SYMPTOMOLOGY, EPIDEMIOLOGY, AND REAL-TIME PCR-BASED DETECTION OF EXOBASIDIUM LEAF AND FRUIT SPOT OF BLUEBERRY CAUSED BY *EXOBASIDIUM MACULOSUM*

by

RUSSELL J. INGRAM

(Under the Direction of Harald Scherm)

ABSTRACT

During the past decade, Exobasidium leaf and fruit spot, caused by the fungus *Exobasidium maculosum*, has emerged from a curiosity to a serious disease causing economic losses to the blueberry industry in the southeastern states. Due to the emerging nature of the disease, little is known about its epidemiology including means of pathogen overwintering, spore dispersal, seasonal timing of infection in relation to host phenology, and disease progression on leaves and fruit in the field. Epidemiological field studies utilizing disease monitoring, spore-trapping, and trap plants were conducted on rabbiteye blueberry (*Vaccinium virgatum*) between 2014 and 2017 to address these knowledge gaps. The symptomology of the disease was reviewed in detail and revealed a previously undescribed symptom type, shoot spots on newly emerging shoots that typically resulted in girdling of the shoot. Monitoring of surface populations indicated that *E. maculosum* overwinters epiphytically on various blueberry tissues tested during dormancy.

Preliminary studies also suggested that reduction of these surface populations is associated with reduced levels of in-season disease, thereby implicating surface populations as a source of primary inoculum. Detailed disease monitoring and trap plant data suggested that primary infection occurs shortly after leaf and flower emergence in the spring, is favored by prolonged rainy periods, and occurs primarily on young and tender tissues. Disease progress curves indicated that the disease progresses monocyclically and that initial fruit infection occurs prior to the presence of sporulating leaf lesions in the field. Weekly spore-trapping revealed airborne inoculum only after leaf lesions producing basidiospores were present, suggesting that the primary inoculum is not airborne. To provide a diagnostic tool for further investigation of aerial spore dispersal of *E. maculosum*, a real-time polymerase chain reaction (PCR)-based assay was developed and evaluated for the detection and enumeration of the pathogen from environmental samples such as the spore trap tapes generated in this study. The assay reliably produced positive reactions from cultures of *E. maculosum* and did not cross-react with non-*Exobasidium* species of fungi. Preliminary application of the assay to spore trap tapes from a Burkard spore sampler corroborated trends observed via alternate spore trapping methods in the field.

INDEX WORDS: Exobasidium leaf and fruit spot, disease cycle, *Exobasidium maculosum*, emerging disease, plant disease epidemiology, rabbiteye blueberry, *Vaccinium virgatum*

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DEDICATION

To my father, I hope that I have made you proud. To my grandparents, thank you for believing in me. To my wife Alina Ingram, thank you for all that you have sacrificed to allow me to pursue my dreams.

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Nothing that we do in life of any worth is done alone. Thank you to all the advisors, mentors, colleagues, and friends that have helped me along the way. Neither my education nor this body of work would have been possible without the efforts and guidance of others.

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CHAPTER 1

INTRODUCTION

Georgia's Blueberry Industry

North America (the United States, Canada, and Mexico) continues to be the largest producer of cultivated blueberries in the world, with production totals accounting for 88.6% of the world's harvested total. The United States alone represented approximately 55% of global production with a farm gate value of $850 million in 2012 (USDA-ERS 2013b). Since 2012, production from other countries such as Chile, Canada, and - most significantly - China has increased substantially and has very quickly placed significant market pressure on the United States' domestic and export markets. However, the United States is still the global leader in blueberry production.

After the high antioxidant capacity of blueberries was discovered and publicized in the late 1990s, the market demand for blueberries has increased dramatically. Since that time, the blueberry industry, which previously occupied a small niche within the greater specialty crop sector, has expanded into global markets and has become the number one fruit crop for the state of Georgia. In order to keep pace with increasing consumer demand, the commercial blueberry industry in North America has undergone a rapid expansion over the past 25 years. Throughout this period of expansion, a large proportion of the increases in both acreage and production have occurred in the Pacific Northwest and southeastern regions of United States. As a result of this expansion, the Southeast, which contains one-third of the total number of blueberry-producing states and

more than one-third of the cultivated blueberry acreage, now represents the largest production area in the United States, reaching a record high of 12,500 harvested hectares in 2016 (USDA-ERS 2013a; Wolfe and Stubbs 2017). Furthermore, in Georgia alone, planted area of blueberries has increased 5-fold while the farm gate value of blueberries has increased 10-fold from approximately $29.6 million in 2002 to $288.9 million in 2016 (Fig. 1.1), making it the most valuable fruit crop in Georgia (Wolfe and Stubbs 2017). Much of the rapid expansion in blueberry production experienced in the Southeast has been driven by factors such as low cost of land, suitable growing environment, a long growing season, and the ability to reach the marketplace earlier than other regions. These factors, along with the constant improvement of cultivars derived from the native rabbiteye (*Vaccinium virgatum*) and the introduced southern highbush (*V. corymbosum* interspecific hybrids), suggests that the blueberry industry in the Southeast will continue to grow in both scale and importance in the near future (Krewer and NeSmith 2002; Scherm and Krewer 2003).

In its infancy, blueberry cultivation in the southeastern United States experienced few disease problems and consequently required very little input of chemical controls for the management of pests and diseases. The low disease pressure observed on cultivated rabbiteye blueberries has largely been assumed to be the result of adaption of this native species to the common pests and environmental conditions of the region. However, as the blueberry industry has increased in size, as cultivation has intensified over the past two and a half decades, and as southern highbush blueberries have become more prevalent, several economically important diseases have increased both the need and usage of fungicides. For example, a survey of Georgia blueberry producers before 1992 indicated

that only 36% of producers applied fungicides at some point during the growing season (Hubbard et al. 1992). A subsequent 1999 survey of Georgia blueberry producers indicated that over 80% of the respondents were applying fungicides two to three times per season (Scherm and Stanaland 2001). Fungicide usage has increased even more during the past two decades on both rabbiteye and southern highbush cultivars.

The considerable increase in fungicide usage was initially due to the proliferation of mummy berry disease caused by *Monilinia vaccinii-corymbosi*, and primarily associated with rabbiteye blueberry cultivars. Although this disease can be an important yield and quality constraint, current fungicide schedules have allowed for satisfactory levels of control and maintenance of crop quality (Scherm and Stanaland 2001) resulting in a dramatic decrease in percent reduction in crop value from 1.5% in 1998 to 0.02% in 2015 (Williams-Woodward 1999; Little 2016). Other fungal and oomycete blueberry diseases of economic importance include Phytophthora root rot caused by *Phytophthora cinnamomi*, flower and twig blight caused by *Botrytis cinerea*, stem blight caused by species within the *Botryosphaeriaceae* complex, and leaf spotting and rust diseases caused by various species of fungal plant pathogens (Scherm et al. 2001).

Exobasidium Leaf and Fruit Spot

Within the past decade, Exobasidium leaf and fruit spot, a new blueberry disease of increasing economic importance, has been reported by producers and researchers causing variable levels of crop loss throughout the southeastern United States. A similar disease, attributed initially to an *Exobasidium* sp. within the *Exobasidium vaccinii* species complex, was reported previously from lowbush blueberry (*V. angustifolium*) in Nova

Scotia (Canada) and from northern highbush blueberry (*V. corymbosum*) in North Carolina (Nickerson and Vander Kloet 1997; Cline 1998). Recent phylogenetic analysis of the causal organism of the disease in the southeastern United States revealed that the pathogen causing Exobasidium leaf and fruit spot on rabbiteye and highbush blueberry represents the new species *Exobasidium maculosum* (Brewer et al. 2014). The organism causing leaf and fruit spots on lowbush blueberry in the northeastern United States and Canada was shown to belong to the same species, although the populations of *E. maculosum* in the two regions were genetically distinct (Stewart et al. 2015).

Exobasidium leaf and fruit spot has become economically important because of the crop losses that result from premature fruit drop as well as the conspicuous green lesions formed on ripened fruit. These lesions render the fruit unmarketable and increase packing costs due to the difficulty of removing infected fruit. Also adding to the increasing concern is that recent reports of Exobasidium leaf and fruit spot have indicated that in cases of high disease pressure, crop losses can reach 60 to 70% or higher (Cline and Bloodworth 2014; Cline et al. 2014).

To date there have been numerous studies on the management of Exobasidium leaf and fruit spot in the southeastern United States. Of the earliest studies that have been conducted, early-season fungicide applications of pyraclostrobin + boscalid, fenhexamid, and especially captan have shown promise in reducing both leaf and fruit disease incidence (Brannen et al. 2014). However, at least one population of *E. maculosum* sampled from a commercial blueberry planting in southern Georgia exhibited high levels of insensitivity to pyraclostrobin + boscalid (R.J. Ingram, unpublished). Another chemical control strategy that has been studied extensively is the use of a late dormant

application of lime sulfur. Field testing results over more than 5 years indicated that the use of a single late-dormant application of lime sulfur or Sulforix (calcium polysulfides) is capable of achieving control levels similar or better than the currently used in-season fungicides (Brannen et al. 2017). The finding that a contact fungicide results in such highly efficacious control has important implications for understanding the overwintering and life cycle of *E. maculosum* and provided an important starting point for a broader investigation into the disease cycle of Exobasidium leaf and fruit spot of blueberry.

Justification and Objectives

Due to the recent emergence of Exobasidium leaf and fruit spot of blueberry, very little is known of the life cycle of *E. maculosum* or the epidemiology of the disease. The few studies that have investigated disease development as part of management trials have noted that infection appears to occur in early spring during the period of bud-break and/or shoot expansion of the crop. Additionally, increased incidence and severity of the disease has been observed in association with conditions such as high humidity, excess moisture, and poor airflow (Brannen et al. 2014, 2017). These observations also noted that the pathogen was capable of sporulating on leaf and fruit lesions, producing basidiospores. Outside of these general observations, there is no information on basic epidemiological features such as pathogen overwintering, the type and source of initial inoculum, monocyclic vs. polycyclic disease progression, stages of host susceptibility, mode of spore dispersal, and when in relation to each other leaf and fruit infection occur. It is the overall goal of this dissertation to begin to address some of these knowledge gaps. Specific objectives are to:

- Better understand the symptomology, impacts, and environmental factors associated with Exobasidium leaf and fruit spot;
- Document the site of overwintering, mode of initial inoculum dispersal, and seasonality of infection associated with the disease;
- Quantify the timing of basidiospore production and dispersal and their role in annual epidemics;
- Synthesize known information of Exobasidium leaf and fruit spot to define the disease cycle associated with this increasingly important pathosystem.

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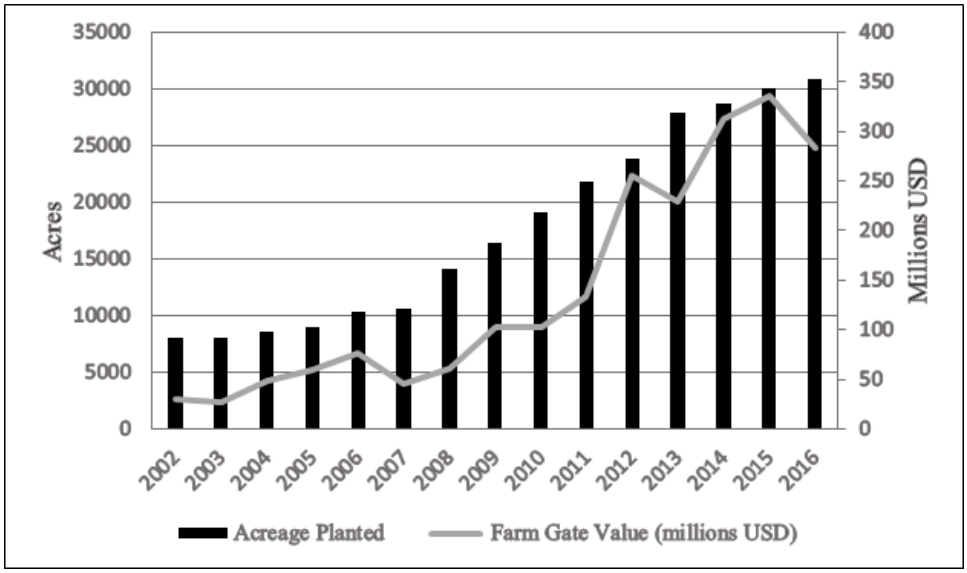

Fig. 1.1. Growth of the Georgia blueberry industry, 2002-2016. Source: CAES Farm gate value reports 2003 to 2017.

CHAPTER 2

LITERATURE REVIEW

The Genus *Exobasidium*

According to Woronin (1867), the first representative description of the genus *Exobasidium* in the literature (Fuckel 1861) originally placed the phytopathogen within Fungi Imperfecti and under the genus name *Fusidium*. A few years later, a taxonomic treatment of the genus provided the initial introduction of the current nomenclature of the genus *Exobasidium* Woron. through the investigation of the epidemiology and description of *E. vaccinii*, causal agent of a leaf gall on *Vaccinium vitis-idaea* near St. Petersburg, Russia (Woronin 1867). The genus *Exobasidium* is morphologically characterized as being dimorphic, with most species having both a dikaryotic mycelial and monokaryotic yeast state. Sexually derived monokaryotic, hyaline basidiospores bear a hilar appendage, are variably septate, have a musiform shape and are borne on sterigmate basidia. Basidia, while variable in number of sterigmata, are only produced on the host plant on a hymenial layer of dikaryotic hyphae. Conidia, the pathogen's yeast state, appear to exist for all but the species *E. vexans*. These conidia, while variable in size, are characterized by being uninucleate, nonseptate, and hyaline, and can be derived from both germinating basidiospores and budding mycelium (Gadd and Loos 1948a; Wolf and Wolf 1952; Graafland 1960).

This enigmatic group of phytopathogens primarily infects a wide diversity of members of the *Ericaceae*, but is also reported to affect hosts within the

Commenlinaceae, *Lauraceae*, and *Theaceae*, most notably the economically important tea plant, *Camellia sinensis* (Sundstrom 1964). While symptom types are diverse and include leaf spots, witches' brooms, flower galls, leaf galls, shoot spots, leaf and shoot enlargements, and reddening/pinking/purpling of plant organs, the types of infections caused by *Exobasidium* spp. may be operationally separated into perennial systemic (e.g., red leaf of blueberry caused by an *Exobasidium* sp. previously considered *E. vaccinii*) and annual nonsystemic infections (e.g., blister blight of tea caused by *E. vexans*) (Gadd and Loos 1948b; Hilborn and Hyland 1956; Nannfeldt 1981). Additionally, an intermediate category of locally systemic infections, such as the leaf galls caused by *E. japonicum* on azalea, could be considered.

The Seminal Work by Graafland (1960) and Sundstrom (1964)

Due to the conspicuous leaf and flower gall symptoms caused by some species, the genus *Exobasidium* has attracted the interest of naturalists and researchers as far back as the seventeenth century (Hoffman 1662). Scientific interest in the genus underwent a renaissance during the 1950s and 1960s, which included what are arguably the most definitive and comprehensive investigations of both the epidemiology and taxonomy of members of the genus *Exobasidium* (Graafland 1960; Sundstrom 1964). Within these combined bodies of work, numerous *Exobasidium* spp. were described, catalogued, and in one case, *E. japonicum* causal agent of azalea leaf and flower gall, a significant part of the disease cycle and epidemiology was described. From his work with *E. japonicum* and complemented by a thorough review of the literature, Graafland (1960) concluded that most members of the genus *Exobasidium* had a narrow host range, and that for all

Exobasidium spp. producing basidiospores and conidia described at that time, both spore types were able to cause disease symptoms on their respective hosts. Also, presented and investigated within this body of work were inoculation trials with basidiospores or conidia of *E. japonicum* showing that inoculation of dormant buds could lead to disease when the buds emerged up to 6 months later. It was unclear, however, whether pathogen spores survived on the surface of buds and/or between bud scales causing infection upon bud break, or whether the fungus infected buds upon inoculation causing a latent infection that resulted in disease symptoms at bud break. It should be noted here that later publications on the nature of overwintering of *E. japonicum* on azalea incorrectly interpreted this earlier work by Graafland (1960) as conclusive evidence that the pathogen was capable of overwintering on the surface of buds, bark, and within bud scales (Coyier and Roane 1986; Wolfe and Rissler 2000). Another important finding from Graafland's (1960) study that has been observed in every case of successful artificial inoculation of *Exobasidium* species leading back to Woronin (1867) is that only the young, tender tissues of host plants are susceptible to infection, and that successful infection only results in conditions of high humidity. Graafland (1960) also documented that the use of lime, lime sulfur, and captan applied at bud break was efficacious at reducing disease incidence in conditions of natural and artificial inoculation.

During investigations into the physiology, morphology, host range, and infectivity of conidia and basidiospores of 19 different *Exobasidium* species, Sundstrom (1964) arrived at several important conclusions with significant relevance to the disease cycle of this phytopathogenic group. Temperature response studies for growth in culture found the lowest maximum temperature allowing growth of *E. myrtilli* at 23°C and the highest

maximum temperature for *E. vexans* at 34°C. The other *Exobasidium* spp. maintained growth of either mycelia or yeast-like conidia up to various temperature maxima within these bounds. Experiments with basidiospores indicated that, while most basidiospores of all other *Exobasidium* spp. tested germinated within hours and/or at the most days, the basidiospores of the species *E. vaccinii-uliginosi* took between 4 and 6 months to germinate. Other important findings derived through inoculation studies confirmed earlier work in that only young and tender tissues were susceptible, monospore cultures of conidia were capable of producing disease symptoms confirming that species within the genus are homothallic, and that while there does appear to be significant evidence of narrow host range for most species tested, at least one "host race" of *E. vaccinii* "oxyc." was capable of producing disease symptoms on all tested hosts (*V. vitis-idaea*, *V. oxycoccus*, *V. uliginosum*, and *Arctostaphylos uva-ursi*) albeit at a much lower disease incidence than was seen with each respective *E. vaccinii* "host race". Because of the combined physiological, morphological, and serological work by Sundstrom (1964), several new *Exobasidium* spp. were described and split out from their former species complexes, resulting in *E. vaccinii* "host races". Many of the *E. vaccinii* "host races" have since been designated as distinct species (Nannfeldt 1981).

Leaf-Spotting *Exobasidium* Species

Since the focus of the current study is on an *Exobasidium* species of the leaf spot variety, it is felt necessary to detail a few other examples of Exobasidium leaf spot diseases on *Vaccinium* spp. hosts. *Exobasidium arescens* is an excellent example of the cause of a leaf spot disease occurring on the host *V. myrtillus* (bilberry). This species has

a symptom progression very similar to that of *E. maculosum*. With both pathogens, small (0.5 cm diameter) leaf spots appearing yellowish to buff and occasionally bright red purple above with a white hymenial layer on the abaxial leaf surface have been observed to sporulate and then rapidly dry, turning gray, black, and becoming necrotic. Other examples of leaf spot pathogens sharing a similar symptomology to that of *E. maculosum* are *E. pachysporum* on *V. uliginosum* (bog bilberry) and *E. rostrupii* on *V. macrocarpon* (American cranberry). Spots caused by the latter species tend be more red in coloration and much smaller than the previously mentioned examples (Nannfeldt 1981). Although there are several other examples of *Exobasidium* spp. associated with plant diseases characterized by leaf spot symptoms, all occur on non-economically or moderately economically important hosts, and therefore virtually nothing is known of their epidemiology and disease cycles.

For blueberry specifically, recent phylogenetic work has shown that the species causing Exobasidium leaf and fruit spot on lowbush blueberry in Canada and the northeastern United States, while belonging to *E. maculosum*, is genetically distinct from populations of the fungus from rabbiteye and highbush blueberry in the southeastern United States (Stewart et al. 2015). This is consistent with inoculation trials conducted by Nickerson and Vander Kloet (1997) with *E. maculosum* from lowbush blueberry, where the authors were unable to reproduce symptoms of the disease on, among other *Vaccinium* spp., *V. vitis-idaea* and more importantly *V. corymbosum*. This finding was not surprising given that both Graafland (1960) and Sundstrom (1964) indicated that many *Exobasidium* spp., including the many species previously classified broadly as *E. vaccinii*, were in fact separate species with narrow host ranges.

