ORANGE CANE BLOTCH OF BLACKBERRY CAUSED BY *CEPHALEUROS VIRESCENS*: CHEMICAL CONTROL AND YIELD LOSSES ASSOCIATED WITH THE DISEASE

by

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(Under the Direction of Phillip M. Brannen and Harald Scherm)

ABSTRACT

Orange cane blotch (OCB), caused by the alga *Cephaleuros virescens*, is prevalent in commercial blackberry plantings in the southeastern United States. The pathogen produces prominent blotches on the surface of canes; affected plant tissue may crack over time, compromising the cane surface. Light and electron microscopy were used to characterize cane damage caused by *C. virescens* in more detail. Algal filaments were observed growing intercellularly and occasionally intracellularly underneath the cuticle and among epidermal layers of host tissue. Field studies at four commercial sites were conducted over 2 years to assess the yield impact of OCB. Fruit numbers per cane decreased with increasing disease intensity, but neither cane diameter nor fruit size were affected. In 3-year field efficacy trials to identify suitable agrichemicals for control of OCB, potassium phosphite was the only product that consistently and significantly suppressed the disease when applied during summer and fall.

INDEX WORDS: Orange cane blotch, orange felt, *Cephaleuros*, fungicide, algicide, *Rubus*, blackberry, filamentous alga

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Bachelor of Science, Abraham Baldwin Agricultural College, 2014

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2017

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May 2017

DEDICATION

This thesis is dedicated to:

-The loving memory of my father who shared his passion for science and taught me to constantly challenge myself and others.

-My Mother who continues to give me unconditional love and support throughout all my endeavors.

ACKNOWLEDGEMENTS

First, I wish to express my sincere gratitude to Phillip M. Brannen for sharing his passion for agriculture. Through his guidance, I have achieved seemingly unattainable goals and continue to raise my expectations for the future. He has taught me that plant pathology requires more than a love for research but also a desire to help others.

I would also like to extend my gratitude to Harald Scherm for his outstanding mentorship. His patience and kind supervision will not be forgotten. This work would not have been possible without his extensive knowledge and expertise in plant pathology.

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CHAPTER 1

INTRODUCTION

The advent of new blackberry (*Rubus fruticosus*) cultivars with desirable production and market qualities has led to a recent expansion of the blackberry industry throughout the United States. For example, the release of new varieties from the University of Arkansas has resulted in an estimated 45% increase in production between 1995 and 2005 (Strik et al. 2007); this production increase has also been driven by perceived health benefits of blackberries, which has, in turn, led to increased commercial value for this crop. In Georgia, both the number of growers and hectares per grower have increased in parallel to the national trend, especially in the last 15 years. Blackberry varieties grown in Georgia differ from those grown in other regions and often vary in fruiting and growth habits and may be thorned or thornless. Popular thornless blackberry varieties grown in the southeastern United States include 'Arapajo', 'Navajo', and 'Ouachita'.

Diseases, including cane diseases, are prevalent in the hot, humid environment of the southeastern Coastal Plains. Many blackberry producers have experienced decline or death of plants and yield losses as a result of cane diseases. Some of these, such as cane blight (caused by the fungus *Paraconiothyrium fuckelii*), are well-described diseases that occur in other more temperate locations as well. However, one of these cane-infecting diseases, orange cane blotch (OCB), has not been well researched and is unique to the Southeast. OCB, caused by an unusual plant pathogen, the alga *Cephaleuros virescens* Kunze, is responsible for symptoms which have caused great concern to blackberry producers in both Florida and Georgia. The algal disease leads to a prevalent orange blotch on blackberry canes, and growers have often associated OCB

symptoms with blackberry decline and eventual death. Although *C. virescens* is the most universally reported algal pathogen of vascular plants (Joubert and Rijkenberg 1971; Wellman 1972; Nelson 2008; Gokhale and Shaikh 2012) and some information has been developed to support the hypothesis that *C. virescens* is the cause for a decline of blackberry canes (Chapman and Good 1983; Holcomb 1986; Holcomb et al. 1998), definitive research has not been conducted to support a pathogenic role of *C. virescens* in the blackberry system.

LITERATURE REVIEW

First report of OCB on blackberry. Although algal plant diseases caused by *C. virescens* have been known to occur in the southern United States for a long time (Holcomb 1975, 1986), the first report of OCB on blackberry was made in Arkansas on the cultivar 'Navajo' in Texarkana. The symptoms and signs reported included cracking of the stem, tissue discoloration, and orange velvet-like growth. The cane was sent to a disease clinic in Loknor in June 1997. The orange felt-like material was identified as sporangiophores and sporangia of the parasitic green alga *C. virescens*. After sporangia were placed in water, zoospores were released. Pathogenicity, however, was not demonstrated and zoospores never developed in culture (Holcomb et al. 1998). The disease was then found in Louisiana at the Calhoun Research Station in north-central Louisiana where it was reported to be more common on thornless varieties (Holcomb et al. 1998).

OCB symptoms and signs. Infected blackberry canes show brightly colored irregular lesions. Immature thalli may be green in color but change to bright orange during sporulation. Prolonged infection may lead to desiccation of infected areas and may result in cracking of tissue. These symptomatic cracks associated with OCB could facilitate secondary infection by other cane pathogens. Producers often report decline of infected canes and in some cases plant death. Heavy

infection is often observed on the basal area of canes, possibly due to the spread of infection through rain splash.

Very little is known about the epidemiology of OCB, but the reported causal agent, *C. virescens*, is thought to damage plants directly (Holcomb et al. 1998). Popular blackberry varieties grown in the southeastern United State have a biennial life cycle; immature primocanes (strictly vegetative), which emerge in early spring, become floricanes the following year when they produce flowers and fruit. Primocanes are believed to be infected in late spring and early summer, with symptoms appearing in late summer and early fall of that same year. Algal lesions continue to expand through the fall, winter, and spring of the following year. To complete the cycle, mature thalli begin to sporulate in late spring, around the time of harvest.

Florida producers consistently report OCB, seemingly causing rapid decline of plants and eventual death, as the primary disease of concern to the blackberry industry in their state. However, this has not been confirmed experimentally. Damage to the cane cortex region has been observed, so girdling of the canes is a possible cause of plant decline. As in other plant species (Suto et al. 2014), it is assumed that *C. virescens* invades the space between epidermal cells under the cuticle; this causes cracking in blackberry canes, possibly providing a site of additional infections by secondary pathogens. Although other cane pathogens such as *Paraconiothyrium fuckelii* and *Pestalotia* spp. have been found in association with OCB, a direct connection has not been proven. However, favorable environmental conditions may lead to extensive colonization by the alga, and when combined with detrimental secondary pathogen infections, decline and dieback of canes is anticipated (Brooks 2004).

Algae as plant pathogens. Our understanding of algae as plant pathogens is complex. Many organisms are grouped into the term “algae” based on morphological features and basic life

history. Algae are in a polyphyletic group, and many are in fact unrelated. The group could be defined as containing organisms other than embryophytic land plants that carry out oxygenic photosynthesis (Cavalier-Smith 2007). Most algae are aquatic, autotrophic, and maintain a simple body structure. Their pathogenic capabilities may sometimes be convoluted by interactions with fungi in a complex symbiotic relationship. The relatively unfamiliar alga *C. virescens* has recently attracted the attention of researchers and crop producers alike. The pathogenicity of this alga is often questioned (Chapman and Good 1983), but intense symptoms and decline of a wide range of hosts raises concerns.

Members of the Trentepohlicaeae family, *Cephaleuros* included, are all filamentous, subaerial algae. Some can be found on inanimate objects such as rocks or dead wood, but many vascular plants can serve as a host for *Cephaleuros* spp. growing either epiphytically or parasitically in tropical or subtropical regions. Large host numbers for *C. virescens* have been reported in the literature. Holcomb (1975) reported 115 host species in Louisiana alone, whereas Batista and Lima (1949) recorded nearly 450 vascular plant hosts in Brazil. Economically important crops such as citrus, coffee, and tea are infected by *C. virescens* (Swingle 1894; Went 1895; Mann and Hutchinson 1907; Winston 1938; Wellman 1965; Golato 1970; Groth 1971; Joulbert and Rijkenberg 1971; Vidhyasekaran and Parambaramani 1971). Confusion has been added to diagnosis of algal diseases by assigning the terms “red rust” and “algal rust”, implying a fungal infection (Mann and Hutcheson 1907; Chapman and Good 1983). Other misidentifications of the disease have also occurred where algal disease was mistaken for coffee rust, powdery mildew, leaf spots, and bacterial canker (Thompson and Wujek 1997; Wellman 1965).

The genus *Cephaleuros* is a member of the phylum Chlorophyta, sister to phylum Streptophyta, containing several green algae as well as land plants (Leliaert et al. 2014).

Classification in Trentepohliales is largely based on the type of sporangia observed (Thompson and Wujek 1997). The unique zoosporangia are supported by a "suffultory cell" used to hold sporangia away from the algal body. Several other unique ultrastructural, cytological, and biochemical characteristics have distinguished Trentepolialean algae from other groups (Chapman and Good 1983; Chapman and Henk 1985; Van den Hoek et al. 1995).

Currently, *C. virescens* is included as a member of the family Trentepohliaceae. Algae in the Tretepohliaceae family are most often recognized by the presence of a yellow to orange-colored pigment, hematochrome. When accumulated in large concentrations, mature algal colonies assume bright coloration. Members of the Trentepohliaceae are also known for having more robust cell walls than those observed in most aquatic green algae (Chapman and Good 1983). In addition to thick cell walls and yellow-orange pigmentation, subaerial algae found in this family also produce zoosporangia.

