THE INFLUENCE OF CRANBERRY FLORAL WAX ON APPRESSORIUM FORMATION IN COLLETOTRICHUM FIORINIAE

by

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ABSTRACT OF THE THESIS

The Influence of Cranberry Floral Wax on

Appressorium Formation in *Colletotrichum fioriniae*

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The effect of cranberry floral wax was tested on appressorium formation rates in *Colletotrichum fioriniae*, a causal agent of fruit rot on cranberry (*Vaccinium macrocarpon*). Signaling mechanisms regulating appressorium induction vary by pathosystem, and offer potential targets for fungal control measures. Although studies have been done on close relatives, appressorium formation triggers have not been definitively identified in the *C. fioriniae* – cranberry system. Surface wax from flowers was extracted in chloroform and some of the chemical constituents of the wax were identified by gas-chromatography-mass spectrometry (GC-MS). Spores of *C. fioriniae* were incubated with surface wax or with pure identified compound from the wax to assess their effect on appressorium formation. Floral wax induced appressorium formation in as little as six hours. The methyl ester of

hexadecanoic acid, a monomer of the plant biopolymer cutin, was identified as a wax component that showed strong stimulatory effects *in vitro*. This is significant, as it points to fungal cutinase production as a potential target of future disease management strategies on cranberry. In addition, no differences in induction were found between waxes extracted from resistant and susceptible host genotypes. Further, although hydrophobicity has been shown to induce appressorial formation in other pathogens, it was ruled out as a stimulus here by tests with hydrophobic paraffin wax. Waxes isolated from cranberry fruit throughout the growing season did not induce appressorium formation, though whether this is due to a lack of stimulatory compounds or the presence of inhibitors was not determined and merits further study.

Acknowledgement and Dedication

I'd like to thank Dr. Peter Oudemans, without whose patronage and guidance none of this research would have been possible. Thanks also to Timothy Waller, Christine Constantellos, and Dr. Thomas Gianfagna, who were all overwhelmingly generous with their time and assistance.

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means or willpower, and without her inspiration and support I would have starved or quit several times along the way.

I dedicate my thesis to anyone who is kept awake at night

by that special kind of madness called curiosity.

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Introduction

The large American cranberry (*Vaccinium macrocarpon* Shear) is an economically important small berry crop native to North America (Eck 1990). The fruits of the plant are processed to make juices, jellies, and relishes, and are also dried and processed for use in cereal, snack food, and health products. Cranberries have several health benefits, including significant antioxidant activity (Xiaojun et al. 2002) and the ability to reduce risk of urinary tract infection among susceptible populations by rendering bacteria unable to cling to the walls of the human bladder (Howell 2007).

Fruit rot is a major cause of loss in cranberry cultivation, sometimes up to 100% if left untreated (Oudemans et. al 1998). Although the fungicide regimens and cultural practices currently in existence do a good job of keeping rot to manageable levels, upcoming restrictions on certain highly effective fungicides are a growing concern for the industry (Personal Communication, Dr. Peter Oudemans). While the causal organisms of fruit rot vary from growing region to growing region and season to season, they are typically kept in check using very similar methods (Oudemans et al. 1998). One causal organism in particular, *Colletotrichum fioriniae* (formerly grouped with *C. acutatum*), is of interest for studying latent infections caused by *Colletotrichum* species (Damm et al. 2012), and more specifically for studying those leading to cranberry fruit rot that is difficult to assess in the field. By better understanding the biology of *C. fioriniae* we can more carefully tailor fungicide programs and management recommendations to reduce losses from fruit rot.

An area of particular interest when discussing the reduction of yield losses due to disease is the interaction between the host and the pathogen at the plant cuticle. The cuticle is the first line of defense for the plant, and the first chance for recognition of a specific host by the pathogen. Thus, understanding the complex interactions which occur when a spore first lands on a plant is of paramount importance in combating phytopathogenic fungi.

With that in mind, these studies aimed to test the effect of cranberry floral cuticle wax on the formation of infection structures in *Colletotrichum fioriniae.* Further, they endeavored to determine if there was a difference in stimulation of infection structure production between wax from genotypes of cranberry that are resistant to fruit rot, and those which are susceptible. The chemical composition of cranberry floral wax was also partially determined and individual chemicals were tested for potential infection structure stimulation. Wax from ovaries and berries over the course of the growing season was tested as well to assess potential ontogenic changes in stimulation. Finally, carnauba and paraffin waxes were tested to determine if surface hydrophobicity was the triggering factor stimulating infection structure formation.

The results clearly show that cranberry floral and ovary cuticular wax induces infection structure formation in *C. fioriniae* in as little as six hours. Further, no correlation was found between cranberry genotype resistance and infection structure formation in two resistant and two susceptible genotypes.

While the wax of ovaries and petals stimulated infection structure formation, berry wax did not. This fact, along with the identification of the

prominent stimulatory compound as hexadecanoic acid methyl ester, which is present in both floral and berry wax suggests the possible presence of inhibitory compounds within the cuticle of the berry.

Surface hydrophobicity, a stimulus that triggers infection structure formation in other pathosystems, was shown to have no effect here; neither synthetic paraffin wax nor carnauba wax induced formation of infection structures.

Chapter 1. Background

Background on Cranberry

The term “cranberry” refers colloquially to several small, berry-bearing plants in the genus *Vaccinium*. However, the main species in commercial cultivation is *Vaccinium macrocarpon*, the large American cranberry (Eck 1990). For the purpose of simplicity, the term “cranberry” hereto refers to *V. macrocarpon*.

V. macrocarpon is a sprawling, evergreen, vine-like, woody perennial that is native to northeast North America. From these vines grow upright shoots that reach 10 to 15 centimeters in length, on which small pink to dark red berries are borne. The plant’s roots are fine and lack root hairs, instead forming relationships with mycorrhizal fungi in the soil to gather moisture and nutrients. Cranberries are acidophilic, naturally growing in peat bogs and wetlands with a pH between 4.0 and 5.0 (Eck 1990).

As of 2012, the majority of cranberry production worldwide took place in the United States with a total acreage estimated by the United Nations Food and Agriculture Organization of around 15,580 hectares. Compared to the total world production of 24,587 hectares, this is roughly 63% of global cranberry acreage. Another 5,764 hectares, or 23% of global production, comes from Canada. The remaining 14% comes primarily from Belarus, Azerbaijan, Latvia, and other parts of eastern Europe (FAOSTAT 2013). These reports do not give numbers for Chilean cranberry production, but two of the largest cranberry operations in Chile, Cran Chile Farms and Cranberries Austral Chile, self-report production of

430,000 barrels and 1500 “tons” (metric, long, or U.S. not specified) respectively (Cran Chile Farms 2015 and Cranberries Austral Chile 2015).

Within the United States, as of 2014, Wisconsin was the largest producer at 58% of US production - roughly 5 million barrels (1 barrel is equal to 100 pounds). Massachusetts is second with around 2.4 million barrels, or 28% of US production, while New Jersey, Oregon, and Washington account for the remaining 14% of cranberry production in the United States (NASS 2015).

At an average price of $30/barrel (2014 US average for processed berries, fresh berries are twice as valuable, but account for less than 4% of total production) this translates to a 266 million dollar industry in the US alone (NASS 2015). Because of the high-value nature of the crop, any loss from disease can be considered economically significant. Losses before the advent of fungicides could reach as high as 100% in certain regions (Oudemans et. al 1998). The development of Bordeaux mixture in the late 19th century marked the beginning of the era of chemical fungicides that have been used with varying success to manage cranberry fruit rot for the last hundred years (Rice-Mahr and Moffitt 1994; Shear and Stevens 1918).

“Fruit rot” is a catchall term used to describe the decay of cranberries caused by a pathogen. Because infection by numerous pathogens produces relatively similar symptoms, it is often impossible to distinguish the causal organism for an individual case of fruit rot without culturing infected berries (Stiles and Oudemans 1998). More than a dozen different species in at least nine genera are responsible for fruit rot of cranberries, either in the field, or during

storage. Though there is some overlap, many are isolated typically from either field-rotted or storage-rotted berries (Caruso and Ramsdell 1995; Oudemans et. al 1998).

Typically a combination of cultural practices and fungicides are used to manage cranberry fruit rot (Bain et. al 1942). There is also evidence for a heritable trait conferring general resistance to fruit rot, which agrees with historically observed anecdotal evidence. However, research into this trait is, at present, inconclusive (Georgi et al. 2013; Johnson-Cicalese et. al 2009; Vorsa et al. 2009). In general, conditions favoring fungal growth and spread should be avoided. Thick, matted vines caused by insufficient pruning or overuse of nitrogen fertilizer hold moisture that can foster fungal growth (Bergman 1953, Caruso and Ramsdell 1995). This can be exacerbated by use of overhead sprinkler irrigation often employed in cranberry culture to protect against frost (Eck 1990; Oudemans et al. 1998).

Background on *Colletotrichum fioriniae* and *C. acutatum*

Colletotrichum fioriniae is an Ascomycete fungi in the Pyrenomycetes that was, until recently, classified as *C. acutatum* (Damm et al. 2012). Thus most of the studies cited here deal with *C. acutatum*, but are surely relevant to the biology of *C. fioriniae. C. acutatum* causes various diseases of significant economic impact on a number of plant species including, but not limited to, cranberry (Oudemans et al. 1998), blueberry (Smith et al. 1996), citrus (Zulfiqar et al. 1996), mango (Arauz 2000), strawberry (Leandro et al. 2001), papaya (Dickman and Patil 1986), peach (Adaskaveg and Hartin 1997), olive (Moral et al.

2009), avocado (Podila et al. 1993), and almond (Adaskaveg and Hartin 1997). Historically it was often misidentified as its close relative *C. gloeosporioides* until genetic analysis became widely available. The two fungi have many overlapping hosts, often present with similar symptoms, and exhibit variable morphological characteristics at a microscopic level, making them difficult to distinguish. They are also the two species of *Colletotrichum* most frequently associated with infection in the literature (Wharton and Dieguez-Uribeondo 2004).