The first report of Exobasidium leaf and fruit spot of blueberry in the southeastern United States was on cultivated *V. corymbosum* (northern highbush blueberry) in commercial plantings in North Carolina in 1997 and 1998 (Cline 1998). This report noted that in 1997, over 25% of the berries from a planting of cv. Wolcott were affected by a sharply delineated green spot on otherwise ripe fruit. Leaf spots appeared as circular, light green amphigenous spots 4 to 7 mm in diameter that were smooth on the adaxial leaf surface and covered with a dense white fungal growth on the abaxial leaf surface. The high incidence of green spots on otherwise ripe berries caused all fruit from this field in that year being unsaleable, resulting in a total loss to the grower. During the description of *E. maculosum* by Brewer et al. (2014) as a distinct species, the following symptomology and morphological characteristics were reported. A hymenial layer was observed on the surface of fruit spots or abaxial surface of leaf spots; leaf spots were noted as circular, chlorotic, 2 to 14 mm in diameter, with a pale yellow to pale green coloration that turned crimson to maroon with age, adaxial surface concave, abaxial surface white to pale yellow, raised and felt-like. Fruit spots were pale green and leathery, turning powdery and pale yellow to crimson or maroon with age. Incidence and total number of spots on leaves were reported to increase toward the interior and lower portions of affected bushes. Host symptoms aside from leaf and fruit spots were not reported. Basidia were noted as 8.5 to 11.5 x 5 to 6.5 µm across, cylindrical, emerging from lower epidermis, straight or slightly curved at base, arranged in clusters of varying stages, 5 to 6 sterigmate. Sterigmata 2 to 3 x 3 µm across and attenuated to 1 to 2 µm at the base. Basidiospores 8 to 10 x 3 to 3.5 µm, hyaline, smooth, ellipsoid to musiform, hooked at the base, distinct knob at hila appendix, often transversely once-septate after

detachment from basidium. Germination occurs at both ends of basidiospores, giving rise to yeast-like conidia or hyphae. On potato dextrose agar, most basidiospores produced conidia that gave rise to more conidia; however, some germinated to form up to 30-µm-long unbranching hyphal filaments. Conidia were noted as 7 to 9 x 1 to 1.5 µm, ellipsoid, hyaline, smooth, aseptate, arising from basidiospores and/or other conidia. Hyphae, 1 to 2.5 µm across, were abundantly observed within the leaf spongy mesophyll and thickened tissue layer throughout the leaf epidermis.

Exobasidium Disease Cycles

Although this group of phytopathogens has piqued the curiosity of researchers for well over a century, thorough investigations into the epidemiology of only three *Exobasidium* spp. are available in the literature, and only one has a well-evidenced and complete disease cycle. The first of these three is the aforementioned *E. japonicum* causing leaf and flower galls on azalea, which although being well described in most aspects of its disease cycle, is still incomplete due to a lack of conclusive evidence of the site of overwintering and source of primary inoculum (Graafland 1960). The second example, the *Exobasidium* sp. causing red leaf of lowbush blueberry, while somewhat lacking in a comprehensive understanding of its epidemiology, has a well-evidenced and complete disease cycle whereby the pathogen has been shown capable of overwintering as a systemic infection in the form of mycelia in the rhizomes of its host, *V. angustifolium.* In the following spring and early summer after new shoots have emerged from infected rhizomes, the pathogen produces a hymenial layer on leaves and shoots of the new tissue, which then produce basidiospores that spread to produce new infections

(Hilborn and Hyland 1956; Lockhart 1958; Nannfeldt 1981). The role of the conidia in this pathosystem is not clear. The last and by far the most well-studied *Exobasidium* sp. is *E. vexans*. Due to the blister blight disease that this pathogen causes on the economically important tea plant, numerous studies have been conducted to determine all aspects of the epidemiology including its disease cycle. This species has a very simplified disease cycle in that it is not dimorphic (i.e., does not produce a yeast-like conidial stage) and that the evergreen and constantly pruned nature of its host in commercial conditions allows continuous cycles of basidiospore infection year-round. Indeed, one of the first infection models, based largely on hours of sunshine, relative humidity and temperature, ever developed for the prediction of a plant disease aimed to predict the incidence of blister blight of tea (Kerr and Shanmuganathan 1966; Shanmuganathan and Arupragasam 1966; Kerr and Rodrigo 1967a, b; Kerr and de Silva 1968). These studies as well as De Weille (1960) showed that *E. vexans* infects and causes disease in conditions of high relative humidity >90%, extended periods of leaf wetness, low number of hours of direct sunshine, and temperatures below 35°C. It was also found that the basidiospores of *E. vexans* will not germinate in low humidity <60%, and that exposure to both UV-B and temperatures in excess of 35°C will kill basidiospores (De Weille 1960). Although numerous studies have been conducted on this particular pathosystem, no complete disease cycle is available due to the inability of past studies to adequately evidence the means of pathogen survival during the unfavorable season when the disease is not active in the field *(*Ajay et al. 2009; Sugha 1997).

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CHAPTER 3

SYMPTOMOLOGY AND EPIDEMIOLOGY OF EXOBASIDIUM LEAF AND FRUIT SPOT OF BLUEBERRY[1]

[1] Ingram, R.J., Allen, R.M., and Scherm, H. 2017. *Acta Horticulturae* 1180:205-214. Reprinted here with permission of publisher, 5/2/2018.

Symptomology and epidemiology of Exobasidium leaf and fruit spot of blueberry

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Abstract

During the past decade, the state of Georgia has become one of the largest producers of cultivated blueberries in the United States. As production has expanded and intensified, several new blueberry diseases have emerged, with Exobasidium leaf and fruit spot, caused by the newly described fungal pathogen *Exobasidium maculosum*, being one of the most recent. The disease affects both rabbiteye and southern highbush cultivars, causing leaf spotting early in the season as well as green spots on ripe fruit that can result in the rejection of the fruit at the packinghouse. The pathogen produces both basidiospores and yeast cells, but its life cycle, including overwintering biology and primary and secondary inocula, is currently unknown. To fill these knowledge gaps, epidemiological field studies that included trap plants, leaf spot demographic surveys on naturally infected field plants, and epiphytic population monitoring were initiated in 2014. Monitoring of surface populations showed that *E. maculosum* is capable of overwintering epiphytically on all blueberry tissues tested during dormancy. Shoot demography and trap plant data suggested that dispersal of primary inoculum occurs shortly after leaf and flower emergence in the spring, and that infection occurs primarily on young and tender tissue and is favored by prolonged rainy periods. Disease progress curves indicated that the disease is active from March through late May, and that initial

fruit and leaf infection may occur simultaneously. Additional observations revealed a novel symptom type, lesions on emerging shoots causing girdling and blighting. Initial results suggest that the disease is monocyclic, but further confirmatory studies are needed to fully elucidate disease epidemiology and pathogen life cycle.

Introduction

During the past 25 years, blueberry cultivation in Georgia has increased exponentially to reach a farm gate value of $335 million in 2014 (Wolfe and Stubbs 2015), making the state one of the largest producers of blueberries in the United States. Much of this growth has been driven by high blueberry prices, a favorable climate allowing for fruit to mature early, and the availability of land well suited to blueberry cultivation (Scherm and Krewer 2003). The expansion of the blueberry industry has been an economic boon for small and medium-sized farms in economically disadvantaged counties in the southern part of the state, where traditional cash crops such as tobacco have declined in both acreage and profitability.

As cultivation of blueberries has expanded and intensified in Georgia, several new disease problems of economic importance have emerged (Brannen et al. 2014), the most recent example of which is Exobasidium leaf and fruit spot. The disease affects rabbiteye (*Vaccinium virgatum*) and southern highbush (*Vaccinium corymbosum* interspecific hybrids) cultivars, causing leaf spotting early in the season as well as green spots on ripe fruit. In the southeastern United States, the disease was first described from North Carolina in 1997 (Cline 1998). In Georgia, the first sporadic occurrence of Exobasidium leaf and fruit spot was noted in 2010, but within subsequent years, both disease

prevalence and intensity increased markedly, leading to widespread losses due to downgrading or rejection of affected fruit at the packinghouse (Brannen et al. 2013). Although initial reports suggested that the causal organism of the disease was *Exobasidium vaccinii sensu lato*, recent phylogenetic analyses revealed that the fungus represents the novel species *Exobasidium maculosum* (Brewer et al. 2014).

Exobasidium maculosum produces basidiospores on the surface of infected host tissues and grows as a yeast in culture. However, knowledge of the pathogen's life and disease cycles is incomplete, hence there are no pathogen biology-based disease control recommendations available at present. Although empirically derived fungicide schedules for management of Exobasidium leaf and fruit spot have been developed and deployed (Brannen et al. 2017), a better understanding of the epidemiology of the disease, particularly the pathogen's oversummering and overwintering sites and sources of initial inoculum, will be critical for estimating disease risk and developing robust, targeted disease management tactics. Also needed to fully understand the impacts of the disease is a more detailed appraisal of symptomology, symptom progression, effects on plant health, and potential for yield loss. The present study was initiated to address these objectives through epidemiological field studies involving the use of trap plants, leaf spot demographic surveys on naturally infected field plants, and epiphytic population monitoring during the dormant season.

Materials and Methods

Disease symptomology. In 2014 and 2015, fungicide-untreated 'Premier' and 'Tifblue' rabbiteye blueberry plants in commercial plantings near Alma (southern

Georgia) and Toccoa (northern Georgia) were monitored weekly for the development and progression of disease symptoms. Monitoring began in early March, coinciding with the initiation of new plant growth following dormancy, and ended in June after fruit harvest. Additional, less frequent surveys were conducted during summer and fall. Microscopic observation of diagnostic basidia and basidiospores was used to confirm the presence of *E. maculosum* on sporulating tissues. Fruit infections typically become visible to the naked eye when fruit turn color; to visualize earlier symptoms, immature, green fruit were washed in 95% ethanol for 1 min to remove the wax layer.

Quantification of overwintering inoculum. Prior fungicide efficacy trials against Exobasidium leaf and fruit spot on rabbiteye blueberry indicated that a single late-dormant application of calcium polysulfides was highly effective at reducing subsequent disease development on leaves and fruit (Brannen et al. 2017). The significant control achieved with a dormant application of this contact fungicide suggested that the pathogen overwinters, at least in part, epiphytically on its host. To quantify epiphytic populations of *E. maculosum*, we collected blueberry tissue samples from an affected planting during the dormant season, and dilution-plated tissue washes onto a semi-selective medium for pathogen enumeration.

At weekly intervals from late dormancy to shoot elongation/full bloom (January through March 2015), 20 shoots formed during the previous year were taken on each sampling date from fungicide-untreated plants in a commercial planting of ‘Premier’ rabbiteye blueberry in Alma, GA. From this random sample, two shoots were pooled to give a set of ten replicates. Leaf buds, flower buds, green bark, and lignified bark were excised from the shoots and placed in 1.5-mL microcentrifuge tubes containing 0.01%

Tween 80. The tubes were then sonicated in a Branson 2200 ultrasonic bath (Branson Ultrasonics Corp., Danbury, CT, USA) for 2 min, followed by vortexing for 30 s and dilution-plating of the suspension in quadruplicate onto M5 semi-selective medium (3 g yeast extract, 5 g peptone, 20 g dextrose, 20 g agar; autoclaved for 30 min and allowed to cool to 55°C before adding 100 $\mu g \cdot mL^{-1}$ chlortetracycline, 50 $\mu g \cdot mL^{-1}$ streptomycin, 7 mL 1N HCL, and a final concentration of 1 $\mu g \cdot mL^{-1}$ Pristine fungicide). Dishes were observed 2 weeks later for characteristic *E. maculosum* colonies (cream to pink, raised, farinose, and with an irregular margin).

Determination of dispersal and infection windows using trap plants. In 2015, a trap plant study was employed to identify the timing of primary inoculum dispersal and windows of leaf infection. At weekly intervals from February to May, three 2-year-old 'Premier' rabbiteye blueberry plants in #1 containers (2.8 L) were placed within a row of previously diseased plants in a commercial 'Premier' rabbiteye blueberry planting in Alma, GA. The trap plants had been standardized in their leaf development prior to field exposure by holding them in cold storage at 5 to 8°C until 10 to 14 days before placement in the field. Plants were subsequently moved into a greenhouse at 22 to 28°C until the majority of leaf buds were at shoot expansion and flowers were close to full bloom. Each set of trap plants was exposed for 7 days in the field before being returned to the greenhouse and placed on a mist bench under shade cloth for 7 days. Plants were observed daily for 2 weeks for the development of leaf spots. Temperature and precipitation data were collected at an agrometeorological station ca. 3 km from the test site to determine potential associations of weather factors with trap plant infection.

Leaf and fruit spot progression in the field. To describe symptom progression and disease phenology, a leaf spot demography study was conducted from March to June 2015 in a commercial 'Premier' rabbiteye blueberry planting in Alma, GA, with a history of the disease. Starting in March, shortly after initial shoot elongation, 72 newly emerging shoots were selected randomly, flagged, numbered, and monitored weekly for the occurrence of leaf spots. Each leaf on each emerging shoot was tracked individually. Once leaf spots were observed, they were classified as either *new* (1 to 3-mm spot, inconspicuous and yellow-green on the adaxial leaf surface and more conspicuous and dull white on the abaxial surface); *mature* (flat to somewhat sunken spot with a light yellow halo around a chlorotic center on the adaxial surface and glossy white on the abaxial surface); *sporulating* (yellow to pink or red spot on the adaxial surface and velvety white, dull sporulation on the abaxial surface); or *post-sporulation* (bright red margins of spots with brown and necrotic centers on both sides of the leaf). Progress curves were generated for each class of leaf spots to visualize the developmental shift and timing of disease as related to host phenology.

To monitor disease development on fruit, weekly random samples of 36 fruit clusters, starting 2 weeks after petal fall and extending until harvest, were observed for the occurrence of symptoms. Fruit spot incidence data were used to create a disease progress curve that was overlaid on the leaf spot demography curves to illustrate the timing of fruit and leaf symptoms relative to host phenology.

Results and Discussion

Disease symptomology. In early spring (late March), small (1 to 3-mm), amphigenous lesions appearing slightly chlorotic on the adaxial leaf surface (Figure 1A) and light green to white on the abaxial surface (Figure 1B) were observed on newly expanded leaves. Lesions continued to expand over the course of 1 to 2 weeks, reaching a diameter of 2 to 10 mm. Prior to sporulation, mature lesions had a characteristic light yellow-green halo that surrounded the yellow center (Figure 1C) which was only visible on the adaxial surface of the leaf. As lesions developed further, the halo disappeared and the center of the lesion turned bright yellow. Also occurring as lesions matured, the abaxial surface of the leaf within the lesion became increasingly white (Figure 1D) as the hyphae of the fungus continued to colonize the mesophyll layer and formed a hymenium. Once the hymenial layer was formed, the emerging immature basidia pushed back the lower epidermis, leaving a velvety, white appearance. Post-sporulation (Figure 1E), lesions gradually became brown and necrotic with a distinct red margin. Leaf lesions that occurred on or near petioles often resulted in leaf drop. Defoliation of heavily diseased leaves appeared to be common but was not quantified. Leaves with one or a few spots did not seem to experience significant defoliation.

On fruit, disease spots commonly occurred on ripe fruit. However, as part of our monitoring studies, we observed symptoms already on very young green fruit, 0.25 to 1.0 cm in diameter. This symptom became apparent only after the waxy layer on developing fruit had been removed, revealing a light green lesion (Figure 2A). As affected fruit began to ripen and darken, the unripened, light green lesions became increasingly conspicuous (Figures 2B and C). When lesions reached maturity, they often appeared

slightly white to gray due to sporulation on the fruit surface. Post-sporulation fruit spots became necrotic, which may enable colonization by secondary pathogens.

A novel symptom type, lesions on young, actively growing shoots, was observed as part of this study. These lesions first appeared as slightly chlorotic spots on one side of the stem, with a small dark point in the center and with or without a darkened margin (Figure 3A). As the lesions developed further and expanded, the affected stem tissue became increasingly white and raised. At maturity, the stem lesions appeared white and velvety due to sporulation of the fungus (Figure 3B). Post-sporulation, lesions became brown or black and necrotic with a distinctive red margin (Figure 3C). Spots then either remained isolated and formed a canker or completely girdled the stem (Figure 3D). In a sample (n=36) of shoots with early stem spot symptoms observed in Alma, GA, in 2015, 72% of stem spots resulted in complete girdling of affected shoots. Subsequent dissection of stem lesions showed that tissue damage extended from the epidermis into the pith of the stem (Figure 3E).

Quantification of overwintering inoculum. Cells of *E. maculosum* were consistently recovered through surface washes from all tissue types tested in early 2015 (Table 1). However, as new shoots and flowers emerged from leaf and flower buds, epiphytic populations declined. The presence of surface populations documented the ability of the pathogen to overwinter epiphytically on its host. The marked decline in inoculum levels on leaf and flower buds after emergence could reflect a dilution effect due to increased surface areas, or it could indicate that new growth is not colonized directly from leaf or flower buds at emergence.

Determination of dispersal and infection windows using trap plants. Trap plant infection occurred consistently from the week of 6 to 13 March through the week of 17 to 24 April 2015 (Figure 4). Interestingly, the incidence of trap plant infection was either very high (>70 spots/plant) or very low (<4 spots/plant), indicating significant variations in either pathogen dispersal and/or environmental favorability for infection. The first trap plant infection periods, 6 to 13 March and 13 to 20 March, occurred prior to symptom development on field plants and coincided with shoot elongation of new leaves in the field. Infection of trap plants prior to symptom development on field plants indicated that dispersal of primary inoculum occurred very early in the growing season, shortly after the emergence of new growth. It is worth noting that only one significant infection period of trap plants occurred later during the sporulation of leaf spots on field plants, and no infection of trap plants occurred when the highest number of spots on field plants were actively sporulating (compare Figures 4 and 5).

When the number of rainy days per weekly exposure period was overlaid onto the trap plant infection data for the same period, an interesting pattern emerged: significant trap plant infection occurred only during periods of 4 or more consecutive rainy days during the exposure week (Figure 4). The association between consecutive rainy days and infection has been documented previously for *E. vexans,* another leaf-spotting *Exobasidium* spp. which causes blister blight of tea (Kerr and Rodrigo 1967). Whereas determining the exact length of time of rainy/overcast conditions necessary for infection by *E. maculosum* will require further research, the strong association with weather may explain the lack of significant infection during peak sporulation of spots on field plants.

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Table 3.1. Weekly colony counts of *Exobasidium maculosum* from surface wash-platings of dormant to emerging leaf buds, flower buds and flowers, green bark, and lignified bark (Alma, GA, 2015).

Tissue sample	log_{10}(CFU/g tissue)							
	26 Jan.	2 Feb.	8 Feb.	12 Feb.	26 Feb.	6 Mar.	12 Mar.	20 Mar.
Leaf buds	N/D[1]	4.3	4.5	4.6	4.1	2.9	0.6	0
Flower buds	N/D	1.7	3.9	3.8	3.7	3.1	0	0
Green bark	3.8	3.9	4.3	4.4	4.4	4.2	3.9	3.8
Lignified bark	4.6	4.1	5.0	4.6	4.5	4.4	N/D	4.2

[1]N/D = not determined.

