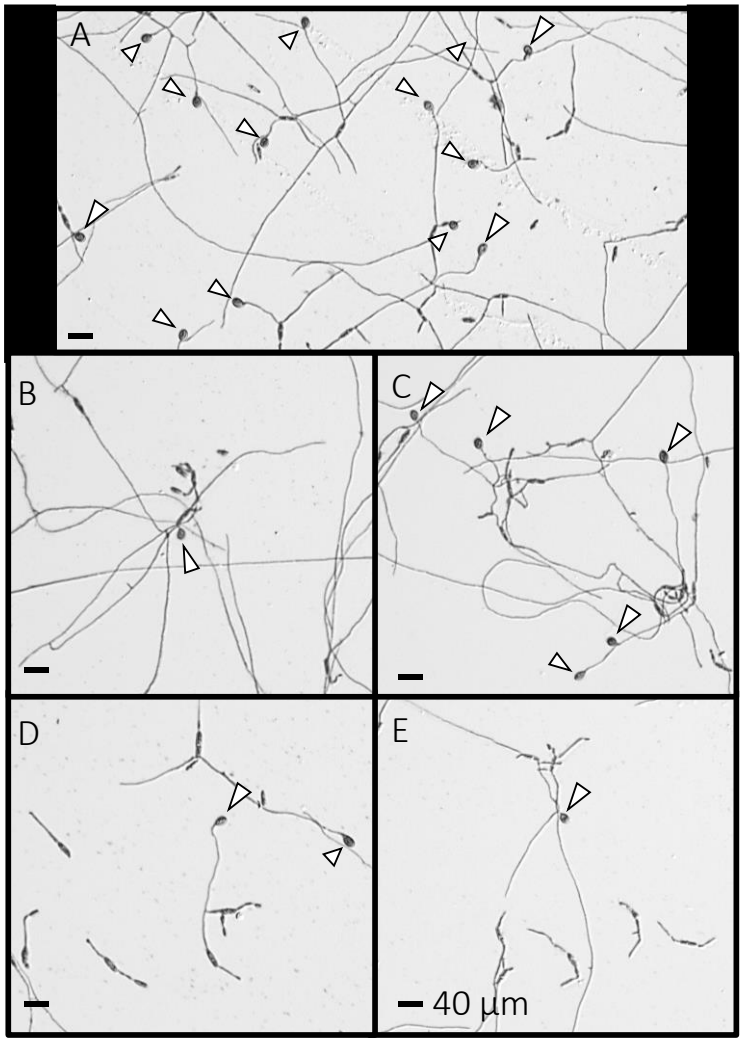


(Figure 5)



Graphical display of data collected using rw-FE in a bioassay (Figure 5). Rainwater run off of cranberry flowers (CB "Flower" rw-FE) and virgin rainwater that had not touched any cranberry plant tissues ("Ground" rw-FE) from a single wetting-event plus a standard *active*, cranberry water-based floral extract (CB *active*-FE) (positive control) and SDW (negative control) were subjected to a water-based coverslip bioassay and evaluated for *C. fioriniae* growth. CB "Flower" rw-FE had the same level of secondary conidiation and appressorium formation as the standard CB *active*-FE at 24 h post-inoculation, indicating that the collection devices were effective in capturing floral stimulants released during a wetting-event. Total conidia is comprised of primary (deposited), conidia and newly formed secondary conidia. Letters indicate significant differences at $P < 0.05$ (LSD), uppercase, total conidia; lowercase, appressoria.

(Figure 6)



Cranberry phenology based ch-FE bioassay, visual inspection (Figure 6). Here cranberry chloroform-based extracts (ch-FE) from multiple growth stages of cranberry ('Stevens') were visually evaluated for the effect of surface waxes on *C. fioriniae* at 24 h post-inoculation. Ovaries collected in June **(A)**, immature fruit collected in July and August **(B, C)**, harvested fruit collected in October **(D)**, and a SDW control **(E)** were inspected for appressorial formation (arrowheads). Ovary ch-FE had the greatest magnitude of appressorial formation indicating that this plant phenology (bloom) is critically important to the lifecycle *of C. fioriniae*.

Discussion:

The bioassays detecting *C. fioriniae* response to floral extracts (FEs) were developed for the blueberry and cranberry fruit rot pathosystems but can be readily adapted to other horticultural crops. The protocol detailed above has been valuable in acquiring many important data sets including, but no limited to; FE effects on multiple isolates of numerous pathogens, time-course information pertaining to fungal growth stages in the presence of various FEs, comparison of extraction techniques, inspection of individual chemicals on *C. fioriniae* growth and differentiation, evaluation of individual flower organ extracts, effects of temperature on *C. fioriniae* while in the presence of FE, effects of phenology dependent wax extractions, and floral rainwater effects. Through the use of these techniques, data generated has also provided a much clearer understanding of *C. fioriniae* life stages and partially elucidates why the bloom period is so critical to the control of many fruit rotting pathogens.

Initially all flowers were processed identically to the *active*-FE, but the extraction process has moved towards using whole flowers. Floral dissection was time consuming and had very little effect on the bioactivity of the resulting FEs. However, individual floral organs can and have been evaluated using this protocol, but great care must be taken to not completely macerate the floral tissues (Supplemental Movie 1, with precautions detailed in step 2.3), as this may result in released fungi-toxic/static compounds into the FE that could distort the microscopic evaluations. Less invasive extractions such as *pass*-FE (Supplemental Movie 2) and rw-FE are now more favorable due to their ease of acquisition. Additionally, these extraction techniques require only vacuum filtration to acquire biologically active floral chemical cues.

The flowers utilized in all extractions were typically refrigerated for 0-3 days prior to extract preparation. A challenge of this protocol is time management of FE turnover (field collection through storage of extracts). This was exacerbated by numerous samples from multiple sources and dates. Frozen flowers have not been evaluated to any real extent, as thawed flowers appear deteriorated and discolored. However, once the water-based FEs have been prepared, repeated freezing and thawing has shown no effect on the bioactivity of the FE, so as long as the FE are quickly refrozen after bioassay preparation (viable 3 year old FE).

Chloroform-based extraction enables the investigation of pathogen responses to three-dimensional floral/fruit surface waxes in a two-dimensional plane via ch-FE evaporation on glass coverslips. However, it is unlikely that the actual crystalline structures of waxes deposited from the ch-FE are identical to the surface from which they were collected. Meaning, supplemental techniques should be implemented if fungal response to specific wax structures in vivo are the main focus of investigation. Chloroform-based extracts need more storage maintenance than the water-based extractions. In addition to keeping the ch-FE extracts in the dark, the PTFE lined cell culture tube caps and parafilm sealing wrap need to be regularly checked for evaporative leaks and replaced as necessary.

The concept of monitoring floral rainwater runoff is rooted in the idea of advancing site-specific disease monitoring tools. The rainwater collection devices can be adapted to many other plant architectures, so long as the collection device captures rainwater that has run off of flowers. This approach provides information on whether or not floral stimulation is present in the field at any given time and can be monitored

throughout the season. Alternatively, collection devices can be deployed at multiple canopy locations to determine how far floral cues have been washed during any given wetting-event. In future experiments, rw-FE will dictate when fungicide applications should begin and when they can safely end. Additionally, by monitoring phenology dependent wax extractions (protocol section 9), the importance of the bloom period to pathogen biology has become even more evident. That section was also included to demonstrate the flexibility of these bioassays; providing methods that allow for side-by-side comparison of host surface waxes that are temporally separated. The data generated using the floral extraction techniques and bioassays, represent tangible indicators of pathogen stimulation, specific chemical classes important to pathogen biology, and targets for future control strategies.

Supplemental movies:

Supplemental Movie 1: Active, water-based floral extracts (*active*-FE).
Supplemental video support following steps 2.3-2.5.1. Blueberry 'Bluecrop' flowers were used.
(LINK: https://www.jove.com/files/ftp_upload/58880/active-FEextraction.mp4)

Supplemental Movie 2: *Passive*, water-based floral extracts (*pass*-FE).
Supplemental video support following steps 3.3-3.4 Blueberry 'Bluecrop' flowers were used.
(LINK: https://www.jove.com/files/ftp_upload/58880/pass-FEextraction.mp4)

Supplemental Movie 3: Deployment of cranberry floral rainwater collection devices.
Supplemental video support following step 6.4.
(LINK: https://www.jove.com/files/ftp_upload/58880/rw-FEdeployment.mp4)

Supplemental figures:

Supplemental Figure 1: Blueberry inflorescence. Blueberry flowers were collected for extractions during full bloom (April-May in New Jersey, USA) (shown 'Bluecrop'). Note the overlap of corollas/ovaries from adjacent flowers and the overall architecture of the inflorescence compared to Supplemental Figure 2 (cranberry upright).

Supplemental Figure 2: Cranberry upright. Cranberry flowers were collected for extractions during full bloom (June-July in New Jersey, USA)(shown 'Stevens'). Note the varied flower stages on a single cranberry inflorescence (upright), and the hooked, water droplet retaining the shape of the corolla.

Supplemental Figure 3: Blueberry rainwater deployment (flower). Completed blueberry floral rainwater collection device, placed directly under a cluster of inflorescences. Note the plastic-coated wire used to vertically orient the device.

Supplemental Figure 4: Blueberry rainwater deployment (stem). Completed blueberry floral rainwater collection device, placed half way down the stem between an inflorescence and the crown of the bush.

Supplemental Figure 5: Blueberry rainwater deployment (crown). Completed blueberry floral rainwater collection device, placed at the base of the bush (crown). Note plastic coated wires can be removed if not necessary.

Supplemental Figure 6: Blueberry rainwater deployment (ground). Completed virgin rainwater collection device, placed adjacent to blueberry bushes.

Supplemental Figure 7: Cranberry rainwater deployment (close-up). Completed cranberry floral rainwater collection device, with two uprights tucked under the neatly crossed wire ties.

Supplemental Figure 8: Cranberry rainwater deployment. Multiple completed cranberry floral rainwater devices deployed in a bog.

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Chapter III

Floral involvement in the disease cycle of *Colletotrichum fioriniae* and other Cranberry Fruit Rot fungi

Introduction:

Fruit rot of blueberry and cranberry are widely distributed across all growing regions in North America and can severely limit production (Oudemans et al. 1998; Polashock et al. 2007). In the case of cranberry, disease is caused by more than ten fungal species of which *Colletotrichum fioriniae* (*C. acutatum* s.l.) (Damm et al. 2012), *Colletotrichum fructivorum* (*C. gloeosporioides* s.l.) (Doyle et al. 2013), *Coleophoma cylindrospora* (*C. empetri* s.l.) (Crous and Groenewald 2016), *Phyllosticta vaccinii*, and *Physalospora vaccinii* are reported most frequently, collectively known as the cranberry fruit rot complex (Doyle et al. 2013; McManus et al. 2003; Oudemans et al. 2005; Oudemans et al. 1998; Polashock 2017; Polashock et al. 2009). In blueberry, fruit rot is most often caused by the anthracnose pathogen *Colletotrichum fioriniae* (*C. acutatum* s.l.) (Damm et al. 2012), as well as other less frequent fruit rotting pathogens such as *Alternaria tenuissima, Botrytis cinerea, Phomopsis vaccinii*, and *Phyllosticta vaccinii* (Polashock 2017).

Cranberry fruit rot pathogens are divided into three broad categories based on the life cycle (Oudemans et al. 1998), of which *C. fioriniae* and *C. fructivorum* fall into Type I field-rot, where fungi overwinter in detritus and older, often woody tissues or persistent fruit pedicels as appressoria, dormant conidia, or hyphal fragments within the dense,

annually flooded cranberry canopy. In blueberry, *C. fioriniae* preferentially overwinters in dormant inflorescence buds (DeMarsay 2005; Wharton and Diéguez-Uribeondo 2004) and these have been implicated as important inoculum reservoirs in apple (Bernardi et al. 1983) as well as sweet and sour cherry (Borve and Stensvand 2006), suggesting a close relationship of primary inoculum with floral tissues and structures. Cranberry uprights, which support the flower buds, are likely overwintering reservoirs for the *Colletotrichum* species. Other fungal species such as *Coleophoma cylindrospora*, *Phyllosticta vaccinii*, and *Physalospora vaccinii* belong to the cranberry fruit rot Type II field-rot category and overwinter as latent infections within living tissues such as vines or cranberry leaves that generally persist for two seasons (Oudemans et al. 2005; Oudemans et al. 1998). Cranberry fruit rot Type III storage-rot species such as 'Black Rot' pathogens *Strasseria geniculata* and *Allantophomopsis lycopodena* typically infect fruit injured during water harvest whereas Types I and II are thought to infect fruit during the bloom period (Oudemans et al. 1998; Schwarz and Boone 1983; Stiles and Oudemans 1999).

The Type I and II pathogens form latent infections on developing fruit that activate after seed maturity resulting in a pre- or post-harvest fruit rot (Cipollini and W. Stiles 1992; Stiles and Oudemans 1999). However, the bloom period is believed to be a key developmental stage for cranberry and several other horticultural crops in relation to effective disease control, as infection rates are highest during this phenology (Polashock 2017; Waller et al. 2018). Additionally, several studies have demonstrated a synchronization of *C. fioriniae* conidial production and the bloom period, further highlighting this developmental stage as a target for disease suppression (DeMarsay 2005; Miles et al. 2013; Wharton 2002). Floral induction of secondary conidiation is a

key step in the epidemiology of citrus post-bloom fruit drop (Agostini et al. 1993; Agostini and Timmer 1994; MacKenzie et al. 2010; Peres et al. 2005) as well as strawberry anthracnose (Leandro et al. 2001, 2003). In addition to secondary conidiation, blueberry floral components significantly shortened the time required for appressoria to form, thus potentially increasing the number of infection periods possible during the bloom period (Hartung et al. 1981; Verma et al. 2007; Waller et al. 2018). Furthermore, these water-mobilized floral chemical cues can be carried some distance during wetting events, such as irrigation or rainfall, synchronizing multiple inoculum reservoirs to host plant phenology (MacKenzie et al. 2010; Waller et al. 2019; Waller et al. 2018). In addition to aqueous floral chemical cues, host plant epi- and intracuticular waxes have been implicated in signaling appressorial formation of numerous plant pathogenic fungi, in addition to commonly reported physical factors (Gager 2015; Liu et al. 2011; Liu and Kolattukudy 1998; Parbery and Blakeman 1978; Podila et al. 1993; Skamnioti and Gurr 2007). The extent at which flower cuticular waxes effect *C. fioriniae* and other members of the cranberry fruit rot pathosystem is currently uncertain, warranting further investigation of the primary interface between host and pathogen.

Cranberry fruit rot is caused by a diverse group of latent pathogens that exhibit similar patterns of infection as blueberry anthracnose (Oudemans et al. 1998; Peres et al. 2005; Polashock 2017; Waller et al. 2019; Waller et al. 2018; Wharton and Diéguez-Uribeondo 2004; Wharton and Schilder 2008). It is likely that the cranberry fruit rot fungi (Types I and II) that infect near bloom time will respond to flower components similar to *C. fioriniae*, whereas the later infecting Type III will not. To test this and further characterize the effects of floral components on multiple cranberry fruit rot pathogens we

utilized four fruit rotting species representing Leotiomycetes (Dermataceae and Phacidaceae) and Sordariomycetes (Glomerellaceae). The potential impact of host plant phenology, host genetic resistance as well as the mobility of floral factors was examined using *Colletotrichum fioriniae* as the primary pathogen species.

Materials and Methods:

Fungal isolates. The primary isolates of *C. fioriniae* used in this research (Table 1) were CB-PMAP182, and BB#10, previously described in Waller et al. (2018). Additional *C. fioriniae* isolates were utilized in one assay; cranberry isolates CB-PMAP184, CB-KPCF16, and CB-KP33 and a blueberry isolate BB-89. Isolates matched conidial and morphological descriptions as well as 99% sequence similarity with *C. fioriniae* type-material CBS 128517 (Gen Bank NR_111747). Four other cranberry fruit rot pathogens were evaluated in one assay including *C. fructivorum* (GL-08-126), *Coleophoma cylindrospora* (CE-15-2), and a 'black rot' pathogen *Allantophomopsis lycopodena* (AL-15-1). Isolates identified as *Coleophoma cylindrospora* are synonymous with *C. empetri* (Crous and Groenewald 2016; Polashock et al. 2009). Single spore isolates were stored on corn-meal agar (CMA) (Becton, Dickinson and Company, Sparks, MD).

High-density sporulation of *C. fioriniae* was induced by streaking conidia onto clarified V8 juice agar (cV8A) (Miller 1955) with no calcium carbonate and a greater concentration of agar (28 g/L) (Waller et al. 2019; Waller et al. 2018). Cultures were incubated at 25˚C in the dark for seven days and spore suspensions were prepared by washing the conidia with sterile deionized water (SDW) from the high-density plates.

Sporulation of *C. fructivorum* was accomplished by the addition of mycelial plugs onto CMA plates, then incubated for 7-10 days at 25˚C in the dark. *C. fructivorum* spore suspensions were prepared by scraping (with sterile loop) newly formed conidia on the surface of CMA into SDW. *C. cylindrospora*, and *A. lycopodena* sporulation was accomplished by the addition of mycelial plugs onto instant potato agar, containing sucrose and coconut water (IPSAC), then incubated for 10-20 days (until sporulation) at 25˚C in the dark. Spores were then scraped into SDW. IPSAC recipe; 50 ml coconut water (no additives or pulp) combined with 10 g instant potato flakes, 10 g sucrose, 15 g agar, and 950 ml SDW prior to double 30 min autoclave cycles separated by 24 h. Spore concentrations were estimated using a hemocytometer and adjusted to 1.0 X 10^5 conidia per ml of SDW, with the exception of fruit infectivity inoculum that was adjusted to 1.0 X 10^6 conidia per ml of SDW.