Life cycle and propagation of *Cephaleuros*. Similar to some land plants, an alternation of heteromorphic generations and a homothallic mating system has been described for algae in the genus *Cephaleuros* (Thompson 1961; Thompson and Wujek 1997). *Cephaleuros viresens* can reproduce sexually and asexually. Sexual reproduction leads to the production of a zygote from which a sporophyte develops (Thompson 1961). Gametangia arise from terminal and lateral cells and can be produced on the same gametophyte thallus that produces zoosporangia. Gametangia can produce 8 to 64 biflagellate isogametes. They are initially spindle-shaped but change form after swimming in a film of water (Thomas and Wujek 1997). Gametangia are generally located beneath the host cuticle and are borne from terminal cells of prostrate filaments (Chapman and Good 1983). Gametes can fuse within the gametangium or externally. The resulting sporophyte located near the parent thallus will produce small meiozoosporangia which release 4 to 8

quadriflagellate meiozoospores after abscission. It has been assumed that the meiozoosporangia will infect normally and produce a typical thallus (Chapman and Good 1983).

Asexual reproduction involves multiple structures that allow the subaerial growth (growth on surfaces above soil, water, snow and ice that are exposed to air) observed in *Cephaleuros*. Terminal and lateral cells in the algal body give rise to the sporangiophores which release zoospores when wet. Each zoosporangium will release 8 to 16 zoospores (Chapman and Henk 1985; Thompson and Wujek 1997). Zoospores have keeled (winged) flagella, unique to Trentepohlian algae (Graham and McBride 1975; Chapman and Good 1983). During wet and humid conditions, the suffultory cell swells and holds the zoosporangium away from the surface, making dispersal more efficient (Lopez-Bautista et al. 2003). Rain, insects, arachnids, wind, mechanical disturbances, and moving water can dislodge the sporangia (Joulbert and Rijkenberg 1971; Thompson and Wujek 1997; Chapman and Good 1983). The dispersal of zoospores from the zoosporangium is a passive process; however, the abscission of the zoosporangia is a result of several morphological developments, also unique to Trentepohlialean algae (Chapman and Good 1983). The zoospores serve as the primary inoculum for the disease. It is important that they land on a suitable surface quickly and begin the formation of a thallus. When released from zoosporangia, zoospores are quadriflagellate, i.e., they are equipped with four flagella; however, these pair off and two individual flagella twist around each other to form two pairs. The zoospores and isogametes are very similar in shape and only differ in size. Isogametes are one-third to one-half smaller compared with zoospores released from zoosporangia (Thomas and Wujek 1997).

A third mode of propagation involving resistant reproductive cells during adverse conditions has also been suggested. During this process, akinetes are formed during dry conditions, which subsequently develop enlarged sporangiophore initials with thick walls (Chapman and Good

1983). Once environmental conditions improve, either a sporangiophore lateral or quadriflagellate zoospores are produced. The process is similar to the formation of gametangia; however, gametangia release biflagellate zoospores, but in this case, quadriflagellate zoospores are produced (Chapman and Good 1983). Both asexual and sexual reproduction can occur over a long period of time (more than 6 months in some cases).

Other species of *Cephaleuros* can be lichenized by ascomycete fungi (Mann and Hutcheson 1907). The algae are thought to damage host plant tissue before the fungus invades (Brooks 2004; Schubert 1981). This further opens the question of a relationship between fungi and *C. virescens* on blackberry canes. The relationships of these organisms should be explored further.

Infection process. After release, zoospores must quickly locate a suitable substrate and begin formation of a thallus (Chapman and Good 1983). Because *Cephaleuros* colonizes plant tissue beneath the cuticle, either the zoospores or the thallus must directly penetrate the surface or utilize a wound in the surface for entry. It is assumed that a short-lived zoospore would not efficiently penetrate the cuticle, and the ability of young thalli to dissolve the cuticle has not been shown; therefore, the mode of entry is currently unknown (Chapman and Good 1983). Interestingly, arachnids and insects that feed on host plants have both been reported to transport zoosporangia of *Cephaleuros* (Chapman and Good 1983). Feeding by these potential dispersal agents could also create a wound for infection. A positive correlation between the number of thalli and the number of insect galls on mango has been reported by Chowdary and Jose (1979), suggesting a role of insects in the transmission and/or infection process.

Necrosis under algal lesions has been observed in the upper epidermal layers; however, in severe cases, the necrosis can extend to deeper layers as well (Chapman and Good 1983). Intracellular penetration has not been clearly demonstrated. In cases where intercellular

penetration was reported, the alga grows among weak or dead cells near the surface. Several authors have suggested that shading, enzymes, or toxins may play roles in the wound response (Joubert and Rijkenberg 1971; Wolf 1930); however, no experimental evidence exists to confirm these hypotheses. In addition to protection from desiccation, the host also provides a suitable habitat, and it is assumed that this alga relies on the plant for water supply and inorganic salts. Host plant hormones may also be required for reproduction, because reproductive structures are not observed in culture (Chapman and Good 1983).

In the case of blackberry, a full disease cycle is thought to require 8 to 9 months to complete, but as mentioned previously, the epidemiology of *C. virescens* on blackberry has not been elucidated. Algal lesions generally appear in late summer and will advance throughout the fall, winter, and following spring. In wet and humid conditions, canes can be largely covered by coalescing thalli, although the efficiency of infection has not been determined. As a pathogen, *C. virescens* is reported to be monocyclic, meaning the zoospores and gametes will land and develop thalli in one plant growth cycle, and dispersal, reproduction, and further infection will occur the following year (Suto and Ohtani 2013).

Geographical distribution. A parasite of higher plants, *C. virescens* causes major disease symptoms in wet, humid, and hot climates, wherever they occur. In fact, climate is the most important factor determining the frequency and severity of disease occurrences (Bentz et al. 2010). Although this species prevails in subtropical and tropical regions such as those in Africa, Australia, China, India, Japan, Central and South America, the West Indies, and the United States, to include the Hawaiian Islands (Brooks 2004; Holcomb 1986; Sinclair and Lyon 2005), some *Cephaleuros* species can exist in more temperate regions (Chapman and Good 1983). It is thought that these regions are the center of abundance and diversity of algae in the Trentepohliales. These algae are

also likely to grow in cooler areas that are adequately humid (Suto and Ohtani 2009). Global warming could possibly be responsible in part for the increase in reports of disease. Increased rainfall, combined with higher temperatures, could push the migration of Trentepohlian algae to other climes. Any regions with temperate climates receiving annual rainfall over 1700 mm would provide a suitable environment for infection (Suto and Othani 2009).

Cephaleuros virescens has been observed on 287 plant species, in 80 of which it has invaded stems (Holcomb 1986). The genus *Cephaleuros* has been described as containing space parasites and pathogens that grow in the space between upper layers of plant tissue, similar to other algae in the order Trentepohliales (Chapman and Good 1983). Algal infections have been observed on several economically important crops (Holcomb 1986; Holcomb et al. 1998; Sinclair and Lyon 2005), including avocado (*Persea americana*), cacao (*Theobroma cacao*), citrus (*Citrus* spp.*)*, coffee (*Coffea arabica)*, grape (*Vitis vinifera)*, common guava (*Psidium guajava)*, litchi nut (*Litchi chinesis)*, magnolia (*Magnolia grandiflora)*, mango (*Mangifera indica)*, oil palm (*Elaeis guineensis)*, pecan (*Carya illinoensis*), pepper (*Piper nigrum)*, rubber (*Hevea brasiliensis*), tea (*Camellia sinensis)*, vanilla (*Vanilla planifolia)*, and some timber-yielding plants (Chakravarty and Mishra 1983; Chapman and Good 1983; Holcomb 1986). Although these crops and ornamental plants have all been reported to be infected by *Cephaleuros* species, Koch's postulates have not always been completed, and the causative agent has not been confirmed in several cases (Chapman and Good 1983). In cases such as avocado, citrus, coffee, and guava, the marketable fruit is damaged once infected by *Cephaleuros*. Although leaf infections may not appear as important, infections on tea, which can have detrimental effects on both physiological and biochemical aspects, can cause significant economic loss (Ponmurugan et al. 2007). Stem infection may be more detrimental than when other organs are affected, because infections could result in partial or

total girdling, affecting translocation of nutrients (Chapman and Good 1983). These types of infections could potentially result in stunted plants, a decreased number of leaves or reproductive structures, or in severe cases, plant decline and death. Traditional blackberry production sites (e.g., in the Pacific Northwest or Northeast) have had lower temperatures and less humidity, and the disease is not observed there. It is only with the expansion of the industry into the Southeast that this disease has become a concern for blackberries.

Host specificity. Although many vascular plants can be infected by *Cephaleuros,* it is clear that the organism does not infect all plants, and several factors must be considered. Young, undamaged leaves are not usually infected, hence thalli are not typically observed on plants with short-lived leaves (Chapman and Good 1983). In contrast, broad-leaf evergreens provide a substrate that can be infected over a long period of time. In order for infection to occur, the alga must penetrate the host cuticle, hence thickness and surface topography of the waxy surface layer may play a role in host susceptibility. After attachment and penetration, the alga must then establish a thallus, a process dependent on host responses.