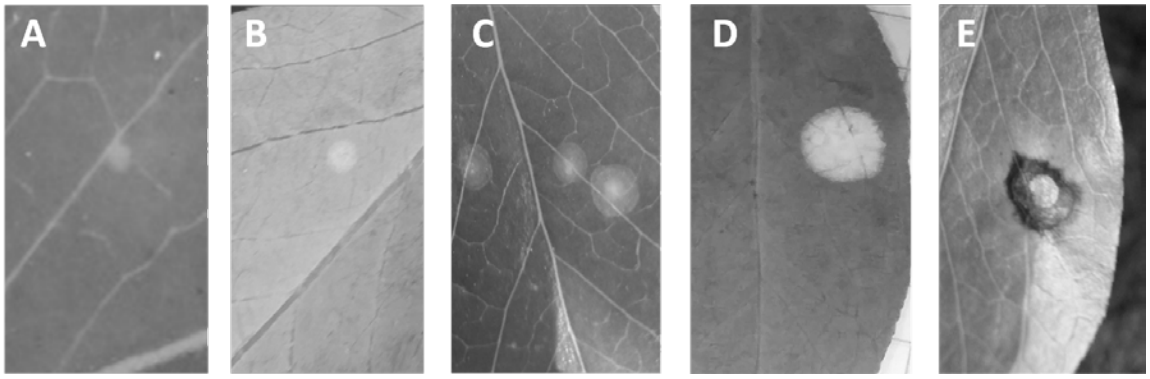

Figure 3.1. Progression of leaf spot symptoms caused by *Exobasidium maculosum* on 'Premier' rabbiteye blueberry. Newly appearing spots as viewed from the upper (A) and lower (B) leaf surface; mature spot (C), upper leaf surface; sporulating spot (D), lower leaf surface; and necrotic spot (E), upper leaf surface.

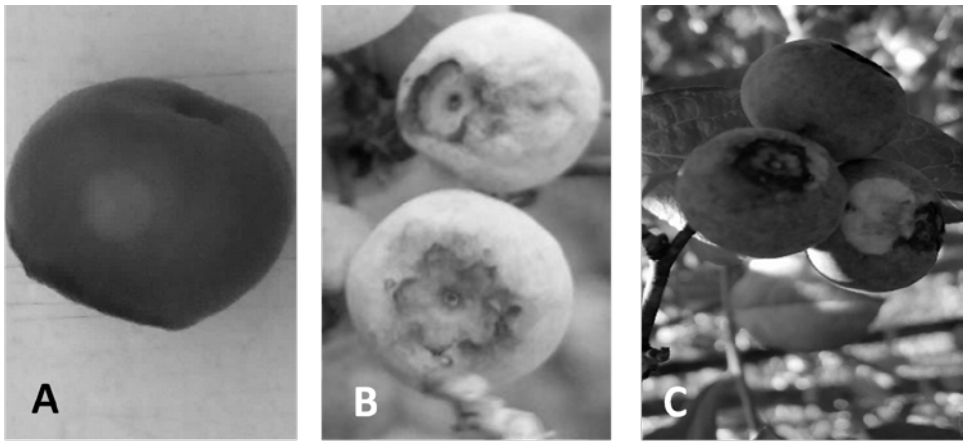

Figure 3.2. Progression of fruit spot symptoms caused by *Exobasidium maculosum* on ‘Premier’ rabbiteye blueberry. Newly appearing spot on young, green fruit (A) visualized following removal of the wax layer with ethanol; sporulating spot on immature berries (B); and characteristic green spot on ripening fruit (C).

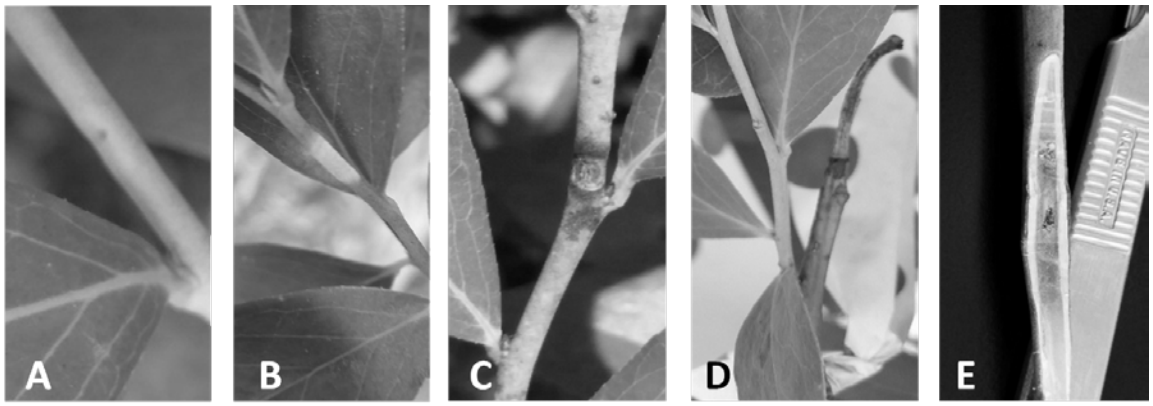

Figure 3.3. Progression of stem spot symptoms caused by *Exobasidium maculosum* on ‘Premier’ rabbiteye blueberry. New infections on young, tender shoots first appear slightly chlorotic with or without a small necrotic lesion in the center (A). As spots develop, they become velvety-white due to the production of basidiospores (B). Subsequently, the center of the spot turns brown while the margin turns bright red (C). Post-senescence, the majority of stem spots result in girdling and blighting of affected shoots (D). Dissection of stem spots reveals that necrosis extends into the pith (E).

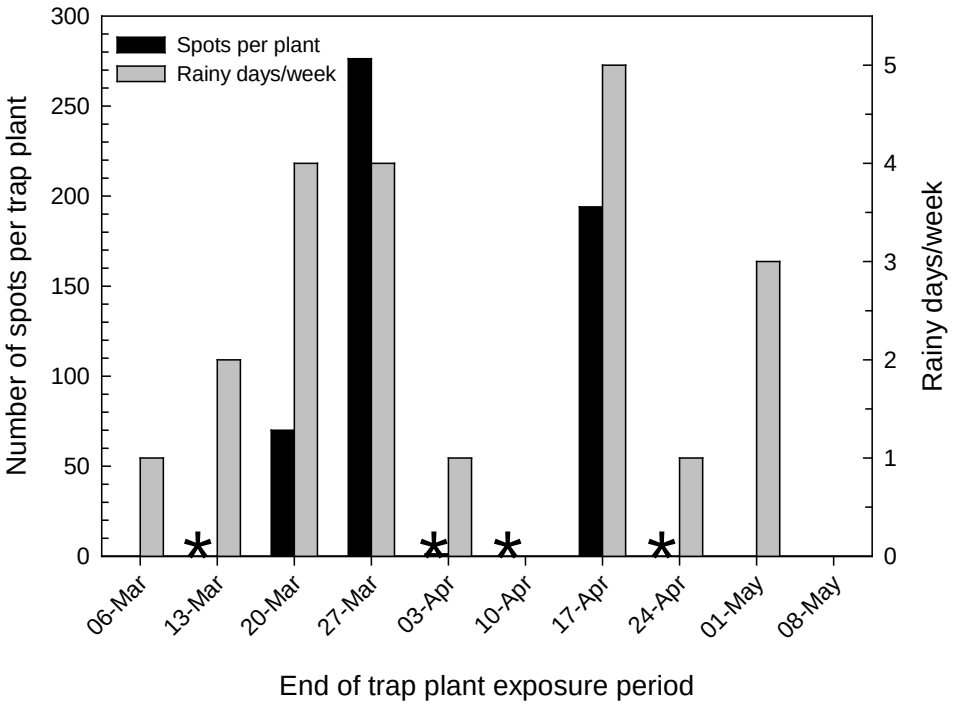

Figure 3.4. Infection of 'Premier' rabbiteye blueberry trap plants (black bars) by *Exobasidium maculosum* during weekly exposure periods in relation to the number of rainy days per week (gray bars) in Alma, GA (2015). Weeks with very low trap plant infection (<2 spots/plant) are denoted with an asterisk (*), whereas those with no symbols (6 March, 1 May, and 8 May) had zero infections.

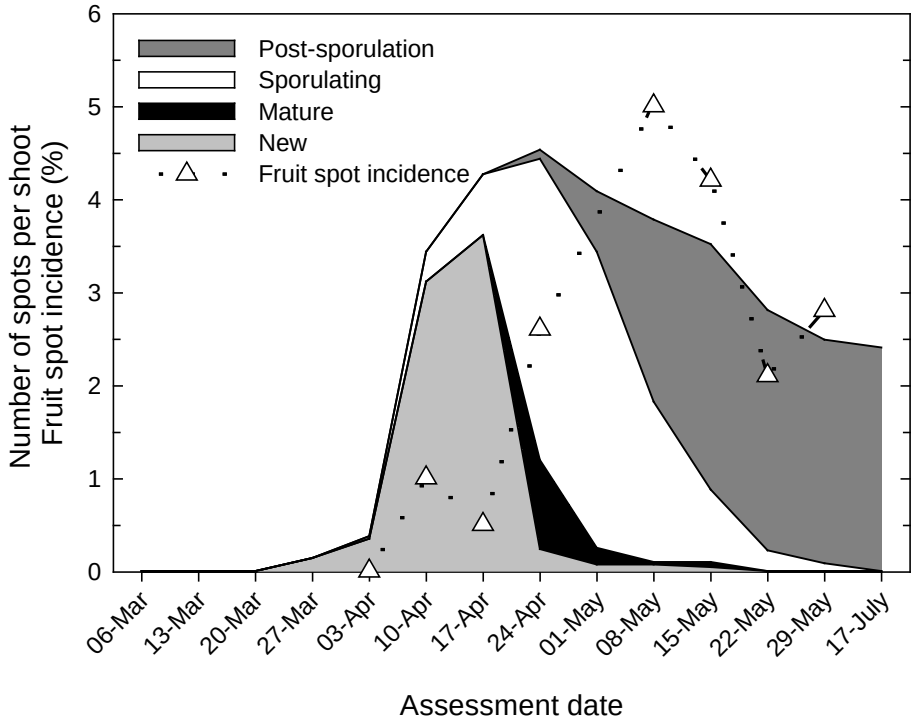

Figure 3.5. Exobasidium leaf and fruit spot progression on ‘Premier’ rabbiteye field plants in Alma, GA (2015). The solid fills denote different leaf spot demography classes, whereas the dashed line indicates fruit disease incidence.

CHAPTER 4

EPIDEMIOLOGY OF EXOBASIDIUM LEAF AND FRUIT SPOT OF RABBITEYE BLUEBERRY: PATHOGEN OVERWINTERING, PRIMARY INFECTION AND DISEASE PROGRESSION ON LEAVES AND FRUIT[1]

[1] Ingram, R.J., Capps, H.D., and Scherm, H. To be submitted to *Plant Disease*.

Epidemiology of Exobasidium leaf and fruit spot of rabbiteye blueberry: Pathogen overwintering, primary infection and disease progression on leaves and fruit

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ABSTRACT

Epidemiological field studies utilizing disease monitoring, spore-trapping, and trap plants were conducted on rabbiteye blueberry (*Vaccinium virgatum*) between 2014 and 2017 to shed light on the epidemiology of Exobasidium leaf and fruit spot, an emerging disease in the southeastern United States caused by the fungus *Exobasidium maculosum*. Wash-plating of field-collected blueberry tissue from the late dormant season through bud expansion showed that the pathogen overwintered epiphytically on blueberry plants in the field, most likely in its yeast-like conidial stage. Agrichemical applications during the dormant season altered epiphytic populations of the pathogen, which correlated directly with leaf spot incidence later in the spring. Disease monitoring of field plants and weekly exposure of potted trap plants revealed that young leaves at the mouse-ear stage were most susceptible to infection, that disease incidence on leaves progressed monocyclically, and that infection periods were associated with rainfall variables such as the number of days per week with ≥1.0 mm of rain or cumulative weekly rainfall. Weekly spore-trapping with an Andersen sampler revealed airborne inoculum only after sporulating leaf lesions producing basidiospores were present in the field, suggesting that the primary inoculum is not airborne. The first symptoms on young, green fruit were observed soon after petal fall (requiring removal of the waxy fruit layer

to visualize lesions), and visible disease progress on fruit was delayed by 1 to 3 weeks relative to that on leaves. Fruit infection of field plants and trap plants occurred before airborne propagules were detected by spore-trapping and before sporulating leaf lesions were present in the field. Hence, this study showed that fruit infections are initiated by the same initial inoculum as leaf infections, but it was not possible to conclusively exclude the possibility of a contribution of basidiospore inoculum from leaf lesions to disease progress on later developing fruit. This is one of only a few studies addressing the epidemiology and disease cycle of a member of this elusive group of phytopathogens in a pathosystem where artificial inoculation has not been possible to date.

Introduction

During the past decade, Exobasidium leaf and fruit spot of blueberry has emerged from a curiosity to a serious disease causing economic losses. The first reports of a spot disease in blueberry caused by an *Exobasidium* species were from unmanaged and managed wild lowbush blueberry (*Vaccinium angustifolium*) fields in Nova Scotia (Canada) in 1997 and subsequently from commercial fields of northern highbush blueberry (*V. corymbosum*) in North Carolina in 1998 (Nickerson and Vander Kloet 1997; Cline 1998). Noted in the report by Nickerson and Vander Kloet (1997) was the sporadic occurrence of whitish to pale-green spots on the leaves of mostly shaded lowbush blueberry plants, whereas Cline (1998) observed morphologically similar spot symptoms on both leaves and fruit of infected northern highbush plants. Both reports, although different in symptomology and host species, attributed the disease to an *Exobasidium* species within the *Exobasidium vaccinii* species complex. During the past

decade, Exobasidium leaf and fruit spot has become particularly prevalent and severe in blueberry-producing regions of the southeastern United States on rabbiteye (*V. virgatum*) and to a lesser extent southern highbush (*V. corymbosum* interspecific hybrids) cultivars (Brannen et al. 2011). Recent phylogenetic analysis has revealed that the causal organism of the disease is a previously undescribed species, *Exobasidium maculosum* (Brewer et al. 2014). Although members of the same species, populations of *E. maculosum* from rabbiteye and highbush blueberry in the southeastern United State are distinct from those on lowbush blueberry in the northeastern United States and Canada (Stewart *et al.* 2015). *Exobasidium maculosum* is distinct from the *Exobasidium* sp. (previously referred to as *E. vaccinii*) causing red leaf disease of lowbush blueberry (Brewer et al. 2014).

A detailed description of the symptomology of Exobasidium leaf and fruit spot of rabbiteye blueberry is presented in Ingram et al. (2017). Commercial crop losses are mainly associated with the fruit-spotting phase of the disease; the green spots fail to ripen, rendering the berries unmarketable and increasing sorting costs in the packinghouse due to the difficulty of removing infected fruit. In addition to leaf and fruit spots, the pathogen can also cause necrotic, girdling lesions on young shoots, which further contribute to yield losses via the reduction of fruiting wood (Ingram et al. 2017). Although formal crop loss estimates are currently lacking, Cline (1998) reports fruit losses of 25% in a severely affected field of northern highbush blueberry in North Carolina.

Information about the epidemiology of diseases caused by *Exobasidium* spp. in general, and of Exobasidium leaf and fruit spot of blueberry in particular, is limited (Cline and Brewer 2017). Leaf symptoms appear early in the growing season (late March

to early April in the southeastern United States), suggesting that young, emerging leaves are infected (Ingram et al. 2017). By early summer, a distinct red margin forms around leaf and shoot spots as they become brown to black, necrotic and occasionally form shot-holes. No new disease symptoms are observed on young leaves that emerge during summer and fall. Fruit symptoms are readily apparent in the field in early summer when the berries turn color, but closer inspection reveals symptoms on green fruit as early as 2 weeks after petal fall especially when the waxy layer of the fruit is removed, e.g., with ethanol (Ingram et al. 2017). The type(s) of inocula causing leaf and fruit spots and the mode of oversummering and overwintering of the pathogen are currently unknown (Cline and Brewer 2017). The disease is controlled readily with a single late-dormant application of liquid lime sulfur (Brannen et al. 2017), a primarily surface-active fungicide, suggesting that the pathogen may overwinter epiphytically on blueberry tissues in the field.

Exobasidium maculosum produces two spore types, once-septate basidiospores and single-celled yeast-like conidia (Brewer et al. 2014). Basidiospores are formed on basidia that are part of a thin hymelial layer on the lower surface of leaves as well as on fruit and stem spots, whereas the conidia are produced by budding at each end of basidiospores. When basidiospores or conidia are plated on culture media, the pathogen grows as a yeast colony, whereas filamentous (hyphal) growth is observed only in infected plant tissue. The role(s) of the two spore types in the disease cycle of Exobasidium leaf and fruit spot are unclear, but there is some information available from other Exobasidium pathosystems. Some species (such as the *Exobasidium* sp. causing red leaf of lowbush blueberry) incite systemic infections in their hosts following basidiospore

infection of young stems (Mims and Nickerson 1986; Nickerson and MacNeill 1987; Nickerson and Hildebrand 2017), whereas others (such as *E. vexans* causing blister blight of tea) produce polycyclic leaf spot epidemics via repeated cycles of basidiospore dispersal and infection (Gadd and Loos 1948). In other examples, such as *E. japonicum* causing leaf galls on azalea, both basidiospores and conidia are capable of infecting young, emerging leaves following dormant bud inoculation (Graafland 1960). The wide range of life histories of Exobasidium diseases warrants closer investigation of the disease cycle specific to *E. maculosum*. It is important to note that, outside of the systemic in planta mycelial overwintering of the *Exobasidium* sp. causing red leaf of lowbush blueberry, to the best of the authors' knowledge no study has been able to clearly evidence and identify the mode of overwintering and therefore provide a complete disease cycle for any disease caused by this group of phytopathogens.

Artificial inoculation experiments using basidiospores and/or conidia of several *Exobasidium* species have produced infections on typically young and tender host plant tissues (Graafland 1960). For example, Nickerson and Vander Kloet (1997) reported reproducing leaf spot symptoms on lowbush blueberry (*V. angustifolium*) by artificial inoculation with conidia of *E. maculosum*. In contrast, artificial inoculation of young leaves or fruit of rabbiteye blueberry with basidiospores and/or conidia of diverse isolates (and combinations of isolates) of *E. maculosum* by different lab groups has not resulted in successful infection (H. Scherm, unpublished; M.T. Brewer, unpublished). Hence, it is currently not possible to shed light on the disease cycle of Exobasidium leaf and fruit spot via artificial inoculation experiments. Based on these considerations, we report here the results of epidemiological field studies to unravel the means of pathogen

overwintering, spore dispersal, seasonal timing of infection, and disease progression in the Exobasidium leaf and fruit spot pathosystem on rabbiteye blueberry. Preliminary results have been published in a proceedings report (Ingram et al. 2017).

Materials and Methods

Field site and plant material. The experiments reported below were conducted between 2014 and 2017 in a commercial rabbiteye blueberry planting with a history of Exobasidium leaf and fruit spot near Alma, GA. The planting consisted of alternating rows of three cultivars (Premier, Climax, and Tifblue), but only cv. Premier, which is highly susceptible to Exobasidium leaf and fruit spot, was used in the study. Plants were mature (>20 years old in 2014) and were left untreated with fungicide throughout the study period (except for the 2017 trial in which agrichemical applications were used to manipulate overwintering surface inoculum densities). All other horticultural and pest management practices followed those recommended for commercial rabbiteye blueberry production in the southeastern United States (Burrack et al. 2013).