Table 1: List of New Jersey cranberry fruit rot (CFR) isolates evaluated.

Pathogen	Isolate	Host[z]	Location
Colletotrichum fioriniae	BB#10	Blueberry	Jenkins Neck
"	BB-89	Blueberry	Jenkins Neck
"	CB-PMAP182	Cranberry	Shamong
"	CB-PMAP184	Cranberry	Hog Wallow
"	CB-KPCF16	Cranberry	Jenkins Neck
"	CB-KP33	Cranberry	Jenkins Neck
Colletotrichum fructivorum	GL-08-126	Cranberry	Hog Wallow
Coleophoma cylindrospora	CE-15-2	Cranberry	Jenkins Neck
Allantophomopsis lycopodena	AL-15-1	Cranberry	Hog Wallow

[z] Large American cranberry (*Vaccinium macrocarpon* Aiton). Highbush blueberry (*V. corymbosum* L.) Isolates were collected from symptomatic fruit from the host listed.

Floral and rainwater collections. Cranberry (*Vaccinium macrocarpon* Aiton) and blueberry (*V. corymbosum* L.) flowers were collected from fungicide-free cranberry or blueberry plantings at P. E. Marucci Center for Blueberry and Cranberry Research and Extension, Rutgers University in Chatsworth, NJ (PEM), during peak bloom over five growing seasons, 2013-2018. Four cranberry genotypes were utilized in this research. Fruit rot susceptible 'Stevens' and 'Mullica Queen', and resistant 'Buds Blues' and 'US89-3' (Johnson-Cicalese et al. 2015; Johnson-Cicalese et al. 2009). Anthracnose susceptible blueberry genotypes 'Bluecrop' and 'Coville', moderately resistant 'Duke', and resistant 'Elliot' were utilized as controls (Polashock et al. 2005; Waller et al. 2018). Rainwater collections as described by Waller et al. (2019) were deployed in several locations in an established 'Stevens' bed at PEM throughout the flower to fruit ontogeny. Each collection device was placed below two flower bearing uprights and collections were obtained during bloom (June), green fruit (July), maturing fruit (August), and harvested fruit (September). Additional sets of collection devices were also deployed to compare rainwater run off of flowering and vegetative uprights as well as rainwater that had not touched plant material.

Preparation of water-based extracts. Flowers were processed using a protocol described in Waller et al. (2018, 2019), with two additional modifications. The cranberry w-FEs were prepared using whole flowers (1g flowers; 9ml SDW) with only peduncles removed, and once flowers had been combined with SDW the samples were vacuum filtered through Whatman # 1 filter paper (Maidenstone, UK). Cranberry 'Stevens' and blueberry 'Bluecrop' water-based leaf extractions (w-Leaf) were also made by combining

1 g of freshly collected mature leaves and 9 ml SDW, then subjecting the sample to vacuum filtration. Rainwater collections were brought in from the field and vacuum filtered as above. Following the water extraction, samples were stored at -20˚C in 15 ml aliquots until use. At least three independent extractions were processed or collected for each water-based extraction.

Preparation of chloroform-based extracts. Chloroform extractions were performed using a previously described protocol (Gager 2015; Waller et al. 2019). Plant material was chloroform extracted by immersing and gently swirling in chloroform for 30 seconds at a weight to volume ratio of 1 g of flowers to 9 ml of chloroform. This mixture was then strained through a stainless steel screen into clean glass tubes, closed with polytetrafluoroethylene (PTFE) lids and stored at 4ºC in darkness. To test for differences between floral organs, flowers were dissected prior to extraction and 1 g of each floral component was then chloroform-extracted. Extractions included whole flowers (entire flowers with only peduncles removed), corollas (entire flower with peduncles and ovaries removed) and ovaries (all other floral organs removed with only the ovaries remaining). As a result of that assay, whole flowers were used for the remaining experiments. Flower to fruit ontogeny-based chloroform extractions of cranberry ‘Stevens’ were collected over three growing seasons at PEM (2015-2017). Extractions were processed as described above and included full bloom (June), green fruit (July), maturing fruit (August), and harvested fruit (September). At least three independent chloroform-based extractions were processed for each treatment type.

Bioactivity of water-based floral extracts (w-FEs). A glass coverslip bioassay as previously described (Waller et al. 2019; Waller et al. 2018) was used to quantify bioactivity of water-based extracts on multiple cranberry fruit rot fungi. This bioassay was used to measure both secondary conidiation and appressorial formation (or germinated conidia if appressoria did not form). Some modifications to the bioassay included the use of different fungal species, extracts, and temperature adjustments. A total of six *C. fioriniae* isolates as well as *C. fructivorum, C. cylindrospora*, and *A. lycopodena* (Table 2) were evaluated with or without cranberry 'Stevens' w-FE (ST w-FE) at 24 h postinoculation. Fungal species were evaluated independently and each trial consisted of two treatments replicated three times in a completely randomized design, except for *C. fioriniae* isolates where a randomized complete block design (blocked by isolate) was applied.

A time-course assay to evaluate the effects of ST w-FE or SDW on *C. fioriniae* (CB-PMAP182) every 6 h over a 24 h period was conducted. The initial reading was performed at 2 h postinoculation to ensure conidial adhesion to the glass surface. Treatments were replicated three times per time point in a completely randomized design. The effect of incubation temperature on *C. fioriniae* (CB-PMAP182) was evaluated at 10, 15, 20, 25, and 30°C (separate incubators) in the presence or absence of ST w-FE at 24 h postinoculation. Treatments were replicated three times per temperature and each temperature-treatment set was individually analyzed in a completely randomized design.

C. fioriniae (CB-PMAP182) response to three common floral nectar sugars was also evaluated using this bioassay. Crystalline sucrose, glucose (alpha-D-glucose (dextrose/corn sugar), and D-fructose were compared to each other as well as a ST w-FE

positive control and a SDW negative control. Each sugar solution (replacing the w-FE portion) was prepared at 300 mM and serially diluted to 100, 10, 1, and 0.1 mM once combined with one volume each conidial suspension and SDW. Inoculated glass coverslips were incubated at 25°C and evaluated at 24 h postinoculation. Treatments were replicated three times in a completely randomized design.

Water-based leaf extractions (w-LE) from cranberry 'Stevens' and blueberry 'Bluecrop' were compared to ST w-FE and SDW controls, to determine if flowers were uniquely stimulatory. Treatments were replicated three times in a completely randomized design.

The ontogeny-based rainwater runoff collected from each device (rw-ontogeny) was stored as above until all samples were collected. A glass coverslip bioassay was then conducted as described above per collection device for a total of three trials. Treatments were replicated three times per device in a completely randomized design, whereas all other water-based bioassays were replicated twice.

Bioactivity of chloroform-based floral extracts (ch-FEs). To evaluate the biological activity of chloroform extracts from multiple sources a modified glass coverslip bioassay was utilized (Waller et al.. 2019). A 33 µl droplet of each chloroform extract was placed on a glass coverslip and allowed to evaporate. A clean glass Van Tieghem cell (8 mm outer diameter / 6 mm inner diameter) contained the droplet to prevent it from spreading across the coverslip. Once the chloroform evaporated and a thin film of residue remained, a 99 µl droplet of inoculum (premixed at a ratio of 33 µl of 1 X 10^5 conidia per ml SDW to 66 µl SDW) was added. The bioassay mixture was incubated at 25°C in the dark until

a 10 µl droplet of lactophenol cotton-blue was applied to the cells, thus stopping growth at pre-determined time points (typically 24 h postinoculation). The Van Tieghem cells were then removed and coverslips were inverted onto a glass slide and observations were made as described previously (Waller et al.. 2018). Different bioassays included the use of multiple CRF pathogens, time points, ch-FE of multiple blueberry and cranberry cultivars, floral organs, ch-Leaf extractions as well as the ontogeny-based chloroform extractions (ch-ontogeny). Treatments were replicated three times in a completely randomized design and all bioassays were replicated twice, except for the ontogeny bioassay which was replicated three times.

Cranberry fruit inoculation with *C. fioriniae* (CB-PMAP182). An inoculation trial was conducted on healthy, non-fungicide treated 'Stevens' mature cranberry fruit collected at PEM in late September (2017) to determine if ST w-FE could increase the occurrence of fruit rot at 2 weeks postinoculation, following an initial 24 h infection period. Fruit were stored at 4 ˚C once harvested for 3 weeks prior to use in the infectivity assay. Each week after collection, fruit were again sorted to remove/discard any obviously infected or soft fruit to reduce the likelihood of background contamination. Plastic sealable containers (12"x5"x3"), lined with sterile, moistened paper towels and sterile plastic netting placed above towels, were used as inoculation chambers (Waller et al.. 2018). Twenty fruit placed into each container, utilizing the plastic netting to stabilize the prolate spheroid shaped fruit, represented a single treatment replicate. A total of five replicates per trial (100 fruit per treatment) were used for each of the four aqueous treatment mixtures: fruit treated with ST w-FE alone, ST w-FE plus *C. fioriniae* (1.0 X

10^6 conidia per ml SDW) conidia, conidia alone, and a SDW only control. Each aqueous treatment mixture was combined prior at a 1: 1: 1 ratio (w-FE: conidia: SDW), adjusting with SDW to retain volume and concentration as per treatment demands. Containers were organized in a randomized complete block design and fruit were inoculated individually with a 20 µl treatment droplet. Containers were then sealed until 22 h postinoculation, when lids were opened and the inoculation droplet was allowed to dry (approximately 2 h, thus providing a 24 h infection period), then resealed to promote high relative humidity. Containers were held at approximately 26 ˚C within greenhouse conditions (October (NJ), 14 h photoperiod) for 2 weeks, when fruit were evaluated for disease development. Entire experiment was conducted twice.

Data analyses. Analyses were conducted using CoStat version 4.0 (CoHort Software, Monterey, CA) (Cardinali 2013) and data were checked for normality using Bartlett's Test for homogeneity of variances. For all coverslip bioactivity assays, analyses were conducted according to Waller et al. (2018) and Gager et al. (2015). *Count data* consisted of counted total conidia (comprised of primary/inoculated, and secondary conidia) and appressoria. In cases where appressoria did not form, conidial germination was recorded (presence of germ tube at least equal in length to the width of the conidia). For each replicate of each treatment (single coverslip) data were collected from 4 or 8 microscope fields at 200x magnification, totaling areas of 3.808 mm^2 and 7.616 mm^2, respectively. The field counts were then summed for each replicate and normalized using a $\log_{10}$ transformation (+0.5 added to compensate for zero count data). A completely randomized 2-way ANOVA was applied to transformed data using experiment (trial) and treatment as

independent variables and either total conidia or appressoria (or germinated conidia, per note above) as the dependent variable. Comparisons of time points or temperatures within the same aqueous treatment mixture utilized a completely randomized 2-way ANOVA applied to transformed data as described above with the modification of time or temperature as an independent variable. Comparisons of *C. fioriniae* isolates were arranged in a randomized complete block design, and a 2-way ANOVA was applied using the same variables as described above with additional blocking by fungal isolate. Count data results were displayed as the back-transformed means divided by the total counted area per mm^2. The *ratio* of appressoria to conidia was calculated as the quotient of total counted appressoria per replicate (single coverslip) divided by total counted conidia per replicate, yielding a proportionate value. Ratio data were then normalized by arcsine transformation (if ratio value < 1.0) or square root transformation (if ratio value > 1.0), and a completely randomized 2-way ANOVA was applied as described above. Ratio back transformed means were displayed without further modification. A post hoc Fishers Least Significant Difference (LSD) test was performed for means separation when necessary. Error bars for total conidia and appressoria counts reflect the standard deviation of count data that had been transformed by the area of a single counted field (0.952 mm^2). Error bars for ratio data reflect the standard deviation of the quotient of count data. A randomized complete block 2-way ANOVA was applied to fruit inoculation data using experiment (trial) and treatment as independent variables and percent infected fruit at 2 weeks postinoculation as the dependent variable, blocking by replicated container sets.

Results:

Cranberry 'Stevens' FE stimulates multiple cranberry fruit rotting pathogens. Cranberry fruit rot pathogens *C. fioriniae*, *C. fructivorum*, *C. cylindrospora*, and *A. lycopodena* were evaluated in the presence of both ST w-FE and ST ch-FE (Fig. 1) for secondary conidiation, appressorial formation, and the ratio thereof at 24 h postinoculation, to determine if FEs were stimulatory (Table 2). All six *C. fioriniae* isolates (four isolates from cranberry and two isolates from blueberry) showed a significant increase in the number of total conidia present with a 2.8-fold increase over the SDW control, indicating secondary conidiation. Appressorial formation also significantly increased from 0.7 in the SDW control to 15.7 appressoria per mm^2 in the presence of ST w-FE. In the presence of ST ch-FE *C. fioriniae* isolates had a significant yet lowered 1.7-fold increase of secondary conidiation over the control. However, appressorial formation was dramatically affected in the presence of ST ch-FE with a 19.6-fold increase over control. The ratio of appressoria to conidia was differentially affected by the two FE types, with ST ch-FE driving growth towards appressorium formation with a ratio value of 0.82 compared to ST w-FE that stimulated more secondary conidiation (0.44). *C. fructivorum* (GL-08-126) had significantly more secondary conidiation in the presence of both ST w- and ch-FEs compared to SDW ($P \leq 0.0001$) and chloroform ($P = 0.0007$) controls, respectively. ST ch-FE stimulated more *C. fructivorum* appressorial formation (10.5-fold increase over chloroform) than ST w-FE (5.0-fold increase over SDW). Additionally *C. fructivorum* in the presence of ST ch-FE had a ratio value greater than 1.0, indicating a single conidium often generated more than one appressorium. Secondary conidiation was not as obvious with *C. cylindrospora* in the

presence of either FE type, yet more conidia were observed in the presence of ST ch-FE (P = 0.0157) with a 1.2-fold change. However, appressorial response to both FEs types was dramatic and similar, with ratio values of 0.77 and 0.60 in response to ST w-FE and ST ch-FE, respectively. *A. lycopodena* did not form secondary conidia or appressoria but did reach 100% germination in the presence of ST w-FE and 22% in response to ST ch-FE.

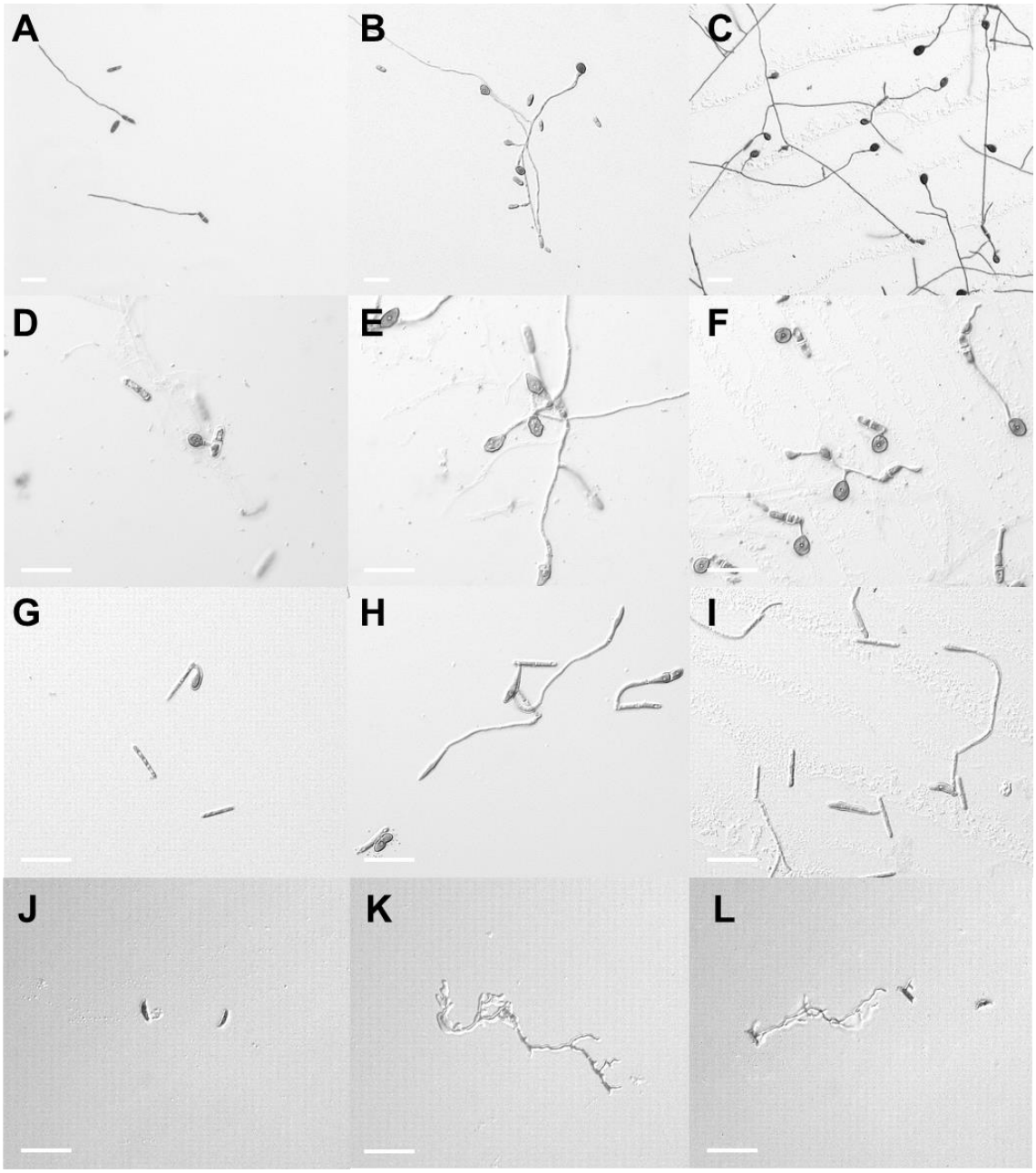

Figure 1. Appressorial formation and secondary conidiation of three Type I and II cranberry fruit rot pathogens (bloom period infections) (Oudemans et al.. 1998); *Colletotrichum fioriniae* (**A-C**), *Colletotrichum fructivorum* (**D-F**), *Coleophoma cylindrospora* (**G-I**), and a Type III mature fruit infecting pathogen, *Allantophomopsis lycopodena* **(J-L)** to both water- (w-) and chloroform-based (ch-) cranberry 'Stevens' (ST) floral extracts (FE) on glass coverslips at 24 h postinoculation (25°C). **A,D,G,J** (first column): Primary (inoculated) conidia in sterile deionized water controls**. B,E,H,K** (Second column): Conidia in the presence of ST w-FE, often forming secondary conidia and appressoria (with the exception of *A. lycopodena*, that formed long, branched hyphae). **C,F,I,L** (third column): Conidia in the presence of ST ch-FE, forming prolific appressoria (with the exception of *A. lycopodena*). Scale bars: 20 µm.