C. acutatum is capable of reproducing sexually, but has never been seen to do so in nature, instead typically reproducing asexually by forming primary conidia in acervuli or secondary conidia on plant surfaces (Peres et al. 2005). *Colletotrichum* species often exhibit a complex lifestyle on many hosts, infecting vulnerable tissue as a biotroph, then lying quiescent for the duration of the growing season until they resume growth and cause disease in a necrotrophic fashion (Prusky 1996, Zulfiqar et al. 1996). This quiescent lifestyle makes rot due to *C. acutatum* difficult to assess in the field, and complicates grower-supplier relationships.

C. acutatum attacks different tissues on different hosts. For instance, on apples it rots fruit but spares leaves (Peres et al. 2005), while on olive it does not usually grow into leaf or branch tissue, though it can cause dieback of these regions, probably by way of a toxin (Moral et al. 2009).

The initial infection is usually achieved by directly penetrating the plant cuticle, rather than invading a wound or natural opening as many pathogens do (Wharton and Dieguez-Uribeondo 2004). To achieve this, *C. acutatum* produces

an infection structure called an appressorium. Appressoria are produced at the end of the germ tube that grows from a germinated conidia. In *C. acutatum* pathogenic to strawberries, it has been shown that the germ tube and initial spore collapse shortly after forming the appressoria (Leandro et. al 2001).

Appressoria are specialized cells that are thick-walled and often melanized, which swell with large amounts of osmotic pressure that is then used to physically rupture the plant cuticle and force a specialized hypha called a penetration peg down into the plant tissue (Dean 1997, Howard et al. 1991). Although the method by which this turgor pressure is generated has not been studied in *C. acutatum*, glycerol was shown as the osmolyte responsible for generating the high turgor pressure employed by *Pyricularia oryzae* (syn. *Magnaporthe oryzae,* syn. *M. grisea*), the causal organism of rice blast disease (de Jong et. al 1997). Appressoria also function as survival structures, allowing pathogens to endure non-ideal growing conditions like hot, dry summer air, cold winter temperatures, lack of adequate substrate nutrients, or the presence of preformed host defense chemicals in young fruit (Emmett and Parbery 1975, Prusky et al. 2013).

There has been some debate over the exact method of entry through the cuticle by appressorium-forming pathogens. While some sources claim that appressorial turgor pressure is the only factor necessary (Howard et al. 1991), others claim the ability to produce digestive enzymes is also required in order to effectively form functional appressoria and possibly penetrate the cuticle (Suzuki et. al 1982, Skamnioti and Gurr 2007).

While a number of studies have shown evidence that isolates of *C. acutatum* taken from different hosts have little host specificity in vitro, many of these studies were done on wounded fruit, and phylogenetic groupings of *C. acutatum* based on molecular analysis seem to be associated with specific hosts (Peres et al. 2005). If cuticle penetration, and therefore appressorium formation, is involved in the mechanism for host specificity, it could help to explain this discrepancy.

The mechanisms responsible for the differentiation of germ tube cells into appressoria have been studied for decades, and vary among pathogens (Kolattukudy et al. 1995). For instance, in *Uromyces* species, appressorium differentiation is initiated by a thigmotropic response to small ridges. *In vivo*, this is triggered when the fungal germ tube comes in contact with the raised edge of a stomatal guard cell, signaling that a vulnerable site has been reached for infection to occur (Hoch et. al 1987). However, in *P. oryzae*, the differentiation of germ cells into appressoria is triggered by several different factors, including substrate hydrophobicity and recognition of cutin monomers or primary alcohols that make up the plant cuticle (Liu et al. 2011, Gilbert et al. 1996).

In several species of *Colletotrichum*, starvation has been proposed as a possible factor responsible for appressorium formation, since one major function of appressoria is that of a survival structure which enables the fungus to lie dormant until its substrate is ideal for metabolization (Emmett and Parbery 1975).

Some limited work has been done on the signals regulating appressorium formation in *Colletotrichum* species. In *C. musae*, a pathogen of banana fruit,

water-soluble leachates from the surface of Cavendish cultivar banana fruit induced appressoria formation. Specifically, an aromatic compound, anthranilic acid, was shown to be the most potent inducer of both germination and appressorium formation (Swinburne 1976).

C. musae was also shown to produce appressoria differently when stimulated by surface wax from the top or bottom of beet leaves. Upon incubation on the adaxial surface one isolate produced appressoria that were sessile, growing immediately out of the germinating spore. In contrast, when incubated on the wax of the abaxial surface, this same isolate formed appressoria primarily at the end of long germ tubes, demonstrating the sensitivity of appressorium-forming machinery to host environmental cues (Parbery and Blakeman 1978). Beet leaf surfaces have also been shown to stimulate appressoria formation in *C. acutatum*, along with strawberry leaf surfaces. That *C. acutatum* was the only *Colletotrichum* species out of six tested that formed numerous appressoria when incubated on these surfaces, but not on glass slides makes it an ideal candidate for studying the effect of various surfaces on appressorium formation (Parbery and Blakeman 1978).

C. musae and *C. gloeosporioides* were also found to form appressoria in response to ethylene, a plant hormone typically involved in fruit ripening (Flaishman and Kolattukudy 1994). This may be a mechanism by which the fungi can sense the vulnerability to pathogens that is often associated with ripening processes.

Loss of epicuticular wax on the abaxial surface of leaves of the legume *Medicago truncatula* caused a marked decrease in infection structure formation by *Colletotrichum trifolii* incubated on the surface of the plant (Uppalapati et al. 2012). Thus, without the chemical and/or physical signals present in the wax, the pathogen is unable to recognize its substrate as a suitable host.

Indeed, mutants of *C. orbiculare* (syn. *C. lagenarium*), the causal agent of cucumber anthracnose, which lacked an ortholog of the *Saccharomyces cerevisiae* PEX13 gene called CoPEX13 which codes for a peroxisome biogenesis factor formed thin appressorial cell walls, could not properly undergo melanization, and showed significantly lower appressorial turgor pressure than the wild-type (Fujihara et al. 2010). In addition, these mutants showed decreased levels of pathogenicity when compared to the wild-type. Since plant cuticles are made largely of fatty acids and fatty acid oxidation (and therefore metabolism) occurs only in the fungal peroxisomes (Poirier et. al 2006) this conclusion suggests a possible connection between substrate metabolism and appressorium formation in *Colletotrichum spp*.

A *C. gloeosporioides* isolate pathogenic to avocado has been shown to form appressoria in response to avocado surface wax chemicals that were dissolved in chloroform and reformed on glass cover slips, in particular to very long chain fatty alcohols made of 24 carbon atoms or greater. However, other *Colletotrichum* species including *C. trifolii* (pathogenic on alfalfa), *C. orbiculare* (pathogenic on cucumber and watermelon), *C. pisi* (pathogenic on peas), *C. capsici* (pathogenic on cotton and peppers), and *C. coccoides* (pathogenic on

tomato), showed no effect when treated with avocado wax (Podila et al. 1993). Only one other species tested, *C. lindemuthianum* (pathogenic on bean), showed similar rates of appressoria formation when incubated on avocado surface wax.

Thus, surface signaling as a mechanism for appressorial induction is possibly a cause of host specificity among *Colletotrichum*. To add to this, waxes obtained from the surfaces of several other plants representing multiple plant families showed no stimulatory effects on appressoria formation by *C. gloeosporioides*. Whether this is due to the absence of stimulators or the presence of inhibitors is not known for certain. Regardless, it hints at a possible mechanism of species specificity among *Colletotrichum* pathogens (Podila et. al 1993).

Ultimately, it would seem that many different appressorial induction mechanisms exist, and that many fungi may rely on a combination of several factors to achieve substrate recognition. By accurately determining these causes in economically important fungi, better and more precise disease management methods may be developed to reduce significant yield loss.

Background on Cuticle Wax

The plant cuticle is the first interface between pathogenic organisms and their plant hosts, and plays a significant role in plant-microbe interactions, especially with regards to pathogens (Jeffree 1986, Lara et al. 2014). Plant cuticles cover nearly all aerial surfaces of land plants, and are separated into two chemically and physically distinct layers (Ensikat et al. 2000, Jeffree 1986).

The inner layer, sometimes referred to as the cuticle proper, is made predominantly of cutin. Cutin is a polyester formed primarily of 16- and 18-carbon fatty acids that composes a large portion of the cuticles found in higher plant species, including cranberry (Heredia 2003, Croteau and Fagerson 1972).

The outer layer is referred to as the epicuticular wax, and is a substance that varies widely in chemical composition and physical structure. Differences in epicuticular wax have been observed in different species (Jeffree 1986, Barthlott et al. 1998, Griffiths et al. 2000, Gulz 1994), different organs on the same plant (Tomaszewski and Zielinski 2014), and even on the same organ over the course of plant development (Avato et al. 1984). Environmental conditions also influence the structure of epicuticular wax (Baker 1974; Baker 1982).

In general, epicuticular wax consists of very-long chain fatty acids and alkanes (Baker 1982), but may also contain triterpenoids, fatty alcohols, and/or a wide array of volatile compounds suspended in this hydrophobic mixture (Croteau & Fagerson 1971a; Croteau & Fagerson 1971b). Because of their crystalline nature, the chemistry of these waxes affects their physical structure and arrangement (Koch and Ensikat 2008). Thus, the microscopic topography of a particular cuticle, which can serve a variety of functions, is directly influenced by its chemical makeup.

The evolution of waxy cuticles was probably originally an adaptation by the first land plants to regulate water loss and gas exchange in their new dry environment (Jeffree 1986). However, since that time, numerous other functions have been incorporated (Yeats and Rose 2013).