Currently, 17 species are included in the genus *Cephaleuros*. As mentioned previously, environmental conditions play a large role in whether successful infections occur. Stressed plants may be more likely to be infected than healthy plants. Plants may be stressed due to drought conditions, lack of nutrients, or infection by other plant pathogens.

Cephaleuros spp. can infect leaves, stems, and fruits. Few studies have been conducted where the same algal species has been examined in terms of morphology, aggressiveness, and host responses across a range of host species. It has been reported that algal species, even the same specimen, can vary with respect to morphology and other characteristics when exposed to different environments (Howland 1929; Nakano and Handa 1984; Thompson and Wujek 1997; Rindi and

Guiry 2002). Comparative studies are needed to determine whether multiple species really differ in morphology or only vary based on host phenotype (Chapman and Good 1983).

Trentpohlialean taxonony. Trentepohlialean algae are most often recognized by their vibrant colors, ranging from orange to yellow and red. The bright pigmentation is due to the buildup of carotenoid compounds in the algal cells (Lopez-Bautista et al. 2003). The environment plays a key role in the amounts of carotenoid pigments present. In the presence of strong light and low nutrients, the amounts of pigmented compounds reach their peak (Abe et al. 1999; Hametner et al. 2014; Ho et al. 1983). Algal colonies may appear greener in shaded areas, meaning that the green coloration of the chloroplasts are not overpowered by the carotenoid pigments, such as beta-carotene or astaxanthin, in the absence of sunlight (Thompson and Wujek 1997). Algae classified in the Trentepohliaceae family are distinguished from other algal groups by characteristics such as the presence of beta-carotene and haematochrome in cells often responsible for the bright orange, red or yellow coloration; lack of pyrenoids in the chloroplast; unique flagellar apparatus; plasmodesmata in transverse cell walls; and differentiated reproductive structures (Chapman and Henk 1983; Roberts 1984).

A system for classification at the genus level was developed in 1939 that relied on certain morphological characteristics (Printz 1939). The genus *Cephaleuros* currently consists of 17 accepted species known for their irregularly branching filaments and ability to coalesce to form colonies below the epidermis or cuticle of vascular plants. Morphological characters previously used for classification, which may not have phylogenetic significance, include size and shape of vegetative cells; development of prostrate parts compared with erect parts of the thallus; branching pattern of filaments; presence or absence of hair-like protrusions; arrangement of sporangiate laterals; size and shape of zoosporangium and suffultory cell; apical cell shape; cell wall color;

presence of corrugations on cell wall; and type of substrate colonized (Rindi et al. 2009; Hariot 1890; De Wildeman 1900; Printz 1921 and 1939; Sarma 1986; Ettl and Gärtner 1995). It is not uncommon to observe significant variation of these morphological characters, and environmental factors appear to have a major influence on the plasticity of species (Howland 1929; Nakano and Handa 1984; Thompson and Wujek 1997; Rindi and Guiry 2002). There is a lack of clarity in other groups of Ulvophycean green algae where classification has been based solely on morphology (Verbruggen et al. 2007; De Clerck et al. 2008).

Fortunately, DNA sequence data for Trentepohlialean algae has become increasingly available in the last decade; however, data is limited to a low number of 18S rDNA and phragmoplastin (*rbc*L) gene sequences (López-Bautista and Chapman 2003; López-Bautista et al. 2003, 2006). 18S rDNA is nuclear DNA and *rbc*L genes are associated with chloroplasts. According to a study by Rindi et al. (2009), *rbc*L provides a better resolution at genus and species levels. Even when partial sequences are used, the *rbc*L gene has provided researchers with successful results when testing phylogenetic hypotheses. 18S rDNA sequences show low phylogenetic resolution and are therefore insufficient for species-level conclusions, and additional genes may be required (Cardon et al. 2008). Morphology of species in *Trentepohlia* and *Cephaleuros* can appear very similar in culture. In both cases, filaments grow freely. The molecular analyses conducted by Rindi et al. (2009) revealed that molecular and morphological circumscription of several major clades of species in *Trentepohlia* and *Pritzina* (genera containing several filamentous algae) were not in agreement. It appears most morphological characteristics (Hariot 1890; DeWildeman 1891, 1900; Printz 1939; Sarma 1986; Ettl and Gärtner 1995) traditionally used for classification of Trentapohlialean species are phylogenetically irrelevant (Rindi et al. 2009).

A suitable DNA marker for the purpose of barcoding could rectify the current situation where species are potentially misidentified. Unfortunately, in the case of Trentepoliales, the *rbc*L gene does not meet requirements necessary for barcoding purposes. The *rbc*L gene is not easily amplified through primers designed from conserved regions of the gene (Rindi et al. 2009). In a study by Rindi et al. (2009), some samples did not amplify at all using *rbc*L primers. Currently, no such genetic marker exists for green algae as a universal DNA barcode, indicating an area requiring additional work. However, multigene studies have provided useful information on the evolution of embryophyte land plants (Nickrent et al. 2000; Shaw and Allen 2000; Karol et al. 2001). A combination of *rbc*L and SSU 18S rDNA genes could be useful in confirming the identity of algal plant pathogens.

***Cephaleuros virescens* as a plant pathogen.** Although algal blotches have been observed on leaves, stems, and fruit of various plant species, the pathogenicity of *C. virescens* has been questioned. Most *Cephaleuros* species are described as obligate parasites, but they have been described as obligate epiphytes by some and occasional parasites by others (Thomas and Wujek 1997). In some cases, investigators thought that these algae were opportunistic pathogens and severe infections only occurred on stressed plants (Thompson, 1959). These algae have been reported to grow subcuticularly and/or intercellularly, causing damage to tissue near the surface (Chapman 1976), and most damage occurs through destruction of epidermal cells. Most *Cephaleuros* species that grow subcuticullarly only cause necrosis under the thalli (Brooks, 2004; Suto and Ohtani 2009).

Early infections may be overlooked due to the presence of immature thalli (Chapman and Good, 1983), which lack the large accumulation of the hematochrome pigment and may not be visible to the naked eye. Few erect branches are found on such young thalli (Chapman and Good

1983), and as the immature thalli are located under the cuticle, timing of infection may be difficult to interpret. Older thalli which have acquired large amounts of the haematochrome pigment will appear bright orange with a velvety appearance during periods of asexual reproduction (Chapman and Good 1983). A large area of infection has been associated with *Cephaleuros* species. In cases where a large surface area is utilized, it is thought that colonies may overlap (Chapman and Good 1983).

Abundant sterile setae (stiff hair-like structures) and fertile erect branches are responsible for the velvety appearance observed on infected plant surfaces (Chapman and Good 1983). Although some branches are visible above the plant cuticle, the prostrate components of the thallus grow subcuticularly. The structures arising from the thallus that comprise the erect parts of the system must break through the cuticle, causing damage to plant tissue (Chapman and Good 1983).

Algal spots and blotches observed on fruits are often smaller and more superficial (Joubert and Rijkenberg 1971; Ogle 1997). Although fruit infection has not been reported in blackberry, readily detectable damage from *Cephaleuros* species is observed on leaves and/or twigs of tea, citrus, and blackberry (Dewdney 2012; Holcomb et al. 1998; Ramya 2013). Reports of other effects on host plants caused by Trentepohliales algae have also included reduction in transpiration and glucose levels. These host plants included mango, guava, citrus, vanilla, and sapota (Dhillon et al. 1992). Algal lesions on host plants have reduced photosynthetic capabilities (Nelson 2008). Another study showed that infected leaves of timber-yielding plants had decreased amounts of nitrogen and phenol compared with infected leaves. Sugars, amino acids, and dry weights of leaves were also higher in those infected. The same study surveyed disease in timber-yielding plants and found that infection was present in angiosperms but not in gymnosperms, possibly indicating non-host resistance (Chakravarty et al. 1983).

Control of orange cane blotch. Several management options have been recommended, to include: (1) bedding and drainage of blackberry sites to reduce excess moisture and humidity, (2) careful pruning and cane selection to open up air movement in the canopy, (3) rapid removal and destruction of old floricanes immediately after harvest, (4) weed control through herbicides, (5) use of plastic mulch and drip irrigation, and (6) application of copper sulfate fungicides. In wet conditions observed in many years, these practices have not been sufficient for disease management in the Coastal Plain region of Georgia.

Recommendations for management of OCB have included applications of copper fungicides, but these have not been effective in Georgia. The use of copper as a management strategy may also have undesired effects on the target crop, as copper injury can occur under poor drying conditions. Also, most labels do not allow application during the summer months when *C. virescens* likely infects.

JUSTIFICATION AND OBJECTIVES

OCB is a problem for producers growing blackberries in warm and humid climates. Brightly colored blotches on blackberry canes have caused concern among producers. However, actual damage due to OCB has not been adequately documented. Currently, the relationship between OCB and blackberry yield is not known. While some management strategies have been recommended, they have not adequately controlled disease in the southeastern United States. Uncertainty regarding the classification of filamentous algae also raises questions concerning the causal agent responsible for OCB. Based on these considerations, the objectives for this study were as follows:

1) Assess the impact of OCB on cane damage and blackberry yield.

2) Develop chemical control recommendations for OCB through testing of disinfectants, algicides, and fungicides in field efficacy trials.
3) Confirm the species of the alga isolated from blackberry canes and compare with algal isolates from blueberry.
4) Determine the utility of herbicides and other agrichemicals applied during the blackberry dormant period as a means to manage OCB.