Table 2: Effects of water (w-) and chloroform (ch-) based cranberry 'Stevens' floral extracts (FE) on four cranberry fruit rot pathogens[u].

Pathogen[v]	Treatment	Conidia	Appressoria	Ratio
Colletotrichum fioriniae[w]	w-FE	36.2 a	15.7 a	0.44 a
	control	13.0 b	0.7 b	0.07 b
Type[y] I: bloom period infections				
	ch-FE	48.9 a	39.1 a	0.82 a
	control	28.6 b	2.0 b	0.08 b
Colletotrichum fructivorum	w-FE	33.4 a	20.6 a	0.62 a
(GL-08-126)	control	21.5 b	4.1 b	0.20 b
Type I: bloom period infections				
	ch-FE	59.0 a	63.9 a	1.09 a
	control	39.6 b	6.1 b	0.16 b
Coleophoma cylindrospora	w-FE	41.2 a	31.6 a	0.77 a
(CE-15-2)	control	37.5 a	5.9 b	0.16 b
Type II: bloom period infections				
	ch-FE	52.6 a	31.8 a	0.60 a
	control	43.1 b	8.2 b	0.19 b

		Conidia	Germinated[z]
Allantophomopsis lycopodena	w-FE	10.3 a	10.3 a
(AL-15-1)	control	9.5 a	0.1 b
Type III: mature fruit infections			
	ch-FE	10.1 a	2.2 a
	control	9.8 a	0.3 b

[u] Analyses were performed on $\log_{10}$-transformed means of total conidia, appressoria or germinated conidia (if appressoria did not form) coverslip count data and arcsine-transformed means (or square root-transformed if ratio value > 1.0) of ratio data generated from the count data. Values are presented as back-transformed means for Ratio and means per square millimeter for conidia, appressoria, and germinated conidia at 24 h postinoculation (25°C). Letters per column per pathogen and extraction group indicate significant differences at $P < 0.05$ according to Fisher's least significant difference test. Control treatment for water-based cranberry 'Stevens' FE (w-FE) is sterile deionized water, control for chloroform-based cranberry 'Stevens' FE (ch-FE) is evaporated chloroform.

[v] Each pathogen and extraction group was evaluated independently via glass coverslip bioassays for total conidia, comprised of primary/inoculated and secondary conidia (Conidia), appressorium formation (Appressoria), and the ratio of appressoria to conidia (Ratio). Isolate codes are in parentheses.

[x] Multiple *C. fioriniae* isolates from cranberry (CB-PMAP182, CB-PMAP184, CB-KPCF, and CB-KP33) and blueberry (BB-89 and BB#10) were evaluated.

[y] Type as described in Oudemans et al.. (1998).

[z] *A. lycopodena* conidia germinated but did not form appressoria.

Corollas contribute the greatest stimulation to *C. fioriniae*. To address which region of the flower contributed greatest to the biological activity observed with *C. fioriniae* response to ST ch-FE, whole cranberry 'Stevens' (ST ch-FE) flowers as well as blueberry 'Bluecrop' flowers (BC ch-FE), were compared to corolla-only and ovary-only chloroform-based extractions. Floral components were evaluated with their respective *C. fioriniae* isolate: CB-PMAP182 (cranberry) or BB#10 (blueberry). Corolla-extract stimulation of secondary conidiation was not different from that of ST ch-FE nor BC ch-FE (cranberry: 44.6 and 43.9 conidia per mm^2, blueberry: 60.4 and 64.8 conidia per mm^2, respectively). Cranberry ST ch-FE and corolla-extract stimulated significantly greater magnitudes of appressorial formation (32.9 and 38.2 per mm^2), followed by the chloroform control (10.5 per mm^2) with ovary-extractions having the least stimulation (4.0 per mm^2). Whole flower BC ch-FE (57.1 per mm^2) stimulated more appressoria than the corolla-extracts (42.0 per mm^2), yet both stimulated significantly more than ovary-extracts (19.2 per mm^2) or the chloroform control (8.8 per mm^2). The ratio values for both species' corolla-extracts were not different than the whole flower extracts (cranberry: 0.88 and 0.77 appressoria per conidia, blueberry: 0.56 and 0.65 appressoria per conidia, respectively), however both were significantly greater than ovary-extracts and the chloroform controls (cranberry: 0.14 and 0.36 appressoria per conidia, blueberry: 0.33 and 0.15 appressoria per conidia, respectively), indicating that whole flower extractions could be safely used to estimate stimulation in the bioassays. All differences per host extract / isolate group were highly significant at $P \leq 0.0001$ according to Fisher's least significant difference test.

Floral extracts reduce wetness period time requirements for secondary conidiation and appressorial formation. *C. fioriniae* isolate CB-PMAP182 was observed in the presence of either ST w-FE (Fig. 2, A-C) or ST ch-FE (Fig. 2, D-F) every 6 h over 24 h to estimate a growth response timeline. *C. fioriniae* secondary conidiation was significantly greater beginning at 18 h in the presence of ST w-FE ($P \leq 0.0001$) and ST ch-FE ($P = 0.0116$) with a 2.0-fold increase over SDW and 1.1-fold increase over chloroform, respectively. At 24 h postinoculation ST w-FE stimulated a 2.8-fold increase in secondary conidiation over the SDW control ($P \leq 0.0001$), whereas ST ch-FE stimulated a significant yet lower 1.3-fold increase over chloroform ($P = 0.0001$). Within the ST w-FE treatment secondary conidiation significantly increased every 6 h between 12-24 h, yet within the ST ch-FE treatment this observation was only made between 18-24 h. Evaluations of the initial conidial concentration per assay were conducted at 2 h postinoculation to ensure conidial adhesion to the glass surfaces, thus facilitating accurate counts of primary inoculated conidia. Interestingly, a low level of *C. fioriniae* appressorial formation (0.3 per mm^2) was observed at this time in the presence of ST ch-FE ($P = 0.0007$) yet was not observed in the ST w-FE. The majority of appressorial formation began at 6 h postinoculation in the presence of both FE types and continued to increase in magnitude over the 24 h period. Although the quantity of appressoria increased, there was no significant difference within the ST w-FE treatment across the 12-24 h timeframe, however within the ST ch-FE treatment all time points were significantly greater than the prior 6 h count. As dramatic secondary conidiation increased in the presence of ST w-FE, the ratio of appressoria to conidia was driven down with the highest ratio value of appressoria to conidia at 12 h and 18 h. Inversely,

increased appressorial formation lead to elevated ratio values for ST ch-FE across time points, with no difference between 12-24 h. Regardless of FE type, ratio values were significantly greater than their corresponding controls beginning at 6 h and 2 h postinoculation for the ST w-FE and ST ch-FE, respectively.

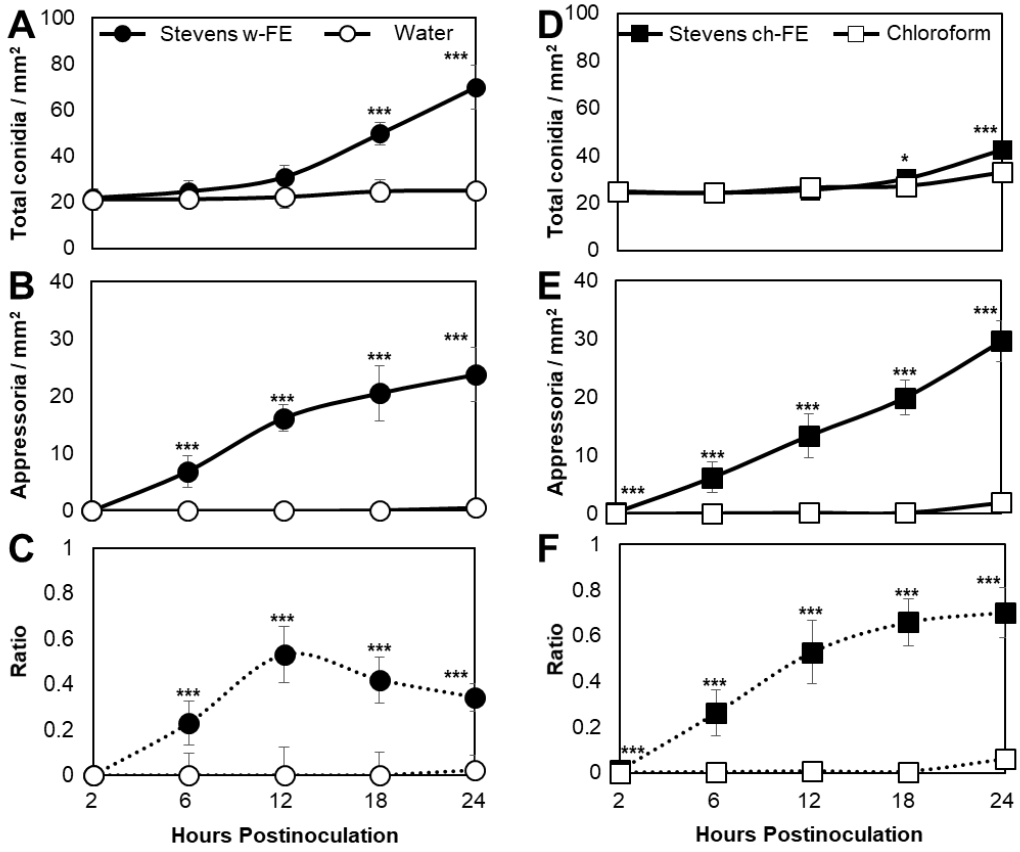

Figure 2. Time-course study of secondary conidiation and appressorial formation of *Colletotrichum fioriniae* (isolate CB-PMAP182) in the presence or absence of cranberry variety 'Stevens' floral extracts (FE). **A-C.** *C. fioriniae* response to 'Stevens' water-based FE (ST w-FE). **A.** Secondary conidiation was evaluated as total conidia (primary / inoculated and newly formed secondary conidia). Primary conidia at 2 h were 21.9 and 21.3 for ST w-FE and water, respectively; P = 0.449. **B.** Appressorium formation in the presence of ST w-FE. **C.** Ratio of appressoria to conidia (appressoria count data divided by total conidia count data) in the presence of ST w-FE. **D-F.** *C. fioriniae* response to ST chloroform-based FE (ST ch-FE). **D.** Secondary conidiation; primary conidia at 2 h were 24.4 and 25.0 for ST ch-FE and evaporated virgin chloroform (chloroform), respectively; P = 0.491. **E.** Appressorium formation in the presence of ch-FE. **F.** Ratio of appressoria to conidia in the presence of ST ch-FE. All trials conducted at 25°C. Error bars for total conidia and appressoria reflect the standard deviation of count data that had been transformed by the area of a single counted field (0.952 mm^2). Error bars for ratio reflect the standard deviation of the quotient of count data, per glass coverslip. Asterisks *, **, ** indicated level of significance of $P < 0.05$, 0.01, and 0.005 according to Fisher's least significant difference test, respectively, in regards to the transformed data. Dotted lines represent calculated data.

Effect of cranberry ST w-FE on *C. fioriniae* at multiple incubation temperatures. Pathogen development is often related to a temperature optimum, leading to question what effect ST w-FE would have on *C. fioriniae* (CB-PMAP182) secondary conidiation, appressorial formation, and the ratio thereof at 24 h postinoculation across multiple incubation temperatures (10, 15, 20, 25, and 30°C) (Fig. 3). At the coldest temperatures 10°C and 15°C, secondary conidiation was not different between ST w-FE and SDW, however at 10°C a low level of appressorium formation was initiated. In the 15-30°C range, appressorial formation in the presence of ST w-FE significantly increased over the SDW control, yet within the ST w- FE treatment there was no difference over the temperature range. In the 20-30°C, range secondary conidiation was significantly greater in the presence of ST w-FE over the SDW control. This growth response in the presence of ST w-FE was more affected by temperature with a significantly greater magnitude of secondary conidiation at 25°C (46.8 conidia per mm^2) and 30°C (46.4), followed by 20°C (31.7), and the 10/15°C group ($P \leq 0.0001$). Increasing secondary conidiation coupled with stable appressorial formation in the presence of ST w-FE initially drove the ratio of appressoria to conidia towards 1:1 at 15-20°C, but fell at 25-30°C due to the significant increase of secondary conidiation over those temperature ranges.

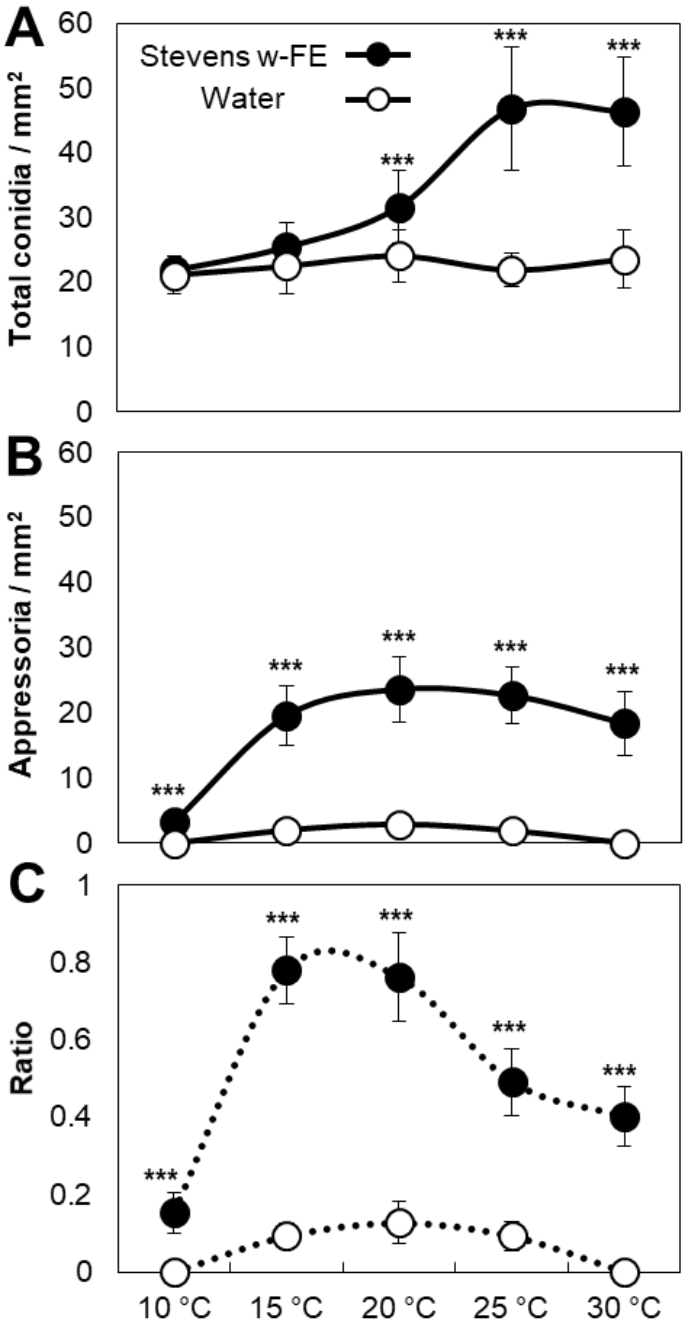

Figure 3. Evaluation of *Colletotrichum fioriniae* (isolate CB-PMAP182) over multiple incubation temperatures with or without water-based 'Stevens' floral extracts (ST w-FE) on glass coverslips at 24 h postinoculation. **A.** Total conidia (primary / inoculated, and secondary conidia). **B.** Appressorial formation. **C.** Ratio of appressoria to conidia. Error bars for total conidia and appressoria reflect the standard deviation of count data that had been transformed by the area of a single counted field (0.952 mm^2). Error bars for ratio reflect the standard deviation of the quotient of count data, per glass coverslip. Asterisks *, **, ** indicated level of significance of $P < 0.05$, 0.01, and 0.005 according to Fisher's least significant difference test, respectively, in regards to the transformed data. Dotted lines represent calculated data. Water = sterile deionized water.