Quantification of dormant-season surface populations. Tissue samples were collected from one or more rows of Premier plants located near the eastern woodline of the planting during the dormant seasons (January and/or February) and during leaf and flower bud expansion (March) in 2014, 2015, and 2016 to determine presence and levels of epiphytic populations of *E. maculosum* by wash-plating. Tissue types tested were leaf buds (from dormant to mouse-ear); flower buds (from dormant through full bloom); green bark from previous year's growth; and mature, woody bark from growth that was 2 years old or older (included in 2014 only). Sampling was conducted weekly by collecting

the distal 10 to 20-cm segments of previous year's shoots (fruitwood) by removing two shoots from both sides of a bush, then skipping a bush, and continuing until a total of 40 shoots had been obtained. Shoot segments were shipped overnight or brought directly to Athens, GA, where they were stored at 4°C and processed within 5 days of collection. Twenty shoot segments were selected randomly, and two segments were combined to give ten experimental units. Using a sterile scalpel, eight leaf buds and eight flower buds were removed from each set of two shoots. Ten milligrams of bark, excluding tissue beneath the bark, were removed using a sterile scalpel. After excision, weights of all tissue samples were recorded and each tissue type placed in a 1.5-mL microcentrifuge tube prefilled with 1 mL of 0.01% Tween 80 in sterile dH_20, with the steps repeated for each of the ten experimental units. At the point in the growing season when flower and leaf buds on collected shoots had broken and reached full bloom and shoot elongation, the volume of the wash solution for these two tissue types was doubled from 1 to 2 mL within a 10-mL tube. All tubes with tissue samples were sonicated in a water bath for 2 min (Branson 2000; Branson Ultrasonics Corp., Danbury, CT) and then vortexed for 30 s immediately before wash-plating 0.1 mL of tissue wash suspension onto the semi-selective medium M5 (Ingram et al. 2017) in quadruplicate for each of the ten tissue-specific experimental units. Aliquots of the tissue wash suspension were placed in -80°C for later molecular confirmation of the accuracy of the visual identification of *E. maculosum* colonies. Petri dishes were placed in a plastic crisper box at 21 to 23°C and 70 to 75% relative humidity for 2 weeks, at which time each dish was reviewed under a dissection microscope (60x) for characteristic *E. maculosum* colonies (cream to pink, raised, farinose, and with an irregular margin; Ingram et al. 2017). Colony counts were

recorded along with the initial weight of tissue placed in each microcentrifuge tube to calculate the number of colony-forming units (CFU) of *E. maculosum* per gram of tissue for each tissue type and sampling date.

For molecular confirmation, previously frozen wash-plating aliquots were spread-plated onto M5 medium, and representative colonies (n = 35) visually identified as *E. maculosum* were sub-cultured on potato dextrose agar amended with 50 mg/L streptomycin. Yeast conidial suspensions from these colonies were subjected to bead-beating DNA extraction and real-time polymerase chain reaction (PCR) using forward primer EXORI-F, reverse primer EXORI-R, and LNA probe EXORJI with reaction conditions as described by Ingram (2018).

Leaf spot development in the field. The study was conducted during the 2015, 2016, and 2017 growing seasons, but the 2017 trial had to be discontinued after a late freeze in mid-March damaged leaf tissue and flowers, resulting in an insufficient fruit crop (Collins 2017). At the mouse-ear stage of leaf bud development (NeSmith et al. 1998) in early March of each year, 72 newly emerged shoots on plants within a row were flagged, with four shoots on each side of every other bush from 1 to 2-m-high tagged at random. These shoots were monitored weekly from late February until June for the occurrence of Exobasidium leaf spots on all leaves within a shoot (n = 4 to 15 leaves per fully expanded shoot).

At each assessment date, leaf spots were classified based on their stage of development (Ingram et al. 2017). New spots were small (1 to 2 mm) and chlorotic without a light yellow halo. Mature spots were yellow on the upper surface of the leaf with a green-yellow halo and a white underside but no visually apparent sporulation.

Sporulating spots were yellow on the upper surface of the leaf with or without a green-yellow halo and a velvety-white underside. Post-sporulating spots were yellow or brown on the upper surface and gray, brown, or black on the underside of the leaf and occasionally associated with a distinct red margin. Disease progress curves were generated for the four leaf spot classes based on the average number of leaf spots per shoot for each assessment date.

Fruit spot development in the field. Starting at petal fall in late March or early April and ending at 40 to 50% ripe fruit in late May or early June in 2015 and 2016, between 32 and 36 fruit clusters were sampled from four field plants within the experimental row by removing four fruit clusters from both sides of every other bush starting at the third bush. This resulted in 93 to 240 fruit per sampling date. Fruit samples were immediately brought into the lab, placed in 4°C, and assessed for fruit spot incidence within 1 week of sampling. All fruit was sprayed with 10% ethanol to remove the waxy surface layer that would otherwise obscure disease spots on green fruit. This effect was only present when the surface of green fruit was still wet with the ethanol spray. Disease progress curves were generated based on fruit disease incidence data.

Early-season airborne spore sampling. From late February to late May or early June in 2015 and 2016, weekly sampling for airborne *E. maculosum* spores was conducted using a battery-operated six-stage viable Andersen sampler (Air Quality Instruments, Franklin, MA). The sampler was placed within the plant row between two bushes, 1.0 m above the ground, fitted with six Petri dishes containing the semi-selective M5 medium (Ingram et al. 2017), and operated for two separate time periods (30 and 60 s) at midnight on the same day of the week each week. Considering the two run times and

the sampler's air suction rate of 28.3 L/min, the detection threshold was calculated as 24 propagules per m^3 of air. After the two exposure periods, Petri dishes were placed in a plastic sleeve and brought to the lab the next day. To allow for colony development, dishes were placed in a partially opened plastic crisper box for 2 weeks (21 to 23°C, 70 to 75% relative humidity). For each sampling date, the sampler run time having countable numbers of *E. maculosum* colonies was selected, and colony counts on all stages corresponding to that run time were taken and converted to CFU/min. Because exact counts were not always possible due to the presence of other fungal colonies, each sampling date was classified for aerial propagule numbers on a semi-quantitative scale as absent (0 CFU); low (1 to 50 CFU); medium (51 to 200 CFU); and high (>200 CFU).

Identification of primary leaf and fruit infection periods using trap plants. Two-year-old potted plants of cv. Premier were obtained from Bottoms Nursery (Concord, GA) and maintained for different periods in a cold room and greenhouse to obtain susceptible leaf or fruit stages were used as trap plants by exposing them to natural inoculum in the aforementioned rabbiteye blueberry planting for weekly intervals during the epidemic in 2015 and 2016. Target stages at the time of field exposure were mouse-ear to early shoot elongation for leaves (NeSmith et al. 1998) and 0.25 to 0.75 cm green fruit diameter for fruit. Because of the different timing of these phenological stages for leaves and fruit on the same plant, separate groups of trap plants were required to monitor leaf and fruit infection. Plants were manipulated to maintain dormancy prior to use by storing them in darkness in a walk-in cold room at 5 to 8°C. Two to three weeks prior to placement in the field for leaf trap plants and 5 to 7 weeks for fruit trap plants, plants were moved from the cold-room into the greenhouse (22 to 28°C, 12 h of light).

Bumblebees (Koppert Natupol Class C hive; Koppert Biological Systems, Howell, MI) were used to pollinate fruit trap plants over a period of 2 weeks by placing two hives within a section of greenhouse containing Premier trap plants and pollinator plants cv. Alapaha and Climax at full bloom.

Leaf trap plants (four or five per week) with the appropriate leaf development stages were exposed weekly from late February to late May in 2015 and 2016. Similarly, fruit trap plants (four per week) were exposed weekly between early March and mid-May in 2016. Trap plants were placed in the field in pot-depth holes located between field blueberry plants within a single row and were left in the field for an exposure period of 7 days. Starting in late April when temperatures began to exceed 27°C, trap plants were prevented from drying out with a custom-made drip watering system. At the end of each exposure period, trap plants were brought back into the greenhouse and placed under a shade cloth on a mist bench (misting rate: 10 s every 1 h). After 1 week, plants were moved to a separate section of the greenhouse and then monitored for symptom development (number of leaf or fruit spots per plant) once a week for 1 month. Trap plants with phenology similar to those exposed in the field at each exposure interval were retained in the greenhouse to serve as controls.

Correlation analysis was used to relate the average number of leaf spots per trap plant during weekly exposure periods from mid-March to mid-May in 2015 and 2016 (n = 10 each year) with select rainfall variables, i.e., the number of days per week with ≥1.0 mm of rain and cumulative weekly rainfall as recorded with a standard agricultural weather station located ca. 3 km from the experimental site.

Susceptibility of leaf stages. The trap plant studies described above were used to determine the susceptibility of different leaf development stages to primary infection by *E. maculosum*. In one set of experiments in 2016, two fruit trap plants having more advanced leaf development stages were used to test the susceptibility of tender leaves vs. those that had already hardened-off. These plants had 15 shoots per plant flagged at the junction of hardened-off (proximal) and tender (distal) leaves. In a separate set of experiments using leaf trap plants from February to mid-May in 2015 and 2016, a total of five shoots per leaf development stage (NeSmith et al. 1998) per plant were flagged to assess the relative susceptibility of stages 3 (green tip), 4 (mouse ear), and 5 (shoot elongation) to infection by *E. maculosum*. At the end of each weekly exposure period, plants were returned to the greenhouse, placed under a shade cloth on a mist bench, and assessed for Exobasidium leaf spots as described previously. Chi-Square contingency table analysis was used to determine associations between leaf development stages and the presence or absence of symptoms on leaves.

Chemical-based manipulation of dormant season surface populations of *E. maculosum* and impact on primary infection. This study was conducted on field plants of Premier rabbiteye blueberry in Alma, GA, during the 2017 growing season. At the late dormant stage of development, a fungicide trial was conducted to assess the impact of reduced or increased surface population levels of *E. maculosum* on the incidence of Exobasidium leaf spot in the field. Treatments included an untreated control, lime sulfur (Lime Sulfur Ultra; OrCal, Junction City, OR; 18.7 L/ha in 467.6 L/ha water) and two horticultural products, hydrogen cyanamide (Dormex; AlzChem, Trostberg, Germany; 7.0 L/ha in 467.6 L/ha water) and mineral oil (Damoil; Drexel, Memphis, TN; 28.2 L/ha

in 935.2 L/ha of water). Previous research had shown that dormant applications of lime sulfur and hydrogen cyanamide decrease disease incidence, whereas dormant oil applied at the same time increased the disease (Scherm et al. 2017), and we hypothesized that this was the case because of an impact of these products on overwintering surface populations of *E. maculosum*. Applications were made on 18 January at the late-dormant stage of plant development using an air-blast sprayer set to 413.7 kPa and fitted with standard cone nozzles. The experiment was designed as a randomized complete block with four replications of four-bush plots within a single row, with two untreated bushes between treatments and an untreated buffer row on either side.

To quantify surface population levels of *E. maculosum*, shoot samples were collected from each plot 1 day prior to application, 2 days after application, 8 days after application, and 59 days after application. Five shoots (10 to 20 cm in length) were excised from both sides of each bush within a plot for a total of 20 shoots per plot. Shoot samples were shipped overnight from Alma to Athens, and all samples were processed as described previously with the only exception being that only green bark was tested in this study.

On 15 May, 20 current-season shoots per plot were collected and assessed for shoot-level incidence of Exobasidium leaf spot by calculating the percentage of shoots with a least one infected leaf. Treatment means were calculated and regressed against log-transformed *E. maculosum* population densities (CFU) determined for the same treatments at the 59-day post-treatment sampling in mid-March (n = 4). No fruit spot assessments were possible in 2017 due to a total crop loss following the mid-March freeze.

Results

Quantification of dormant-season surface populations. Epiphytic populations of *E. maculosum* (in its yeast stage on culture) were consistently recovered by wash-plating from blueberry tissues sampled between early February and late March at the experimental site (Fig. 4.1). Population densities were variable, ranging from below 10 to above 10^4 CFU/g of fresh tissue; the lowest densities were generally observed on leaf and flower buds later during the sampling period (late February and beyond), presumably because of a dilution effect as leaf and flower tissues expanded rapidly following bud break. Consistently high numbers (above 10^3 CFU/g) were recovered from green bark throughout the sampling period (Fig. 4.1), whereas numbers on mature bark (included only in 2014) were more variable; no colonies of *E. maculosum* were recovered from mature bark tissue during the first sampling date in 2014.

Thirty-three of the sample of 35 colonies identified morphologically as being characteristic of *E. maculosum* were confirmed as such by real-time PCR (*data not shown*). The two negative colonies were re-evaluated by amplifying their 5.8s ITS regions by conventional PCR using the universal fungal primers ITS1-F and ITS4; a BLAST search of the sequenced amplicons revealed that these colonies were *E. maculosum* as well (identity = 99%), suggesting they were false negatives in the real-time PCR. Thus, visual assessment based on colony morphology was highly accurate in identifying the fungus from tissue wash-platings.

Leaf and fruit spot development in the field. The first leaf spots on field plants appeared during the period of shoot expansion in late March to early April in both 2015 and 2016 (Fig. 4.2). Subsequent disease progression on leaves followed that of a

monocyclic disease, as suggested by single peaks for the four leaf spot classes (new, mature, sporulating, post-sporulation). The first sporulating leaf spots (with visible presence of basidiospores) were observed in early to mid-April, and this class made up the majority of all leaf spot classes between late April and mid-May. Thereafter, spots became necrotic, no longer supporting visible sporulation. The decrease in the average number of leaf spots per shoot from May onward (Fig. 4.2) was due to defoliation of severely affected leaves (Ingram et al. 2017).

Progression of fruit disease incidence was similar across the 2 years (Fig. 4.2). The first spots on green fruit were observed in early to mid-April, 1 to 3 weeks after the first observation of spots on leaves. Subsequently, fruit spot incidence increased to peak in early May, followed by a decrease in the proportion of symptomatic fruit prior to harvest, most likely due to premature drop of affected fruit (Ingram et al. 2017).

Early-season airborne spore sampling. Data collected with the Andersen sampler produced semi-quantitative information on the relative abundance of airborne propagules (presumably basidiospores) of *E. maculosum* during specific weekly sampling periods (Tables 4.1 and 4.2). No airborne spores were detected prior to or during the emergence of the first new leaf spots on field plants. The first presence of airborne spores was recorded after the appearance of sporulating leaf spots in the field, and the highest spore densities generally were associated with those periods when sporulating spots made up the majority of the four leaf spot classes (Tables 4.1 and 4.2).

The first fruit spots on field plants occurred 1 week after airborne propagules were first detected with the Andersen sampler in 2015, whereas fruit spot detection preceded spore detection by 2 weeks in 2016 (Tables 4.1 and 4.2). Airborne spores were

still detected at the end of the sampling period in late May or early June after the number of sporulating leaf spots on field plants had declined sharply.

Identification of primary leaf and fruit infection periods using trap plants. Infection of potted trap plants during weekly exposure periods occurred 1 or 2 weeks prior to the appearance of the first leaf spots in the field (Tables 4.1 and 4.2); hence, assuming an incubation period of approximately 1 to 2 weeks in the field, this suggests that leaves on field plants and trap plants were infected by the same initial inoculum. Across the 2 years, the highest levels of leaf infection on trap plants were generally noted between late March and mid-April, although there was some week-to-week variation. Considerable leaf infection occurred in both years prior to the presence of sporulating leaf spots on field plants and prior to the detection of airborne propagules with the Andersen sampler (Tables 4.1 and 4.2). Conversely, no additional leaf infection on trap plants occurred from early May onward, despite the presence of high numbers of airborne *E. maculosum* spores as recorded by the Andersen sampler during that period. No symptoms of Exobasidium leaf spot occurred on control trap plants (not placed in the field) in either year.

The average number of leaf spots per trap plant during weekly exposure periods from mid-March to mid-May correlated positively with rainfall variables in both years, i.e., the number of days per week with ≥1.0 mm of rain in 2015 ($r = 0.755$, $P = 0.0116$) and cumulative weekly rainfall in 2016 ($r = 0.761$, $P = 0.0106$).

Fruit infection on trap plants was investigated only in 2016 (Table 4.2). Potted plants conditioned in the greenhouse to have early fruit development stages were exposed in the field weekly between 4 March and 20 May 2016. Overall fruit spot incidence was

low during exposure periods resulting in infection, ranging from 0.75 to 2 affected fruit per plant (Table 4.2). The first fruit infection occurred on plants exposed during the week ending 25 March, which also corresponded to the first period of leaf infection on the separate batch of trap plants used to monitor leaf infection. The last fruit infection on trap plants occurred during the exposure period ending 15 April, which was prior to the appearance of sporulating leaf lesion on field plants and prior to the detection of airborne propagules with the Andersen sampler (Table 4.2). Only relatively young, green fruit smaller than 0.5 cm in diameter at the time of exposure became infected (*data not shown*). No additional fruit infection on trap plants occurred from late April onward, despite the presence of high numbers of sporulating leaf lesions on field plants and of the consistent presence of airborne *E. maculosum* propagules as recorded by the Andersen sampler during that period. No symptoms of Exobasidium fruit spot occurred on control trap plants (not placed in the field).

Susceptibility of leaf stages. On fruit trap plants where tender (distal) and hardened-off (proximal) shoot sections were tagged and monitored over time, leaf infection was significantly more likely on tender sections than on hardened-off sections on the same shoot (Chi-Square = 51.1, $P < 0.0001$, df = 1; Table 4.3). When specific leaf development stages were tagged prior to exposure of leaf trap plants, leaf stages from late green tip to shoot elongation were found susceptible to infection, whereby those at the shoot elongation stage were most likely to be infected (Chi-Square = 36.5, $P < 0.0001$, df = 2; Table 4.4).

Chemical-based manipulation of dormant-season surface populations and impact on primary infection. One day prior to the application of the dormant spray

treatments, average epiphytic population densities of *E. maculosum* on green bark were similar across the four treatments, ranging from 2.1 to 4.4 x 10^3 CFU/g tissue (Fig. 4.3). Two and eight days after application, population densities declined for lime sulfur, remained relatively constant for hydrogen cyanamide, and increased for dormant oil and the untreated control (up to ~1.5 x 10^4 CFU/g). At the last sampling date 59 days after treatment (in mid-March, when primary infection would be expected to occur based on disease progress data obtained in the previous 2 years), pathogen populations were highest for dormant oil, lowest for lime sulfur, and intermediate for hydrogen cyanamide and the untreated control (Fig. 4.3). Although Exobasidium leaf spot pressure was low in 2017 (likely associated with dry spring weather and damage of susceptible leaf tissue in the mid-March freeze), disease incidence in the treatment plots mirrored the previously collected pathogen population density data in that shoot-level leaf spot incidence was highest in the dormant oil treatment and lowest in the lime sulfur treatment (Fig. 4.4). Indeed, there was a significant positive correlation between log-transformed *E. maculosum* population densities in mid-March and shoot-level leaf spot incidence in mid-May across the four treatments ($r = 0.978$, $P = 0.0221$, $n = 4$).

Discussion

This study represents the first investigation of the disease cycle of *E. maculosum* causing Exobasidium leaf and fruit spot of blueberry in the southeastern United States. Whereas it is often preferable to describe disease cycle components using artificial inoculation trials, previous efforts to reproduce symptoms on rabbiteye blueberry by inoculation with basidiospores and/or conidia of *E. maculosum* have proven unsuccessful.

To circumvent this constraint, we applied an empirical approach, conducting epidemiological field studies with natural inoculum to shed light on the means of pathogen overwintering, spore dispersal, seasonal timing of infection and disease progression by *E. maculosum*.

Using this approach, we documented epiphytic overwintering of *E. maculosum* on blueberry tissues during the dormant season through early spring. During those periods, the pathogen was recovered from all plant surfaces tested, although population densities were more variable for some tissue types than for others. We presume that *E. maculosum* was most likely present on the surface of these tissues as yeast-like conidia, which is generally considered the saprophytic stage of *Exobasidium* (von Arx et al. 1982). We also showed that a decrease (increase) in such surface populations following agrichemical applications during the dormant season was associated with reduced (increased) incidence of primary infection of leaves in treated field plants later in the spring. Furthermore, spore-trapping with the Andersen sampler indicated that airborne basidiospores of *E. maculosum* were not present in detectable densities within the study area prior to the first appearance of leaf and fruit spots on field plants affected by the disease, thus indicating that primary inoculum is not introduced via airborne inoculum from potential disease reservoirs outside the field.

Taken together, the most parsimonious explanation for the above observations is that primary infection of emerging leaves and young, green fruit in the spring occurs from overwintered surface inoculum of *E. maculosum* (presumably yeast-like conidia), either by inoculum splashing or via direct infection from inoculum on infested buds as leaves are emerging and expanding. The latter scenario is less plausible, given that we

would not have been able to observe symptoms of primary leaf infection on trap plants simultaneously with those on field plants if primary infection had been due to bud contamination with the pathogen.