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CHAPTER 2

YIELD RESPONSE TO ORANGE CANE BLOTCH OF BLACKBERRY GROWN IN THE GEORGIA COASTAL PLAINS[1]

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ABSTRACT

Orange cane blotch (OCB), an algal disease on commercial blackberry plants in the southeastern United States, has been an increasing concern among producers. The causal agent, *Cephaleuros virescens*, produces brightly colored green to orange lesions on blackberry stems, but proof of actual damage and impact on crop yield has not been documented. Naturally-infected stem sections were viewed using transmission and scanning electron microscopy to evaluate cane damage. Surface abrasions, intercellular growth, and occasional intracellular growth were observed on the surface and epidermal layers. Field studies at four commercial sites over 2 years were conducted to assess the impact of OCB on yield in 'Oachita' blackberry plants not treated with algicidal chemicals. Neither cane diameter nor fruit size were impacted by severity of OCB; however, fruit number decreased with increasing OCB intensity in a non-linear manner, thereby resulting in reduced yields. Application of potassium phosphite consistently suppressed the disease, resulting in increased yield.

Additional keywords**:** orange felt, parasitic algae, yield response.

INTRODUCTION

In recent years, university breeding programs have released blackberry (*Rubus fructicosus*) cultivars adapted to warmer climates, allowing an increase in blackberry production in the southeastern United States, primarily in the Coastal Plains region. Traditional blackberry production areas do not experience such warm temperatures and high humidity as those observed in the southern states. As a result of these climatic conditions, cane diseases are a major concern for blackberry production in the Southeast. One disease in particular, orange cane blotch (OCB), has become prevalent in the Coastal Plains, causing great concern to producers. Commercial varieties have been largely selected based on consumer appeal; however, several of these cultivars are highly susceptible to OCB.

OCB, also known as orange felt, is caused by the parasitic alga, *Cephaleuros virescens* Kunze. The first report of OCB on blackberry was from Texarkana, AR, on a commonly planted cultivar, 'Navajo'. Reported symptoms and signs included stem cracks, discoloration of tissue, and velvet-like orange blotches on canes. Sporangiophores were reported as the source of the felt-like appearance. Zoospores were released when these reproductive structures were placed in water; however, the zoospores did not germinate in vitro, and pathogenicity was not confirmed (Holcomb 1986). A subsequent report involved blackberry canes at the Calhoun Research Station in northern Louisiana (Holcomb et al. 1998).

Cephaleuros virescens thrives in warm, wet, and humid environments. Investigators initially believed that the alga was opportunistic and that heavy infection was limited to stressed plants (Thompson 1959). Although the pathogenic capability of *C. virescens* has been controversial, it has been hypothesized that algal infection can lead to girdling of canes and, in severe cases, cane decline and death. In some reports the alga is considered an obligate epiphyte,

while others characterize the species as an occasional or obligate parasite (Thomas and Wujek 1997). However, it is now apparent that several factors are involved in host specificity (Chapman and Good 1983) and environmental interactions. Due to the fact that *C. virescens* can infect a large number of host species on different organs, it is likely that the alga does not have taxonomic restrictions and that other factors such as cuticle thickness, host response, and surface topography should be considered when reviewing host-parasite interactions (Chapman and Good 1983).

While *C. virescens* is prevalent on blackberry canes in the Coastal Plains, it has also been reported to infect nearly 300 vascular plant species at other locations (Holcomb 1986). Infections of 115 species have been recorded in Louisiana alone (Holcomb 1975). *Cephaleuros virescens* is not limited to one tissue type, and at least 80 of these host relations were stem invasions similar to those observed on blackberry canes. Stem infections are often considered more detrimental to the plant, because translocation of nutrients could be hindered and partial or total girdling of the cane is possible (Chapman and Good 1983). Resulting downstream effects for the host plant could include a decrease in the number of leaves or reproductive structures, stunted plants, or even plant death.

Epidemiological knowledge is limited for diseases incited by *C. virescens*; however, researchers have assumed that plants are damaged directly by the alga (Holcomb et al. 1998). Infections by *C. virescens* in other plant species demonstrate an invasion of the space between the epidermal cells underneath the cuticle, causing damage near the plant surface (Suto et al. 2014). This particular growth pattern could be responsible for the typical cracking of blackberry canes observed in the field. A major concern with cracked tissues caused by OCB is potential invasion by other cane pathogens. When favorable conditions persist in the field, a combination of substantial algal colonization and presence of detrimental secondary infections could result in

death and dieback of canes (Brooks 2004). Some subaerial algae (algae growing on surfaces above ground and exposed to air) have been reported to decrease transpiration and glucose levels of citrus, guava, mango, sapota, and vanilla plants (Dhillon et al. 1992).

In the field, stem lesions of OCB on blackberry appear in late summer or early fall and may increase in size throughout the fall, winter, and spring of the following year. Primocanes (initial vegetative canes) emerge in late spring during the time algal lesions begin to sporulate on the floricanes (flower and fruit-bearing canes). It is assumed that zoospores spread from floricanes to new primocanes during this timeframe; hence, old floricanes are removed and destroyed immediately after harvest as a cultural disease management task. Reproduction in *Cephaleuros* algae has been described as an alternation of heteromorphic generations with homothallic mating, a system similar to that of land plants (Thompson 1959; Thompson and Wujek 1997). While the life cycle of *Cephaleuros* algae has been reported in general terms, the biennial growth pattern of blackberries may further complicate the disease cycle.

Recommendations to manage OCB in the field include strategic site drainage and appropriate row bedding, selective pruning to increase air movement in the canopy, removal of symptomatic canes immediately after harvest, effective weed control, use of plastic mulch in combination with drip irrigation, and application of agrichemicals with activity against algae. While control measures have been recommended, the yield impact of OCB has not been studied previously. The objectives of this study are to investigate the intensity of damage on diseased canes, assess yield losses due to OCB, and determine the utility of potassium phosphite to achieve disease reduction and yield increase.

MATERIALS AND METHODS

Observation of cane damage by SEM and TEM. Field samples of symptomatic canes were collected from Berrien, Clinch and Lanier counties for microscopic examination of cane damage through electron microscopy (EM). A sterile razor blade was used to cut thin cross-sections of infected canes in the field and immediately placed in a fixative (5% EM grade glutaraldehyde and potassium phosphate buffer) to prevent degradation of materials. Samples were then submitted to the Georgia Electron Microscopy (GEM) facility at the University of Georgia for further processing.

Some tissues were processed following a standard chemical fixation protocol in preparation for scanning electron microscopy (Glidewell and Mims 1979; Mims 1981). Tissue was fixed overnight at 4°C in a 1:1 mixture of 5% EM grade glutaraldehyde and 0.1M potassium phosphate buffer (pH 6.8 or 7.2), followed by washing 3 times for 15 min in potassium phosphate buffer at 4°C. A postfix mixture of 1:1 2% osmium tetroxide and buffer was subsequently applied for 2 h at 4°C. Samples were washed twice in double-distilled water (ddH_2O) for 15 min each at 25°C. Next, the samples were dehydrated using a graded ethanol series (10, 25, 50, 75, 95, 100, and 100%), each step requiring 15 min. Samples were then critical point-dried and placed into a desiccator before being mounted on stubs and stored in the desiccator again. Samples were coated with gold palladium and stored in the desiccator for future use. Samples were viewed on an SEM microscope (Zeiss 1450EP, Carl Zeiss MicroImaging, Thornwood, NY) at 15kV.

Additional samples were processed using a transmission electron microscopy (TEM) protocol (Glidewell and Mims 1979; Mims 1981) for plant material using Spurr's resin. Samples were fixed overnight in 5% (v/v) glutaraldehyde and potassium phosphate buffer (pH 7.2) at 4°C. Samples were then washed in potassium phosphate buffer 2 or 3 times each for 15 min at 4°C.

Next, samples were stained in 2% (v/v) OsO_4 for 2 h at 4°C. Distilled water (dH_2O) was used to rinse the samples twice for 15 min each at room temperature. Aqueous uranyl acetate (0.5%) was used for *en bloc* staining overnight before rinsing with dH_2O twice for 15 min each again. Dehydration steps were carried out next, using a series of graded ethanol (25, 50, 75, 95, and 100%) concentrations for 15 min each. Samples were incubated in acetone and Spurr's resin for 8 h in each of the following steps: 1) 75% acetone and 25% Spurr's, 2) 50% acetone and 50% Spurr's, 3) 75% acetone and 25% Spurr's, 4) 100% Spurr's, and 5) 100% Spurrs. Next, samples were embedded in Permanox petri dishes and allowed to rest in molds for 45 min. Molds were then placed in an oven for polymerization at 60°C for 24 h. Blocks were trimmed with razor blades and 70-nm sections were cut with a Diatome diamond knife on a Reichert-Jung ultramicrotome and placed on grids before staining with uranyl acetate and lead citrate. The grids were viewed with a transmission electron microscope (JEOL JEM 1011, Tokyo, Japan). Thick sections (1 µm) were cut and stained with 1% toluidine-blue for light microscopy.