Impact of common floral nectar sugars [100 mM – 0.1 mM] on *C. fioriniae* secondary conidiation and appressorium formation. Three common floral nectar sugars (sucrose, glucose, and fructose) were evaluated at 24 h postinoculation to investigate possible nutritional impacts on *C. fioriniae* (CB-PMAP182) secondary conidiation and appressorium formation (Fig. 4). All sugars were evaluated at 100, 10, 1, 0.1 mM final concentration. With the exception of fructose (1 and 0.1 mM), all sugars regardless of type or molarity, stimulated more secondary conidiation than SDW (negative control) but less than ST w-FE (positive control). Appressorial formation was effected differently throughout the changing molarities and sugar types, but was at the greatest magnitude in the presence of ST w-FE. Although not highly significant, two general trends emerged; appressorium formation increased as sucrose molarity decreased and as glucose/fructose molarity increased, as well as increasing secondary conidiation with sugar concentrations.

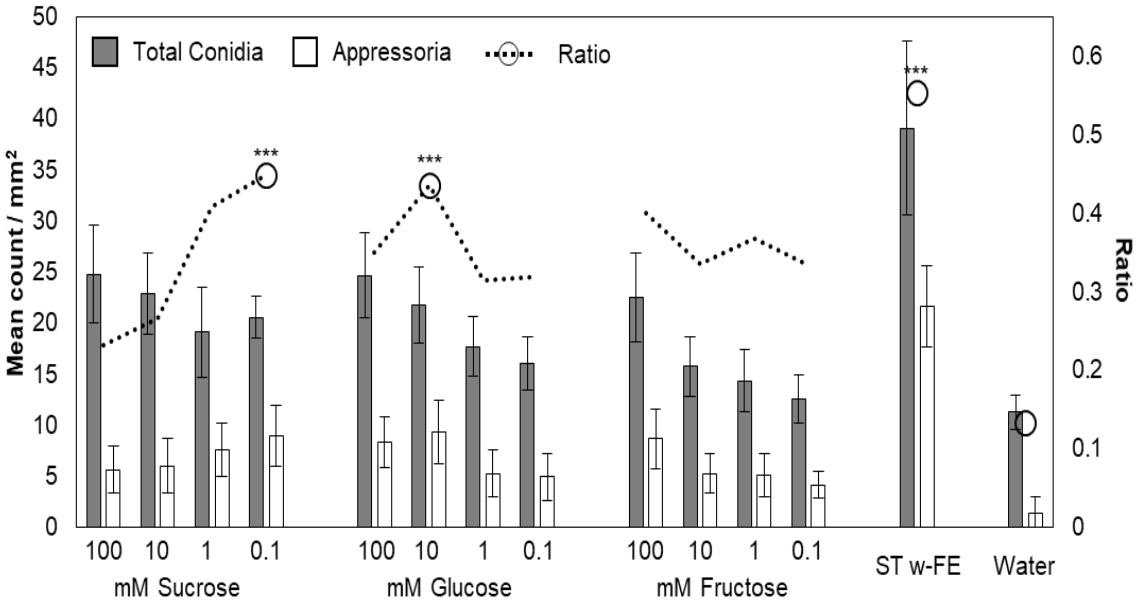

Figure 4. Comparison of three common floral nectar sugars over a range of molarities, cranberry 'Stevens' water-based floral extract (ST w-FE), and sterile deionized water (SDW) effects on secondary conidiation, appressorium formation and the ratio thereof on *Colletotrichum fioriniae* (isolate CB-PMAP182) at 24 h on glass coverslips (25°C). Sugars solutions (sucrose, crystalline), glucose (alpha-D-glucose; dextrose (corn sugar)), fructose (D-fructose)) were prepared at 3 times concentration starting at 300 mM and serially diluted to reflect 100, 10, 1, 0.1 mM once combined with one volume each conidial suspension and sterile deionized water (water). Error bars for total conidia and appressoria reflect the standard deviation of count data that had been transformed by the area of a single counted field (0.952 mm^2). Calculated ratio of appressoria to conidia (appressoria count data divided by total conidia count data) in the presence of each sugar or ST w-FE were displayed as dotted lines per sugar. Circles with asterisks indicate instances where the ratio values of specific sugar molarities and ST w-FE were not significantly different at $P < 0.05$ according to Fisher's least significant difference test. Circle above water indicates the baseline ratio value in the control.

Cranberry and blueberry genotypes ch-FE effect the ratio of *C. fioriniae* appressoria to conidia differently depending on host. Cranberry and blueberry genotypes with known levels of field rot susceptibility (Johnson-Cicalese et al. 2015; Johnson-Cicalese et al. 2009; Polashock et al. 2005) were evaluated at 24 h postinoculation to determine if this susceptibility could be related to ch-FE activity in vitro (Table 3, similar to a previous report concerning blueberry w-FE (Waller et al.. 2018). Susceptible blueberry 'Bluecrop' and 'Coville' as well as moderately resistant 'Duke' had a significantly greater ratio of *C. fioriniae* (isolate BB#10) appressoria to conidia compared to resistant 'Elliot', which was still greater than the chloroform control. Susceptible cranberry genotypes 'Stevens' and 'Mullica Queen' did not affect *C. fioriniae* (isolate CB-PMAP182) differently than resistant 'Buds Blues' and 'NJ-89-3'. However, all cranberry genotypes ch-FE were significantly more stimulatory than the chloroform control.

Table 3 Comparison of chloroform-based floral extracts from multiple blueberry and cranberry cultivars on *Colletotrichum fioriniae*[x].

Crop	Cultivar	Fruit rot susceptibility[y]	Ratio[z]	
Blueberry	*Coville*	Susceptible	0.72	a
	Bluecrop	Susceptible	0.61	a
	Duke	Moderate Resistant	0.67	a
	Elliot	Resistant	0.35	b
	Chloroform	…	0.21	c
Cranberry	*Stevens*	Susceptible	0.48	a
	Mullica Queen	Susceptible	0.49	a
	Buds Blues	Resistant	0.42	a
	NJ 89-3	Resistant	0.50	a
	Chloroform	…	0.02	b

[x] Two *C. fioriniae* isolates were evaluated; BB#10 and CB-PMAP182 from blueberry and cranberry fruit, respectively. [y] Blueberry anthracnose and cranberry fruit rot susceptibility rankings were based on Polashock et al. (2005) and Johnson-Cicalese et al. (2015), respectively. [z] Analyses were performed on arcsine-transformed means of ratio data (ratio of appressoria to conidia) 24 h postinoculation (25°C). Values are presented as back-transformed means. Letters per crop indicate significant differences at $P < 0.05$ according to Fisher's least significant difference test.

Stimulation of *C. fioriniae* to leaf material dependents on extraction type utilized. A series of bioassays were evaluated at 24 h postinoculation on glass coverslips to investigate the effects of water- and chloroform-based leaf extractions on *C. fioriniae* (CB-PMAP182) secondary conidiation, appressorial formation, and the ratio thereof (Table 4). There was a greater magnitude of appressorial formation in the presence ST ch-FE, yet was not significantly different from either of the chloroform-based leaf extractions (ch-Leaf) from blueberry or cranberry. However, this greater magnitude of appressoria coupled with a significant increase in secondary conidiation, led to a greater ratio of appressoria to conidia in the presence of ST ch-FE over both of the ch-Leaf extracts followed by chloroform controls ($P \leq 0.0001$). Secondary conidiation was also observed in the presence water-based leaf extractions (w-Leaf) from blueberry and cranberry, albeit at a significantly lower magnitude than ST w-FE. Blueberry and cranberry w-Leaf did not affect appressorial formation as dramatically as ch-Leaf extractions but did have significantly more appressoria than the water controls per square millimeter. Consequently the ratio of appressoria to conidia was greater in the presence of ST w-FE compared to the other water-based treatments ($P \leq 0.0001$).

Table 4: Response of *Colletotrichum fioriniae*[x] to blueberry and cranberry leaf extractions.

Crop	Extraction type[y]	Ratio[z]	
Blueberry	w-Leaf	0.09	b
Cranberry	w-Leaf	0.08	b
Cranberry	w-FE	0.52	a
-	Water	0.01	c
Blueberry	ch-Leaf	0.64	b
Cranberry	ch-Leaf	0.68	b
Cranberry	ch-FE	0.81	a
-	Chloroform	0.01	c

[x] *C. fioriniae* isolate CB-PMAP182 was used for this bioassay.
[y] Water-based extractions (w-) of blueberry 'Bluecrop' leaves, and cranberry 'Stevens' leaves (w-Leaf) and flowers (w-FE). Chloroform-based extractions (ch-) of blueberry 'Bluecrop' leaves, and cranberry 'Stevens' leaves (ch-Leaf) and flowers (ch-FE). Sterile deionized water (Water) and evaporated chloroform (Chloroform) were utilized as negative controls.
[z] Analyses were performed on arcsine-transformed means of ratio data (ratio of appressoria to conidia) generated from total conidia and appressoria count data at 24 h postinoculation (25°C). Values are presented as back-transformed means. Letters per extraction type indicate significant differences at $P < 0.05$ according to Fisher's least significant difference test.

Flowers are the source of *C. fioriniae* stimulation in rainwater runoff from cranberry uprights. Field rainwater collections were implemented to determine if rainwater that had run off of vegetative uprights with only leaves (no floral development) were as stimulatory as runoff collected from flowering uprights (Waller et al. 2019) (Table 5). Rainwater runoff of flowering uprights stimulated the greatest magnitude of secondary conidiation with a 4.7-fold increase over vegetative upright runoff and 1.4-fold increase over ST w-FE. Rainwater that had not runoff plant material, vegetative upright runoff, and the SDW water control did not stimulate secondary conidiation. Appressorial formation as well as the ratio of appressoria to conidia in the presence of flowering upright runoff was not different from ST w-FE, where both were significantly greater than the other collections and water controls ($P \leq 0.0001$).

Table 5: Response of *Colletotrichum fioriniae*[x] to rainwater runoff collected from flowering or vegetative cranberry 'Stevens' uprights.

Rainwater collections[y]	Ratio[z]	
Stevens - flowering upright	0.46	a
Stevens - vegetative upright	0.01	b
Rainwater	0.02	b
Stevens - w-FE	0.53	a
Water	0.01	b

[x] *C. fioriniae* isolate CB-PMAP182 was used for this bioassay.
[y] Rainwater that had run off of two flower bearing cranberry 'Stevens' uprights (inflorescences) or two vegetative (no floral tissues) uprights were collected and compared to rainwater that had not come into contact with any plant material (rainwater) as well as a positive control (water-based cranberry 'Stevens' flower extract (w-FE)) and a negative control (sterile deionized water (Water)).
[z] Analyses were performed on arcsine-transformed means of ratio data (ratio of appressoria to conidia) generated from total conidia and appressoria count data at 24 h postinoculation (25°C). Values are presented as back-transformed means. Letters per extraction type indicate significant differences at $P < 0.05$ according to Fisher's least significant difference test.

Full bloom is the most stimulatory stage of the flower to fruit ontogeny. Ontogeny-based chloroform extractions (ch-ontogeny) as well as corresponding rainwater collections (rw-ontogeny) were evaluated at 24 h postinoculation to determine what stage of the flower to fruit ontogeny was the most stimulatory to *C. fioriniae* (CB-PMAP182). Both ch-ontogeny and rw-ontogeny collections acquired during bloom stimulated the greatest ratio of appressoria to conidia compared to those collected during sequential stages of fruit maturation (Fig. 5). Secondary conidiation was significantly greater in all ch-ontogeny samples compared to chloroform controls ($P = 0.0008$), however the bloom through fruit maturation extractions were not different from each other ($P = 0.5775$). There was an interaction between trial and treatment ($P = 0.0006$) for secondary conidiation in the presence of rw-ontogeny sampled from the different collection devices, however the trend of bloom collections stimulating the greatest magnitude of secondary conidiation over fruit maturation collections (average: 1.4-fold increase), followed by SDW (water) controls, was consistent ($P = 0.0006, 0.0001, 0.0001$). Bloom ch- and rw-ontogeny collections stimulated a significantly greater magnitude of appressorial formation compared to their respective fruit maturation samples in all trials. Fruit ch- and rw-ontogeny samples were not different per extraction or collection type, respectively, in terms of appressorial formation, but as a group were consistently more stimulatory than the controls.

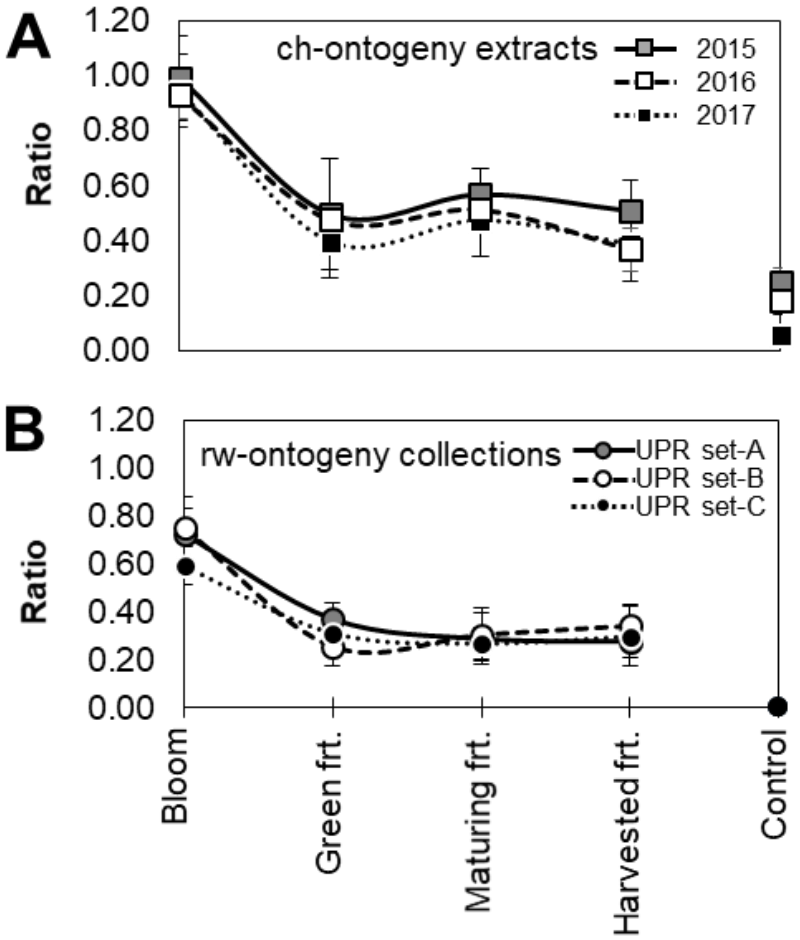

Figure 5. Response of *Colletotrichum fioriniae* (isolate CB-PMAP182) in the presence of sequential flower to fruit ontogeny based chloroform extractions (ch-ontogeny) and rainwater collections (rw-ontogeny) on glass coverslips at 24 h postinoculation (25°C). **A.** Ratio of appressoria to conidia (appressoria count data divided by total conidia count data) in the presence of cranberry 'Stevens' chloroform extractions collected over three years at each bloom to fruit maturation stage; Bloom (June), Green fruit (frt.) (July), Maturing frt. (August), and Harvested frt. (September). Evaporated chloroform constituted the control for each ch-ontogeny trial. **B.** Ratio of appressoria to conidia in the presence of cranberry 'Stevens' rainwater collections, where each collection device separately monitored the development of two flowering upright sets (UPR set-A/B/C) through sequential flower to fruit ontogeny stages. Sterile deionized water constituted the control for each rw-ontogeny trial. All bioassay trials were conducted once the final Harvested frt. extractions or rainwater collections were collected. Error bars for ratio reflect the standard deviation of the quotient of total conidia and appressoria count data, per glass coverslip.

Cranberry ST w-FE moderately enhanced *C. fioriniae* (CB-PMAP182) infection on mature 'Stevens' fruit. Prior assays demonstrated an increase in appressorial formation in response to ST w-FE, leading to question if w-FE could enhance infection on healthy, fungicide free, 'Stevens' cranberry fruit. Conidia in the presence of ST w-FE induced a significantly greater level of rot-symptomatic fruit (9.5%) compared to conidia in the absence of FEs (3.5%), treatment $P \leq 0.0001$. The two control treatments that did not contain conidia in the infection droplet had very low levels of symptomatic fruit, (ST w-FE alone (1%) and SDW alone (0.5%)), indicating a low level of background infections. Data were collected at 2 weeks postinoculation for each trial and the data were combined due to the lack of statistical differences between the two trials ($P = 0.7667$), blocks ($P = 0.0802$), nor interactions thereof ($P = 0.2984$).