Leaf spot progress curves on field plants followed that of a monocyclic disease, as suggested by single peaks for the four leaf spot classes (new, mature, sporulating, and post-sporulation). Indeed, during maximum production of basidiospores in the field (high proportion of sporulating lesions on field plants and high spore densities documented with the Andersen sampler), both infection of leaf trap plants and appearance of new leaf spots on field plants were virtually absent. This lack of new leaf infections during peak basidiospore production and dispersal clearly was not due to a lack of susceptible tissue, given that trap plants consistently had appropriate leaf development stages (mouse-ear to early shoot elongation) whenever they were exposed in the field. An alternative explanation is the transition to unfavorable environmental conditions during late April to early May when infection on trap plants and new lesions on field plants dropped off markedly. It is well established that members of the genus *Exobasidium* are particularly sensitive to high temperatures in culture and in the field (Graafland 1960; De Weille 1960), and physiological studies with isolates of a range of *Exobasidium* spp. showed that none of the species tested could be cultured at temperatures above 29^{o}C (Graafland 1960; Sundstrom 1964). This appears consistent with our field data from 2016, where mean daily maximum temperatures were consistently close to 29^{o}C or higher during and after the week ending 6 May, at which time no more infection on trap plants occurred (Table 4.2). In 2015, however, a different pattern was observed in that leaf infection of trap plants ceased during the weeks ending 24 April and 1 May despite the prevalence of

moderate temperatures (mean daily maximum temperatures of 26.0 and 24.5ºC) and the occurrence of multiple rainy days during those weeks (Table 4.1). Thus, unfavorable temperatures alone are insufficient to explain the lack of leaf infection during peak basidiospore dispersal periods of *E. maculosum* in this study.

With regard to fruit infection, disease progress curves on field plants showed that the first fruit spots can be observed on small, green fruit within 1 to 3 weeks of petal fall (requiring removal of the waxy layer to visualize these early spots). The infections causing these symptoms would have had to occur around petal fall, before the presence of sporulating leaf spots in the field and before airborne propagules of *E. maculosum* were detected with the Andersen sampler. The most parsimonious explanation for these observations is that the same primary inoculum causing leaf infections also causes early fruit infections. This is supported by the fruit trap plant study in 2016, where plants forced in the greenhouse to have small, green fruit during field exposure were infected at the same time as leaf trap plants having susceptible leaf stages. Subsequent disease progression on fruit on field plants is more difficult to explain, in part because of variability in fruit disease incidence over time associated with abscission of some of the affected fruit. The delayed peak in fruit spot incidence compared with leaf spot incidence could be due to a longer incubation period on fruit or a delayed primary infection period on fruit vs. leaves associated with the hardening-off (and decreased susceptibility) of leaf tissue as susceptible young green fruit become available in the field. Alternatively, it may be possible that basidiospores produced on leaves cause some of the later infections on fruit, given the temporal overlap among sporulating leaf lesions, *E. maculosum* airborne propagule dissemination as documented with the Andersen sampler, and the emergence

of fruit spots in the field. However, this is not supported by the trap plant study where no fruit infections were observed during the basidiospore dissemination period. Hence, only successful artificial inoculation trials where basidiospores are used to infect young, still green fruit will provide conclusive evidence as to whether or not basidiospores produced on leaves are capable of infecting fruit during the same season.

Based on the above considerations, the role of basidiospores in this pathosystem appears to be less one of contributing to reinfection during the same growing season and more one of enabling sexual recombination and serving as dispersal agents until infection occurs again at the beginning of the next growing season. Basidiospores of *Exobasidium* spp., in general, are hyaline, and exposure to UV radiation and temperatures in excess of 29°C are fatal to most species (Graafland 1960; Sundstrom 1964; Visser et al. 1961; De Weille 1960), hence oversummering and subsequent overwintering of the pathogen as basidiospores is highly unlikely. Instead, it is more plausible that the yeast-like conidia are serving this function. When basidiospores of *E. maculosum* are released onto culture media, they readily bud to produce yeast colonies. Although the longevity of the conidia of *E. maculosum* has not been investigated, inoculation experiments using monospore cultures of conidia of the related *E. japonicum* on dormant azalea buds showed that the pathogen remained capable of producing symptoms for up to 6 months after inoculation (Graafland 1960); however, it was not clear whether the pathogen remained an epiphyte during that period, causing infection when the buds broke; or whether the bud scales were infected latently following inoculation with symptoms only becoming apparent during bud break 6 months later. This led Graafland (1960) to hypothesize that the pathogen was capable of persisting on buds and/or within budscales until such time as new infections

could occur at bud break and leaf development. For *E. maculosum*, our tissue plating studies documented that the pathogen is consistently present on the surface of blueberry plants during the dormant season, thereby providing conclusive evidence that this species is capable of effective epiphytic survival.

A review of the literature suggests that one common feature among diseases caused by *Exobasidium* spp. is that only young, tender host tissues are susceptible to infection (Graafland 1960; HIlborn and Hyland 1956; Nickerson and Vander Kloet 1997; Sundstrom 1964; De Weille 1960; Wolf and Wolf 1952). This was confirmed in the present study for leaf and fruit infection by *E. maculosum*. In other aspects of pathogenesis and epidemiology, the genus *Exobasidium* appears to be highly variable. The type of infection produced ranges from localized spots or blisters (such as with *E. maculosum* and *E. vexans*) to locally systemic galling (e.g., *E. japonicum*) and fully systemic infection (such as with the *Exobasidium* sp. causing red leaf of lowbush blueberry) (Nannfeldt 1981). Variability is also observed among species in the roles played by the different spore types. The majority of *Exobasidium* species produce both basidiospores and conidia, and in all cases where artificial inoculation has been successful with those species, both spore types are capable of infecting (Graafland 1960; Sundstrom 1964). *Exobasidium vexans* represents an anomaly in that this species only produces basiodiospores and appears to have lost its saprophytic stage. The near opposite appears to be the case for *E. maculosum*, where the saprophytic stage dominates the annual disease cycle and the basidiospores may primarily serve the purpose of dispersal (along with increasing genetic variability), with limited or no contribution to the within-season epidemic. This latter life strategy is very similar to that of ascomycetes within the

genus *Taphrina* (e.g., *T. deformans* causing peach leaf curl), where the sexual spores are released during a brief period in the spring to produce yeast-like conidia, which – following epiphytic oversummering and overwintering – incite a single cycle of primary infection the following spring (Mix 1935). The striking similarity in general life history strategies between *Exobasidium* and *Taphrina* has been noted more than 150 years ago by Woronin (1867) when he erected the genus *Exobasidium*, and has been reiterated by Graafland (1960) and von Arx et al. (1982) in their reviews of the genus. These two fungal groups may also share other similar epidemiological characteristics in that infection only occurs under narrowly specific environmental conditions of temperature, humidity, rainfall, and plant phenology (Graafland 1960; Rossi et al 2006; De Weille 1960).

Exobasidium leaf and fruit spot of blueberry in the southeastern United States can be controlled remarkably well with a single late-dormant application of liquid lime sulfur (Brannen et al. 2017). Our results, documenting the importance of epiphytic primary inoculum and the monocyclic nature of disease progression (at least on leaves), provide a biological foundation for this empirically derived disease management tactic. Our results also suggest that there may be potential to manipulate other cultural, horticultural, or pest management practices during the epiphytic survival phase of the pathogen to further mitigate the impacts of the disease (Scherm et al. 2017). In the context of optimizing disease management, additional research is also needed to quantify the contribution of basidiospores to fruit infection, which will require development of artificial inoculation protocols for this pathosystem where efforts to accomplish artificial inoculation have been unsuccessful to date.

Acknowledgements

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Table 4.1. Progress of Exobasidium leaf spot on field plants cv. Premier, presence of airborne spores, infection of trap plants cv. Premier, and weekly temperature and precipitation in a blueberry planting near Alma, GA, in 2015.

	Date[a]															
Variable	27 Feb.	6 Mar.	13 Mar.	20 Mar.	27 Mar.	3 Apr.	10 Apr.	17 Apr.	24 Apr.	1 May	8 May	15 May	22 May	29 May		17 Jul.
Day of year	58	65	72	79	86	93	100	107	114	121	128	135	142	149		198
New spots per shoot on field plants[b]	0	0	0	0	0.14	0.35	3.11	3.61	0.24	0.07	0.07	0.04	0	0		0
Sporulating spots per shoot on field plants[b]	0	0	0	0	0	0.03	0.32	0.65	3.24	3.18	1.72	0.78	0.22	0.08		0
Airborne spore density[c] (CFU/min)	0	0	0	0	0	0	1-50	51-200	51-200	>200	1-50	1-50	1-50	1-50		ND
Leaf spots per trap plant[d]	0	0	0.25	52.5	207.3	1.00	0.25	145.5	0.25	0	0	0	0	ND		ND
Days with ≥1.0 mm rain per week	2	2	2	3	4	0	0	5	2	3	0	0	2	2		2
Cumulative rainfall (mm per week)	48.0	23.1	24.6	24.9	15.2	0.3	0.0	38.6	21.1	50.5	0.0	0.0	17.3	19.1		19.3
Mean daily maximum temperature (C)	14.6	19.6	22.8	24.6	22.8	22.3	28.4	26.2	26.0	24.5	26.6	31.6	31.9	30.4		35.0

[a] Date denotes the end of the weekly exposure period for trap plants, the end of the weekly summary period for weather variables, and the weekly assessment date for disease variables and spore sampling.

[b] See Fig. 2 for complete disease progress curves.

[c] Determined with a six-stage Andersen sampler operated separately for 30 and 60 s on each sampling date and categorized into four classes based on total number of *Exobasidium maculosum* colony-forming units (CFU) obtained in the sampler per minute. ND = not determined.

[d] Average of four potted trap plants exposed weekly. ND = not determined.

Table 4.2. Progress of Exobasidium leaf spot on field plants cv. Premier, presence of airborne spores, infection of trap plants cv. Premier, and weekly temperature and precipitation in a blueberry planting near Alma, GA, in 2016.

	Date[a]														
Variable	26 Feb.	4 Mar.	11 Mar.	18 Mar.	25 Mar.	1 Apr.	8 Apr.	15 Apr.	22 Apr.	29 Apr.	6 May	13 May	20 May	27 May	3 Jun.
Day of year	57	64	71	78	85	92	99	106	112	119	126	133	140	147	155
New spots per shoot on field plants[b]	0	0	0	0	0	0.10	1.03	2.90	1.63	0.11	0.03	0.03	0.04	0.03	0
Sporulating spots per shoot on field plants[b]	0	0	0	0	0	0	0	0	0.43	3.08	4.31	3.15	1.19	0.58	0.07
Airborne spore density[c] (CFU/min)	ND	0	0	0	0	0	0	0	1-50	>200	0	>200	1-50	1-50	0
Leaf spots per trap plant[d]	ND	0	0	0	14.0	207.3	102.5	0.75	11.5	5.75	0	0	0	ND	ND
Fruit spots per trap plant[d]	ND	0	0	0	2	1	0	0.75	0	0	0	0	0	ND	ND
Days with ≥1.0 mm rain per week	1	1	0	2	3	2	3	2	1	1	4	0	2	2	1
Cumulative rainfall (mm per week)	14.2	13.2	0.8	7.9	26.7	71.1	101.3	21.1	24.4	3.8	41.9	0.0	81.8	0.8	5.8
Mean daily maximum temperature (C)	22.5	20.1	23.2	27.5	22.8	24.5	24.6	22.7	24.2	28.6	29.1	28.9	29.1	29.8	32.3

[a] Date denotes the end of the weekly exposure period for trap plants, the end of the weekly summary period for weather variables, and the weekly assessment date for disease variables and spore sampling.

[b] See Fig. 2 for complete disease progress curves.

[c] Determined with a six-stage Andersen sampler operated separately for 30 and 60 s on each sampling date and categorized into four classes based on total number of *Exobasidium maculosum* colony-forming units (CFU) obtained in the sampler per minute. ND = not determined.

[d] Average of four potted trap plants each for monitoring leaf or fruit infection exposed weekly. ND = not determined.

Table 4.3. Contingency table analysis[a] showing numbers of leaves on trap plants cv. Premier with or without symptoms of Exobasidium leaf spot on marked shoot segments (n = 30) that were either hardened-off (lower section of the shoot) or tender (upper section of the shoot) in a blueberry planting near Alma, GA, April 2016.

Shoot segment	Asymptomatic leaves	Symptomatic leaves
Tender	46	28
Hardened-off	166	5

[a] Chi-Square = 51.1, $P < 0.0001$, df = 1.

Table 4.4. Contingency table analysis[a] showing numbers of leaf clusters with or without symptoms of Exobasidium leaf spot on trap plants cv. Premier on which different leaf development stages were marked prior to weekly exposure periods in a blueberry planting near Alma, GA, 2016.

Leaf development stage at exposure	Asymptomatic leaf clusters following exposure	Symptomatic leaf clusters following exposure
Late green tip	44	3
Mouse-ear	71	13
Shoot elongation	49	45

[a] Chi-Square = 36.5, $P < 0.0001$, df = 2.

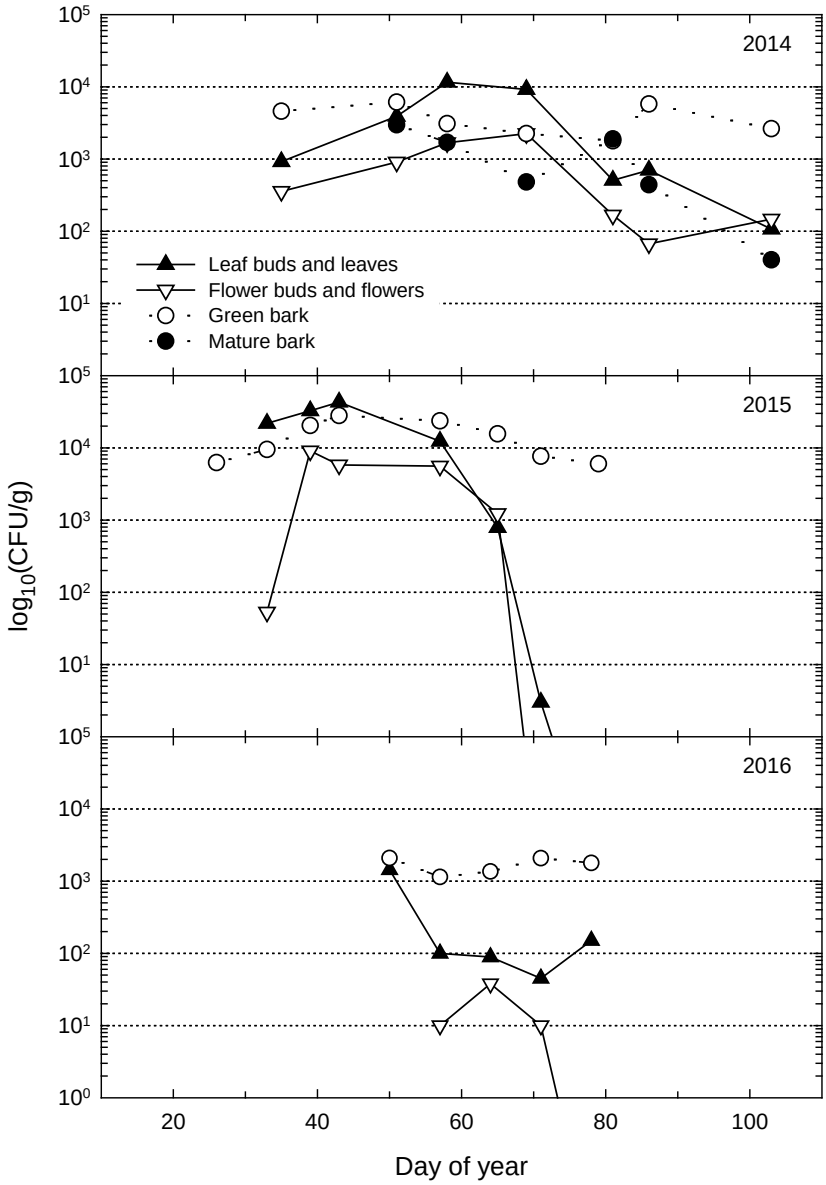

Figure 4.1. Epiphytic population densities of *Exobasidium maculosum*, determined by dilution-plating onto semi-selective medium, on different tissues of field-grown Premier rabbiteye blueberry plants during winter and early spring of 2014 to 2016. Values are means of ten experimental units, each obtained by pooling tissue samples obtained from two shoots. CFU = colony-forming units. No colonies of *E. maculosum* were recovered from mature bark on the first sampling date in 2014.

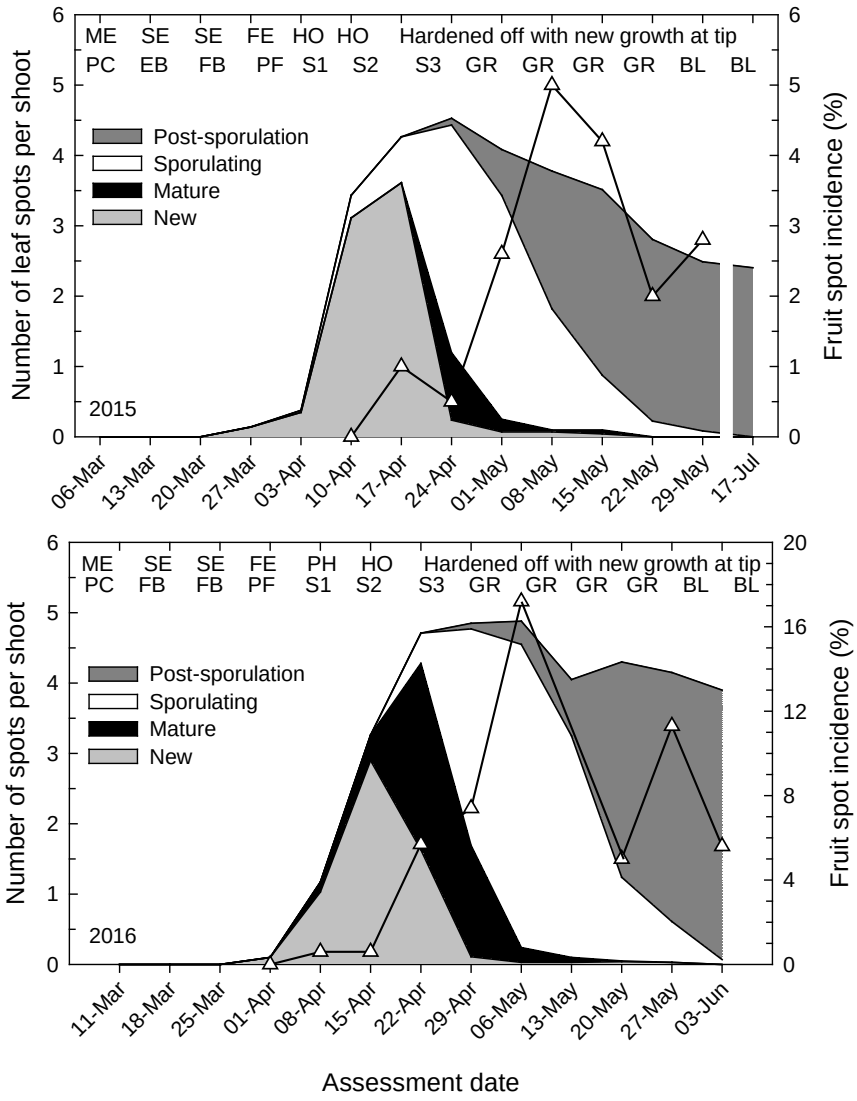

Figure 4.2. Progress of Exobasidium leaf spot (stacked curves) and fruit spot (open triangles) on current-season shoots of field-grown Premier rabbiteye blueberry plants during spring and early summer of 2015 (top) and 2016 (bottom). At each assessment date, the relative proportion of pre-sporulating (new or mature), sporulating, and post-sporulating (necrotic) leaf lesions is presented. Values are means of 72 shoots per assessment date for leaf spot progression and 93 to 240 fruit per date for fruit spot progression. For each assessment date, the most common leaf development stages are indicated at the top of each graph as follows: ME = mouse ear, SE = shoot expansion, FE = fully expanded shoot, PH = lower leaves on shoot hardened off, HO = all leaves on

shoot hardened off. Similarly, the most common flower development stages are indicated as PC = pink corolla, FB = full bloom, PF = petal fall, S1 = small green fruit 0.25 to 0.50 cm diam., S2 = 0.50 to 0.75 cm, S3 = ≥1.0 cm, GR = fully expanded green fruit, BL = blue fruit.