OCB impact on blackberry yield. Disease progression was assessed in the spring of 2015 over a 3-month period at three commercial blackberry sites in Berrien, Echols, and Lanier counties in Georgia. The popular blackberry cultivar, 'Ouachita', was used for disease progress trials. Mature plants (~6 years old) remained unsprayed for field studies. One-hundred primocanes were marked with field tape at all locations. Percent of algal cane coverage for both sides of canes was recorded at five dates. Canes were assessed on dates as follows: Berrien County – 19 March, 3 April, 17 April, 30 April, and 11 May; Echols County – 20 March, 2 April, 17 April, 30 April, and 15 May; Lanier County – 20 March, 3 April, 17 April, 30 April, and 14 May. On the same day as the last assessment, blackberry fruit were harvested from each marked cane while still red in color, and cane diameters were recorded using digital calipers (Mitutoyo, Aurora, IL). Quantitative data

was collected for berry number, and weight per cane was measured with a digital balance (Ohaus, Parsippany, NJ).

A similar method was used to assess disease progression in the following growing season, but disease assessments were initiated in the fall of 2015 and continued through the spring of 2016 in Berrien, Clinch and Lanier counties. Again, 100 canes of relatively uniform size were selected at each site and algal coverage was assessed (as percent cane coverage with symptoms) throughout the growing season. Canes were assessed on dates as follows: Berrien County – 19 October and 9 November 2015 and 5 January, 13 March, 29 April, and 18 May 2016; Clinch County – 9 November 2015 and 5 January, 11 March, 29 April, and 17 May 2016; Lanier County – 16 October and 6 November 2015 and 5 January, 11 March, 29 April, and 16 May 2016. As in the previous crop year, cane diameters were measured, and berries were removed from marked canes on the last rating date (a few weeks before commercial harvest); yield was quantified by recording berry numbers and weight, all as described previously.

Yield response to application of potassium phosphite. Potassium phosphite (ProPhyt, Helena Chemical, Collierville, TN) is a systemic fungicide with activity against downy mildews, Phytophthora diseases, and several other diseases in orchards, vineyards, row crops, and vegetables. Because of the activity against several major groups of pathogens, it was assumed that the chemical may have activity against the alga, *C. virescens*, and multiple years of testing confirmed this (Browne 2016). Blackberry plants in Berrien and Lanier counties were used to further test the utility of potassium phosphite and the impact on disease control and yield. Treatments included potassium phosphite and an untreated control and were arranged in a randomized complete block design with five replications. Applications were made in Lanier and Berrien counties on 7 August, 28 August, 25 September, 16 October, 30 October, and 20

November 2015. A minimum of one plant was skipped between spray plants to minimize plot-plot spray drift. A CO_2 backpack sprayer (R & D Sprayers, Opelousas, LA) was used for applications, and treatments were applied until runoff. Percent of cane covered by algae was recorded in Berrien County on 19 October and 9 November 2015 and 13 March, 29 April, and 20 May 2016; in Lanier County, data was collected on 16 October and 6 November 2015 and 11 March, 29 April, and 16 May 2016. Berries were harvested at the last assessment date while they were red in color. Number of berries and total fruit weight per cane were recorded, and cane diameters were documented.

Data analysis. Cane disease severity values over time were converted to areas under the disease progress curve (AUDPC), in units of percent-days, for all trials. Individual berry weight was calculated based on berry number per cane and total fruit weight per cane. Since total fruit weight and berry numbers were closely correlated (with *r* values ranging from 0.89 to 0.96; *data not shown*), only fruit numbers and individual berry weights were subsequently analyzed. The two yield parameters were plotted against AUDPC or AUDPC divided by cane cross-sectional area; the latter conversion was done as a means of standardization to account for potential effects of cane cross-sectional area on yield (Archbold et. al 1989). Nonlinear regression analysis (SigmaPlot v. 11.0; Systat Software, San Jose, CA) was used to explore the relationship between the number of berries and AUDPC per cane cross-sectional area.

RESULTS

Observation of cane damage by SEM and TEM. Electron microscopy demonstrated the extent of algal colonization of infected canes. Algal filaments under the surface gave rise to large numbers of fertile erect branches that penetrated the blackberry cane surface causing micro abrasions over large areas (Fig. 2.1). Figure 2.2 shows a single sporangiophore breaking through

the surface, creating a small wound. All cells of algal origin (sterile and fertile branches) contained intact chloroplasts with thylakoid membranes. Fertile branches compromised the integrity of the protective waxy layer on the blackberry surface. A build-up of additional organisms of unknown origin was noted at several locations at breaks where sporangiophores pushed through the surface (Fig. 2.2). Intercellular growth of algal filaments was observed in infected canes (Fig. 2.3 and 2.4). Algal filaments grew between plant cells and breaking apart whole layers of epidermal tissue. Whole plant cells were completely surrounded by algal filaments that alter the natural orientation of plant tissue. Intracellular algal growth was also observed (Fig. 2.5 A-C). Invading algal cells with clearly defined chloroplasts were photographed growing inside of host plant cells encompassed by external filaments.

OCB impact on blackberry yield. There were generally no significant correlations of total fruit number per cane, individual fruit weight, or stem cross-sectional area with AUDPC across the six trials with the exception of a few locations with a large amount of scatter (Table 2.1). However, significant nonlinear relationships emerged in all but one location when fruit number per cane was regressed against AUDPC divided by cane cross-sectional area (Fig. 2.6).

In 2015, disease severity was highest in Echols County and lower in Lanier County and Berrien County (Fig. 2.6 A-C). Nevertheless, all test sites demonstrated a statistically significant yield decrease with increasing disease (AUDPC per cane cross-sectional area) without regard to disease pressure. Correlation coefficients of non-linear regression were low, ranging from 0.34 to 0.42, but *P*-values ranged from 0.0001 to 0.0004. Similar results were observed in Berrien and Lanier counties in 2016 (Fig. 2.6 D and E). In contrast, the data collected in Clinch County showed no significant relationship between fruit number per cane and AUDPC per cane cross-sectional

area (Fig. 2.6 F). It must also be noted, however, that Clinch County had abnormally high disease levels compared with other counties in 2015 and 2016.

Yield response to applications of potassium phosphite. When data from the two trials were combined, disease levels (AUDPC) were significantly decreased in canes treated with potassium phosphite ($P = 0.0002$; Fig. 2.7 A-D). Although potassium phosphite was only applied in late summer and fall, the chemical appeared to have activity through the winter and early spring, affecting the entire epidemic. When averaged across both locations, the decrease in disease resulted in increased yield for treated canes ($P = 0.1$; Fig. 2.7 E and F), although differences at individual locations were not significant.

DISCUSSION

Plant-pathogenicity of algal species has been a subject of significant controversy. In the past, actual damage caused by algal infections has not been clearly described, especially in the case of stem infections. Previous investigators believed that *Cephaleuros* algae were opportunistic pathogens, and severe infections were observed only on stressed plants (Thompson, 1959); however, infections on blackberry canes in the southeastern United States appears evenly dispersed in fields, suggesting the alga is more dependent on environmental conditions than on plant stress.

Previously, little was known about the pathogenesis of OCB on blackberry, but it was assumed that the alga could damage plants directly (Holcomb et al. 1998). During sporulation, large numbers of sporangiophores erupt through the surface of canes. Each fertile branch makes its own tear in the protective waxy cuticle on the surface, creating a wound. This study confirmed the intercellular growth of algal filaments similar to algal diseases reported in other plant species

(Suto et al. 2014). This intercellular growth separates epidermal layers near the surface, possibly compromising tissue structure.

In addition to intercellular growth, the alga may also be capable of intracellular growth, a growth habit that has not been clearly observed in algal systems. Electron micrographs from this study showed invasion of plant cells by structures having chloroplasts with thylakoid membranes and presumed to be of algal origin. Although host plant cells appear to be invaded by algal filaments, it cannot be determined whether the alga directly penetrates host cells or only invades after host cells are compromised. In this scenario, algal filaments may only be able to invade weakened host cells.

Plant diseases involving infection of young stems could result in partial or complete girdling of the cane and impairment of nutrient translocation (Chapman and Good 1983). Based on the growth patterns observed in this study, algal filaments appear to have the ability to grow in the intercellular spaces around the entire cane surface. Heavy infection observed at the base of canes may be a result of zoospores being washed down towards the ground. These heavily infected areas are of particular concern because the pathogen may girdle the cane in an area that would impact main and lateral shoots from which flowers and fruit arise. In this context, one would expect the disease to influence yield.

In this study, yield was impacted by OCB in 5 out of 6 locations assessed. Decreased total fruit weight per cane corresponded to a decreased number of berries per cane; a pattern that was consistent under varying disease pressure. Disease severity varied among locations, and variable disease pressure could be a result of variable precipitation or lack of proper management in previous years. Sporulating algal lesions are present on floricanes, from which inoculum infects

primocanes that produce the blackberry crop for the following year; therefore, if the disease is not adequately controlled in the previous year, the subsequent crop may have more severe infections.

No relationship existed between individual berry weight and amount of disease; therefore, fruit size did not appear to be affected by higher amounts of disease. Cane diameter was not affected in 5 of the 6 sites suggesting that cane development was not affected by disease. Clinch County in 2016, however, did show a relationship between cane diameter and disease. This could be a result of higher disease pressure than that observed at other locations.

Application of potassium phosphite was shown in this study to suppress OCB. *Cephaleuros virescens* grows beneath the waxy layer of the blackberry cane, a layer that may provide protection against contact chemicals. Potassium phosphite, however, is a systemic fungicide with dual modes of action. While it is known to have a direct effect on fungal pathogens, it also activates immune responses in plants (Rebollar-Alviter et al. 2012). An increase in yield was also observed in canes treated with potassium phosphite. It can be concluded from this study that increasing levels of disease will result in decreased yield, so it may be assumed that the increase in yield observed in canes treated with potassium phosphite was a direct result from the decreased disease due to applications throughout the summer and fall.