Discussion:

All pathogens evaluated in this study were strongly affected by floral extracts (FEs). Type I and II cranberry fruit rot field-rot pathogens (*C. fioriniae*, *C. fructivorum*, and *C. cylindrospora*) (Oudemans et al. 1998) formed secondary conidia and appressoria in the presence of both water- and chloroform-based FEs. It appears that these pathogens respond in a similar fashion as *C. fioriniae* in the blueberry pathosystem (Waller et al. 2018), where FEs reduced the time necessary to form appressoria and increased the rate of infection when co-inoculated with *C. fioriniae* conidia. Temperature was found to have a significant effect on *C. fioriniae* conidiation when in the presence of ST w-FE. Additionally, ST w-FE also allowed appressorial formation at much lower temperatures, implicating the importance of bloom specific epidemiological information in the disease

cycle of *C. fioriniae*. Based on both chloroform extractions and rainwater collections throughout the flower to fruit ontogeny it appeared that the bloom period was the most stimulatory phenology to the development of fungal infections. These observations coupled with common blueberry and cranberry recommendations, focused on initiating fruit rot management strategies during the bloom period (Polashock 2017), point to the importance of flowers in the initial stages of the disease cycle of *C. fioriniae* and other fruit rotting fungi.

Secondary conidiation in response to water-mobilized FEs has been linked to increased inoculum loads and the activation of inoculum reservoirs in at least three crops affected by members of the *C. acutatum* species complex (Agostini et al. 1993; Agostini and Timmer 1994; Borve and Stensvand 2006; Leandro et al. 2001, 2003; MacKenzie et al. 2010; Peres et al. 2005). In citrus post bloom fruit drop (Citrus PFD) sucrose-rich floral signals are thought to be "washed" through the canopy during periods of frequent rain, activating dormant appressoria on persistent calyces (buttons) or leaves to sporulate (MacKenzie et al. 2010). Sucrose is the predominate sugar associated with many Ericaceous species' nectar, often accounting for 80 percent of total sugars (Moquet et al. 2015; Stiles and Freeman 1993), has proven to be a potent nutritional source for many *Colletotrichum* spp. (Agosteo et al. 2015; MacKenzie et al. 2010; Manandhar et al. 1995). Undoubtedly floral sugars contribute to the energetics of *C. fioriniae* development, however, conidia in the presence of ST w-FE (estimated to have a maximum bioassay concentration of 3.9 mM sucrose (Cane and Schiffhauer 1997), produced a greater magnitude of secondary conidia and appressoria at comparable sugar molarities, thus increasing the probability of other stimulants in the water-mobilized FEs.

Signals triggering appressorial formation are difficult to separate from one another and typically include a combination of perceived surface topography (thigmotaxis), wettability, or hard surface recognition in addition to chemical cues present at the epicuticular wax interface of susceptible tissues (Gilbert et al. 1996; Hoch et al. 1987; Liu et al. 2011; Liu and Kolattukudy 1998; Uppalapati et al. 2012). Initially, it was suspected that *C. fioriniae* would form appressoria in response to the concentric rings/ridges deposited as the ch-FE dried, following the same thigmotactic response as the rust fungus *Uromyces appendiculatus* (Hoch et al. 1987), however this was quickly dismissed due to apparently random distribution of appressoria within the Van Tieghem cells. The importance of hydrophobicity in the bioassays, was diminished due to the lack of *C. fioriniae* appressorial formation in the presence hydrophobic paraffin and carnauba waxes (Gager 2015). Additionally, blueberry cultivars with assumed equivalent hydrophobic forces as well as specific sugars at equal molarities (osmotic pressures), affected *C. fioriniae* growth differently, suggesting fungal activation by specific chemical compounds. Recognition of host surfaces are critically important to many *Colletotrichum* spp. (Dickman et al. 2003; Dickman and Patil 1986; Parbery and Blakeman 1978; Podila et al. 1993). For example, the ability *C. trifolii* to infect *Medicago truncatula* was dramatically reduced on leaves with disrupted epicuticular wax production (Uppalapati et al. 2012), thus hindering perception of host by deregulating recognition of epi- and intracuticular waxes associated with cutinase production and cuticle penetration ((Dickman et al. 2003; Dickman and Patil 1986; Koller et al. 1991; Woloshuk and Kolattukudy 1986).

Avocado fruit surface waxes dissolved in chloroform have been shown to stimulate appressorial formation of *C. gloeosporioides* s.l., citing very long chain fatty acid and alcohols as stimulants (Podila et al. 1993). In a preliminary study Gager (2015) found the methyl ester of a major cutin monomer to stimulate *C. fioriniae* appressorium formation, in addition to the presence of nonacosane in cranberry waxes extracted with chloroform. The presence of nonacosane and presumably its derivative nonacosan-10-ol, is interesting as this compound forms crystalline wax tubules (Barthlott et al. 2017; Barthlott et al. 2016; Koch and Ensikat 2008; Zeisler-Diehl et al. 2018) that could be broken free from the epicuticular wax matrix, carrying with it non-water-soluble stimulatory compounds during wetting events similar to the waxy "bloom" on plum and blueberry fruits that can be destroyed by only the force of a raindrop (Barthlott et al. 2017). These points taken together likely point to the composition of w-FE and ch-FE as more similar than different. In agreement with this, *C. fioriniae* response to susceptible and resistant blueberry cultivars w-FE (Waller et al. 2018) and ch-FE was very similar, as well as observed similarities between cranberry ST w-FE and ch-FE stimulation of the Type I and II pathogens evaluated. Here water-, rainwater-, and chloroform based extractions are thought to contain many of the same stimulatory compounds albeit at different concentrations as the water-based extractions may simply dislodge non-water-soluble stimulants imbued in the crystalline epicuticular wax matrix whereas chloroform-based extractions strip these same compounds as well as the remaining majority of epi- and intracuticular waxes from host surfaces.

The leaf extractions presented an opportunity to observe a differential response to non-floral stimulation of *C. fioriniae* where w-Leaf extractions did not stimulate a

dramatic increase in appressorial formation whereas ch-Leaf did. This response may be a result of surface physical and chemical composition whereby leaf crystalline wax structures were less likely to be removed by water, as compared to flower extractions of both Types that are highly stimulatory. Surface waxes can vary greatly between plant species, different organs of an induvial plant, or the same organ over a time scale or through plant development (Avato et al. 1984; Barthlott et al. 2017; Griffiths et al. 2000; Gulz 1994; Jeffree 1986; Tomaszewski and Zielinski 2014). This could also explain why rainwater runoff captured from vegetative cranberry uprights was five-times less stimulatory than flowering uprights, where a different organ (flower) with a higher propensity to shed epicuticular constituents was present in addition to leaves. The ontogeny collections and extractions captured stimulatory compounds during bloom, however as the fruit matured stimulatory compounds may have become less abundant or available as a result of the changing epicuticular wax composition, be that chemically and/or structurally. Regardless of the mechanism of stimulation, the ontogeny samples mark a significant intersection between host and pathogen development, pointing to the importance of the bloom period in terms of both pathogen biology and disease management timing.

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Chapter IV

Characterization of fatty acid derivatives from blueberry and cranberry cuticular waxes and their effects on *Colletotrichum fioriniae*

Introduction

The large American cranberry (*Vaccinium macrocarpon* Aiton) and highbush blueberry (*V. corymbosum* L.) are important ericaceous fruit crops native to the United States (Eck 1990; Polashock 2017). The productivity of these crops can be severely limited by fruit rotting fungal pathogens and one pathogen, *Colletotrichum fioriniae,* is particularly important to both crop systems (Oudemans et al. 1998; Polashock 2017; Waller et al. 2019b; Waller et al. 2018). This pathogen, previously classified as *C. acutatum* (Damm et al. 2012), is the causal agent of both blueberry anthracnose as well as a member of cranberry fruit rot complex (McManus et al. 2003; Polashock et al. 2009; Waller et al. 2018). Critical timing for disease control is most successful when fungicides are applied during the bloom period for both crops, as studies have indicated the longer fungicide applications are delayed past peak bloom, the greater disease severity becomes (Oudemans et al. 1998; Polashock 2017). The factors related to plant phenology that affect the disease cycle of *C. fioriniae* are now being resolved. Previous research has shown that floral signals are potent stimulators of secondary conidiation as well as appressorium formation (Leandro et al. 2003; Waller et al. 2019b; Waller et al. 2019; Waller et al. 2018) and it is likely that waxy plant cuticles play a role in the observed phenology based stimulation patterns (Auyong 2015; Kolattukudy et al. 1995; Liu et al.

2011; Podila et al. 1993). In the current study, several tissue types from both blueberry and cranberry were subjected to a fatty acid analysis using the GC-FAME protocols (Midi Inc., Newark, DE) to identify the compounds most important to *C. fioriniae* appressorial formation, further defining the relationship between bloom period infecting fungi and host derived signals.

Appressoria are the infection structures formed by many plant pathogens, including *Colletotrichum,* and provide the rigidity necessary for the penetration peg to puncture the host cuticle (Wheeler 1975). Appressoria are typically dome- or mitten-shaped, highly melanized structures that form at the tips of germ tubes, or sessile to the conidium (Parbery and Blakeman 1978; Ryder and Talbot 2015). The infection period is complete following appressorium formation and the host cuticle breached. In the presence of floral signals, *C. fioriniae* appressoria form at a greater rate and more abundantly, although the active components responsible for this response are unknown (Waller et al. 2019; Waller et al. 2018). Rainwater collections showed that active compounds can be mobilized within the canopy via water (Waller et al. 2019) during the bloom period (Waller et al. 2019b). Since components of the plant can affect critical stages of the infection process including a reduction of the infection period temporal requirements, widening the temperature range for appressorial formation, as well as increasing inoculum load (Waller et al. 2019b; Waller et al. 2018), it may be very beneficial to further describe this phenomenon and elucidate the mechanisms.

The cuticle is a logical source of chemical stimulation since it is the outermost barrier a pathogen's appressorium must breach to gain access into plant cells. Plant cuticles are a biopolymer composed of the polyester cutin, comprised of hydroxylated

and epoxy-hydroxylated scaffolding C_{16} and C_{18} fatty acid derivatives (linked via ester bonds). Intracuticular waxes (principally very long chain (VLC) fatty acids, fatty alcohols (primary and secondary), esters, alkanes, and aldehydes, with lower amounts of other chemical classes such as ketones, flavonoids, and triterpenes) are impregnated in the remaining voids (Barthlott et al. 2017; Barthlott et al. 2016; Zeisler-Diehl et al. 2018). On the surface of the cuticle sits the morphologically variable epicuticular wax layer. This layer is composed of two-dimensional plates, or three-dimensional crystalline wax structures typified by the presence of secondary alcohols; most notably nonacosane-10-ol that forms hydrophobic wax tubules (Barthlott et al. 2017). Some of the compounds present in cuticular waxes have been identified as either triggering appressorial formation or cutinase production (Auyong 2015; Dickman et al. 2003; Kolattukudy et al. 1995; Podila 1988; Podila et al. 1993). Numerous fungal pathogens (and saprophytes) produce serine esterases (Chen et al. 2013), collectively known as cutinases, which hydrolyze cutin into n-hydroxy-monomeric fatty acids, facilitating either direct penetration or softening of the cuticle prior to appressorial penetration (Chen et al. 2013; Koller et al. 1991; Skamnioti and Gurr 2007; Woloshuk and Kolattukudy 1986). In the current study, floral compounds stimulating *C. fioriniae* appressorial formation have been identified.

Chloroform extractions of various host plant tissue types allows for comparison of the entire suite of epi- and intracuticular waxes. The bioactivity of these extracts on *C. fioriniae* and other cranberry fruit rot pathogens provides the basis of bioassay directed methods for determining the identity of the active components. When comparing water- and chloroform-based leaf and floral tissues extractions, chloroform-based extractions from both tissue types stimulated appressorial formation, in contrast, only the water-

based floral extracts were stimulatory and water extracts from leaves were non-stimulatory (Waller et al. 2019b). This suggests stimulation may be more a function of the cuticular and epicuticular structure rather than the chemical composition alone. The primary objective of this research was to identify which components stimulate appressorial formation, and why floral tissues are more stimulatory than other plant tissues. Understanding this would allow for the development of practical applications aimed at disrupting pathogen recognition of host, or deregulation of pathogen synchronization to host plant phenology among others.

Materials and Methods:

Cuticular wax extractions. Cranberry (*Vaccinium macrocarpon* Aiton) and blueberry (*V. corymbosum* L.) tissues were collected from fungicide-free cranberry or blueberry plantings at P. E. Marucci Center for Blueberry and Cranberry Research and Extension, Rutgers University, Chatsworth, NJ (PEM), during peak bloom over five growing seasons, 2013-2018. Two blueberry ((BB)) cultivars were characterized; anthracnose susceptible 'Bluecrop' (BC) and resistant 'Elliot' (ELL) (Polashock et al. 2005; Waller et al. 2018) as well as three cranberry ((CB)) varieties; field fruit rot susceptible 'Stevens' (ST) and more resistant 'Buds Blues' (Bd. Bl.) and 'US89-3' (89.3) (Johnson-Cicalese et al. 2015; Johnson-Cicalese et al. 2009). Extractions were considered by host species as well as in three groups; flower, vegetative, and fruit. Flower extractions; (CB): ST corolla-only (-FLW), ST-whole flower extract (ch-FE); ST water-based floral extract (w-FE) then extracted with chloroform (ST w-FE (CHL EXT)), Bd. Bl.-FLW, 89.3-FLW; (BB): BC-FLW, BC ch-FE, BC w-FE (CHL EXT), ELL-FLW. Vegetative extractions;

CB: ST leaves (-LVS), ST developing flowers (-Hook), ST flowering inflorescence (-UPR); BB: BC-LVS, BC developing buds at stage T4 (-T4 Bud), ELL-T4 Bud. Fruit extractions; CB: ST immature (Im.) and mature (Mat.) fruit (-FRT); BB: BC-Mat. FRT, ELL-MAT. FRT. Materials were collected at the respective peak bloom or maturity level, per sample type, and were all processed utilizing a 1 g tissue to 9 ml chloroform extraction ratio following procedures previously detailed (Gager 2015; Waller et al. 2019) and were stored at 4°C in darkness. All extractions were performed in triplicate.

Preparation and characterization of blueberry and cranberry cuticular waxes. All chloroform-based extractions were analyzed using two distinct protocols. First, detection of natively present fatty acid methyl esters (n-FAMEs) was accomplished by direct injection of chloroform samples. Next, the chloroform samples were saponified and methylated to detect methyl esters of fatty acid derivatives (FAMEs). Prior to saponification, cuticular waxes were suspended in chloroform (1 ml) were evaporated to dryness, then re-suspended in the saponification solution (1 ml of sodium hydroxide-methanol) and placed into a water bath of 100°C for 30 minutes following the GC-FAME protocol (Midi Inc., Newark, DE). Methylation of samples was completed by acid methanolysis in HCl-methanol at 80°C for 10 min, with fatty acid methyl esters (FAMEs) finally extracted into hexane-methyl tertiary butyl ether, washed with aqueous NaOH. The lipid-containing hexane phase was then removed and stored at 4°C in darkness until analyzed. All derivatized FAMEs and n-FAMEs where analyzed in triplicate, using an Agilent 6890 series Gas Chromatograph with a flame ionization detector (injection and detection temperatures of 280°C), and an oven temperature program as follows: held at

70°C for 2 min, ramp 15°C per minute to 300°C, then held for 10 minutes. Unit was equipped with HP Ultra 2 phenyl methyl silicone fused capillary column 25 m x 0.2 mm i.d., film thickness 0.33mm), as well as an automated sampler and computer with associated software. Identification of FAMEs based on their retention times was further confirmed via a coupled Agilent 5973 series Mass Spectrometer (Agilent Technologies, Inc., Santa Clara, CA.) equipped with a HP-5MS capillary column (60 m x 0.25 mm i.d., 0.25 μm film thickness) utilizing helium as the carrier gas. Peak naming was accomplished with the use of Microbial ID, Inc. external calibration standard, comprised of a mixture of the straight chained saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and five hydroxyl- fatty acids. External calibration standard was sampled every 10 injections to validate consistency between sample batches. Compound identity was corroborated against the NIST Mass Spectral Library.

Preparation of fatty acids and fatty acid methyl esters (FAMEs) for use in the bioassay. All compounds evaluated in the bioassay were originally purchased as pure fatty acid chemical standards (Sigma-Aldrich, St. Louis, MO) and were either directly suspended in chloroform (MolBio. grade) or methylated as described above, then dried and re-suspended in chloroform to acquire the fatty acid methyl esters (FAMEs). Compounds were initially prepared at 100 mM concentration, then adjusted with chloroform to bioassay concentration demands. Compounds identified in the direct injection sampling (n-FAME) were considered for the fatty acid methyl ester treatment concentrations in the bioassay, whereas fatty acid derivatives identified through saponification and methylation were considered for the fatty acid treatment

concentrations. Bioassay concentrations were identified as follows, peak area recorded from the GC-FAME protocol for each compound per extraction replicate was recorded and the average taken from non-zero data. The average peak area was then transformed into an estimated µM concentration per compound per extraction type via comparison to an external standard (C16:0 methyl ester, discrete increments [0.1 – 100 µM]) / peak area set, thus proportionately transforming the peak area in a linear relationship to molarity estimates (R-squared between observed peak area and µM C16:0 methyl ester increments = 0.997). These estimates were then used to determine the final µg per ml concentration to determine an appropriate concentration of paraffin as a control treatment. Paraffin concentration was set to 1.0 µg per ml suspended in chloroform (Fisher Scientific, Fairlawn, NJ). Paraffin was selected to represent the supposed, biologically inert, very long chain (VLC) alkane mixtures commonly associated with plant cuticles.