For example, the inner surface wax of carnivorous pitcher plants in the *Nepenthes* genus are arranged in overlapping tiles that break off easily, making it difficult for insects to climb out. (Jeffree 1986). The wax of the sacred lotus plant *Nelumbo nucifera* repels contaminants and channels water in a self-washing mechanism that keeps the plant clean (Barthlott and Neinhuis 1997).

Perhaps one of the most common secondary features of epicuticular wax is defense against pathogens. In addition to providing a physical barrier that requires pathogens to evolve methods of breaching directly or navigating to susceptible areas like stomata, the epicuticular waxes of many plants contain chemicals that act as defensive compounds to inhibit or kill invading pathogens.

In one study, the cuticular wax of asian pear was shown to inhibit germination and mycelial growth of *Alternaria alternata,* the causal agent of Alternaria rot of pears (Yin et al. 2011). In another study, wax isolated from the leaves of beetroot plants showed an inhibitory effect on germination of *Botrytis cinerea* spores (Blakeman and Sztejnberg 1973). However, beetroot leaves have been shown to stimulate appressoria production in *C. acutatum*, further illustrating the variable responses to plant surfaces between pathogens (Parbery and Blakeman 1978).

Inhibition of fungi or bacteria in one form or another by plant waxes or compounds contained therein has been observed in numerous plants including sweet cherry wax on *Monilinia fructicola* (Biggs and Northover 1989), *Ginko biloba* wax on *M. fructicola* and *Stemphylium sarcinaeforme* (Johnston and Sproston 1965), and blueberry leaf wax on *Staphylococcus aureus*, *Listeria*

monocytogenes, *Salmonella typhimurium*, and *Escherichia coli* (Yang et al. 2014). In addition, two commonly-occurring pentacyclic triterpenoid compounds found in plant cuticles, oleanolic acid and ursolic acid, have demonstrated antibacterial activity against numerous bacteria, particularly those that are Gram-positive (Wolska et al. 2010). Both of these compounds have been shown to exist in cranberry fruit cuticle wax (Croteau and Fagerson 1971a).

One study of particular interest showed that several phenolic acids had a significant negative effect on the production of cutinase by *M. fructicola*, a major economic pathogen of stone fruit that causes brown rot disease on peaches (Bostock et al. 1999). In particular, benzoic acid, quinic acid, and several derivatives thereof showed strong inhibitory effects on cutinase production. This is noteworthy in the context of cranberry pathology, as cranberries have long been known to contain high amounts of benzoic acid, which is especially concentrated in the epicuticular wax layer (Croteau and Fagerson 1971b), and .

Chapter 2. Experiments

Introduction

The purpose of these experiments was to determine what effect cranberry cuticle wax may have on the formation of appressoria in *Colletotrichum fioriniae,* to determine the chemical components of this wax, and to test individual pure chemicals for bioactivity in order to determine what, if any, chemical signals are involved in appressorial signaling at the plant surface.

Knowledge gained by studying the behavior of appressorium formation in the *C. fioriniae* - cranberry system is potentially applicable to other *Colletotrichum* systems, and possibly even other appressorium-forming pathogens like *P. oryzae* that cause widespread economic damage on various crops around the world. In thoroughly understanding the nuanced mechanisms by which infection structures like appressoria form, better, more specific management strategies can be employed to combat *Colletotrichum,* be they chemical, cultural, or biological.

Materials and Methods

Wax Extraction

Cranberry flowers were obtained from beds grown at the Rutgers University Philip E. Marucci Blueberry and Cranberry Research Station in Chatsworth, New Jersey, United States of America. Four genotypes - Stevens, Mullica Queen, Budd's Blues, and US89-3 - were used in this trial. Flower petals were first manually separated from ovaries using tweezers. Surface wax was isolated from the petals and ovaries separately by immersing and gently swirling them in chloroform (Fisher Chemical Company, assay grade, ≥ 99% pure, for molecular biology, peroxide-free) for 30 seconds at a weight to volume ratio of 1g of plant matter to 9mL of chloroform. This mixture was then filtered through a small stainless steel screen to remove floral debris and stored in darkness at 4ºC.

General Bioassay Procedure

To test bioactivity of waxes, an 8mm diameter (6mm internal diameter) Van Tieghem cell was placed on a glass cover slip (Fisher Scientific, #1, 22cm x 22 cm) to create a small, open-topped incubation chamber to which 33 µL of chloroform extract was added. The chloroform was then allowed to evaporate, leaving behind a layer of surface wax. For control treatments, pure chloroform was substituted for chloroform wax extract. Following this, 66 µL of distilled water and 33 µL of *C. fioriniae* spore suspension were added (Fig. 1).

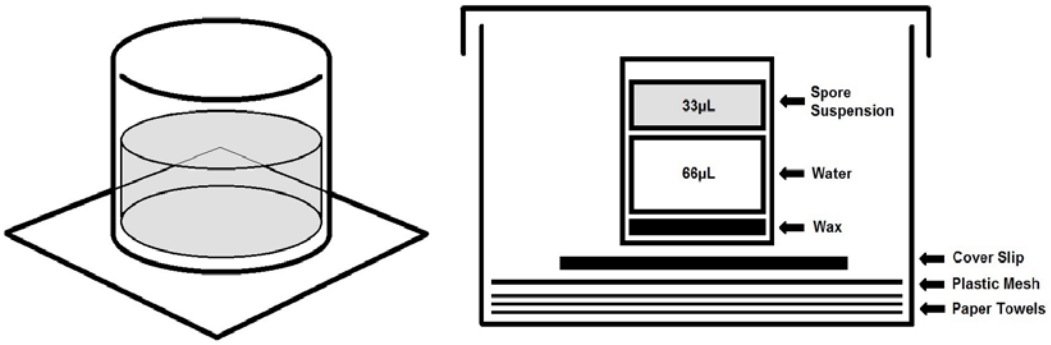

Figure 1. Diagram of Van Tieghem cell assay chamber.

Spore suspension was prepared with *C. fioriniae* (isolate PMAP182) grown for 7 days at room temperature on Clarified V8 media without calcium carbonate. Media was made with 30g Agar (Fisher Scientific, granulated, molecular genetics grade), 200mL clarified V8 (Campbell's Soup Company) and 800mL distilled water. Clarified V8 was prepared by centrifuging 250mL V8 juice at 7500rpm for 10 minutes. Spores were removed from the culture using a loop and suspended in sterile, room-temperature, distilled water. The spore concentration was measured using a hemacytometer and adjusted to $1x10^5$ spores/mL.

Each replicate of cover slips was then incubated in a petri dish (Fisherbrand 100mm x 15mm) at 25°C in darkness for 24 hours. Petri dishes contained three layers of paper towels soaked in distilled water to prevent desiccation, and a thin layer of plastic mesh to raise cover slips off the surface of the wet towel.

Growth was then stopped, and fungi stained by adding 15 µL of lactophenol cotton blue dye to each cover slip. Cover slips were mounted on glass slides (Fisher Scientific, precleaned, 25mm x 75mm x 1mm) and assessed. For each slide, 8 fields at 200x magnification were analyzed, and numbers of ungerminated conidia, germinated conidia, and appressoria were counted. Appressorial formation rates were calculated as a ratio of number of appressoria divided by total combined number of germinated and ungerminated conidia. This approach was chosen to account for the non-homogenous spore distribution caused by the attachment of spores to the wax layer present within the area occupied by the Van Tieghem cell and the fact that many appressoria formed on the ends of long germ tubes that grew into or out of the field, making it impossible to determine if specific germinated conidia had produced appressoria or not.

All experiments were performed twice, with three distinct biological replicates of plant tissue collected for each treatment. In the time course study, only the petal extract from the Stevens genotype was used.

Time Course Study

A time course was run comparing wax treatments to a non-treated control over 24 hours using the general procedure described above to determine when appressorial formation occurred and to determine a standard length of time for further assays. Five timepoints were established at 0, 6, 12, 18, and 24 hours. The results of this time course indicated that 24 hours was an adequate amount of time to see significant differences in appressorial formation rates, and was thus chosen as the standard time for the future assays.

Genotype Study

In the second study, petal extracts from all four genotypes were compared to one another, as well as to a non-treated control after 24 hours using the general procedure given above. Because resistance to field rot is an unknown heritable trait found in some genotypes (Georgi et al. 2013), two genotypes traditionally recognized as susceptible - Stevens and Mullica Queen - and two genotypes considered resistant - Budd's Blues and US 89-3 (Vorsa et. al 2009) - were chosen to test the potential involvement of floral wax as a resistance mechanism.

Ontogeny Study

In the third study, ovaries and berries from various points in the growing season were examined using the general assay procedure given above to test potential ontogenic changes in berry wax effect. Ovaries were collected from Stevens cranberry plants in bloom on 17 June 2014 and mechanically separated from petals using tweezers. Early season berries were collected from a different plot on 25 July, and late season berries were collected from this plot as well on 22 August. "Harvest" berries were collected from the same plot as early and late season on 19 September, concurrent with other cranberry harvests in the area. In early season, late season, and harvest treatments, concerns over the lower surface area to weight ratio of berry tissue to floral and ovary tissue prompted the concentration of early season, late season, and harvest extracts to 10x initial concentration by evaporation in a fume hood.

GC-MS Analysis & Chemical Component Study

Chloroform extracts of petal tissue for the four genotypes used in the genotype study were analyzed using a GC-MS (HP 6890 GC and HP 5973 MS; Hewlett Packard Co., Palo Alto, CA, USA) equipped with a 30 m HP-5ms capillary column with an inner diameter of 0.25 mm. The GC was programmed to begin at 80°C for 2 min, followed by a ramp to 320°C at 10°C/min with a 2 min hold at 320°C. Compounds were initially identified by NIST mass spectra library and confirmed with authentic standards. Compounds positively identified were methyl ester of hexadecanoic acid (Ultra Scientific Analytical Solutions), methyl ester of linoleic acid (MP Biomedicals, original source: safflower oil), methyl ester of α-linolenic acid (ACROS Organics), and nonacosane (SPEX CertiPrep) (Fig. 2). Also tested were eicosanol (ACROS Organics), which proved to be a misidentification and ethlyl linoleate (MP Biomedicals), for which the chemical standard was impure.