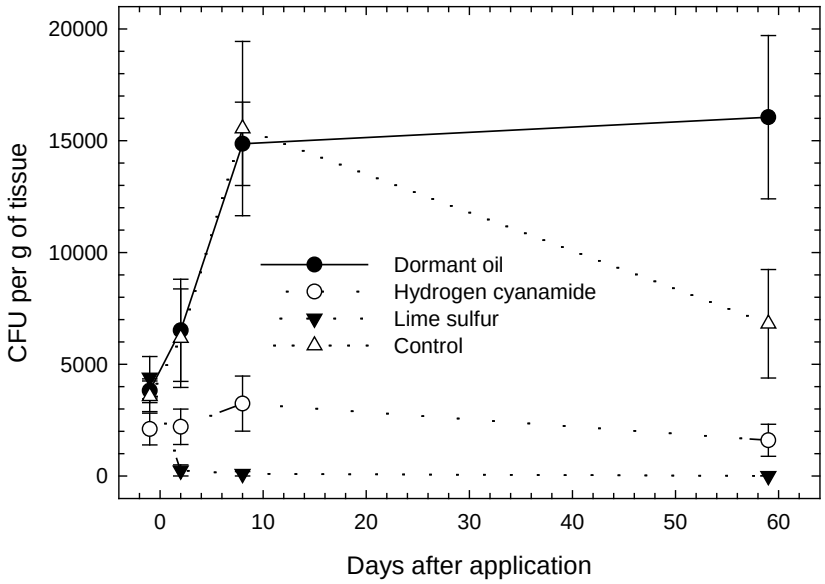

Figure 4.3. Epiphytic population densities of *Exobasidium maculosum*, determined by dilution-plating onto semi-selective medium, on green bark of field-grown Premier rabbiteye blueberry plants following application of different dormant treatments on 18 January 2017 (day 0). Values are means and standard errors of four replicate plots, each with tissue collected from ten shoots per plot. CFU = colony-forming units.

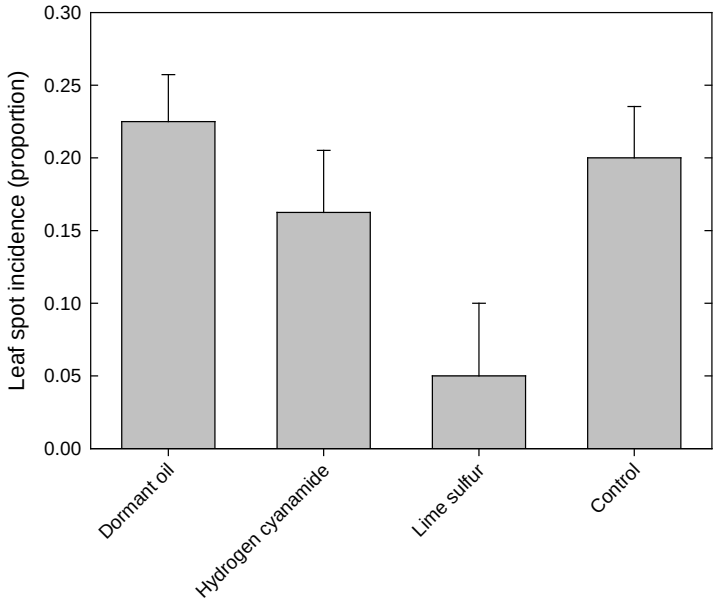

Figure 4.4. Shoot-level incidence of Exobasidium leaf spot on current-season shoots of field-grown Premier rabbiteye blueberry plants in mid-May 2017 following application of different dormant treatments on 18 January 2017. Values are means and standard errors of four replicate plots, each with disease assessed on 20 shoots per plot.

CHAPTER 5

REAL-TIME POLYMERASE CHAIN REACTION-BASED DETECTION AND QUANTIFICATION OF *EXOBASIDIUM MACULOSUM* FROM SPORE TRAP TAPES[1]

[1] Ingram, R.J., Martin, K.F., and Scherm, H. To be submitted to *Journal of Phytopathology*.

Real-time polymerase chain reaction-based detection and quantification of *Exobasidium maculosum* from spore trap tapes

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Abstract

A real-time polymerase chain reaction (PCR)-based assay was developed to facilitate enumeration of airborne basidiospores of *Exobasidium maculosum*, causal agent of Exobasidium leaf and fruit spot of blueberry, from spore trap tapes collected with a Burkard 7-day volumetric spore sampler. The assay utilized primers EXORI-F and EXORI-R, targeting the ITS 5.8s region of the genome and resulting in a 188-bp amplicon, along with a fluorescent LNA probe EXORJI. Primer and probe design were based on the complete ITS region sequences from more than 60 isolates of *E. maculosum* deposited in the NCBI nucleotide database. Initial *in silico* testing of the primer set revealed that the only organisms with sequence complementarity >90% were within *E. maculosum*, and a BLAST search of the primers within the genus *Exobasidium* resulted in 100% coverage of all deposited *E. maculosum* isolates and no matches of other *Exobasidium* spp. Evaluation of the assay with DNA from 55 isolates of *E. maculosum* from cultivated or wild blueberry hosts, one isolate each of *E. rostrupii* and *E. ferrugineae*, and isolates of 14 other fungal species commonly found on blueberry or in the environment showed that the assay reliably detected 51 of the 55 *E. maculosum* isolates, whereby one of the four negatives was an isolate from Nova Scotia, Canada, that was previously shown to belong to a distinct genetic sub-population within *E.*

maculosum. None of the 14 non-*Exobasidium* taxa produced a positive reaction. When the assay was evaluated with Melinex spore trap tape segments to which yeast conidial suspensions of *E. maculosum* had been added and from which DNA had been extracted by beat-beating, tape segments harboring ~100 cells or higher could be quantified reliably. Analysis of spore trap tapes collected in a rabbiteye blueberry planting during an epidemic of Exobasidium leaf and fruit spot between late February and early June 2016 by real-time PCR indicated patterns of basidiospore disseminations consistent with disease observations in the field and with semi-quantitative spore enumeration by an Andersen sampler at the same time.

INTRODUCTION

While real-time polymerase chain reaction (PCR) has a wide range of applications in biology and genetics, its use in the detection and quantitation of phytopathogens has proven to be a specific, rapid, and reliable diagnostic and investigative tool in the field of plant pathology. In cases where species-specific primers can be developed, this tool becomes of even greater benefit due to the ability to detect and quantify levels of a pathogen from environmental samples. This negates the need to obtain pure cultures, of particular importance with obligate parasites. Furthermore, even if selective media is available for pathogen enumeration, real-time PCR provides a less time-consuming means of detection. Other benefits also exist with organisms that are either very difficult to visualize, as is the case with most bacteria, or visually nondescript, as is the case with some fungal spores (Vincelli and Tisserat 2008) such as the basidiospores of *Exobasidium maculosum*, cause of Exobasidium leaf and fruit spot of blueberry. Even in

instances where the spores of a fungal species are easily identifiable, real-time PCR may still be a more advantageous technique due its ability to accurately quantify spore levels and the much-reduced time that it takes to accomplish this task compared with microscopy.

One of the more useful applications of real-time PCR in the assessment of environmental samples of plant pathogens is the quantification of dispersal trends of fungal propagules in epidemiological studies. Past investigations have used the method to effectively identify the timing, magnitude, and - in some cases - the distance of dispersal of economically important fungal phytopathogens such as *Mycosphaerella graminicola* causal agent of Septoria tritici blotch of wheat, *Monilinia fructicola* causal agent of brown rot of stone fruits, and *Blumeria graminis* f. sp. *tritici* causal agent of wheat powdery mildew (Duvivier et al. 2013; Luo et al. 2007; Cao et al. 2016). One comprehensive example of the application of this tool is found in the work of Duvivier et al. (2013). In this study the researchers used Burkard volumetric spore samplers located at various sites of intense wheat production throughout Belgium and developed and applied a species-specific real-time PCR protocol to identify spatio-temporal trends associated with dispersal of the airborne ascospores of *M. graminicola*. The dispersal trends observed proved to be important because they documented a previously unrecognized role of aerially dispersed ascospores as inoculum late in the wheat growing season. Prior to their investigation, ascospores of *M. graminicola* were thought to contribute to the epidemic on wheat only at the beginning of the growing season, but with this new understanding of the contribution of late-season aerially dispersed ascospore

inoculum, the development of further targeted disease management practices was initiated as a result.

Past studies utilizing similar volumetric spore samplers have investigated the epidemiology and the aerial dispersal of the basidiospores of the *Exobasidium vexans*, a relative of *E. maculosum* (Kerr and Shanmuganathan 1966). While successful at identifying environmental factors relating to peak dispersal, this study employed microscopy for quantifying basidiospores on spore trap tape. As discussed by Calderon et al. (2002), the use of microscopy as a method of quantification in extended spore trapping studies is both time-consuming and may be inaccurate compared with PCR-based methods.

Due to the relatively nondescript morphology of the basidiospores of *E. maculosum*, our previous attempts at accurately quantifying these propagules from spore tape samples using microscopy have met with limited success. For these reasons it was felt important to develop a rapid, reliable, and sensitive real-time PCR protocol capable of detecting and quantifying *E. maculosum* from environmental and - more specifically - spore tape samples. As has been seen in past studies, having a tool of this type is critical to a thorough understanding of the biology and epidemiology of the pathosystem under study. Specifically, one of the lingering questions from the research on Exobasidium leaf and fruit spot of blueberry to date is when airborne basidiospores of *E. maculosum* are being disseminated in relation to the timing of leaf and fruit infection and relative to the presence of sporulating lesions in the field (Ingram 2018). Through the development of a species-specific real-time PCR protocol it is hoped that greater temporal resolution can be acquired that will allow for the description of the role of aerially dispersed basidiospores

in the annual epidemic. In addition, such an assay will also provide a much-needed diagnostic tool for the rapid and simple confirmation of the presence of the pathogen from environmental and/or mixed cultures containing the organism.

Materials and Methods

Primer design. The primers EXORI-F (Forward-5’ CTC TTC CTC TAC ATT CTT 3’) and EXORI-R (Reverse-3’ CAC ATT ACT TAT CGC ATT 5’), targeting the ITS 5.8s region of the genome and resulting in a 188-bp amplicon, along with a fluorescent LNA probe (15 DNA bases + 4 LNA bases) EXORJI (5 agt Tcg Ctc Agt Ggc acc t 3’), were developed for detecting *E. maculosum* via real-time PCR. The primer set and probe were designed using Beacon Designer 3.0 software (Premier Biosoft, Palo Alto, CA) and tested with the complete ITS region sequences from more than 60 isolates of *E. maculosum* deposited in the NCBI nucleotide database by Brewer et al. (2014) and Stewart et al. (2015). Due to the presence of highly conserved regions compared with other available regions at the species level, the sequence corresponding to the 5.8s ITS region of *E. maculosum* isolate (Seq ID: gb|KR262255.1) previously deposited in the NCBI database by Stewart et al. (2015) was selected as the basis for primer and probe development. Search parameters within Beacon Designer 3.0 were set to default settings with the exception of a probe length maximum of 20 and the extension of the BLAST (Altschul et al. 1990; Ye et al. 2012) search to all fungi. In the BLAST search of fungi (taxid 5706), bacteria (taxid 2) and eukaryotes (taxid 2759), the only organisms with sequence complementarity >90% to the EXORI-F/ EXORI-R primers were within *E. maculosum*. A separate BLAST search of the primer set within the genus *Exobasidium*

(taxid 5406) resulted in 100% coverage of all deposited *E. maculosum* isolates and no matches of other *Exobasidium* spp.

Initial specificity testing using conventional PCR. To confirm the specificity of the EXORI-F and EXORI-R primer set to *E. maculosum*, conventional end-point PCR was conducted with DNA extracted from 13 isolates of *E. maculosum* (obtained from cultivated *Vaccinium virgatum*, cultivated *V. angustifolium*, wild *V. corymbosum*, or wild *V. eliottii*); one isolate each of *E. rostrupii* from cultivated *V. angustifolium* and *E. ferrugineae* from wild *Symplocus tinctoria*; and isolates of other fungi including *Alternaria alternata*, *Aureobasidium pullulans*, *Botrytis cinerea*, *Colletotrichum acutatum*, *Fusarium oxysporum*, *Monilinia vaccinii-corymbosi*, *Lasliodiploida theobromae*, *Neofusicoccum ribis*, *Penicillium* sp., *Pestalotia vaccinii*, *Pichia* sp., *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Sporobolomyces* sp. (Table 5.1). DNA extractions were conducted using DNeasy Plant Mini Kits (Qiagen, Germantown, MD), and the DNA concentration was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and adjusted to concentrations between 15 and 25 ng/µL. End-point PCR was conducted, in triplicate, using a Bio-Rad T100 thermocycler with the following conditions: lid 105°C, 95°C 5 min, (95°C 15 s, 53°C 15 s, 70°C 45 s) x 30, 70°C 5 min, hold at 4°C. Each reaction contained 2 µL of extracted DNA, 1 µL of EXORI-F (10 µM), 1 µL EXORI-R (10 µM), 21 µL of Millipore ddH_2O, and an illustra PuReTaq Ready-To-Go PCR bead (GE Healthcare Bio-Sciences, Pittsburgh, PA) in a 0.2-mL tube. The resulting PCR products along with a 100-bp ladder were loaded on a 1.5% agarose gel containing 5% ethidium bromide and run at 90 V for

45 min. Gels were imaged using a BioSpectrum 300 Imaging System (UVP, Upland, CA) exposed at 540 to 640 nm and assessed for the presence of bands around 200 bp in length.

Specificity testing using real-time PCR. More extensive specificity testing was conducted using real-time PCR with primers EXORI-F and EXORI-R and probe EXORJI. Testing included 14 non-*Exobasidium* isolates, 55 isolates of *E. maculosum* from various hosts and tissues, and one isolate each of *E. rostrupii* and *E. ferrugineae* (Table 5.1). DNA was extracted using DNeasy Plant Mini Kits (Qiagen) or – in the case of most *E. maculosum* isolates – by beat-beating a yeast conidial suspension (standard concentration adjusted to an optical density of 0.10 to 0.12 in 0.01% Tween 80) using two 40-s Fast-Prep cycles in a Mini-Beadbeater-1 (BioSpec Products, Bartlesville, OK). Each PCR reaction included 5 µL of DNA extract (~15 ng/µL), 10 µL of Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, MA), 0.8 µL of primer EXORI-F, 0.8 µL of primer EXORI-R, 0.4 µL of probe EXORJI (10 µM) with a 5' 6-FAM fluorescent reporter dye and 3' Iowa BlackFQ quencher (Integrated DNA Technologies, Coralville, IA), and 3 µL of ddH2O in an Applied Biosystems 0.1-mL MicroAmp Fast 96-well Reaction Plate (ThermoFisher Scientific) and sealed with MicroAmp TriFlex 3 x 32-Well PCR Reaction Plate Clear Adhesive Film. Cycle conditions in the Applied BioSystems StepOnePlus Real-Time PCR System (ThermoFisher Scientific) were 95°C for 2 min followed by cycling between 95°C for 15 s and 50°C for 30 s up to a cycle threshold (*Ct* value) of 45. The settings in the thermocycler software were altered for TaqMan polymerase, reporting by a TaqMan fluorescent probe, and set to fast run, whereas the *Ct* value and all other settings were left

at the default. All samples were run in triplicate using DNA of *E. maculosum* isolate EXOA as a positive control and ddH_2O as a negative control.

Standard curves for cell suspensions and spore trap tapes. *Exobasidium maculosum* isolate FM18ss was grown for 2 weeks on potato-dextrose agar amended with 50 µg/L streptomycin. Yeast conidia of the pathogen were washed off the plates with 0.01% Tween 80, and a stock solution of 2.08×10^8 cells (optical density 0.12) was made in 1.5-mL microcentrifuge tubes and confirmed by hemacytometer counts of serial dilutions. To develop the standard curve for cell suspensions of *E. maculosum*, serial dilutions were subjected to beat-beating using two 40-s Fast-Prep cycles in a Mini-Beadbeater-1, whereby samples were placed on ice for 2 min after each cycle. DNA extracts of each sample (5 µL) were submitted, in triplicate, to real-time PCR using the aforementioned settings. The resulting *Ct* values were plotted against cell concentration per reaction to generate a standard curve, and linear regression analysis was used to develop a model predicting cell concentrations from *Ct* values.

To develop the standard curve for *E. maculosum* on spore trap tapes, 0.1 mL of each of the aforementioned cell suspensions was deposited on Melinex polyester spore tape segments (2.5 x 1.9 cm) coated with a thin layer of petroleum jelly. Spore tape sections (three replicates per concentration) were allowed to dry for 48 h and DNA of *E. maculosum* was extracted using a protocol modified from Kaczmarek et al. (2009). Briefly, each Melinex tape segment was placed in a sterile 2-mL screw-cap tube with 0.02 g of glass beads (size 425 to 600 µm; Sigma-Aldrich, St. Louis, MO) to which 0.1 mL of 0.01% Tween 80 were added. Samples were subjected to two 40-s Fast-Prep DNA extraction cycles and placed on ice for 2 min after each cycle. DNA extracts (5 µL) of

each sample were subjected, in triplicate, to real-time PCR using the aforementioned settings, and a standard curve was derived as described above.

Application of real-time PCR to quantify spore dissemination in the field. From the last week of February until the first week of June in 2016, a Burkard 7-day volumetric spore sampler (Burkard Scientific, Uxbridge, Middx., UK) was operated in a commercial planting of rabbiteye blueberry (*Vaccinium virgatum*) with a history of Exobasidium leaf and fruit spot near Alma, GA. The spore sampler was placed on a platform 1 m high within a row of plants cv. Premier 1 m from each adjacent bush, and powered by a 12-V marine battery that was exchanged weekly. The rotating drum was fitted with a strip of Melinex tape and the tape covered manually with a thin layer of petroleum jelly. At the end of each weekly exposure period (midnight of the seventh day), the drum was replaced and the exposed drum brought back to the laboratory where the Melinex tape was removed and cut into seven daily sections. Each of these sections was then cut in half to yield 14 tape segments (2.5 x 1.9 cm) representing 12-h periods from 12 AM until 12 PM and 12 PM until 12 AM for each day. The tape segments were then placed individually in 2-mL screw cap tubes using sterile tweezers, with the petroleum jelly-covered portion of the tape facing inwards. The segments were of such a length that there was no overlap of the tape within the tube. One-hundred microliters of 0.01% Tween 80 were added to each tube, and DNA extraction was conducted using two 40-s Fast Prep cycles as described above. DNA extracts of each sample (5 µL) were subjected to real-time PCR using the aforementioned settings, with DNA of *E. maculosum* isolate EXOA (15 ng/µL) as a positive control and ddH_2O) as a negative

control. *Ct* values were converted to spore densities (spores per m^3 per day) using the Melinex tape standard curve and considering the spore sampler's flow rate of 10 L/min.

Results

Properties, specificity, and sensitivity of the PCR reaction. The melting temperatures of the primers EXORI-F and EXORI-R, and of the probe EXORJI were 65, 68, and 68.1ºC, respectively. The predicted amplicon was 188 bp in length. Initial specificity testing using conventional PCR indicated that the primer set consistently produced a single band close to 200 bp in length, with no dimer band, for all tested *E. maculosum* isolates except E15-28AS1, an isolate obtained from *V. angustifolium* in Nova Scotia, Canada. *Exobasidium ferrugineae* from common sweetleaf (*Symplocus tinctoria*) produced a negative reaction, whereas *E. rostrupii* from cranberry (*V. macrocarpon*) produced a positive reaction. No amplification was observed from any of the 14 non-*Exobasidium* fungi (Table 5.1).