Fungal isolate and coverslip bioassay trial. *C. fioriniae* isolate CB-PMAP182 was isolated from an infected cranberry fruit at a commercial farm near Hog Wallow, NJ. Single spore isolates were stored on corn-meal agar (CMA) (Becton, Dickinson and Company, Sparks, MD). High-density sporulation of *C. fioriniae* was induced by streaking conidia onto V8 juice agar (cV8A) (Miller 1955) with no calcium carbonate and a greater concentration of agar (28 g/L). Cultures were incubated at 25˚C in the dark for 4 days and spore suspensions were prepared by washing the conidia with sterile deionized water (SDW) from the high-density plates for each replicate of the bioassay (Waller et al. 2019b; Waller et al. 2019; Waller et al. 2018). Spore concentrations were estimated using a hemocytometer and adjusted to 2.0 X 10^5 conidia per ml of SDW. The

bioactivity of individual fatty acids and methyl ester derivatives on *C. fioriniae* secondary conidiation, appressorial formation, and the ratio of appressoria to conidia, was measured using a glass coverslip bioassay (Waller et al. 2019). The µM concentration of each compound was determined by the maximum molarity estimated from the GC-MS analysis (Table 1). A standard chloroform-based cranberry 'Stevens' floral extract (ST ch-FE) was compared to a chloroform extracted, water-based 'Stevens' FE (ST w-FE (CHL EXT)). A chloroform-only and paraffin (1µg / ml) negative control were also utilized. A 33 µl droplet of each compound was placed inside a glass Van Tieghem cell (8 mm outer diameter / 6 mm inner diameter) and allowed to evaporate. Once evaporated, a 99 µl droplet of inoculum (premixed at a ratio of 33 µl of 2.0 X 10^5 conidia per ml SDW to 66 µl SDW) was added to the Van Tieghem cell, thus initiating the bioassay. For bioassay replication, a separate fungal culture provided conidia for the inoculum mixture. Bioassays were incubated at 25°C in the dark until a 10 µl droplet of lactophenol cotton-blue was applied to the cells, thus stopping growth and semi-preserving the fungi at 24 h postinoculation. The Van Tieghem cells were then removed and coverslips were inverted onto glass slides and 4-field observations were made as described in Waller et al. (2019b).

Data analysis. Epicuticular extractions were broken into three classifications (that included both blueberry and cranberry); flower, vegetative, and fruit to determine the appropriate bioassay concentration for each chemical compound. The estimated maximum molarity per group, as per peak area transformation described above, was recorded as well as the standard deviation between the constituents of that group,

regardless of host species. Molarity estimates for both n-FAMEs and FAMEs were input into Morpheus heat maps (Broad Institute, https://software.broadinstitute.org/morpheus), and were clustered according to one minus Pearson's coefficient hierarchical clustering within the program for both FAME groups. Height of cladogram indicated similarity between compound occurrences (x-axis) or between chemical compositions of the various extractions (y-axis).

Multiple regression analyses were performed utilizing XLSTAT (Addinsoft, Inc.) statistical package. These included comparing the ratio of appressoria to conidia (Ratio) for select cranberry extractions to either the molar concentration or percent FAME of total FAMEs, as well as comparisons between the individual compounds, total carbon number and bioassay molarities on the individual bioassay responses (secondary conidia, appressoria, and ratio thereof). Note, ST ch-FE (flower) ratio data were collected in the current study's bioassay whereas the ST-LVS (leaf), ST-Im. FRT and ST-Mat. FRT (fruit) ratio data were obtained from Waller et al. (2019b), using the same bioassay procedures. Correlation matrixes (Pearson) were also generated using this set of ratio data (Addinsoft, Inc.) and were imported to, and visualized with, Morpheus heat maps.

Bioassay count data results were displayed as the back-transformed means divided by the total counted area per mm^2. The ratio of appressoria to conidia was calculated as the quotient of total counted appressoria per replicate (single coverslip) divided by total counted conidia per replicate, yielding a proportionate value (Ratio), that were displayed without further modification. Error bars reflect the standard deviation of count data that had been transformed by the area of a single counted field (0.952 mm2).

Results:

Identification of native fatty acid methyl esters (n-FAMEs) and fatty acid methyl ester derivatives (FAMEs). Compounds identified through the GC-FAME protocol comprised the majority of the detectable fatty acid derivatives quantifiable by this technique (C9:0-C20:0) in both the n-FAME (Fig. 1A) and FAME (Fig. 1B) samples. Cranberry (CB) floral tissues appeared to be the richest in n-FAMEs with all CB floral samples possessing the majority of detected n-FAMEs, with the exception of (CB) ST w-FE (CHL EXT) that only possessed n-decanoate. The greatest abundance of an n-FAME was n-linoleate, especially in the cranberry variety 'US89-3' with an estimated 3.8 µM concentration. Blueberry (BB) samples were almost completely devoid of n-FAMEs with the exception of n-linoleate and n-linolenate found in (BB)ELL-FLW and (BB)BC-LVS, respectively.

The derivatized FAME samples were, as expected, much more complex in terms of number of compounds and µM concentrations. Decanoic acid derivatives (C10:0) were present in all samples, and were particularly elevated in blueberry samples, peaking in blueberry (BB) 'Bluecrop' (BC) leaves with an estimated 3.9 µM concentration. Derivatives of hexadecanoic (C16:0), octadecanoic (C18:0), and C:10 fatty acids were the only compounds present in all tissue types, including both species' w-FE (CHL EXT) which indicated the presence of non-water-soluble compounds in water extractions, linking the extraction types. CB floral samples were also generally higher in C16:0 and linoleic fatty acid derivatives (C18:2) compared to other tissue types as well as blueberry samples as a whole. Hierarchical clustering revealed a series of general trends, predominantly within the FAMEs as the n-FAMEs were more loosely associated due to

less robust observations. Clustering was observed amongst the host species, tissue types (floral, vegetative, fruit), and interestingly amongst extraction types as the w-FE (CHL EXT) extractions clustered together for both species. Interestingly, varieties that differed in field disease susceptibility (Johnson-Cicalese et al. 2015; Johnson-Cicalese et al. 2009; Polashock et al. 2005) did not cluster together, as evident by comparing (CB)ST-FLW to (CB)Bd.Bl.-FLW and (BB)BC-FLW to (BB)ELL-FLW, field fruit rot susceptible to resistant, respectively. The peak area data generated through the GC-FAME protocol, dictated the estimated maximum µM concentration and standard deviation per sample group (floral, vegetative, fruit) for all compounds (n-FAMEs, FAMEs) that were later evaluated in the bioassays (Table 1). However, some of the n-FAMEs were detected at such low levels (n-dodecanoate and n-tetradecanoate) or not at all (n-nonanoate and n-oleate), and were not evaluated in the bioassays in either their methyl ester or fatty acid form, with the exception of dodecanoic fatty acid (C12:0) due to its derivatives abundance in the FAME samples.

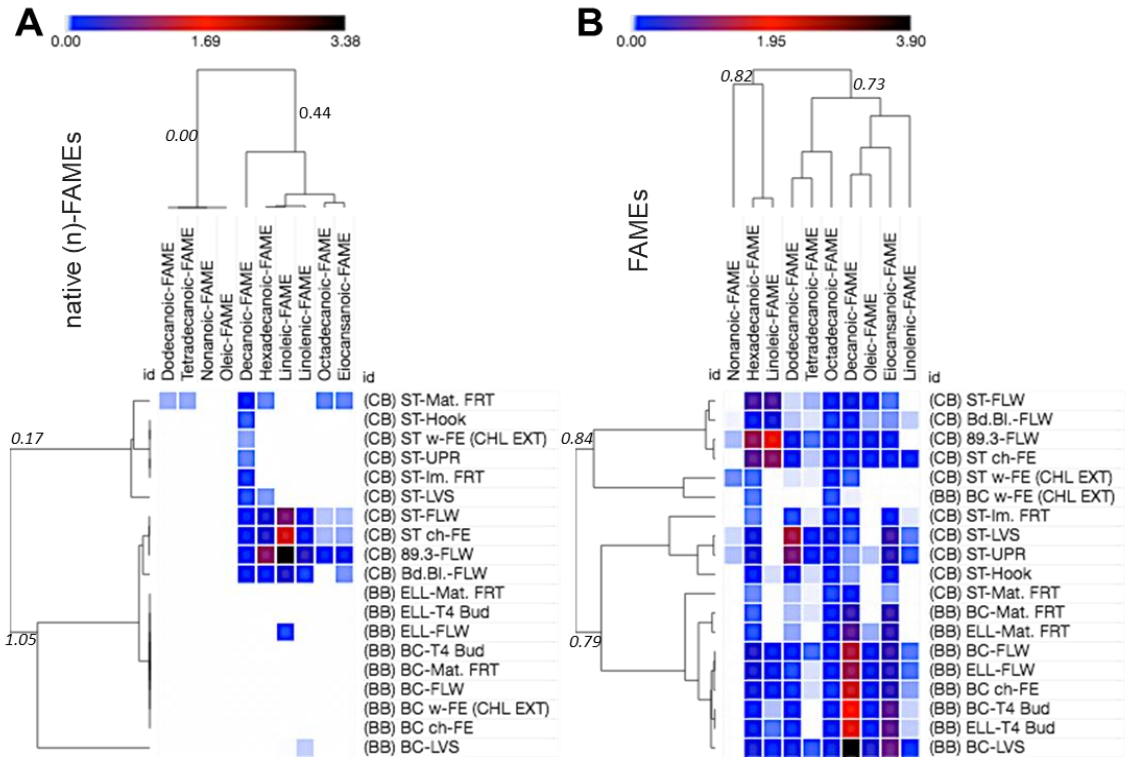

Figure 1. Heat map of the estimated µM concentration of each native fatty acid methyl esters (n-FAMEs) and fatty acid derivatives (FAMEs) identified from blueberry (BB) and cranberry (CB) epicuticular waxes from flowers, vegetative tissues, and fruit. **A.** Direct sampling of chloroform-based extractions processed at a 1 g tissue to 9 ml chloroform ratio to identify n-FAMEs. **B.** FAME sampling after fatty acid saponification and methanolysis of each sample type. One minus Pearson correlation hierarchical clustering performed for each group separately, italic numbers indicate similarity distances (heights) for each cluster's first two nodes, note clustering by host plant and tissues extracted. Displayed heat maps generated via Morpheus (Broad Institute).

Abbreviations: corolla only floral extracts (-FLW), whole flower extract (ch-FE), chloroform extraction of water-based floral extracts (w-FE (CHL EXT)), leaves (-LVS), immature (Im.) and mature (Mat.) fruit (-FRT), developing (stage: 'T4') blueberry buds (-T4 Bud), developing cranberry flowers (stage: 'hook') (-Hook), developing cranberry uprights (inflorescence) (-UPR). Cultivar abbreviations: 'Stevens' (ST), 'Buds Blues' (Bd. Bl.), 'US89-3' (89.3), 'Bluecrop' (BC), 'Elliot' (ELL).

Table1: Fatty acid derivatives (FA) and native fatty acid methyl esters (n-FAME) identified from blueberry and cranberry flower, vegetative, and fruit cuticle chloroform extractions[v] via GC-MS with estimated[w] µM concentrations detected and utilized in a separate glass coverslip bioassay

Compound[x]				Maximum [µM] across samples [y]						[Bioassay][z]
FA	n-FAME	Lipid #	RT (min)	Flower	+/-	Veg	+/-	Fruit	+/-	[µM]
Decanoic		C10:0	9.21	0.27	0.07	3.90	0.57	1.58	1.59	2.0
	Decanoate			0.16	0.07	0.08	0.04	0.16	0.08	0.2
Dodecanoic		C12:0	10.97	1.31	0.48	0.12	0.04	0.25	0.10	2.0
	Dodecanoate			0	0	0	0	0.04	0.01	-
Hexadecanoic		C16:0	13.91	1.20	0.40	0.46	0.18	0.35	0.13	1.0
	Palmitate			0.99	0.33	0.05	0.02	0.06	0.03	1.0
Linoleic		C18:2	15.04	1.80	0.65	0.20	0.10	0.14	0.06	2.0
	Linoleate			3.37	1.13	0	0	0	0	3.0
Linolenic		C18:3	15.09	0.21	0.08	0.24	0.11	0.41	0.18	0.2
	Linolenate			0.46	0.15	0.02	0.01	0	0	0.5
Octadecanoic		C18:0	15.20	0.29	0.07	0.16	0.03	0.25	0.07	0.2
	Stearate			0.14	0.05	0	0	0.06	0.03	0.1
Eicosanoic		C20:0	16.37	0.66	0.22	0.62	0.24	0.91	0.43	0.6
	Arachidate			0.19	0.06	0	0	0.06	0.03	0.2

[v] Chloroform based extractions were prepared for all tissues at a 1 g to 9 ml volume ratio.
[w] Recorded peak area was compared to an external standard (C16:0 methyl ester, discrete increments [0.1 – 100 µM]) / peak area set to transform data to estimated molarity concentrations.
[x] Peak naming was accomplished with the use of Microbial ID, Inc. external calibration standard, and checked against NIST Mass Spectral Library. Retention time, RT.
[y] All blueberry and cranberry extraction samples were separated into flower, vegetative (Veg) and fruit, with estimated maximum molarity recorded for each group per compound listed.
[z] Indication of selected bioassay treatment concentration as an approximate reflection of the maximum molarity observed. Bioassays were performed following Waller et al. (2019a) protocols.

Effects of fatty acid derivatives on *C. fioriniae* secondary conidiation and appressorial formation and the ratio thereof. Bioassays utilizing non-derivatized fatty acids (FA) as well as fatty acid methyl ester derivatives (FAMEs) revealed *C. fioriniae* specificity towards individual compounds (Fig. 2). Secondary conidiation was not drastically affected by the majority of compounds, with the exception of decanoic acid that had a significantly greater number of total conidia than the chloroform control, comparable to 'Stevens' chloroform-based FE (ST ch-FE). However major differences in appressorial formation were observed in the presence of various compounds, both FAs and FAMEs. Notably both forms of hexadecanoic and linoleic acids stimulated prolific appressorial formation as well as methyl stearate (but not octadecanoic fatty acid) and eicosanoic acid (but not methyl arachidate), which were all comparable to ST ch-FE. Importantly, ST w-FE (CHL EXT) was not significantly different than the standard cranberry floral extraction ((CB)ST ch-FE), and both were significantly more stimulatory than the chloroform control. Paraffin did stimulate a small increase in total conidia, however appressorial formation and the ratio of appressoria to conidia was not greatly affected, indicating that fungal recognition of specific compounds, not the presence of hydrophobic forces, was the main stimulus. In comparison to stimulation, some of the compounds appeared to be inhibitory or at least non-stimulatory. Methyl decanoate, dodecanoic fatty acid, both forms of linolenic and octadecanoic fatty acid were all not significantly different to the chloroform control in terms of both appressorial formation and the ratio of appressoria to conidia.

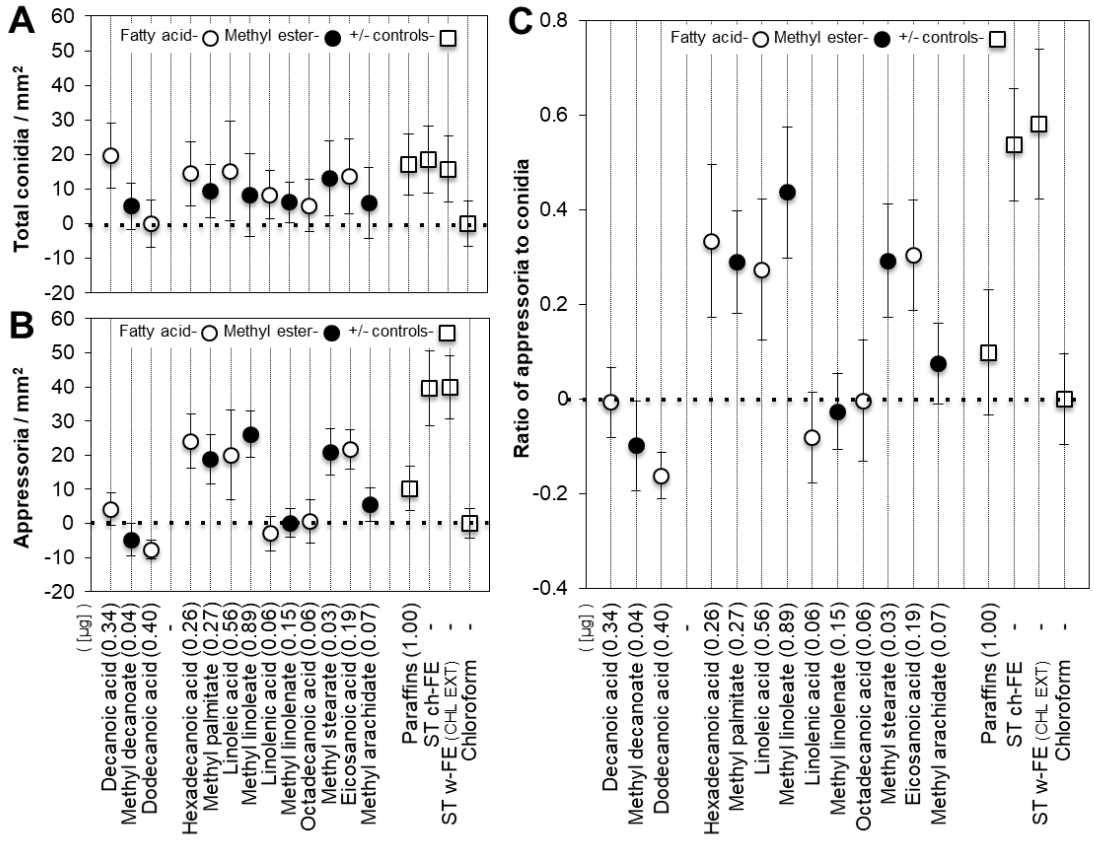

Figure 2. Evaluation of *Colletotrichum fioriniae* (CB-PMAP182) in the presence of fatty acids, associated methyl ester derivatives, paraffins, cranberry 'Stevens' chloroform-based floral extract (ST ch-FE) and chloroform extraction of ST water-based (w-) FE on glass coverslips at 24 h postinoculation, at 25°C, subtracted from the chloroform control data. **A.** Total conidia (primary / inoculated and newly formed secondary conidia). **B.** Appressorium formation. **C.** Ratio of appressoria to conidia (generated from appressoria count data divided by total conidia count data). Error bars reflect the standard deviation of count data. Compound amount [µg] used in parenthesis.