In the fourth study, pure chemical standards corresponding to fractions of petal wax determined by coupled gas chromatography-mass spectrometry were tested for individual effect on appressoria formation in *C. fioriniae*. Chemicals tested were methyl ester of hexadecanoic acid, methyl ester of linoleic acid, methyl ester of alpha-linolenic acid, and nonacosane. The linoleic and linolenic acid were purchased as free acids and methylated.

Compound Name	Chemical Formula
Methyl Ester of Hexadecanoic Acid	$C_{17}H_{34}O_2$
Methyl Ester of Linoleic Acid	$C_{19}H_{34}O_2$
Methyl Ester of α-Linolenic Acid	$C_{19}H_{32}O_2$
Nonacosane	$C_{29}H_{60}$

Table 1. Compounds in cranberry floral wax positively identified by gas chromatography - mass spectrometry.

Methylation was achieved as follows: Free acids were mixed with 1N sulfuric acid in methanol in glass test tubes. The air inside the tubes was then blown out with helium and the tubes capped to prevent oxidation. The mixture was heated to 80ºC for 90 minutes, then cooled at -18°C for 10 minutes. 1.5 mL of 0.9% sodium chloride solution and 200µL of hexane were then added, and the mixture was vortexed for 20 seconds, followed by 2 minutes of centrifugation at 2000 rpm. The upper hexane phase was then extracted, giving a solution of methylated linoleic or linolenic acid in hexane. Although the exact concentration of the solution is not known, it was sufficient enough to produce very prominent peaks when subjected to GC-MS.

These suspensions were then diluted to 1/100 concentration in hexane for the bioassay. Again, the final working concentration was not known, but was enough to leave a residue upon evaporation of the solvent similar to the other compounds tested. The nonacosane was initially suspended in dichloromethane at a concentration of 1 mg/mL for the GC-MS analysis, and was diluted with chloroform to .01 mg/mL for the bioassay.

The hexadecanoic acid was initially suspended at 1 mg/mL in chloroform for the GC-MS analysis and diluted with more chloroform to a concentration of .01mg/mL for the bioassay. All four suspensions were treated the same way as the chloroform floral extracts; 33 µL were pipetted into the Van Tieghem cell / glass cover slip setup and the solvent was allowed to evaporate off, leaving a thin sheen of oil or wax on the slide within the bounds of the Van Tieghem cell. To this, 66 µL of sterile, distilled water was then added, followed by 33 µL of *C. fioriniae* spore suspension prepared as previously described to a concentration of $1x10^5$ spores / mL. This was then incubated in darkness at 25ºC for 24 hours. In this study, hexane was used as a control instead of chloroform, as chloroform had already been shown to be unreactive with regards to appressorial formation, while hexane had not been assessed.

Hydrophobicity Study

In the fifth study, paraffin wax synthesized from petroleum (Fisher Scientific) was used to test the commonly reported claim that surface hydrophobicity is sufficient for appressorium induction. Paraffin wax was chosen for several reasons; One common measure of hydrophobicity is the contact angle formed by a drop of water on the surface of the substance in question. In general, hydrophobic materials are those that cause formation of an angle 90° or greater, while anything less than 90° is considered hydrophilic (Appendix C: Contact… 2009).

Some plant waxes exhibit superhydrophobicity *in vivo*, like the leaves of the sacred lotus which exhibit contact angles of over 150°. However, this has

more to do with wax ultrastructure than chemistry, since plant waxes tend to be hydrophilic, probably due to the number of hydrophilic hydroxyl and carboxyl functional groups they possess (Wagner et al. 2003). In contrast, synthetic paraffin lacks any functional groups and is made entirely of straight-chain hydrocarbons. It has also been shown to be hydrophobic, with a contact angle around 100° (Ray and Bartell 1953). In addition, its uniform chemical nature negates the possibility of inhibitors that might be present in plant-derived waxes.

Paraffin was therefore suspended in chloroform at a concentration of 1 mg/mL and applied in the same manner as the floral extracts in the previous studies. In addition, a positive control of Stevens genotype cranberry floral wax extract was applied. These two treatments were incubated for 24 hours with a non-treated control and appressorial formation rates were compared as in the general procedure given above.

Statistical Analysis

Two-way t-tests assuming unequal variances were preformed on time course data in Microsoft Excel. Two-way ANOVAs were run on genotype, ontogeny, chemical standard, and hydrophobicity data in CoStat statistical software (CoHort company) to test for variation between the two experimental runs and potential interaction effects between run and treatment (Appendix A). When no interaction effect was seen, one-way ANOVAs were run (Appendix B) followed by Student Newman Keuls post-hoc tests to determine significantly different treatment groups.

Results

Time Course Assay

The Stevens genotype time course study showed higher, statistically significant levels of appressoria formation in the extract treatment at all time points except 0 hours when analyzed with a two-tailed t-test assuming unequal variances at the .05 confidence level in Microsoft Excel (Table 2 and Fig. 2). After 24 hours, appressorial formation rates had reached over 0.9.

Time Point	NTC App. Ratio	WAX App. Ratio	p - value
0h	0	0	n/a
6h	0	.099	4.15×10^{-3}
12h	0	.377	3.04×10^{-4}
18h	.04	.588	5.7×10^{-7}
24h	.06	.908	6.98×10^{-8}

Table 2. Appressorial formation over 24 Hours in *Colletotrichum fioriniae* in response to cranberry floral wax and non-treated control. Ratio of appressoria counted to total germinated and ungerminated conidia counted (App. Ratio) at 0, 6, 12, 18, and 24 hours after inoculation with chloroform extract of cranberry floral wax or chloroform control on glass coverslips. Cranberry floral wax treatment (WAX) showed significantly higher appressorial formation rates than non-treated chloroform control (NTC) at all time points except 0h.

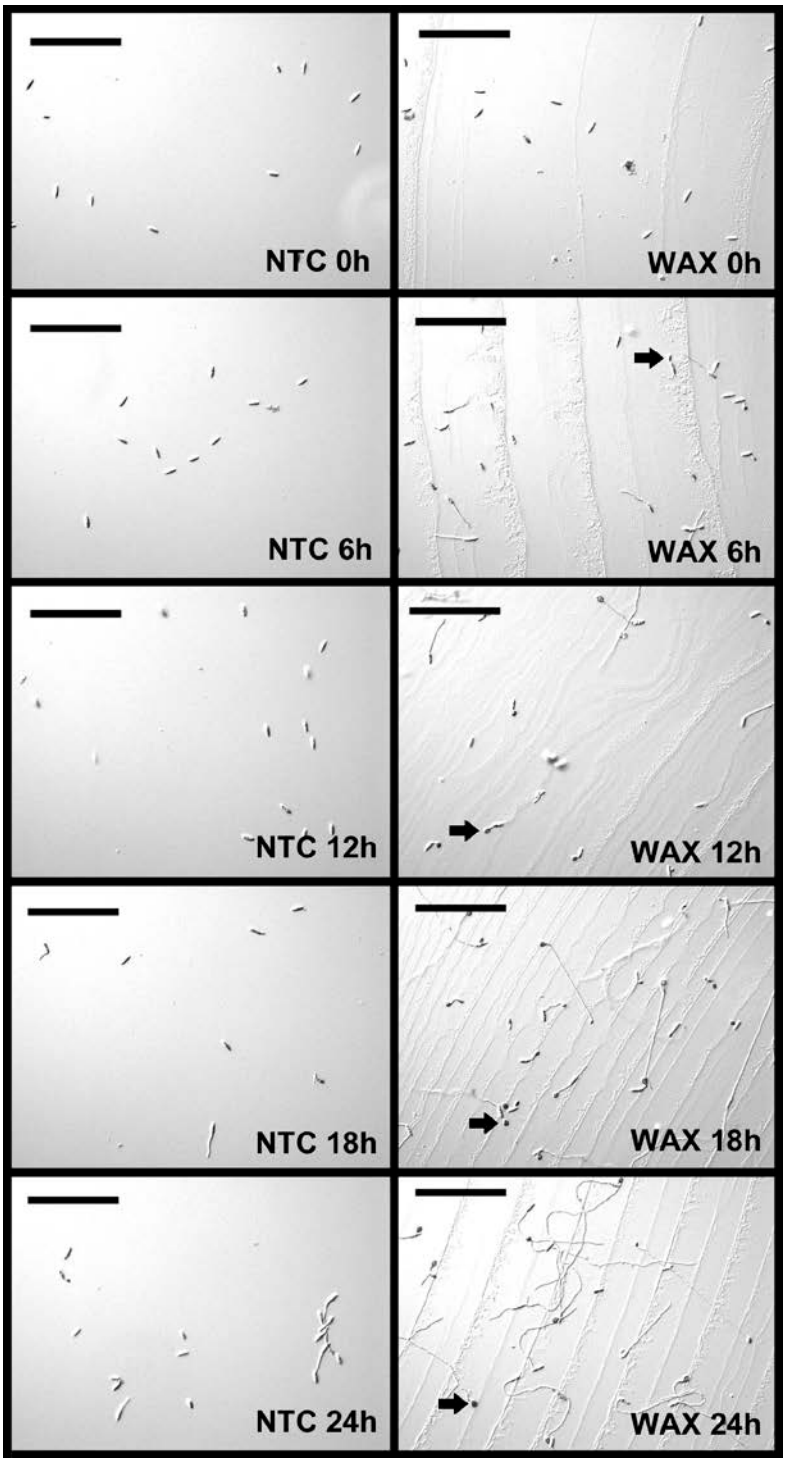

Figure 2. Appressorium formation at 200x magnification in *Colletotrichum fioriniae* over 24 hours in response to cranberry floral wax and chloroform control. **NTC:** non-treated chloroform control **WAX:** chloroform wax extract treatment. Scale bars are 100 µm. Arrows indicate appressoria.