Our results show that OCB is a damaging disease of blackberries in the southeastern United States. Surface and internal damage was observed through light and electron microscopy. A negative impact of increasing disease severity on yield was demonstrated. Producers can suppress the disease through applications of potassium phosphite, thereby increasing yields. More work should be conducted on this alga in terms of epidemiology and damage through intracellular growth. Algal filaments have been observed in this study to grow inside of host cells; however, it cannot be clearly determined if the alga is directly penetrating or merely a secondary invader,

requiring additional studies on damage caused. Timing of applications should be explored further to increase the utility of potassium phosphite as a management tool.

ACKNOWLEDGMENTS

Funded in part by the National Raspberry and Blackberry Association. We thank the commercial blackberry growers and University of Georgia Extension agents who participated in this study.

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Table 2.1. Correlation coefficients (*r*) and probability values (*P*) for the association of cumulative orange cane blotch severity (area under the disease progress curve) with the yield parameters fruit number per cane, individual berry weight, and stem cross-sectional area on blackberry in six trials in southern Georgia, 2015 and 2016.

	Fruit number		Berry weight (g)		Stem cross-sectional area (cm^2)	
Site and year	*r*	*P*	*r*	*P*	*r*	*P*
2015						
Berrien	0.1202	0.2385	0.1339	0.1886	0.0704	0.4911
Echols	0.0708	0.5027	0.0078	0.9413	0.0496	0.6386
Lanier	0.0582	0.5672	0.0930	0.3599	0.0722	0.4774
2016						
Berrien	0.0004	0.9971	0.0607	0.5524	0.1225	0.2294
Clinch	0.4415	<0.0001	0.3759	0.0001	0.3983	<0.0001
Lanier	0.4667	<0.0001	0.4383	<0.0001	0.0423	0.6776

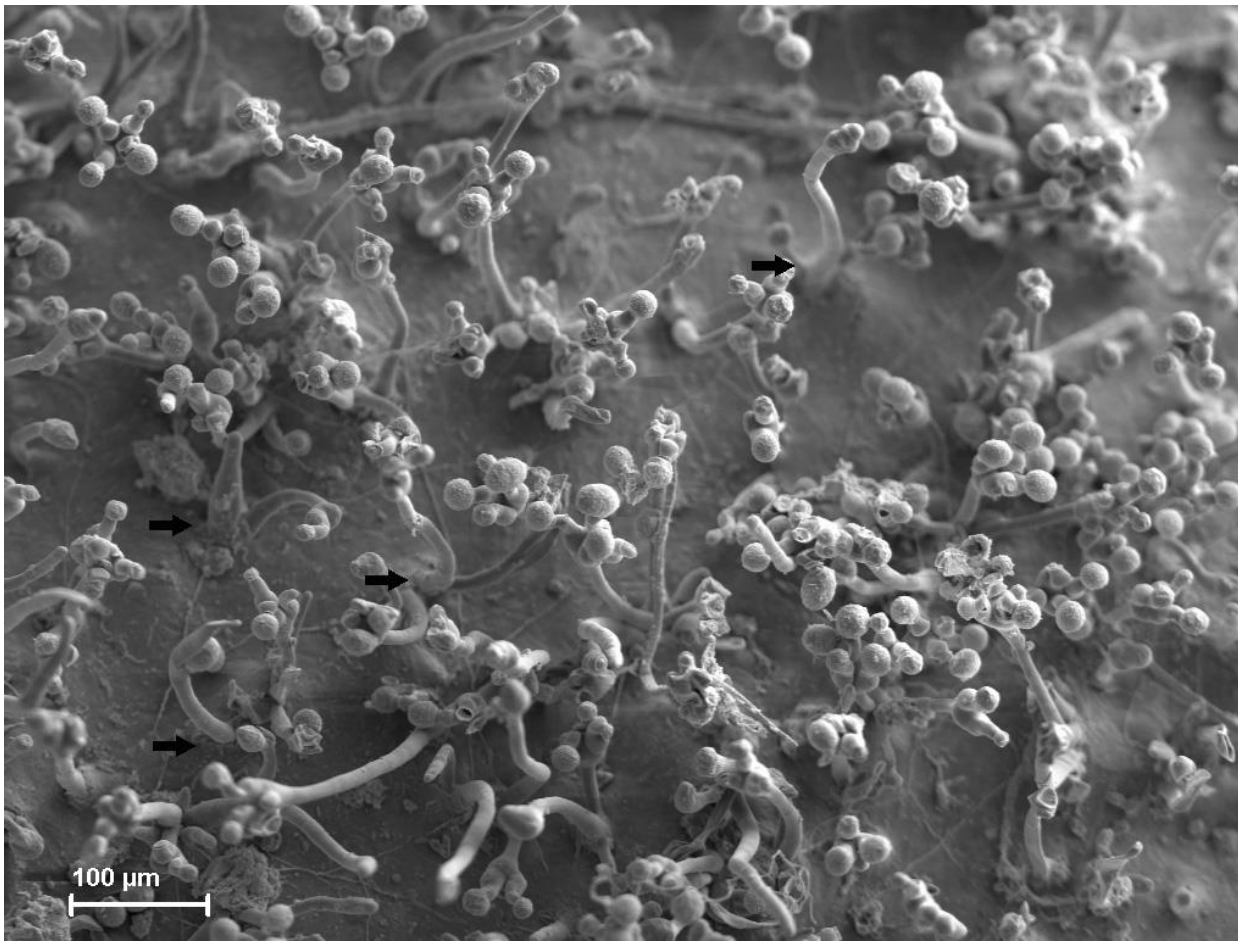

Figure 2.1. SEM micrograph of sporulating algal lesion on infected blackberry cane showing numerous sporangiohores (large arrows) of *Cephaleuros virescens* emerging from the affected tissue. Each sporangiophore bears multiple sporangia.

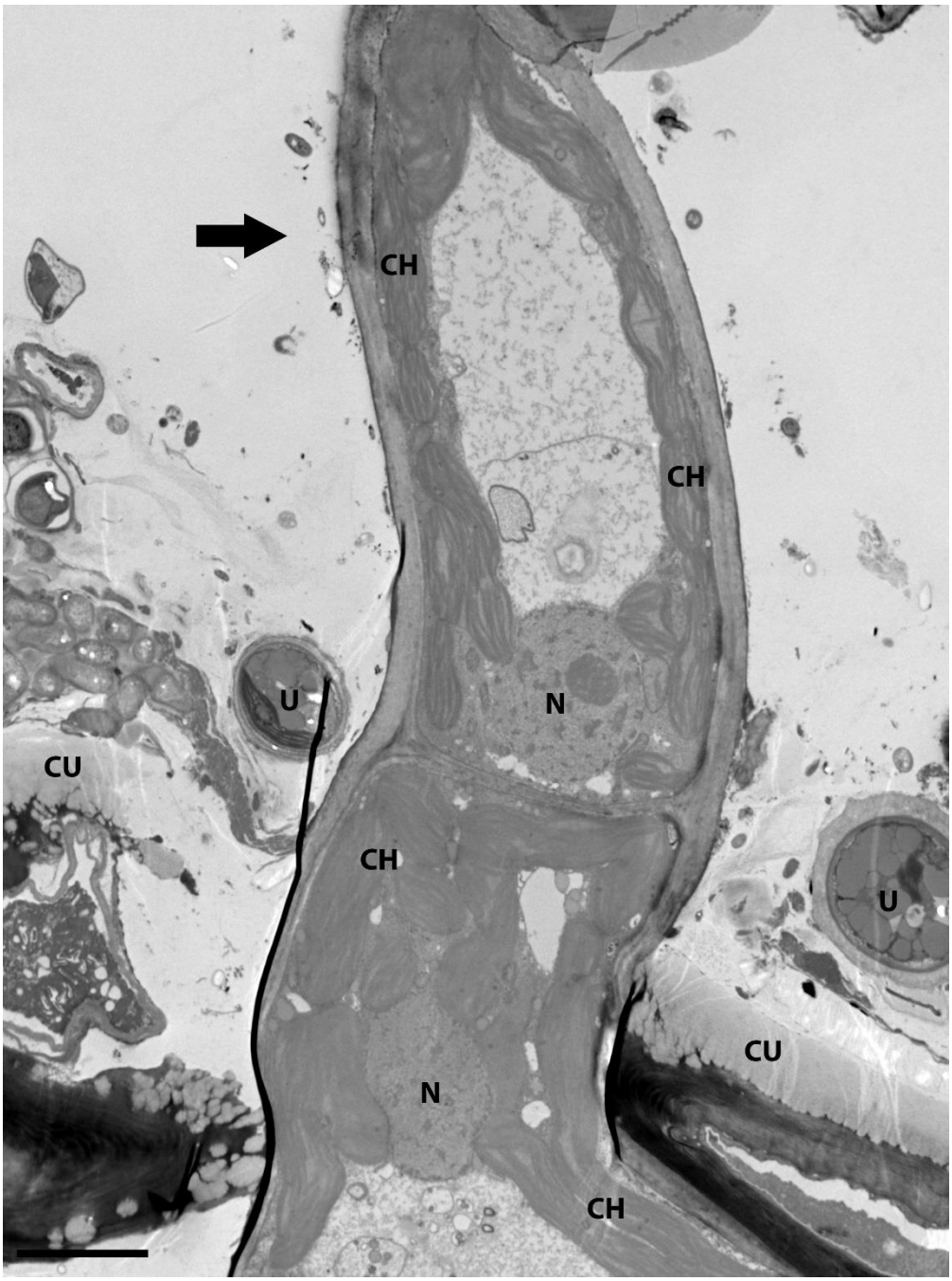

Figure 2.2. TEM micrograph of a *Cephaleuros virescens* sporangiophore (arrow) emerging from an infected blackberry cane, causing a break in the cane surface and cuticle (CU). The sporangiophore shows clearly defined chloroplasts (CH) and nuclei (N). Algal cells are separated by a crosswall (CW). Unidentified organisms (U) are located near the cuticle break. Scale bar = 4 µm.