More extensive sensitivity testing using real-time PCR with primers EXORI-F and EXORI-R along with probe EXORJI confirmed the high specificity of the assay. Using a cycle threshold of *Ct* = 36, all of the 14 non-*Exobasidium* fungi were negative (either not detected or above the threshold), whereas 51 of 55 *E. maculosum* isolates were positive (Table 5.1); one of the negative isolates (EmVC15TN) was previously detected using conventional PCR. Similar to the result obtained with conventional PCR, *E. maculosum* isolate E15-28AS1 from *V. angustifolium* in Nova Scotia did not amplify with real-time PCR, whereas *E. ferrugineae* and *E. rostrupii* produced negative and

positive reactions, respectively. All water controls remained negative and the positive controls produced strong signals (*Ct* = 19 to 23).

The standard curves relating log-transformed *E. maculosum* cell density to *Ct* value were linear and highly significant ($P \leq 0.0025$) over the range of ~10^8 to 10^2 conidial cells per reaction for beat-beating DNA extraction from both cell suspension and Melinex spore tapes (Fig. 5.1). Cell concentrations lower than 10^2 did not consistently produce *Ct* values below 36. Based on the slopes of the two standard curves, real-time PCR detection from Melinex tape was more efficient than from a cell suspension, especially at lower cell densities (Fig. 5.1). The linear regression models predicting log-transformed density *D* of *E. maculosum* (in cells per reaction) from Ct value were:

- for quantification from cell suspensions: $\log_{10}(D) = 9.164 - 0.1955\ Ct$ ($P = 0.0025$; $r^2 = 0.968$);
- for quantification from spore tape: $\log_{10}(D) = 10.21 - 0.2676\ Ct$ ($P = 0.0007$; $r^2 = 0.986$).

Application of real-time PCR to quantify spore dissemination in the field. The calculated detection limit for enumerating *E. maculosum* from spore trap tapes was ~14 spores per m^3 per day. The first new (pre-sporulation) Exobasidium leaf spots in the field were detected the week ending 1 April 2016, and the first leaf spots with visible basidiospore sporulation were observed the week ending 22 April (Ingram 2018). Based on weekly spore trap tapes collected with the Burkard sampler, extracted by beat-beating, and analyzed by real-time PCR, no aerially disseminated spores of *E. maculosum* were detected between 26 February and 7 April. The first detection of spores occurred on 8 April, which was followed by sporadic spore capture over the next 2 weeks at densities

typically below 10^2 per m^3 per day (Fig. 5.2). This was followed by more consistent and higher spore numbers through the end of April and May, recording spore densities typically between 10^4 and 10^5 per m^3 per day.

Discussion

The real-time PCR protocol developed in this study was both accurate and reliable with no detection below the predetermined cycle threshold (*Ct* = 36) of non-*Exobasidium* fungi and only one false positive for *E. rostrupii*. The true positive rate of detection for in vitro cultures of *E. maculosum* was 52 out of 55 (94.5%), with one of the three isolates (E15-28AS1) producing a negative reaction having been obtained from *V. angustifolium* in Nova Scotia, Canada; recent phylogenetic analyses of *E. maculosum* suggested that these Canadian isolates of *E. maculosum* are distinct from those in the southeastern United States and may in fact constitute a different species (Stewart et al. 2015). Thus, a negative reaction with the real-time PCR for *E. maculosum* would not be unexpected for this isolate.

Given the favorable specificity and reliability of this assay, it has significant potential to serve as a rapid and accurate tool for the detection and/or diagnosis of *E. maculosum* from environmental samples. Although further validation will be needed before it can be used to confirm the presence of *E. maculosum* in planta, the evidence from this study suggests that the assay may be useful for quantifying the pathogen from surface washes and/or spore suspensions, thereby allowing reduction in time to diagnosis and greater accuracy than association by symptoms or cultural identification alone.

When combined with spore trapping, the real-time PCR protocol was shown to be useful in the detection of aerial dispersal periods of basidiospores of *E. maculosum*. Dispersal periods suggested by the real-time PCR data corroborate disease progress trends observed by Ingram (2018). In that study, the first date that Exobasidium leaf spots were observed in 2016 on field plants was 1 April, which was 1 week prior to the first date of low-level detection of aerially dispersed spores by real-time PCR. Of even greater interest is that the first date that leaf spots in the field were visually identified as sporulating (22 April) was also the first date that spore trapping combined with real-time PCR identified significantly high levels of aerially dispersed *E. maculosum* spores within the planting. Further corroborating this trend is that the semi-quantitative Anderson spore sampler employed by Ingram (2018) also first detected aerial dispersal of *E. maculosum* on this date. Although the real-time PCR-based Burkard spore trapping method has currently only been applied to data from 1 year, its application over multiple years could prove to be useful in further investigation of the disease cycle and life cycle of this pathogen.

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Table 5.1. Specificity testing of primer set EXORI-F and EXORI-R for species-specific detection of *Exobasidium maculosum* using conventional end-point polymerase chain reaction (PCR) or real-time PCR with LNA probe EXORJI.

Species	Isolate ID	Host	Location	End-point PCR[a]	Real-time PCR[a]
Alternaria alternata	09-087-1	*Citrullus lanatus*	N/A	-	*Ct* > 36
Aureobasidium pullulans	A-1	*Vaccinium virgatum*	N/A	-	-
Botrytis cinerea	BotCin	Unknown	N/A	-	-
Colletotrichum acutatum	C-2	*V. virgatum*	N/A	-	*Ct* > 36
Fusarium oxysporum	FO71499	N/A	N/A	-	*Ct* > 36
Lasliodiplodia theobromae	LasiDip	N/A	N/A	-	-
Monilinia vaccinii-corymbosi	Sto309Cl1	*V. virgatum*	N/A	-	*Ct* > 36
Neofusicoccum ribis	NeoCoc	N/A	N/A	-	-
Penicillium sp.	Penc1	Ambient	Athens, GA	-	-
Pestolotia vaccinii	P-1	*V. virgatum*	N/A	-	-
Pichia sp.	E14-EMES-73452	*Azalea* sp.	N/A	-	-
Rhizoctonia solani	RS87	N/A	N/A	-	-
Sclerotium rolfsii	S.4.97	N/A	N/A	-	-
Sporobolomyces sp.	RedYe1	*V. virgatum*	Alma, GA	-	*Ct* > 36
Exobasidium ferrugineae	E15-21AS2	*Symplocos tinctoria*	N/A	-	-
E. rostrupii	E12-CNJ1-1	*V. macrocarpon*	N/A	+	+
E. maculosum	E15-28AS1	*V. angustifolium*	Nova Scotia, CAN	-	-
E. maculosum	EmVC15TN	*V. corymbosum*	Roan Mtn, TN	+	-
E. maculosum	ExVE15TN	*V. elliottii*	Roan Mtn, TN	+	+
E. maculosum	EXOA	*V. virgatum*	Alma, GA	+	+
E. maculosum	E15Tss	*V. virgatum*	Toccoa, GA	+	+
E. maculosum	E13-A4S1	*V. virgatum*	Alma, GA	+	+
E. maculosum	E13-A5S1	*V. virgatum*	Alma, GA	+	+
E. maculosum	E13-A8S1	*V. virgatum*	Alma, GA	+	+
E. maculosum	E13-A9S1	*V. virgatum*	Alma, GA	+	+
E. maculosum	E13-A10S1	*V. virgatum*	Alma, GA	+	+
E. maculosum	E13-A11S1	*V. virgatum*	Alma, GA	+	+
E. maculosum	E13-A14S1	*V. virgatum*	Alma, GA	+	+
E. maculosum	E13-A17S1	*V. virgatum*	Alma, GA	+	+
E. maculosum	FM S.S. 18	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	Jan 24. 6.16	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	Jan 24. 6.16.A	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	Jan 24. 6.16.B	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	Jan 24. 6.16.C	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.2.15 FB	*V. virgatum*	Alma, GA	Not tested	+

E. maculosum	4.2.15 FB.A	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.2.15 FB.B	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.2.15 FB.C	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.2.15 FB.D	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.2.15 FB2	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.3.15 LB	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.3.15 LB.A	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.3.15 LB.B	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.3.15 LB.C	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.3.15 LB.D	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.4.15 LB	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.4.15 FB	*V. virgatum*	Alma, GA	Not tested	*Ct* > 36
E. maculosum	4.4.15 FB.A	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.4.15 FB.B	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.4.15 FB.C	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.4.15 FB.D	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	4.4.15 FB2	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.3.15 GB1	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.3.15 GB1.1	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.3.15 GB2	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.3.15 GB3	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.4.15 GB1	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.4.15 GB1.1	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.4.15 GB2.1	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.4.15 GB2.2	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.4.15 GB3	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.4.15 GB3.1	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.4.15 GB4	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	5.4.15 GB5	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	GB 5.15 2	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	14 LB	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	14 FB	*V. virgatum*	Alma, GA	Not tested	*Ct* > 36
E. maculosum	FBL(BA)L05ss	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	E13-BA1ss	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	EXO15.2.GBss	*V. virgatum*	Alma, GA	Not tested	+
E. maculosum	LBLBA1.006	*V. virgatum*	Alma, GA	Not tested	+

[a] Positive (+) and negative (-) signs indicate amplification or lack thereof, respectively. Reaction run for 45 cycles with real-time PCR, where a cycle threshold (*Ct* value) of 36 or below was considered positive.

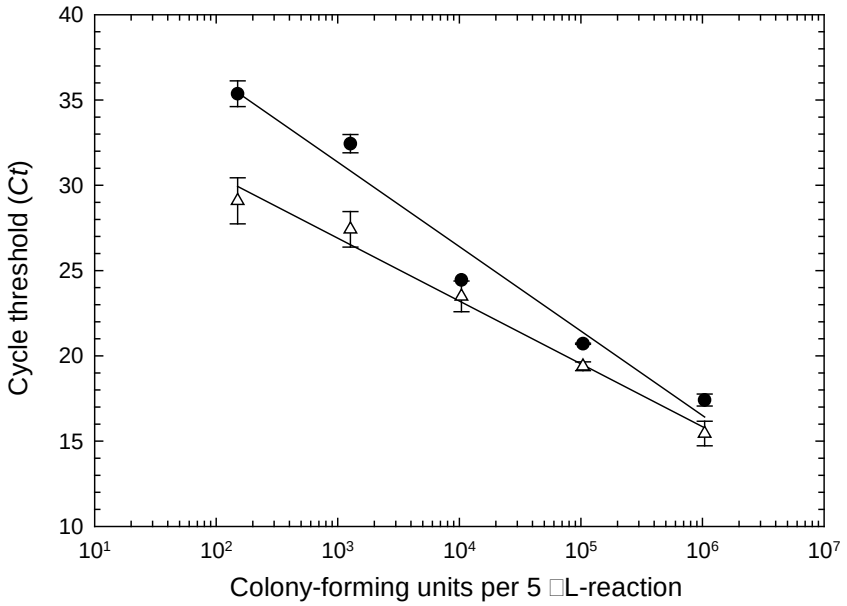

Figure 5.1. Real-time PCR-based standard curves relating cycle threshold to *Exobasidium maculosum* yeast conidial density in 0.01% Tween 80 suspension (•) and on Melinex spore trap tape (Δ) following DNA extraction by beat-beating. Values are means and standard errors of three technical replicates.

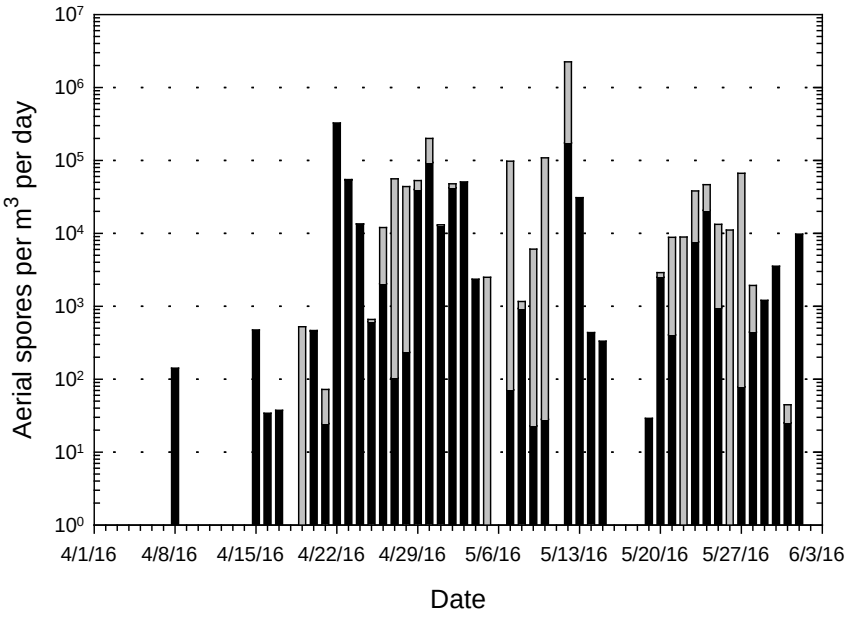

Figure 5.2. Atmospheric *Exobasidium maculosum* basidiospore densities in a commercial planting of rabbiteye blueberry near Alma, GA, as determined with a Burkard volumetric spore sampler and real-time PCR using primer set EXORI-F and EXORI-R with LNA probe EXORJI. The first date of detection was on 8 April 2016; data from the earlier sampling dates (26 February through 31 March) are not shown because of the consistent absence of spores during this period. Black bar: 12-h period from 12 AM to 12 PM; gray bar: 12-h period from 12 PM to 12 AM.

CHAPTER 6

EXOBASIDIUM LEAF AND FRUIT SPOT OF BLUEBERRY[1]

[1] Ingram, R.J., Oliver, J.E., Brannen, P.M., and Allen, R.M. To be submitted as an UGA Extension Circular.

Exobasidium leaf and fruit spot of blueberry

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Introduction

As cultivation of blueberries has expanded and intensified in Georgia, several new disease problems of economic importance have emerged, one of the most recent examples of which is Exobasidium leaf and fruit spot (Fig. 6.1). This early-season disease affects several cultivars of rabbiteye (*Vaccinium virgatum*) and southern highbush (*Vaccinium corymbosum* interspecific hybrids) species, causing spots on susceptible young, tender leaves, shoots, and on fruit (Fig. 6.2). In the southeastern United States, the disease was first described in North Carolina (1998). In Georgia, the first sporadic occurrence of Exobasidium leaf and fruit spot was noted in 2010. Although initially not of major concern, subsequent growing seasons resulted in reports of marked increases in both disease prevalence and intensity, leading to widespread losses due to downgrading or rejection of affected fruit at the packinghouse. The disease is caused by the plant-pathogenic fungus *Exobasidium maculosum*, a new species described in 2014.

Symptoms

Beginning in early spring (late March to early April), small (1 to 3-mm), slightly yellow spots develop on the upper leaf surface of newly expanding leaves (Fig. 6.3A); on

the lower leaf surface, the spots are initially light green to white (Fig. 6.3B). Over the course of 1 to 2 weeks, spots expand, reaching a diameter of 2 to 10 mm. Prior to sporulation, mature leaf spots have a characteristic light yellow-green halo surrounding a yellow center (Fig. 6.3C) and are primarily visible on the lower surface of the leaf. As spots develop further, the halo disappears and the center turns yellowish green. Once mature, these spots become increasingly white (Fig. 6.3D) as the fungus sporulates on the underside of leaves and on the surface of infected fruit and shoots. One of the diagnostic characteristics of the disease is the velvety, white appearance on the underside of sporulating leaf spots. Post-sporulation, spots gradually become brown and necrotic with a distinct reddish-brown margin (Fig. 6.3E). Leaf spots that occur on or near petioles often result in defoliation. Bushes with a high disease severity experience significant defoliation, whereas blueberry bushes with leaves containing only one or two spots do not defoliate as frequently. Defoliation is generally more pronounced within the lower half of the bush.

Characteristic Exobasidium fruit spots are easily recognized on ripe fruit, and with careful observation symptoms may also be found on young green fruit, 0.25 to 1.0 cm in diameter. It is important to note that the light-green fruit spot symptom on young, green fruit is usually only observable via removal of the surface wax layer (Fig. 6.4A). As affected fruit begin to ripen and darken, the immature light-green spots become increasingly obvious (Fig. 6.4B and C). When spots reach maturity, they often appear slightly white to gray due to sporulation on the fruit surface. As fruit ripen and turn blue, fruit spots become increasingly conspicuous and are noted as having a distinctly green or magenta appearance (Fig. 6.4C). Post-sporulation, fruit spots become necrotic, which

may enable colonization by secondary pathogens. There is also evidence to suggest that severe infection may cause premature fruit drop. While the exact level of premature fruit drop is not known, one study estimated that the incidence of in-bush infected fruit decreased by over 50% due to premature fruit drop prior to harvest. Infected fruit are also generally smaller than non-infected fruit.

While less prevalent than the leaf and fruit spot symptoms, spots also form on young, actively growing shoots early in the season. These shoot spots cause damage to new shoots due the progressive necrosis of tissue that often results in girdling of the infected shoot. Shoot spots first appear as slightly chlorotic lesions on one side of young, tender current-season shoots, containing a small dark point in the center, along with or without a darkened margin (Fig. 6.5A). As shoot spots develop further and expand, infected shoot tissue becomes increasingly white and raised. At maturity, shoot spots appear white and velvety due to sporulation of the fungus (Fig. 6.5B). Post-sporulation, shoot spots become brown to black and necrotic with a distinctive reddish-brown margin (Fig. 6.5C). Shoot spots then form a canker that in some cases completely girdles the shoot (Fig. 6.5D). In a sample (n = 36) of shoots with early shoot spot symptoms observed in Alma, GA in 2015, 72% of shoot spots resulted in complete girdling of affected shoots. Subsequent dissection of shoot spots showed that tissue damage extended from the epidermis into the pith of the stem (Fig. 6.5E). At this time there is no evidence to suggest that the pathogen overwinters in the affected shoots.

Causal Organism

The basidiomycete fungus *E. maculosum* produces two spore types - sexual basidiospores (Fig. 6.6A) and asexual yeast-like conidia (Fig. 6.7A). The basidiospores

are produced on fruiting structures called basidia (Fig. 6.6B) that can be found on all symptomatic tissues once disease spots reach maturity. County extension offices can diagnose this disease either directly through in-office examination or via shipment of fresh leaf and fruit samples to extension diagnostic clinics for diagnosis by a UGA Plant Disease Clinic diagnostician. Incubation of samples has not been effective at inducing sporulation, and therefore microscopic diagnosis of the disease is most easily conducted by identifying characteristic basidiospores and basidia found on the underside of leaves within actively sporulating disease spots (Fig. 6.6A and B). In the absence of sporulating spots, diagnosis can be conducted via culturing on semi-selective media and/or by using species-specific real-time polymerase chain reaction (PCR) primers. Note: *E. maculosum* has a characteristic raised, wrinkled, and cream to pink colony morphology when in culture on potato dextrose agar and the semi-selective medium, M5 (Fig. 6.7B).

Disease Cycle and Causal Conditions

Exobasidium leaf and fruit spot of blueberry is an early-season, cool-weather disease. In southern Georgia, leaf symptoms typically first appear in late March to early April, following leaf emergence and full bloom. By the end of June (post-harvest), almost all leaf spots will have turned necrotic, and although the pathogen may still be present on plant surfaces, the disease is no longer active in the field. Multi-year data has indicated that *E. maculosum* overwinters on the surface of buds and shoots. The overwintering and subsequent source of primary inoculum is most likely the yeast-like conidia (Fig. 6.7A). The window of new infections extends from just after bud break, usually early March, until early April; however, this window can vary according to plant developmental stage

and the occurrence of conditions favorable for disease development. Initial symptoms appear 1 to 2 weeks after infection of recently emerged leaves and young, green fruit. Within 1 to 2 weeks after the appearance of symptoms, spots develop to maturity and sporulate to produce clear, curved basidiospores (Fig. 6.6A). In other related *Exobasidium* spp. it has been suggested that the basidiospore is the infective spore; however, in the case of *E. maculosum* causing Exobasidium leaf and fruit spot of blueberry, this has not been found to be the case. Season-long monitoring over several years indicated that the majority of new infections occurred prior to the appearance of sporulating leaf spots in the field. Furthermore, during periods when sporulation of disease spots was the highest, there were often little or no new infections occurring on leaves or fruit. Artificial infection trials, along with microscopy, will need to be conducted to confirm this hypothesis, but current evidence suggests basidiospores primarily serve as the means of long-distance aerial dispersal. Once basidiospores are dispersed, they then likely bud to produce the yeast-like spores that oversummer and overwinter on the surface of blueberry bushes.