Genotype Assay:

The floral wax genotype assay showed no significant differences between appressorial formation rates induced by different genotypes after 24 hours using a one-way ANOVA followed by a Student-Newman-Keuls post hoc test at the .05 confidence level. All genotypes were, however, significantly different from the non-treated control (Figs. 3 and 4).

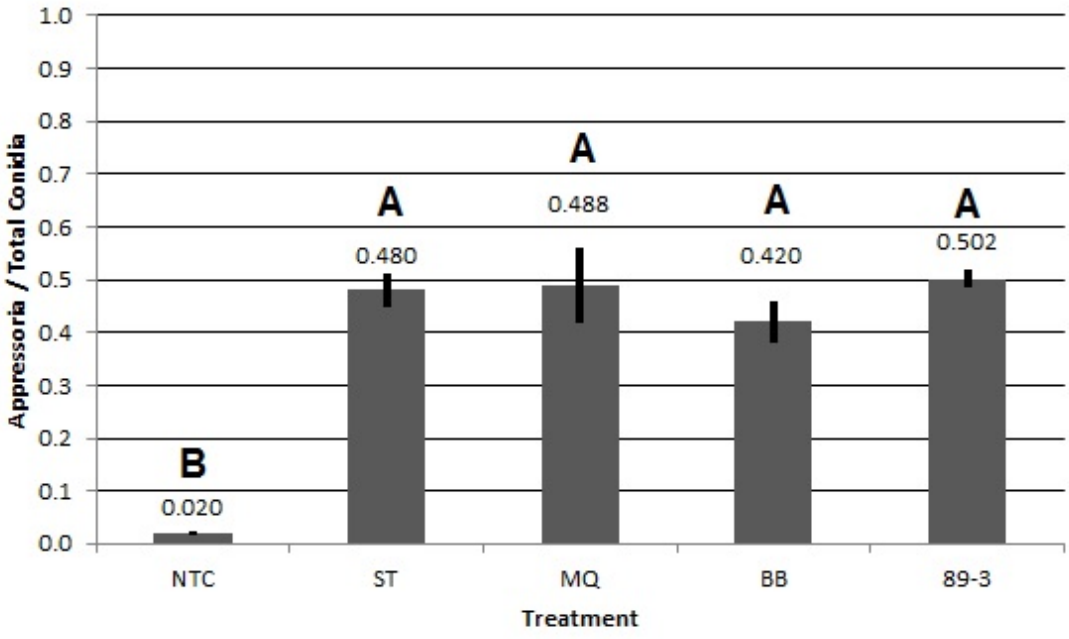

Figure 3. Appressorial formation after 24 Hours in *Colletotrichum fioriniae* incubated with floral wax from resistant and susceptible cranberry genotypes. Ratio of appressoria counted to total germinated and ungerminated conidia counted. **NTC:** non-treated control, **BB:** Budd's Blues (resistant), **89-3:** US89-3 (resistant), **ST:** Stevens (susceptible), **MQ:** Mullica Queen (susceptible). Letters indicate statistically significant groups.

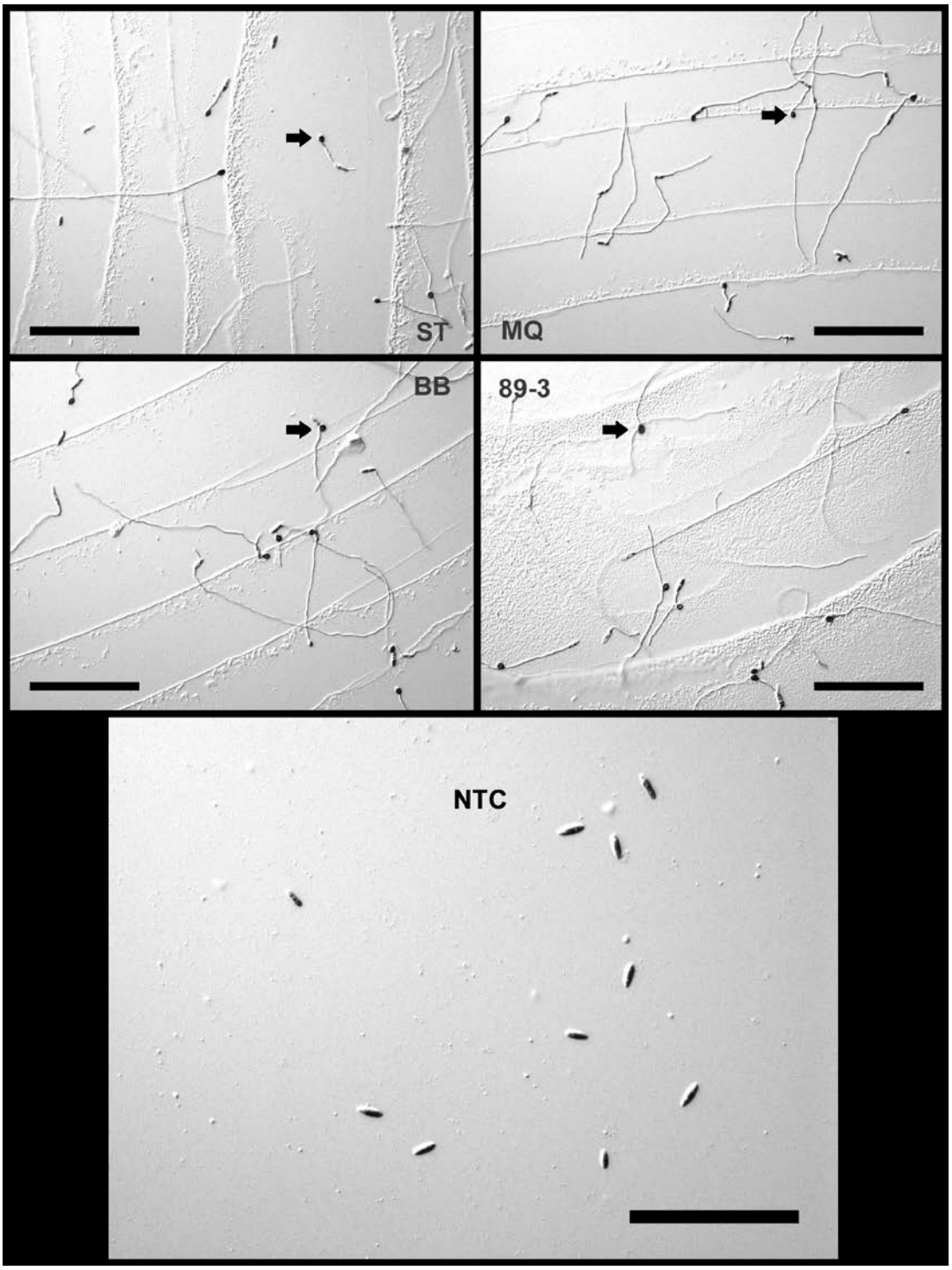

Figure 4. Appressorial formation at 200x magnification after 24 hours in *Colletotrichum fioriniae* incubated with floral wax from two resistant and two susceptible genotypes. **ST:** Stevens (susceptible), **MQ:** Mullica Queen (susceptible), **BB:** Budd's Blues (resistant), **89-3:** US89-3 (resistant). Scale bars are 100 µm. Arrows indicate appressoria.

Ontogeny Assay:

The ontogeny assay showed that while wax obtained from young ovaries had a significant positive effect on appressorial formation in relation to a non-treated control, early season, late season, and harvest time berry wax treatments did not. However, the harvest treatment was significantly different from the late season (Figs. 5 and 6). Data was analyzed with an arcsine transformation followed by a two-way ANOVA and a Student Newman Keuls post hoc test at the .05 confidence level.

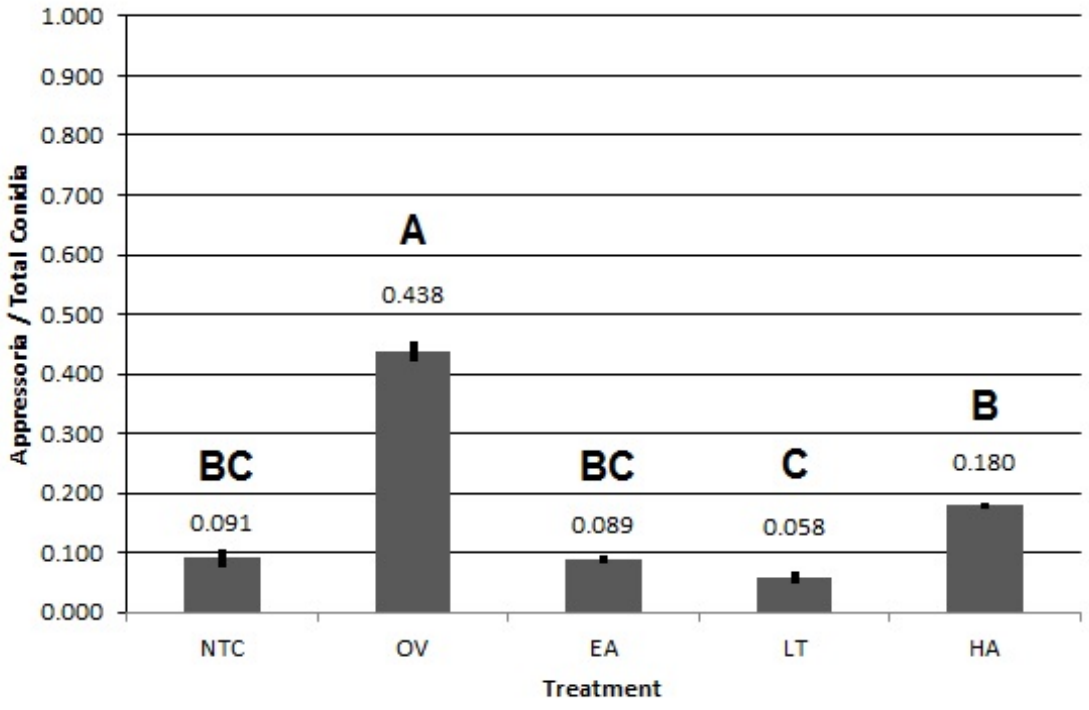

Figure 5. Appressorial formation after 24 Hours in *Colletotrichum fioriniae* incubated with cranberry fruit wax isolated from ovaries and berries over the growing season. Ratio of appressoria counted to total germinated and ungerminated conidia counted in spores incubated with surface wax from immature ovaries (**OV**) collected 17 June and berries collected on 25 July (Early Season - **EA**), 22 August (Late Season - **LT**), and 19 September (Harvest - **HA**) compared to each other and to a non-treated control (**NTC**). Letters indicate statistically significant groups.