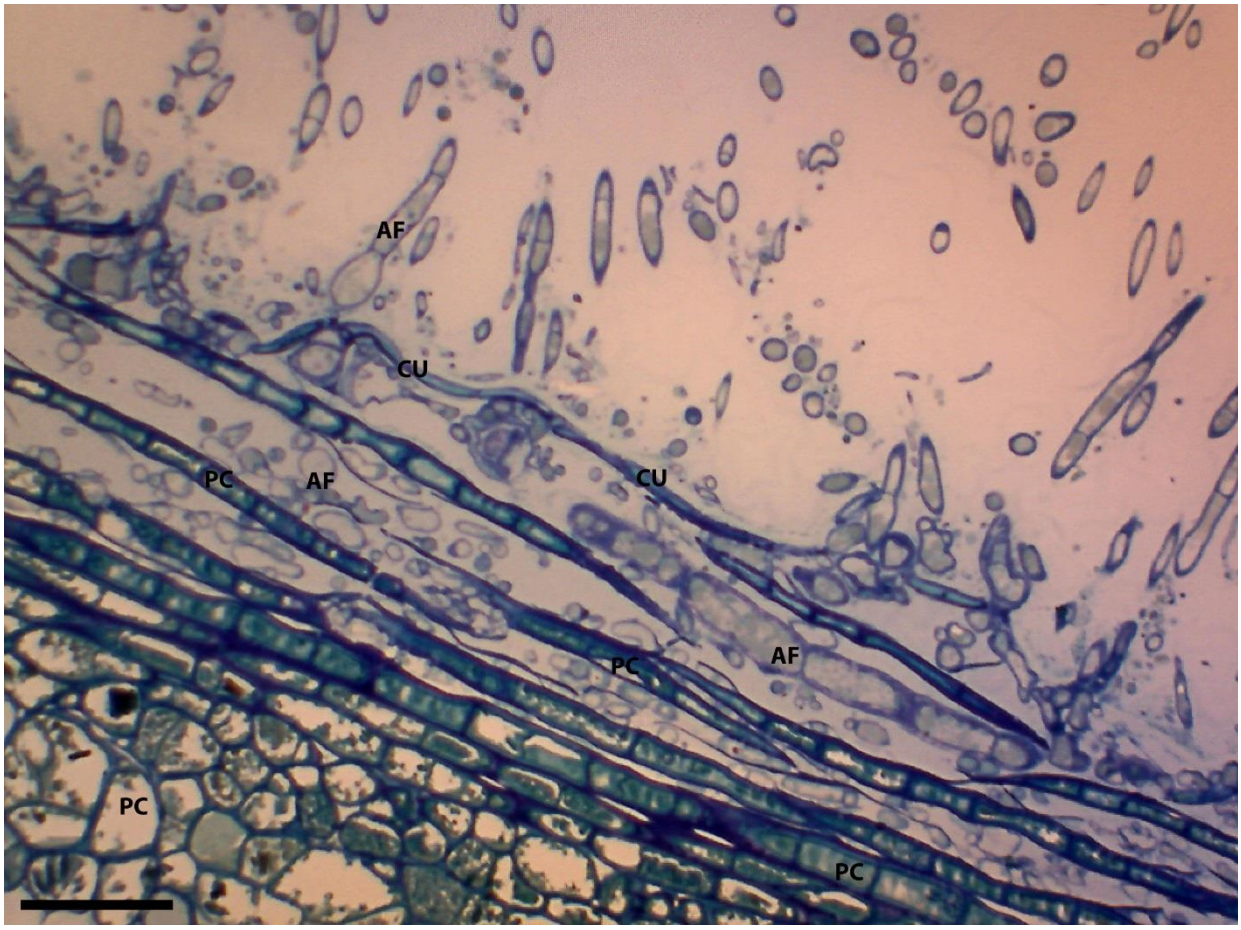

Figure 2.3. Light micrograph of blackberry cane surface infected by *Cephaleuros virescens*. Algal filaments grow intercellularly, disrupting layers of plant epidermal tissue. AF, algal filament; CU, plant cuticle; PC, plant cells; scale bar = 20 µm.

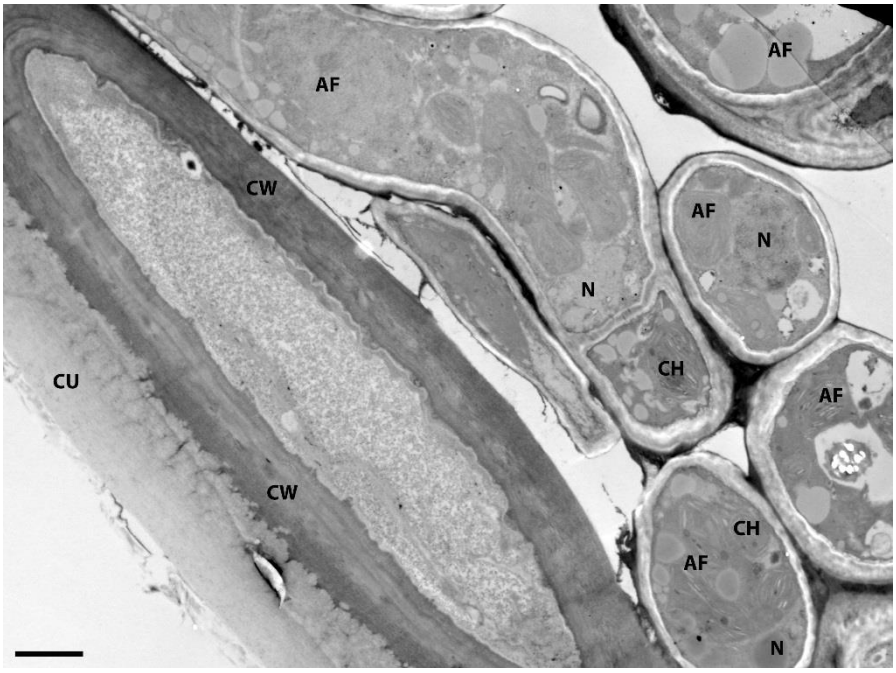

Figure 2.4. TEM micrograph demonstrating intercellular growth of *Cephaleuros virescens* in a blackberry cane. Algal filaments grow between host plant cells, causing physical damage. AF, algal filament; CH, algal chloroplast; CU, plant cuticle; CW, plant cell wall; N, algal nucleus. Scale = 2 µm.

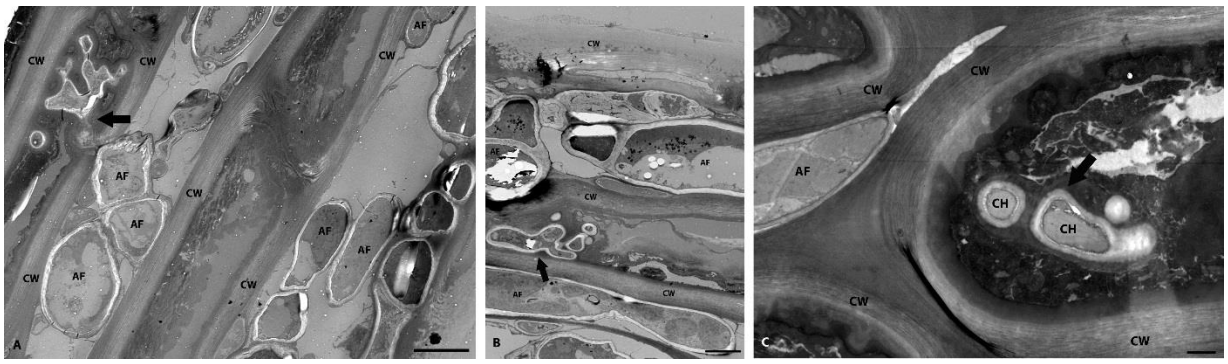

Figure 2.5. TEM micrographs of intracellular growth of *Cephaleuros virescens* (arrows) inside blackberry cane cells. Invading cells show clearly defined algal chloroplasts in C (arrow). AF, algal filament; CW, plant cell wall. Scale bar (right corner) = 6, 4, and 1 µm for A, B, and C, respectively.