Infection of new growth has been associated with extended periods of rain and/or cloudy weather (>3 days). The association of infection with extended rain events may suggest that the primary inoculum for the disease is splash-dispersed. Susceptibility trials have also indicated that only young leaves, prior to hardening off, and young green fruit are susceptible to infection. The stage of susceptibility for shoot infection is not known, but it is assumed to be specific to young, unlignified shoots.

Cultural Controls

Although no specific cultural controls have been investigated for Exobasidium leaf and fruit spot of blueberry, management strategies for other economically important *Exobasidium* spp., such as the causal agent of blister blight of tea *Exobasidium vexans*, suggest that removal of trees on field perimeters and increasing drainage to reduce standing water in problematic locations can be beneficial in reducing disease. Any practice which increases air flow and results in rapid drying of stems, leaves, and fruit will likely result in less infection; among these, adequate summer pruning and winter basal pruning are critical. Use of drip or emitter irrigation will not wet foliage or fruit, whereas overhead irrigation may prove to be problematic due to the possibility of splash dispersal of the pathogen and the potential to create ideal conditions for new infections. Although a comprehensive and reliable list of susceptible versus resistant varieties has not been developed, there appear to be significant differences in the susceptibility of specific varieties to *E. maculosum*. Whereas 'Premier' and 'Tifblue' are known to be particularly susceptible rabbiteye varieties, the southern highbush varieties ‘Star’ and ‘Legacy’ have also been reported to experience significant disease on occasion. Although we do not have proof to date, there is no reason to assume that the field-to-field movement of *E. maculosum* spores on contaminated equipment would not occur; as such, thorough cleaning of equipment (harvesters, hedgers, tractors, etc.) is encouraged before moving to another field site.

Chemical Controls

Current recommendations suggest the application of calcium polysulfide (lime sulfur and similar products) during the late-dormant period of plant development (approximately 2 weeks prior to bud break; blueberry phenology stage 2). Several studies in Georgia have indicated that even a single application of lime sulfur during this period of development has the potential to almost completely control in-season disease on both leaves and fruit. The effectiveness of lime sulfur at controlling Exobasidium leaf and fruit spot is likely due to the fungicide's ability to markedly decrease infective spores harbored on the surface of dormant plants. Other fungicides, such as captan or fenbuconazole, have been shown to be moderately effective at providing in-season disease protection when applied at leaf bud break and during bloom. This in-season application window overlaps with that of chemical control of mummy berry (caused by *Monilinia vaccinii-corymbosi*), hence application of captan and fenbuconazole between leaf bud break and the end of bloom can serve a dual purpose. It is important to note that there has been a report of *E. maculosum* resistance to the fungicide Pristine (boscalid + pyraclostrobin) in at least one commercial field in southern Georgia. Given that resistance does occur, the application of Pristine alone for mummy berry management is not recommended for varieties susceptible to Exobasidium leaf and fruit spot; instead, apply Pristine in a tank-mix with captan (check labels carefully, as Pristine labels may preclude tank-mixing in some states). In addition to mummy berry sprays, two to three additional sprays of captan during early cover sprays may be advisable for particularly susceptible varieties. Application of dormant oil for scale insects may actually increase the incidence of Exobasidium leaf and fruit spot of blueberry; in cases where susceptible varieties are

sprayed with oil, it is critical that a good Exobasidium fungicide program is in place. Always follow the instructions on the label, and contact your local UGA Extension County Office for management updates and specific chemical recommendations.

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Figure 6.1. Leaf spot symptom on lower canopy of rabbiteye blueberry cv. Premier in Alma, GA.

Figure 6.2. Mature leaf spot and shoot symptom on leaf and shoot of rabbiteye blueberry cv. Premier in Alma, GA.

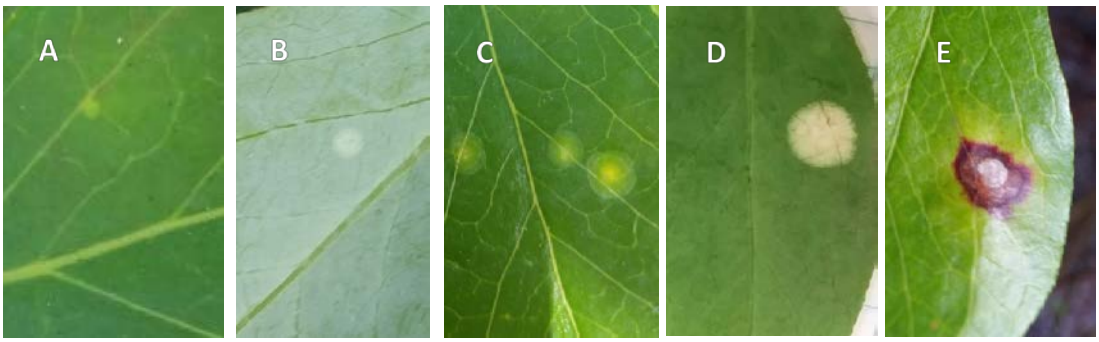

Figure 6.3. Leaf spot symptoms at the four different stages of development on ‘Premier’ rabbiteye blueberry. *New* spots are very small (2 mm in picture), lack a halo on the upper surface (A) and appear dull white (B) on the lower surface. *Mature* spots have a characteristic halo on the upper surface (C) and appear glossy white on the lower surface. *Sporulating* spots are observed with or without a halo on the upper surface and a velvety-white appearance on the lower surface (D). *Post-sporulation,* the margin of spots turns a reddish brown, and the center of spots becomes brown and necrotic (E).

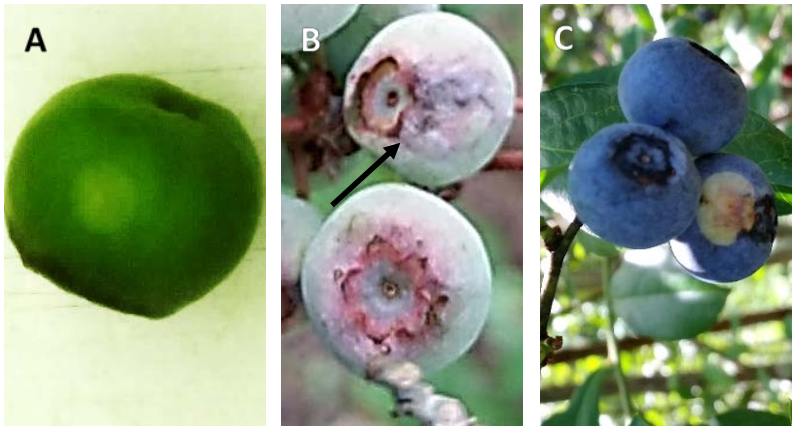

Figure 6.4. Fruit spot symptoms at three different states of development on “Premier’ rabbiteye blueberry. Faint fruit spot on a young (≤1 cm diameter) green berry, visible only after removal of the waxy layer (A). Sporulation (black arrow) of *E. maculosum* on unripened berries (B). As fruit begin to ripen in early summer (May – June) fruit spots become increasingly conspicuous due to their green and occasionally magenta coloration (C).

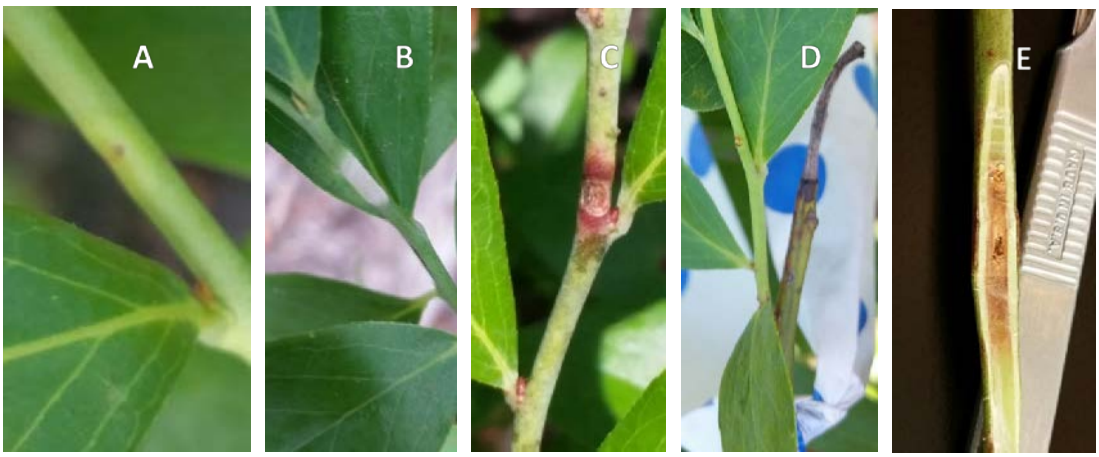

Figure 6.5. Shoot spots caused by *E. maculosum* on ‘Premier’ rabbiteye blueberry. New infections on young, tender shoots first appear slightly chlorotic with or without a small brown to black lesion in the center (A). As shoot spots progress, they become velvety-white due to the production of basidiospores (B). Post-sporulation, the centers of spots become necrotic and brown while the margin turns a bright reddish-brown (C). Post-senescence, the vast majority of shoot spots result in the girdling and blighting of affected shoots (D). Dissection of shoot spots reveals that necrosis extends into the pith of affected shoots (E).

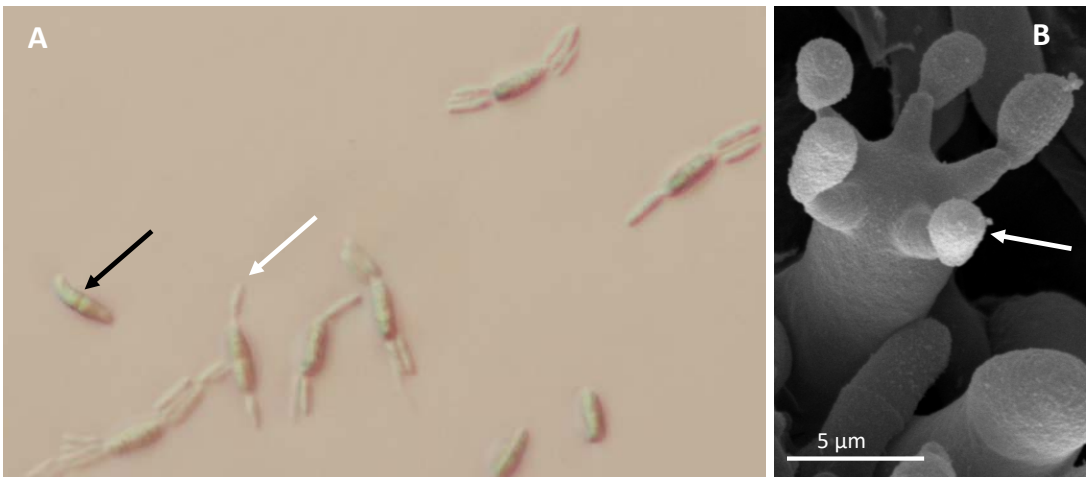

Figure 6.6. Basidiospores (~7 to12 µm in length) of *E. maculosum* germinating on each end to produce yeast-like conidia (~3 to 6 µm in length), 400x and SEM image of basidium. Basidiospores are curved and have a characteristic single cell wall or septum that divides the spore in two (black arrow). Yeast-like conidia are much smaller and lack the septum present in basidiospores (white arrow) (A); basidium (~8 to 12 µm in length), with characteristic sterigmata (white arrow) SEM (B) (Photos: M. Brewer, Department of Plant Pathology, University of Georgia).

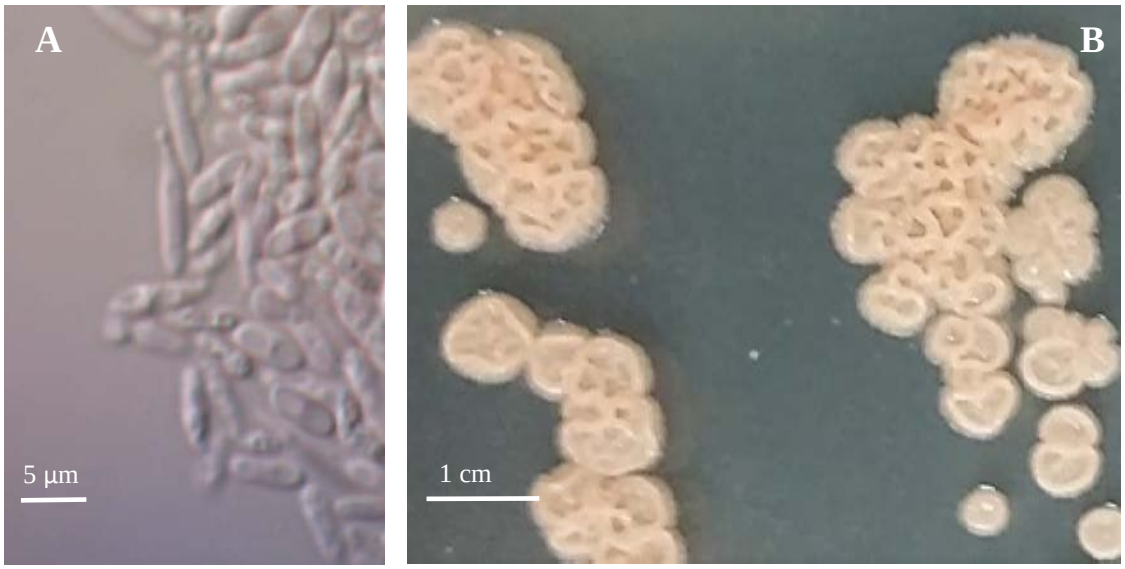

Figure 6.7. Yeast-like conidia of *E. maculosum*, 1000x (A); raised cream to pink-colored, wrinkled colony of *E. maculosum* in culture on potato dextrose agar medium, 20x (B).

CHAPTER 7

CONCLUSIONS

The epidemiology of Exobasidium leaf and fruit spot of rabbiteye blueberry caused by *Exobasidium maculosum* was comprehensively investigated in this study with particular attention being placed on garnering information that has the potential to impact the understanding of current and development of future disease management practices. As part of this study the symptomology of the disease was carefully catalogued to provide a more complete understanding of the total impact of Exobasidium leaf and fruit spot on blueberry plants in commercial settings. Well-recognized symptoms such as leaf and fruit spots were chronicled with greater resolution regarding their development over time, and new symptoms such as lesions on and girdling of young shoots, significant premature defoliation of severely affected plants, and premature fruit drop prior to ripening were also added to the list of symptoms associated with the disease. To the best of the author's knowledge this is the first published report of these symptoms being associated with Exobasidium leaf and fruit spot of blueberry. These findings are also important for assessing the impact of the disease in that previous loss estimates for Exobasidium leaf and fruit spot were based on quality reduction of fruit assessed at harvest. The evidence presented in this study suggests that such harvest estimates most likely underestimate the impact of the disease due to the high frequency of affected fruit that experience premature fruit drop prior to harvest. Although not quantified in the

study, the observations of shoot girdling and premature defoliation are concerning because they have the potential to cause plant stress which may lead to reduced yields.

Our findings concerning the disease cycle of Exobasidium leaf and fruit spot indicated that the infection and development of disease on rabbiteye blueberry in south Georgia occurs from late March to late April. After this period, disease symptoms can be found in the field but the development of new symptoms does not appear to occur after early May. This may be due to temperatures exceeding 30°C at that time and the fact that this is a cool-season disease. The pathogen was also found to infect only young and tender tissues such as emerging leaves, fruit, and shoots. In the case of leaves, once these organs reached a more developed, hardened-off stage, they were no longer susceptible to infection. As normal development of blueberry plants in south Georgia occurs during March to late April with minimal new growth until after harvest in June and July, this may be one of the reasons that further disease development is not observed after this primary period. However, the possibility that the disease is truly monocyclic, at least on leaves, as a result of the pathogen's life cycle cannot be excluded at this time.

One of the key findings of our study impacting current and future management of the disease is that *E. maculosum* is capable of epiphytic overwintering as surface inoculum on blueberry plant surfaces including leaf buds, flower buds, and the bark of previous year's shoot growth and current year's fruitwood. To the best of the author's knowledge, this is the first published research conclusively documenting the ability of any *Exobasidium* sp. to overwinter epiphytically on its host. The importance of this finding was reinforced by the further evidence suggesting that a reduction in levels of surface inoculum was linked to a reduced incidence of disease within the same season.

This information has implications for disease management in that it provides a logical explanation for the mode of action of the currently recommended, empirically derived management practice of applying the contact fungicide lime sulfur in the dormant season. By documenting the ability of lime sulfur to reduce the surface populations of *E. maculosum* and suggesting a link between surface populations and resulting disease, we can now state with confidence that the currently recommended disease management practice provides disease control by targeting the site of pathogen overwintering to reduce levels of surface inoculum. Additionally, this finding will allow future disease management practices to be developed based on an understanding of the biology of the pathogen. Another major finding relating to the epidemiology of Exobasidium leaf and fruit spot is that the disease appears to be largely monocyclic and associated with primary inoculum that is not aerially dispersed. These two points are important because they suggest that the majority of new symptoms each year are the result of a single but punctuated primary infection period and not the result of aerially dispersed basidiospores introduced into the field that then result in multiple cycles of the disease in the same season. By identifying these characteristics, we have increased the understanding of how the disease develops and spreads.

As a final part of our study, it was felt necessary to develop a rapid and reliable diagnostic tool which could be used for the detection of *E. maculosum* and confirmation of the pathogen from environmental samples and potentially mixed cultures. To accomplish this, a real-time polymerase chain reaction (PCR)-based assay using the specific primers EXORI-F and EXORI-R along with the LNA fluorescent probe EXORJI was developed. After initial testing and optimization, the assay was shown to be capable

of detecting low levels of *E. maculosum* yeast conidia without reacting to other fungi commonly observed on blueberry. When applied to environmental samples acquired through weekly spore trapping from late February through early June 2016, the assay was capable of discriminating between periods with and without aerial dispersal of *E. maculosum* basidiospores as well as periods of high and low dispersal. Although our findings regarding basidiospore dispersal patterns were restricted to a single year and therefore preliminary, the use of real-time PCR applied to environmental samples in this way provides a useful tool to future research endeavors that seek to describe the aerial spore dispersal of *E. maculosum*. Through the development of this assay, there is potential for crop protection professionals to utilize this tool, alongside morphological identification and classification of symptoms, to provide diagnoses of Exobasidium leaf and fruit spot with greater confidence.

In summary, Exobasidium leaf and fruit spot of rabbiteye blueberry in south Georgia is a cool-season disease with monocyclic disease progression on leaves. The pathogen *E. maculosum* overwinters on the surface of its host, disperses from there in a non-aerial manner, and infects newly developing young tender blueberry plant growth in early spring. The disease produces symptoms that include spots on leaves, fruit, and shoots and in some cases causes premature defoliation and fruit drop. Basidiospores produced on leaf and fruit lesions during the epidemic serve primarily the purpose of dispersal, although their role in causing fruit infection cannot be excluded based on the evidence obtained to date. Significant control of the disease can be achieved by decreasing surface populations of *E. maculosum* through dormant-season applications of the contact fungicide lime sulfur. A real-time PCR-based assay utilizing the specific

primers EXORI-F and EXORI-R along with the fluorescent probe EXORJI has shown promise as a rapid and reliable diagnostic tool for detecting *E. maculosum* and potentially helping to diagnose Exobasidium leaf and fruit spot of blueberry.