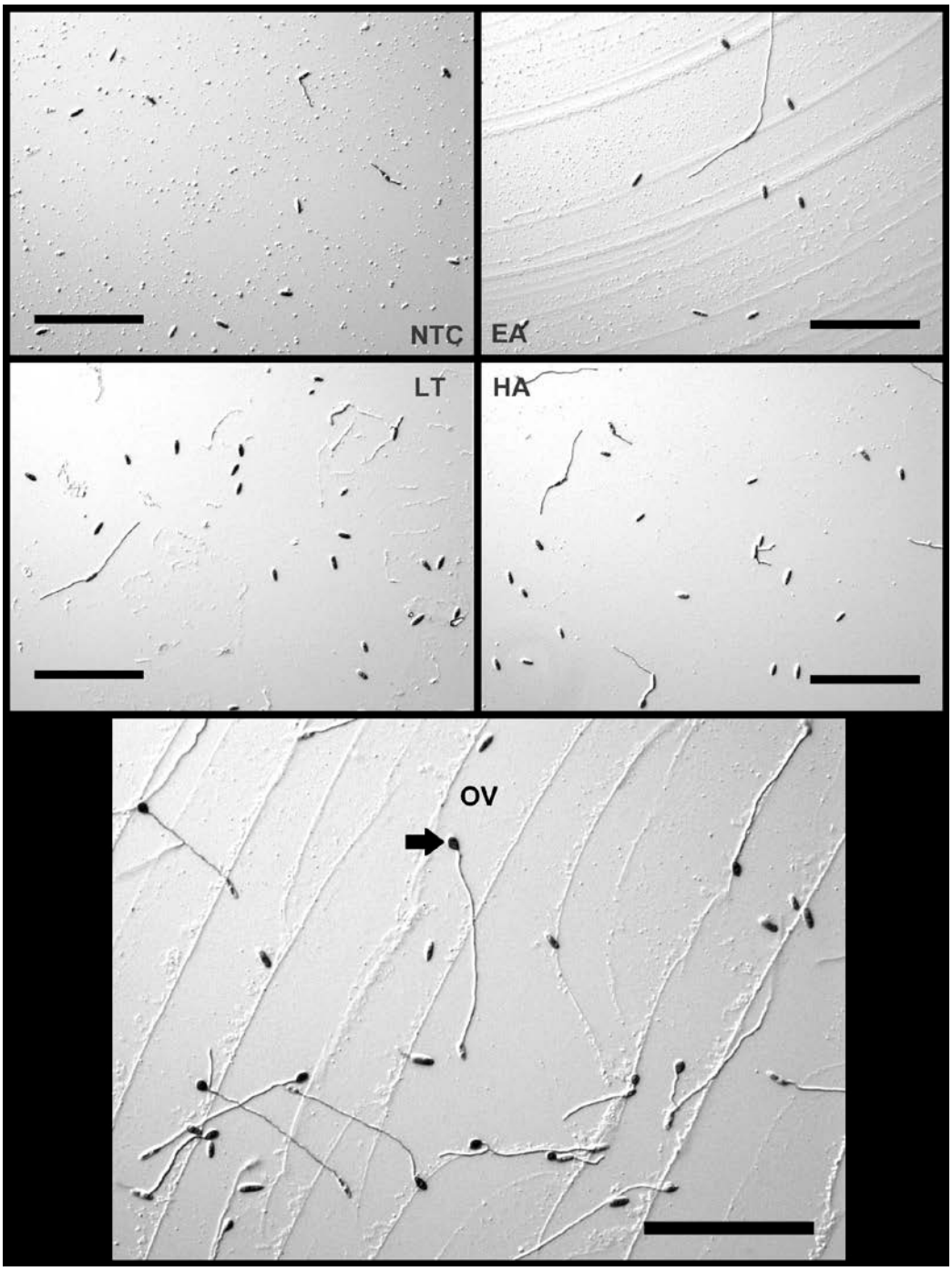

Figure 6. Appressorial formation at 200x magnification after 24 Hours in *Colletotrichum fioriniae* incubated with cranberry fruit wax isolated from ovaries and berries over the growing season. Ovaries (**OV**) collected 17 June and berries collected on 25 July (Early Season - **EA**), 22 August (Late Season - **LT**), and 19 September (Harvest - **HA**) non-treated control (**NTC**). Scale bars are 100 µm. Arrow in OV indicates appressorium.

Pure Chemical Standard Assay:

The pure chemical standard assay results showed that the methyl ester of hexadecanoic acid significantly stimulated appressorial formation, while none of the other chemical standards showed any more activity than the hexane control treatment when analyzed with an arcsine transformation followed by a two-way ANOVA and a Student Newman Keuls post hoc test at the .05 confidence level (Figs. 7 and 8).

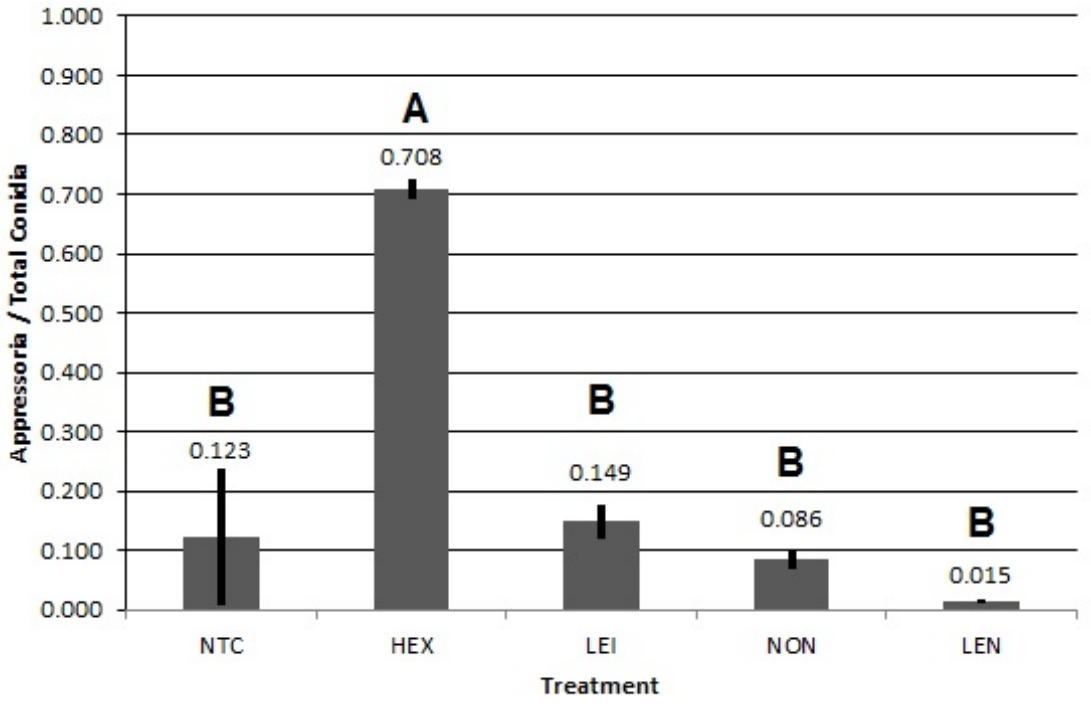

Figure 7. Appressorial formation after 24 hours in *Colletotrichum fioriniae* incubated with four pure chemical standards corresponding to peaks found in GC-MS analysis of cranberry flower chloroform extracts. Ratio of appressoria counted to total germinated and ungerminated conidia counted. **NTC:** hexane non-treated control, **HEX:** methyl ester of hexadecanoic acid, **LEI:** methyl ester of linoleic acid, **NON:** nonacosane, **LEN:** methyl ester of linolenic acid. Letters indicate statistically significant groups.