Correlations between compounds and the bioactivity observed in selected cranberry extractions. In order to determine the factors affecting the ratio of *C. fioriniae* appressoria to conidia, linear regressions as well Pearson correlation matrixes, displayed as Morpheus heat maps (Broad Institute), were performed by plotting specific bioassay data sets (secondary conidiation, appressorial formation, and the ratio thereof) against total carbon number or compound concentration, expressed as estimated µM concentration, percent compound per total n-FAME/FAME observed, or the entire suite of compounds per extraction sample (Table 2) (Table 3). No correlation was found between carbon number and ratio of appressoria to conidia for the selected cranberry extracts (ST flower, leaves, immature fruit and mature fruit). Additionally, when the entire composition of the extractions was compared to the individual bioactivity responses, the correlations were again extremely low. Generally, R-squared and the standardized regression coefficient (r), indicative of positive or negative correlations, were low, however some trends were observed. When individual n-FAME and FAME's estimated µM concentrations were regressed against the ratio data for the selected cranberry extractions hexadecanoic (C16:0), linoleic (C18:2), and linolenic (C18:3) acids were found to have the highest positive correlation to increased bioactivity. Continuing on this approach, the ratio data set of selected cranberry extractions was compared with percent FAME per total FAME per extraction or the observed µM concentration per FAME per extraction was utilized to generate correlation matrixes.

The generated matrixes provided a useful visual tool, indicating both positive and negative correlations between each compound with respect to *C. fioriniae* bioactivity and amongst compounds (Fig. 3). In agreement with the previous observation, there was a

high, positive correlation amongst hexadecanoic (C16:0), linoleic (C18:2), and linolenic (C18:3) fatty acid derivatives in both molarity and percent FAME to bioactivity. Bioactivity was negatively correlated to (C18:0) and decanoic (C10:0) fatty acid derivatives µM abundance. However, in terms of percent FAME per sample type, octadecanoic (C18:0) and decanoic (C10:0) acids were positively correlated to bioactivity, due to these compounds increased abundance in the less stimulatory vegetative and fruit extractions.

Table 2. Correlation between the estimated concentrations ([ ]) of fatty acid methyl ester derivatives (FAMEs) and native FAMEs (n-FAME) to the ratio of *Colletotrichum fioriniae* (CB-PMAP182)[x] appressoria to conidia (bioactivity).

ID[y]	Variables		Correlation description	R-squared (r)[z]	
	Dependent	Independent		FAME	n-FAME
1	Ratio (bioactivity)	Carbon number	All FAs and FAMEs	0.25(0.50)	-
			Stimulatory > control	0.06(-0.25)	-
			Non-stimulatory ≤ chloroform	0.03(0.18)	-
2	Total conidia	Bioassay [µg]	(Bioactivity responses)	0.13(0.36)	-
	Appressoria			0.37(0.61)	-
	Ratio			0.40(0.63)	-
3	Ratio (bioactivity)	Sample [µM]	Decanoic-FAME	0.09(0.30)	0.00(0.02)
			Dodecanoic-FAME	0.25(0.50)	0.29(-0.54)
			Hexadecanoic-FAME	**0.79(0.89)**	**0.55(0.74)**
			Linoleic-FAME	**0.51(0.71)**	**0.51(0.71)**
	Cranberry		**Linolenic-FAME**	**0.85(0.92)**	**0.51(0.71)**
	FLW, LVS, FRT		Octadecanoic-FAME	0.31(0.56)	0.02(-0.15)
			Eiocansanoic-FAME	0.30(0.55)	0.00(-0.05)

[x] Bioassays were performed following Waller et al. (2019a). Ratio data was a proportionate value, calculated as the quotient of total counted appressoria divided by total counted conidia.

[y] Identification code (ID). (1) Regression of total carbon number against ratio data for all compounds utilized in the bioassay (listed under ID 3). (2) All compounds utilized in bioassay against each bioactivity response. (3) Estimated µM concentration ([µM]) of each FAME and n-FAME against bioactivity ratio data for cranberry 'Stevens' (ST) chloroform-based floral extracts (FLW), and bioactivity data from Waller et al. (2019b) for ST leaf (LVS), ST immature and mature fruit (FRT) extractions that were chemically analyzed in the current study.

[z] Linear regressions via XLSTAT (Addinsoft, Inc.) statistical package were performed to determine R-squared and the standardized regression coefficient (r).

Table 3: Estimated[x] concentrations (µM and percent compound per total identified) of fatty acid derivatives (FAMEs) in select cranberry 'Stevens' chloroform based extractions and the ratio (Ratio[y]) of *Colletotrichum fioriniae* (CB-PMAP182) appressoria to conidia in the presence of respective extraction.

Bioactivity		Fatty acid derivative lipid number						
Extraction[z]	Ratio	C10:0	C12:0	C16:0	C18:2	C18:3	C18:0	C20:0
		Percent FAME per total extraction FAMEs						
Whole flower	0.79	6.03	6.20	30.66	40.99	5.54	4.32	6.25
Immature fruit	0.29	19.86	17.81	12.53	0.00	2.48	19.68	27.65
Mature fruit	0.31	38.55	9.02	15.96	0.00	0.00	24.09	12.38
Leaves	0.68	2.92	48.67	10.94	0.00	2.32	10.69	24.46
		Total µM per FAME per extraction						
Whole flower	0.79	0.17	0.17	0.85	1.13	0.15	0.12	0.17
Immature fruit	0.29	0.09	0.08	0.06	0.00	0.01	0.09	0.13
Mature fruit	0.31	0.13	0.03	0.06	0.00	0.00	0.08	0.04
Leaves	0.68	0.08	1.31	0.29	0.00	0.06	0.29	0.66

[x] Recorded peak area was compared to an external standard (C16:0 methyl ester, discrete increments [0.1 – 100 µM]) / peak area set to transform data to estimated total µM concentrations per compound. Percentage of total fatty acid methyl ester was calculated for each compound per group (n-FAME or FAME) per extraction set.

[y] Bioassays were performed following Waller et al. (2019a). Ratio data was a proportionate value, calculated as the quotient of total counted appressoria divided by total counted conidia.

[z] All extractions were characterized in the current study, however the leaf and fruit ratio data were referenced from Waller et al. (2019b). Whole flower = (CB) ST ch-FE.

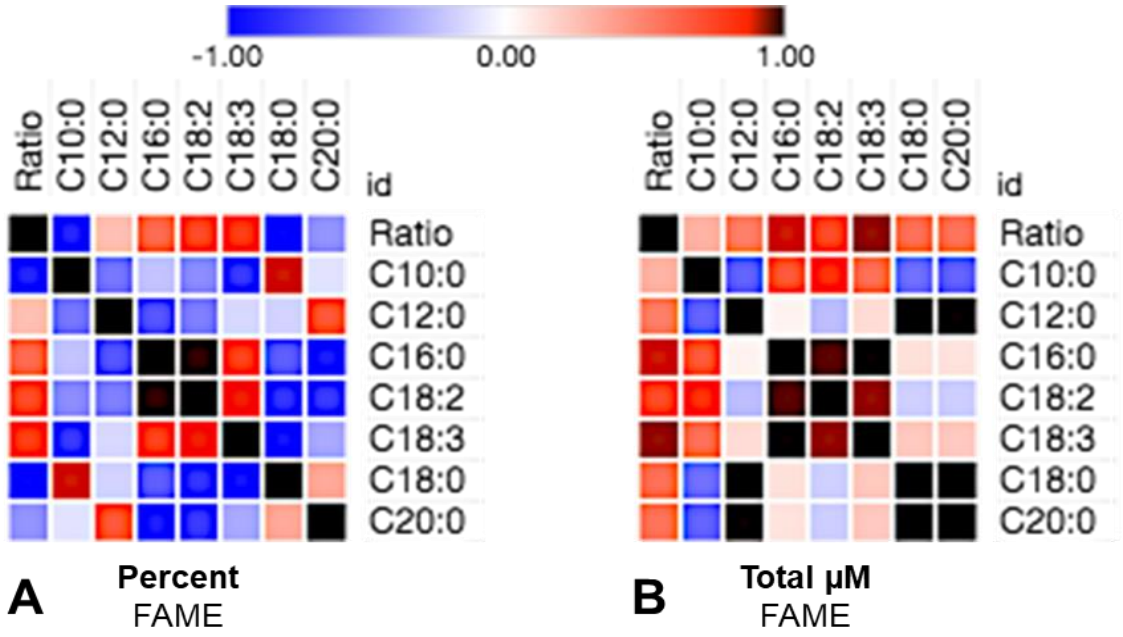

Figure 3. Heat map display of the correlation matrixes (Pearson) between identified fatty acid derivatives (FAMEs) and the set of ratio data from *Colletotrichum fioriniae* appressoria to conidia (Ratio) in the presence of selected cranberry 'Stevens' (ST) chloroform extractions. The ratio data set consisted of whole flower ratio data (present study) and ratio data from Waller et al. (2019b) for ST leaf, immature and mature fruit. **A.** Correlation between percent individual FAMEs of total FAMEs and ratio. **B.** Correlation between estimated [µM] of individual FAMEs and ratio. Correlation matrixes were prepared using XLSTAT (Addinsoft, Inc.) statistical package and displayed heat maps were generated via Morpheus (Broad Institute).

Discussion

Floral extractions (water- and chloroform-based) from both blueberry 'Bluecrop' (BC) and cranberry 'Stevens' (ST) have previously been shown to stimulate *C. fioriniae* secondary conidiation and appressorial formation (bioactivity) (Waller et al. 2019b; Waller et al. 2019; Waller et al. 2018), yet whether these extractions had similar chemical compositions and the overall pathogen activation mechanisms remained unclear. The data presented in the current study quantifies one class of cuticular compounds found on the vast majority of plants worldwide (Barthlott et al. 2017; Buschhaus et al. 2007; Fernández et al. 2016; Van Maarseveen and Jetter 2009; Zeisler-Diehl et al. 2018); C9:0 – C20:0 fatty acids and their derivatives. Through comparing individual compound's

occurrence paired with the glass coverslip bioassay data, one of the main cuticle constituents, hexadecanoic fatty acid (C_{16}) (and derivatives), is likely an important source of *C. fioriniae* stimulation. Additionally this compound, among others were found in both water- and chloroform-based extractions, implicating a probable connection between the chemical composition and observed fungal activation with the respective floral extractions types. The GC-FAME protocol (Midi Inc., Newark, DE) and subsequent hierarchical clustering (one minus Pearson's coefficient) of samples' estimated µM concentrations of fatty acid derivatives (FAMEs) revealed commonality at an extraction, species, and tissue type level. Octadecanoic (C18:0) and decanoic (C:10) fatty acid derivatives, found in the water-based extractions of both species, were negatively correlated to *C. fioriniae* bioactivity, whereas hexadecanoic acid derivatives were positively correlated, helping to deduce which compounds were likely involved in pathogen activation. The native fatty acid methyl esters (n-FAMEs) and fatty acid derivatives (FAMEs) descriptions presented here for the various blueberry and cranberry tissue types were not previously available. The inclusion of both host species and extraction types represented a comparative tool to deduce which compounds were most important to *C. fioriniae*. Moreover, this study represents a foundation to further explore factors and different cuticular compound classes that *initiate* or *impede* important disease cycle events of *C. fioriniae* and other bloom period infecting fungi.

Methyl palmitate isolated from cranberry floral wax was previously shown to stimulate appressorial formation (Gager 2015), however the presence of this specific compound or other n-FAMEs within the floral wax remained ambiguous thus spurring the investigation. Indeed this compound, as well as numerous other n-FAMEs were

identified most often in the cranberry floral extractions, yet were virtually nonexistent in the blueberry extractions or the water extractions (w-FE (CHL EXT)) from both host species. Given that for both species, FEs affected *C. fioriniae* growth and the overall low abundance of methyl esters, it appeared unlikely that these compounds were an important source of pathogen stimulation.

However, the evidence did suggest the importance of fatty acid derivatives or the fatty acids themselves in the disease cycle of *C. fioriniae*. Appressorial formation in response to hexadecanoic acid was logical, as the biopolymer cutin, comprised primarily of scaffolding C_{16} (hexadecanoic) and C_{18} (octadecanoic) hydroxy- and epoxy-fatty acids, that have specifically been shown to stimulate certain fungi, act as the barrier a pathogen must overcome to gain entry into the host (Dickman et al. 2003; Dickman et al. 1989). Dickman et al. (2003) demonstrated that cuticular compounds 9,12-octadecanoic (linoleic) acid; 9,10,16-trihydroxyhexadecanoic (aleuritic) acid, 8,16-dihydroxyhexadecanoic acid stimulated a lipid induced protein kinase (LIPK), a gene required for normal appressorium development (and pathogenicity) of *Colletotrichum trifolii* on alfalfa, however appressorium formation was not observed when incubated with non-cuticular 16-hydroxyhexadecanoic acid, meaning the pathogen was able to discern host surface constituents (cuticle) and induce LIPK genes accordingly. When LIPK was overexpressed, copious appressoria were formed often appearing to stack upon each other (Dickman et al. 2003), similar to previous observations with *C. fioriniae* and *C. fructivorum* in the presence of blueberry and cranberry FEs (*current study*; Waller et al. 2019b; Waller et al. 2018), indicating the probable role of this lipid-mediated cascade in the disease cycle events of other *Colletotrichum* species. In the current study,

hexadecanoic and linoleic fatty acids were highly stimulatory to *C. fioriniae* and their abundance was positively correlated to increased bioactivity, thus the detected fatty acid derivatives were possibly occurring as di- or tri- hydroxyhexadecanoic acids and 9,12-octadecanoic (linoleic) acid within the samples evaluated. In a different pathosystem, researchers Woloshuk and Kolattukudy (1986) found that *Fusarium solani* f. sp. *pisi* produced cutinase 30-40 minutes post incubation with cutin the constituents 10,16-dihydroxyhexadecanoic acid, 9,10,18-trihydroxyoctadecanoic acid, and crude cutin hydrolysates (Woloshuk and Kolattukudy 1986). Cutinase gene transcripts greatly increased in the presence of exogenous cutin, suggesting that fungal spores expressed low levels of cutinase that initially degraded the cuticle, thereby liberating monomeric cuticle hydrolysates, stimulating the constitutive production of cutinase that softens the cuticle allowing for direct fungal penetration (Kolattukudy et al. 1995). The same conclusion was drawn when investigating the role of cutinase in pre-penetration events of apple leaves by *Venturia inaequalis* appressoria (Koller et al. 1991). Dickman et al. (2003) took this model a step further; postulating that once *C. trifolli* conidia land on a host surface (cuticle) the endogenous cutinase hydrolyses the polyester into its constituent *n*-hydroxy-fatty acids, subsequently triggering appressorial formation through the LIPK protein-kinase cascade, thus linking cutinase production and appressorial formation. It is plausible then that *C. fioriniae* responds to cutin hydrolysates (and their fatty acid parental compounds) under field conditions in the same fashion, by stimulating genes involved in both cutinase and appressorial formation.

Since hexadecanoic fatty acid derivatives stimulate appressorial formation, why then is there a discrepancy between the bioactivity observed between floral, vegetative,

and fruit extractions when this compound is omnipresent in plant waxes (Barthlott et al. 2017; Buschhaus et al. 2007; Fernández et al. 2016; Van Maarseveen and Jetter 2009; Waller et al. 2019b; Zeisler-Diehl et al. 2018)? The answer may lie in the balance of stimulatory and inhibitory compounds present in epi- and intracuticular waxes. A related pathogen, *C. gloeosporioides* s.l., produced prolific appressoria when incubated with synthetic fatty alcohols ranging from C_{22}-C_{32} (approximately 4% of total waxes initially characterized from avocado fruit waxes). However, when the pathogen was incubated with other species' cuticular waxes with equivalent or greater concentrations of fatty alcohols, appressorial formation was inhibited. The authors assumed the presence of inhibitory compounds possibly functioning as a mechanism of host-specificity, ultimately underscoring the importance of species/organ cuticular composition (Podila et al. 1993). In the same study, the hydrocarbon fraction (62%) failed to stimulate *C. gloeosporioides* s.l., reiterating the importance pathogen recognition of specific compounds over other factors such as hydrophobicity. Mirroring this, paraffin waxes at 1 µg/ml (chain lengths, C_{20}-C_{40}) failed to stimulate *C. fioriniae* appressorial formation greater than specific fatty acid monomers at lower µg concentrations and chain lengths in the current study.

Although chain length was not directly correlated to bioactivity, positive and negative correlations amongst compounds to *C. fioriniae* bioactivity were observed, providing insight into the selective stimulation by floral extractions. Appressorium inducing hexadecanoic and linoleic fatty acid derivatives were positively correlated to bioactivity and higher in flowers as a whole compared to vegetative and fruit tissues. Whereas, appressorium inhibiting decanoic fatty acid derivatives were most abundant in the vegetative and fruit tissue samples, and negatively correlated to bioactivity.

Generally, extraction types with greater abundance of hexadecanoic and linoleic fatty acid derivatives (flowers) stimulated more *C. fioriniae* bioactivity than extraction types (vegetative and fruit) with higher levels of decanoic and octadecanoic fatty acid derivatives, which were positively correlated to each other and negatively correlated to bioactivity. Thus suggesting hexadecanoic acid as the most likely stimulant within this group, as all of these compounds (except linoleic) were identified from both the w-FEs and ch-FEs of both species. Taken together, the balance of each compound's abundance likely affects bioactivity, as is likely the case when comparing flower to fruit extractions where hexadecanoic fatty acid derivatives are at proportionately lower concentrations.

When comparing water- and chloroform-based leaf and floral tissues extractions, chloroform-based extractions from both tissue types stimulated appressorial formation, in contrast, only the water-based floral extracts were stimulatory and water extracts from leaves were non-stimulatory (Waller et al. 2019b). It is speculated that the epicuticular topography may affect the richness of compound availability to *C. fioriniae* at the host cuticle interface. Often, developing tissues are initially very hydrophobic, later transitioning to more hydrophilic as the organ ages, citing the erosion of three-dimensional epicuticular crystalline structures such as nonacosan-10-ol tubules that initially impart hydrophobicity through this physical characteristic (Barthlott et al. 2017; Barthlott et al. 2016). Interestingly, the non-stimulatory (Gager 2015) parental compound nonacosane was identified in all ericaceous samples evaluated in the current study (*data not shown*), yet may suggest the presence of nonacosan-10-ol tubules on younger tissues such as flowers. The chloroform-based extractions remove the majority of the epi- and intracuticular waxes, meaning nonacosane-10-ol could exist as either the three-

dimensional (crystalline projections) form or in an eroded state (filling the voids of the C_{16} and C_{18} cutin polymer matrix) depending on the stage of organ ontogeny (Barthlott et al. 2017; Barthlott et al. 2016), as cuticular waxes are generally known to change over this period (Avato et al. 1984; Barthlott et al. 2017). Accordingly, if young tissues, such as ephemeral flowers have copious hydrophobic crystals/tubules, then these crystalline tubules could act as carriers of stimulants when eroded during wetting events along with imbued stimulatory compounds such as the scaffolding C_{16} fatty acid derivatives. Also this inherently less compacted epicuticular matrix, as compared to an epicuticular film, may allow for greater physical disruption of the cuticle as is the case with *Dudleya* spp. that are covered by unstable tubular crystals that are destroyed by only the force of a rain drop (Barthlott et al. 2017; Barthlott and Wollenweber 1981), possibly explaining the discrepancy between leaf and floral water extractions. Following this concept, crystalline tubules on flowers and developing ovaries would increase the surface area (with imbued stimulants) accessible to pathogens as compared to an epicuticular film, or eroded surface, thus providing more stimulation per linear area. Waller et al. (2019b) demonstrated that rainwater that had come into contact with cranberry flowers was more stimulatory than all other stages of the flower to fruit ontogeny. In light of previous studies, it is plausible that each growth stage of the flower to fruit ontogeny may have a unique pattern of dislodge-able epicuticular waxes and accompanying fatty acid derivatives, ultimately affecting the rainwater bioactivity observations.

These data taken together provide a series of compounds that stimulate or impede pathogen development, representing a foundation towards understanding the exact factors triggering disease cycle events in *C. fioriniae.* However, the current study was narrowly

focused on a subset of one class of cuticular compounds, fatty acids. Fatty alcohols (including primary and crystal forming secondary alcohols), wax esters (ester bound fatty acids and fatty alcohols), cyclic compounds (pool of antifungal compounds), ketones (crystal forming), and flavonoids (bioactive compounds) should all be explored utilizing this approach of linking compound identification to bioassay techniques as discoveries could lead to very important resources for constantly evolving disease management strategies. Fungicide applications with the inclusion of stimulatory compounds could trigger unanimous activation of inoculum reservoirs thereby making each application more efficient, or using hexadecanoic acid to stimulate the inoculum reservoir during non-fruit bearing seasons in a perennial crop, sequentially depleting the inoculum reservoir as carbohydrate-rich fruit would be unavailable for prolific sporulation events, possibly limiting the need for repeated fungicide applications. Ultimately, a better understanding of the host interface and subsequent chemical activation of fruit rotting pathogens will allow for field-based applications aimed at more efficiently controlling *C. fioriniae* and other pathogens with critical disease control during the bloom period.

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Conclusion

Floral signals play an important role in the disease cycle of *Colletotrichum fioriniae* and other fruit rotting pathogens

The factors responsible for pathogen stimulation during the bloom period were characterized and identified in the current study through the use of directed glass coverslip bioassays. Floral extracts (FEs) from blueberry and cranberry were found to affect *C. fioriniae* and other latent cranberry fruit rotting pathogen's disease cycles by influencing germination, appressorial formation, and secondary conidiation. Secondary conidiation in response to floral signals had been previously observed in two related pathosystems (Leandro et al. 2001; MacKenzie et al. 2010). However, appressorial formation in response to floral signals was unique to this research, signifying an important development in phytopathology (Waller et al. 2019; Waller et al. 2018). More specifically, floral signals isolated through water- (w-FE) or chloroform-based (ch-FE) extraction techniques, dramatically increased both the rate and magnitude of secondary conidiation and appressorial formation. *C. fioriniae* conidia in the presence of floral signals were able to form appressoria at lower temperatures and secondary conidiation increased with temperature (25 - 30˚C), ultimately providing better epidemiological information related to the initial disease cycle factors. Increased appressorial formation was shown to correlate to infection, as conidia in the presence of host floral signals were able to confer greater levels of disease than conidia alone. *C. fioriniae* was selected as a representative pathogen as it readily infects both blueberries and cranberries, thus

providing a robust multi-host platform that allowed for comparisons between host architecture (bushes versus lianas), flower morphology (fused corolla or cleft petals), and bloom period timings (spring versus early summer) (Polashock 2017). Additionally, other ericaceous species w-FEs including *Kalmia latifolia* L., *Lyonia mariana* (L.) D. Don, and *Rhododendron maximum* L. stimulated both disease cycle responses (conidiation / appressoria), suggesting commonality amongst ericaceous floral signals (Waller et al. 2018). Diverse cranberry fruit rot (CFR) pathogens were evaluated as well. *Colletotrichum fioriniae* (Type 1) (Damm et al. 2012), *C. fructivorum* (Type I) (Doyle et al. 2013) and *Coleophoma cylindrospora* (Type 2) (Crous and Groenewald 2016) that infect during bloom then remain dormant until fruit/seed maturation, were highly stimulated by FEs, whereas *Allantophomopsis lycopodena* (Type 3), a necrotrophic pathogen that infects and destroys mature fruit (Oudemans et al. 1998) did not, underscoring the importance of flowers in disease cycles where synchronization of pathogen-host phenology is equally beneficial to both parties, allowing both cranberry seed maturation and ultimately liberation (Cipollini and W. Stiles 1992).

The w-FE and rainwater collections from both crops suggested floral signal mobility within the canopy as well as from corollas to ovaries (Waller 2018, 2019b). Many *Colletotrichum* spp. are well adapted to rain-splash dispersal, where the water droplet volume, contents (plant signals), and external environmental conditions dictate the duration of an infection period, i.e. time until droplet desiccation (Madden et al. 1993; Madden et al. 1996; Miles et al. 2013; Verma et al. 2006; Yang et al. 1990). Additionally *C. fioriniae* preferentially overwinters in floral buds and has been shown to sporulate in synchronization with blueberry bloom (DeMarsay 2005; Miles et al. 2013; Wharton

2002). Taken together, rain-splash dispersed conidia are likely ‘splashed’ from flowers to adjacent ovaries or inflorescences thus transferring stimulatory floral signals to the infection court, reducing the temporal stringency of the wetness period. Although not all pathogens were evaluated in all bioassays, the disease cycle components documented in the current study for *C. fioriniae* provide a framework to better understand *C. fructivorum* and *Coleophoma cylindrospora* lifestyles as these pathogens are activated by and subjected to the same set of factors (Waller et al. 2019b).

Water-based extractions were suggested to be partially composed of water-soluble/polar compounds such as floral nectar sugars (principally sucrose) and unique to this research, dislodged/mobilized (non-water-soluble) epi- and intracuticular waxes comprised of derivatives of hexadecanoic, octadecanoic, and decanoic fatty acids among others as well as larger alkanes and alcohols (Chapter 4). In the citrus post bloom fruit drop-*Colletotrichum* spp. pathosystem, it was concluded that sucrose ‘washed down’ through the canopy activating persistent appressoria to germinate and begin sporulation, functioning as the main stimulatory floral signal (MacKenzie et al. 2010). Nectar sugars sucrose, glucose, and fructose (Moquet et al. 2015; Stiles and Freeman 1993) stimulated secondary conidiation of *C. fioriniae*, corroborating the findings by MacKenzie et al. (2010), across multiple molar concentrations, thus avoiding osmotic variation (Waller et al. 2019b). However, sugars alone could not explain the magnitude difference in appressorial formation between sugars and the standard ‘Stevens’ w-FE, implicating the presence of additional stimulants. Another line of evidence for multiple chemical stimulants came from the blueberry cultivar assay where w-FE from all cultivars evaluated stimulated secondary conidiation whereas only w-FEs from susceptible

cultivars stimulated prolific appressorial formation (Waller et al. 2018). A general trend of increased secondary conidiation in the presence of w-FE compared to more appressorial driven response in the presence of ch-FE, again pointed to the diversity of compounds present in the FEs. This trend, or main effect of the extraction type, was quantitatively evaluated using the ratio of appressoria to conidia as a metric of bioactivity (ratio). For example, when comparing w-FE to ch-FE within the 24 h time-course study (Waller et al. 2019b; Waller et al. 2019) as secondary conidiation began to greatly increase in the presence w-FE the ratio value decreased suggesting w-FE provided more nutritional components (sugars) necessary for replication. Conversely, In the presence of ch-FE lower levels of conidiation coupled to consistently increasing appressorial formation drove the ratio value towards a 1:1 relationship, suggesting ch-FE provided much more focused chemical stimulation of appressorial formation.

Chloroform was utilized as a means of transferring flower, vegetative, or fruit epi- and intracuticular waxes to a glass coverslip, elucidating the early growth stages of conidia after landing on susceptible host organs (i.e. flowers, ovaries, developing fruit) (Waller et al. 2019b; Waller et al. 2019). Appressorial formation was the most obvious bioactivity response with all fungal isolates evaluated with the exception of *A. lycopodena,* again suggesting a close relationship of latent pathogens to floral signals (Waller et al. 2019b). As previously, noted FEs decreased the time needed for *C. fioriniae* to form appressoria, nowhere was this more apparent than in the presence of cranberry ‘Stevens’ ch-FE where appressorial formation began at 2 h postinoculation (25˚C), indicating almost immediate pathogen recognition of host floral signals (Waller et al. 2019b). Previous research concluded that *Colletotrichum acutatum* s.l. needed a wetness

period between 10-12 h (at 11 to 27˚C) to form appressoria capable of infecting blueberry fruit, whereas the same pathogen on the surface of almond blossoms produced appressoria at 3 h (at 15-20˚C) compared to 24 h on almond leaves (at 15-20˚C) (Hartung et al. 1981; Verma et al. 2007). This example coupled with observations from the current study, again promote floral signals / surfaces as highly stimulatory disease cycle factors affecting the early stages of *C. fioriniae* development.

The compositions of numerous blueberry and cranberry chloroform-based extractions as well as two representative w-FEs were partially characterized via the GC-FAME protocol (Midi Inc., Newark, DE), identifying fatty acid derivatives (FAMEs), native fatty acid methyl esters (n-FAMEs), and a limited set of alkanes, alcohols, and a ketone (data not shown). Hierarchical clustering of compound occurrence amongst extractions elucidated similarities amongst species, tissue types (specifically floral tissues), and extraction type chemical composition. This study represented the first FAME characterization of the blueberry and cranberry cuticles analyzed. One of the more interesting observations of this research was the presence of non-water-soluble FAMEs, isolated from w-FEs from both blueberry and cranberry. Thus validating the hypotheses that multiple chemical floral signals are present in any given extraction and that the different extraction types (w-FE versus ch-FE) contain many of the same compounds, as originally implied by overlapping bioactivity responses.

C. fioriniae appressorial response to hexadecanoic fatty acid implicated the presence of 8,16-dihydroxyhexadecanoic acid or 9,10,16-trihydroxyhexadecanoic acid forms within the chloroform extractions, as these compounds are common cuticle scaffolding compounds (Barthlott et al. 2017) that have been shown to stimulate both

appressorial formation and cutinase production in at least *C. trifolii*, *Fusarium solani* f. sp. *Pisi* and *Venturia inaequalis* (Dickman et al. 2003; Dickman et al. 1989; Kolattukudy et al. 1995; Koller et al. 1991; Woloshuk and Kolattukudy 1986). These pathogens' conidia, and likely *C. fioriniae* and other cranberry fruit rotting pathogens, are thought to possess basal cutinase activity (Serrano et al. 2014). Meaning that once a conidium lands on a susceptible tissue, a pool of cutin monomers / hydrolysates can be generated from the host cuticle, subsequently recognition of these hydrolysates (through modified sex pheromone receptors (Braunsdorf et al. 2016; Turrà et al. 2015)) initiates constitutive cutinase production (Kolattukudy et al. 1995; Woloshuk and Kolattukudy 1986) and/or appressorial formation (through the LIPK protein-kinase cascade in *C. trifolii*) (Koller et al. 1991). Many of these hydrolysates are derived from hexadecanoic fatty acid, characterized from w-FEs and ch-FEs, thus suggesting a link between cutinase production and appressorial formation of *C. fioriniae* to floral signals.

Selective stimulation afforded by floral samples may be related to variations of compound abundance or the balance between stimulatory/inhibitory cuticular compounds present within the different tissue types. Appressorial-inducing hexadecanoic and linoleic fatty acid derivatives were observed in all tissues characterized, yet were more concentrated in the floral samples. In comparison, appressorial-inhibiting decanoic fatty acid derivatives were more abundant in the less bioactive vegetative and fruit samples. Additionally, there was virtually no difference between cultivars with ranging fruit rot susceptibilities, indicating that nuanced genotype effects were either not discernable through the approach utilized or that compounds not identified in the current study were more important in distinguishing cultivar susceptibility.

An interesting discrepancy observed when comparing water- and chloroform-based leaf and floral extractions suggested that the availability of bioactive compounds mobilized in water (and thus infection droplets) could be a function of each tissues current epicuticular crystalline state. Older tissues with prior-eroded crystalline structures (Barthlott et al. 2017; Barthlott et al. 2016; Barthlott and Wollenweber 1981), such as fruit and mature leaves, would therefore shed less stimulatory compounds, whereas ephemeral floral tissues with intact crystalline structures would be more prone to shed epicuticular waxes with imbued stimulants / scaffolding hexadecanoic acid derivatives. This suggestion was further qualified by the observation of biologically active rainwater that had runoff of flowers (specifically) as well as the ontogeny-based rainwater collections where *C. fioriniae* activation was highest with samples collected during bloom, with diminishing stimulation through fruit maturation (Waller et al. 2019b; Waller et al. 2019). Ultimately, the richness of chemicals available to pathogen stimulation found in an infection/rainwater droplet during the bloom period may be higher than at other times of the growing season.

The data and commentary present here repeatedly implicate floral signals as key disease cycle components of *C. fioriniae* in both blueberry anthracnose and cranberry fruit rot (along with other cranberry fruit rot pathogens). The paired extraction-bioassay methods represent innovative techniques of plant signal acquisition, as well as a set of dynamic tools to build upon our current understanding of fungal pathogen biology. The descriptions of time and temperature effects on *C. fioriniae* in the presence of host signals highlight the importance of including this type of epidemiological information in disease forecasting and on-site decision making approaches. Additionally, a pragmatic suite of

compounds have been identified that could be utilized to deregulate pathogen-host phenology synchronization, or synchronization of pathogen development to well-timed fungicide applications, in either case allowing those attempting to control fruit rotting pathogens more control of the temporal components of the disease cycle.

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