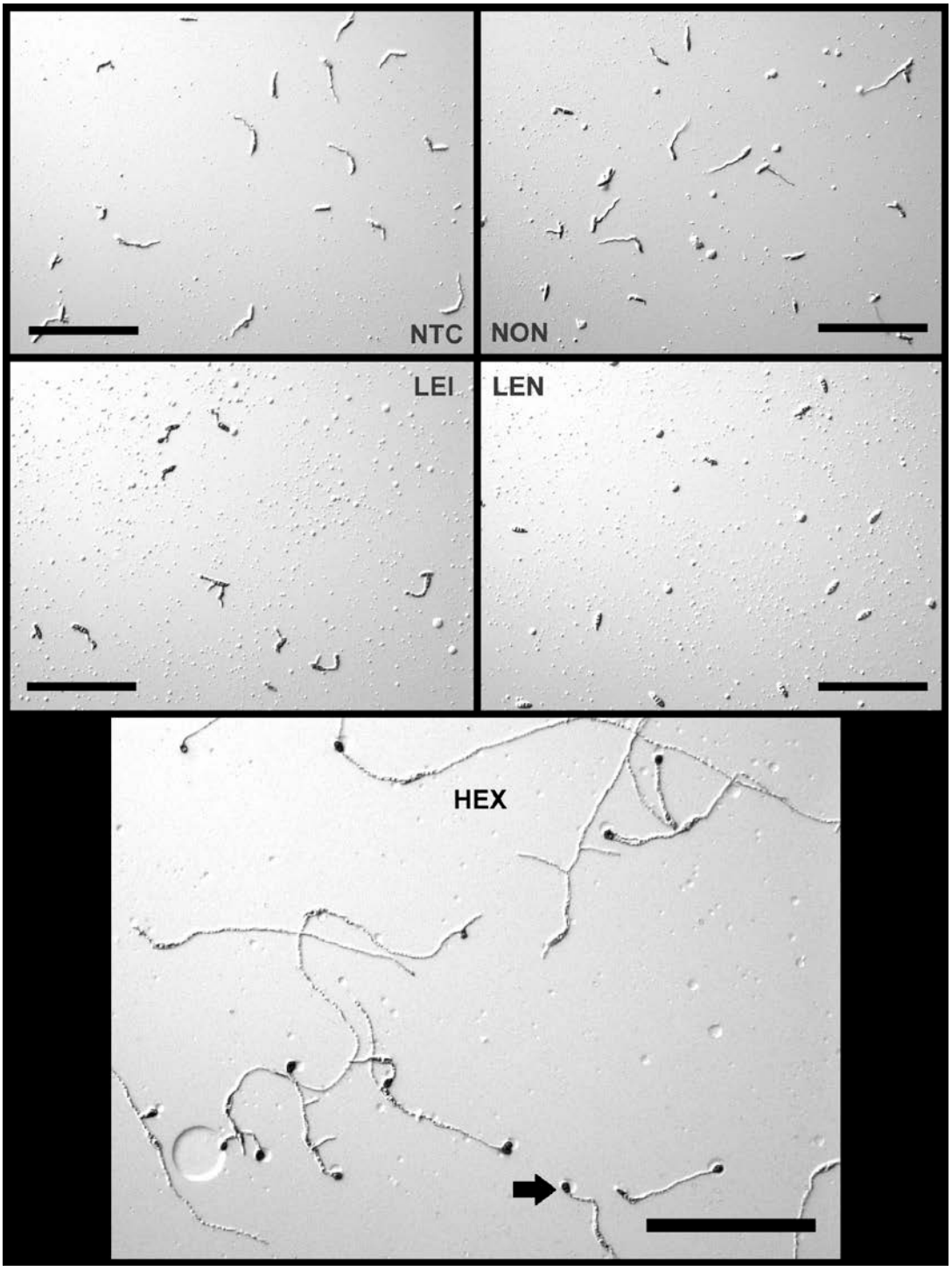

Figure 8. Appressoria formation at 200x magnification after 24 hours in *Colletotrichum fioriniae* incubated with four pure chemical standards corresponding to peaks found in GC-MS analysis of cranberry flower chloroform extracts. **NTC:** hexane non-treated control, **HEX:** methyl ester of hexadecanoic acid, **LEI:** methyl ester of linoleic acid, **NON:** nonacosane, **LEN:** methyl ester of linolenic acid. Scale bars are 100 µm. Arrow in HEX indicates appressorium.

Hydrophobicity Assay:

There was no significant difference between the control treatment and the paraffin treatment when analyzed with an arcsine transformation followed by a two-way ANOVA and a Student Newman Keuls post hoc test at the .05 confidence level. There was, however, a significant difference between the positive wax control and the other two treatments (Figs. 9 and 10).

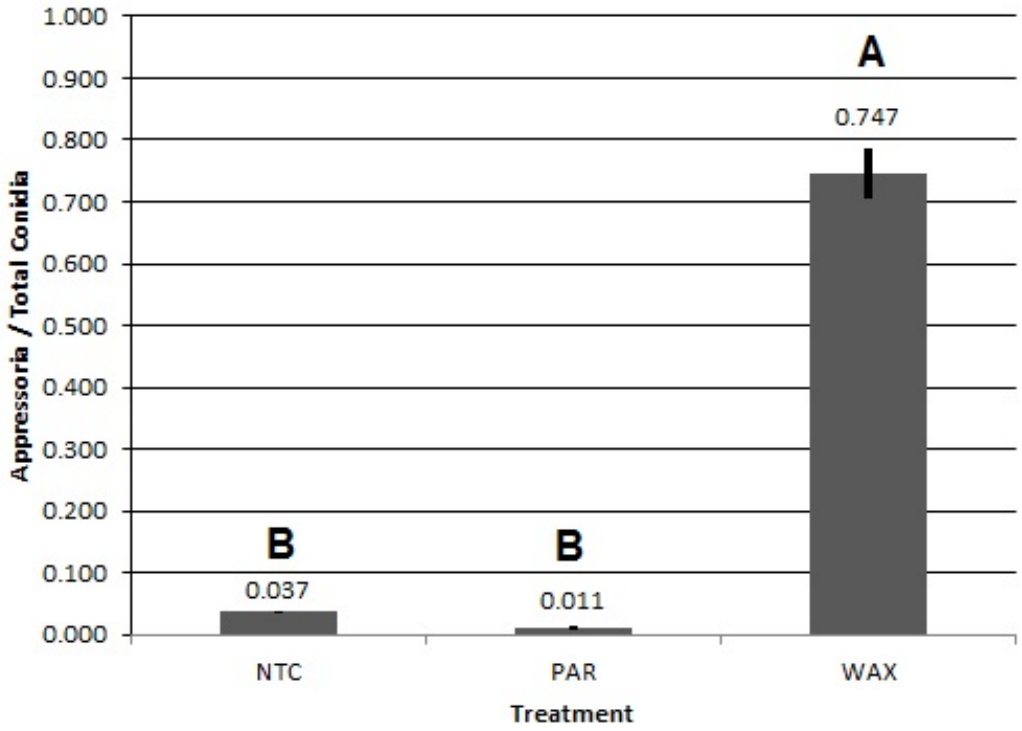

Figure 9. Appressorial formation after 24 hours in *Colletotrichum fioriniae* incubated with hydrophobic paraffin wax compared to non-treated (negative) and inductive floral wax (positive) controls. **NTC:** non-treated control, **PAR:** Paraffin, **WAX:** cranberry floral wax. Paraffin treatment is not significantly different from NTC, while wax treatment is significantly higher than both. Letters indicate statistically significant groups.

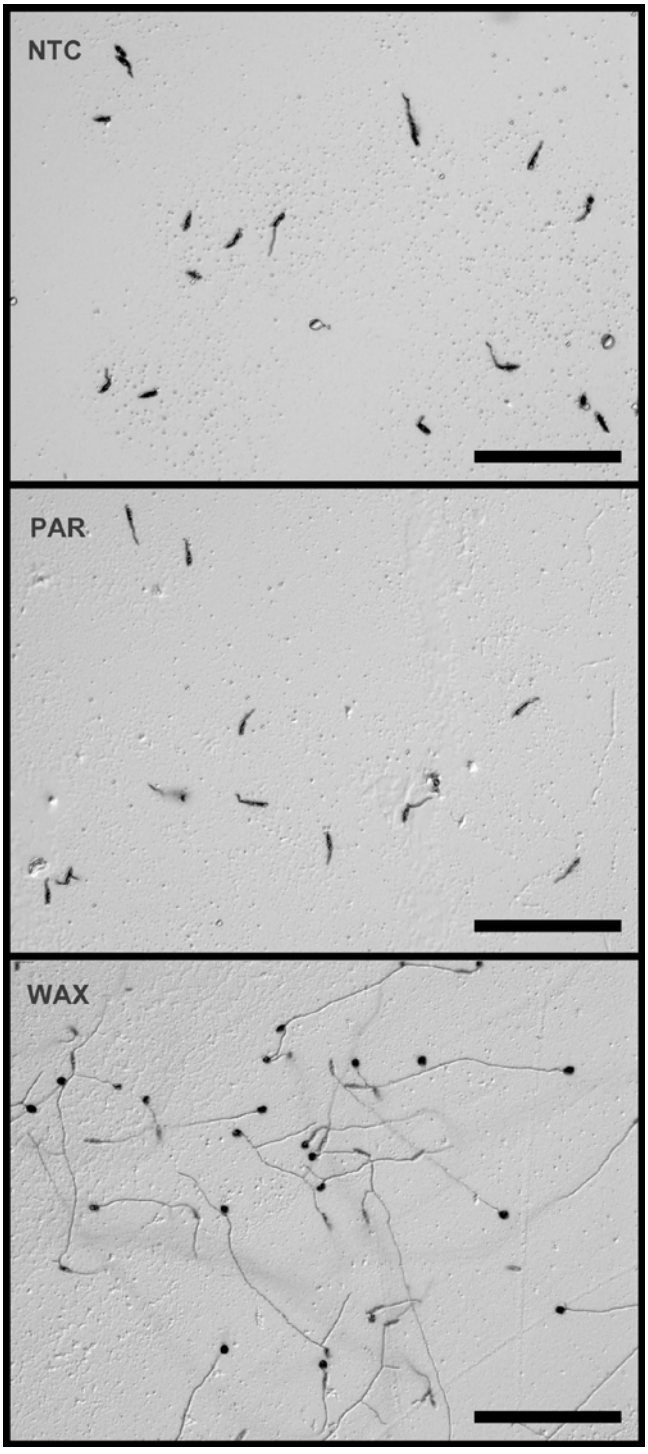

Figure 10. Appressorial formation at 200x magnification after 24 hours in *Colletotrichum fioriniae* incubated with hydrophobic paraffin wax compared to non-treated (negative) and inductive floral wax (positive) controls. **NTC:** non-treated control, **PAR:** Paraffin, **WAX:** cranberry floral wax. Scale bars are 100 µm.

Chapter 3. Discussion

No definitive cause could be found to explain the variation in maximum appressorial formation rates between the various experiments. This merits further study, as it could uncover more mechanisms governing the formation of appressoria which might potentially be exploited to protect plants. In addition, the data presented here only represents one set of biological replicates due to a lack of materials. Further replications of these studies are planned for the near future.

That it only takes 6 hours to induce appressorial formation in *C. fioriniae* using cranberry floral surface wax (Table 2 and Fig. 2) is significant in light of the short wetness periods after rain or irrigation which the pathogen must utilize in order to germinate and form appressoria and agrees with the results of another study of *C. acutatum* on strawberry (Leandro et al. 2003).

While the results of the genotype study cannot support the conclusion that floral wax plays a role in rot resistance (Figs. 3 and 4), more genotypes should be tested before drawing definitive conclusions. Further, berry wax of different genotypes was not tested in this study due to the lack of available materials mentioned above, and, as previously stated, could possibly differ in the composition of its epicuticular wax, or even specific cutin monomers. Therefore, analysis and testing of berry wax among genotypes is a productive avenue of future research.

The stimulation of appressorial formation by ovary extracts may mean several things. Although there is no way to guarantee that the ovaries were not

simply contaminated by compounds from the petal wax, the important thing to note is that regardless of whether the stimulatory effect of ovaries was endogenous or exogenous, some compound was present on the ovaries during the period of time when fungicides are most effective in the field, and therefore the time that is theorized to be the most vulnerable period of cranberry fruit to infection by fungal pathogens (Oudemans et al. 1998).

Thus, cranberry flower (be it petal, ovary, or both) wax is most likely a signal to germinating *C. fioriniae* spores that they have landed on a suitable host for colonization. The apparent lack of stimulation seen in early season, late season, and harvest berry waxes (Figs. 5 and 6) could either be due to a lack of stimulatory compounds, or possibly, as is the case in a number of other previously mentioned pathosystems, the presence of inhibitory compounds that develop as the fruit matures. Indeed, in the previously mentioned study of appressorium induction by avocado wax on *C. gloeosporioides*, addition of non-host plant surface waxes to normally inductive avocado wax treatments inhibited appressorium formation by 80%, suggesting the presence of inhibitory compounds that may play a role in host specificity (Podila 1993).

The response of *C. fioriniae* to hexadecanoic acid (Figs. 7 and 8), one of the main components of the biopolymer cutin, in the plant cuticle is perhaps the most significant finding of this study. It corroborates the findings in several other previously-mentioned pathosystems: that cutin dissolution and recognition are integral parts of appressorium formation.

For instance, *C. trifolii*, the causal agent of alfalfa anthracnose, has been shown to produce a specific protein kinase in response to cutin or cutin monomers called lipid-induced protein kinase, or LIPK (Dickman et. al 2003). This kinase has a profound effect on appressoria formation: when LIPK was overexpressed, a multitude of abnormal appressoria were produced. When the LIPK gene was disrupted, germination was greatly inhibited and appressorium formation was nonexistent. Thus, cutin, and cutin monomers have a direct effect on appressorium formation in *C. trifolii*.

P. oryzae also depends on the degradation of cutin to form appressoria. It has been shown to possess a gene called Cut2 that codes for a cutinolytic enzyme essential to development of and penetration by appressoria (Skamnioti and Gurr 2007). A Cut2 deficient mutant produced significantly fewer appressoria on inductive hydrophobic membranes as compared to the wild type, and showed morphological defects. That these appressorium formation rates and morphologies were rescued by application of exogenous cutin monomers further supports the idea that detection of such monomers, and by inference the ability to degrade cutin, is an integral signaling step in the differentiation of germ tubes into appressoria for *P. oryzae*.

Similarly, another study found that *Fusarium solani (*Teleomorph *Haemonectria haematococca*) produced cutinase in response to the presence of cutin or cutin monomers (Woloshuk and Kolattukudy 1986). The authors theorize that there is a small amount of cutinase produced in the spore walls that is used as an indicator to test a substrate for cutin content. If there is cutin present, it is

degraded, releasing monomers, which trigger further cutinase production. Indeed, a previous study showed that the ability to degrade cutin was essential to the pathogenicity of *F. solani* on pea plants (Köller et al. 1982).

A cutinase-deficient isolate of *F. solani* was significantly less virulent on intact pea stems, but was able to infect wounded stems. Further, the virulence of the isolate was rescued by application of exogenous cutinase and other cell-wall-degrading enzymes. These findings were initially challenged by a study which seemed to show cutinase deficient mutants capable of breaching intact stems (Stahl et al. 1994), but the original hypothesis was vindicated upon discovering that *F. solani* possesses multiple cutinase genes, only one of which was knocked out in the refuting study. This experiment showed that disruption of all cutinase-producing genes in *F. solani* drastically reduced its pathogenicity, forcing it to infect primarily through stomata, rather than directly breaching the cuticle (Rogers et. al 1994).

In a more closely related pathosystem, a cutinase-deficient mutant of a *C. gloeosporioides* isolate normally pathogenic on papaya was shown to be unable to infect fruit with intact cuticles. However, once the cuticle was breached by wounding, the pathogen was once again able to penetrate and colonize the host tissue (Dickman and Patil 1986). Conversely, *Mycosphaerella*, a plant pathogen that is normally only able to penetrate papaya fruit if the cuticle is breached by wounding, was able to infect directly through the cuticle when a cutinase production gene from *Fusarium solani* f. sp. *pisi* was inserted into its genome (Dickman et al. 1989).

Further supporting these results is the fact that several organophosphate pesticides typically used for treating insect problems also prevented infection of papaya by *C. gloeosporioides*, and that these compounds were antagonistic towards purified *C. gloeosporioides* cutinase in vitro (Dickman et al. 1983). In fact, different blends of cutinases were produced by two isolates of *C. gloeosporioides* pathogenic on citrus when induced by the presence of cutin monomers (Liyanage et al. 1993).

The results of the studies presented here on cranberry, particularly the effect of the cutin monomer hexadecanoic acid on appressorium formation, set against the backdrop of the aforementioned research suggest a future course of research that involves the study of cutinase as a possible mechanism of virulence and/or species specificity in *C. fioriniae*, as well as research into chemicals that could potentially inhibit cutinase production, more specifically those that may be present in the wax of cranberry fruit, including benzoic acid. It is also worth noting that although the methyl ester of hexadecanoic acid is a strong stimulant of appressorial formation, others could certainly exist.

Regardless, the fact that rice blast and anthracnose, two of the most economically significant diseases worldwide, both seem to share cutin monomers as a common signaling molecule involved in host substrate recognition should not be taken lightly by plant breeders and fungicide developers. Finding a way to prevent appressorium formation may be a key to developing new fungal management strategies for controlling disease.

Appendix A. Two-Way ANOVA Tables

GENOTYPE

Source	df	Type III SS	MS	F	P
Model	9	2.2345	0.2483	10.9748	<0.0001
Treatment	4	1.8443	0.4611	20.3814	<0.0001
Run	1	0.3329	0.3329	14.7165	0.001
Interaction Trt x Run	4	0.0573	0.0143	0.6329	0.6448
Error	20	0.4525	0.0226		
Total	29	2.6870			

ONTOGENY

Source	df	Type III SS	MS	F	P
Model	9	0.9280	.1031	9.2305	<0.0001
Treatment	4	0.8896	0.2224	19.9083	<0.0001
Run	1	0.0002	0.0002	0.0147	0.9047
Interaction Trt x Run	4	0.0383	0.0096	0.8567	0.5065
Error	20	0.2234	0.0112		
Total	29	1.1515			

CHEMICAL STANDARD

Source	df	Type III SS	MS	F	P
Model	9	2.8104	0.3123	9.5971	<0.0001
Treatment	4	2.5165	0.6291	19.3351	<0.0001
Run	1	0.0759	0.0759	2.3337	0.1423
Interaction Trt x Run	4	0.2180	0.0545	1.6749	0.1952
Error	20	0.6508	0.0325		
Total	29	3.4612			

HYDROPHOBICITY

Source	df	Type III SS	MS	F	P
Model	5	3.1183	0.6237	49.7087	<0.0001
Treatment	2	3.0004	1.5002	119.5728	<0.0001
Run	1	0.0525	0.0525	4.1864	0.0633
Interaction Trt x Run	2	0.0654	0.0327	2.6057	0.1149
Error	12	0.1506	0.0125		
Total	17	3.2689			

Appendix B. One-Way ANOVA Tables

GENOTYPE

Source	df	Type I SS	MS	F	P
Model	4	1.8443	0.4611	13.6794	<0.0001
Treatment	4	1.8443	0.4611	13.6794	<0.0001
Error	25	0.8427	0.0337		
Total	29	2.6870			

ONTOGENY

Source	df	Type I SS	MS	F	P
Model	4	0.8896	.2224	21.2319	<0.0001
Treatment	4	0.8896	0.2224	21.2319	<0.0001
Error	25	0.2619	0.0105		
Total	29	1.1515			

CHEMICAL STANDARD

Source	df	Type I SS	MS	F	P
Model	4	2.6594	0.6648	17.7638	<0.0001
Treatment	4	2.6594	0.6648	17.7638	<0.0001
Error	25	0.9357	0.0374		
Total	29	3.5950			

HYDROPHOBICITY

Source	df	Type I SS	MS	F	P
Model	2	3.2266	1.6133	107.4386	<0.0001
Treatment	2	3.2266	1.6133	107.4386	<0.0001
Error	15	0.2252	0.0150		
Total	17	3.4519			

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