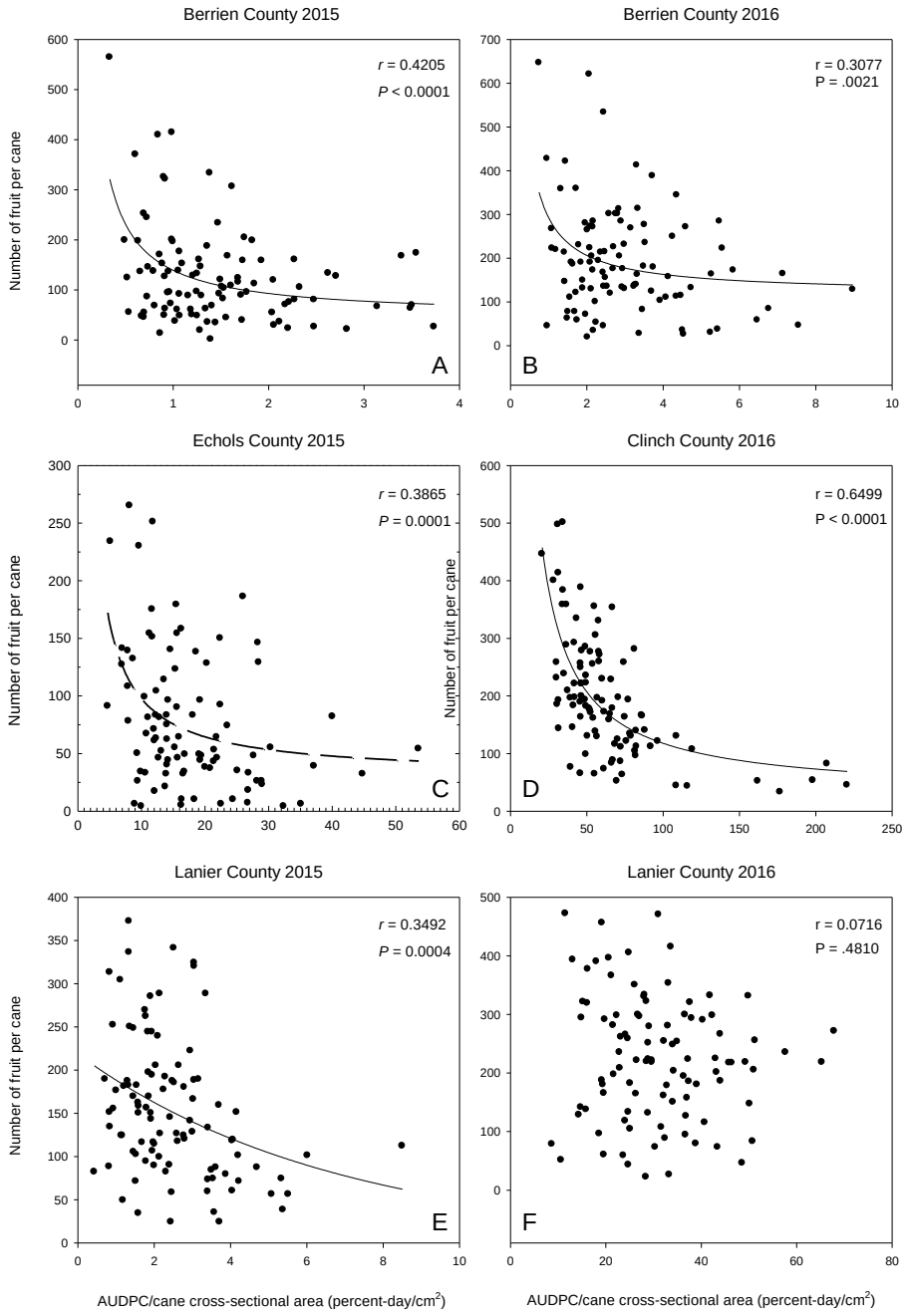

Figure 2.6. Scatter plots relating blackberry yield (number of berries per cane) to cumulative orange cane blotch severity divided by cane cross-sectional area (AUDPC/cane area) in six field trials in southern Georgia. *r* and *P* values are from nonlinear regression analysis.

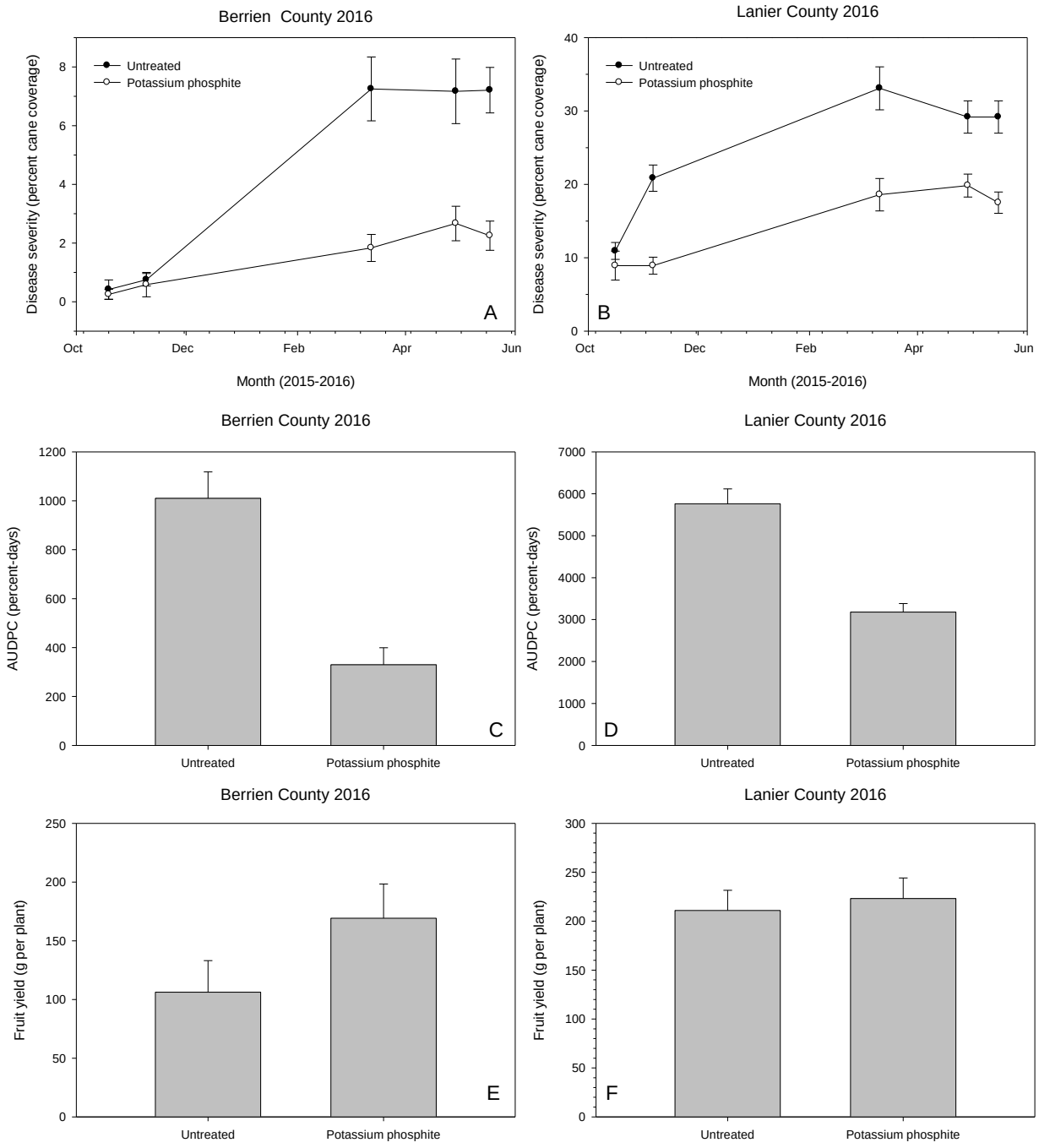

Figure 2.7. Orange cane blotch disease progression (A, B), area under the disease progress curve (C, D), and yield (E, F) in blackberries treated with potassium phosphite compared with the untreated control at two sites in southern Georgia in 2016. Six applications of potassium phosphite were made at ~3-week intervals from August through November. Values are means and standard errors of five replicates at each site.

CHAPTER 3

EVALUATION OF DISINFECTANTS, ALGICIDES, AND FUNGICIDES FOR CONTROL OF ORANGE CANE BLOTCH OF BLACKBERRY IN THE FIELD

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[2]Frances B. Browne, Phillip M. Brannen, Harald Scherm, Jeremy R. Taylor, Justin S. Shealey, Leigh A. Fall, and Edward D. Beasley. To be submitted to: *Crop Protection*

ABSTRACT

The alga *Cephaleuros virescens* causes orange cane blotch (OCB), a serious disease of blackberry in the southeastern United States. Field efficacy trials with diverse algicides, disinfectants, and fungicides were conducted over a 3-year period at two locations on 'Ouachita' thornless blackberry. Foliar treatments, applied during the summer and fall, included ametoctradin + dimethomorph, calcium polysulfide, calcium polysulfide + surfactants, captan, chlorothalonil, copper hydroxide, copper hydroxide + hydrogen dioxide, fluazinam, fluopicolide, hydrogen dioxide, mancozeb, mancozeb + copper hydroxide, mandipropamid, mefenoxam, mefenoxam + copper hydroxide, mefenoxam + mancozeb, potassium phosphite, potassium phosphite + captan, potassium phosphite + copper hydroxide, oxathiapiprolin and diluted sodium hypochlorite. Potassium phosphite was the only chemical that provided consistent and significant disease suppression. Management of OCB will be difficult in light of the limited number of labeled applications of potassium phosphite allowed and the potentially long infection period of the pathogen.

Keywords: blackberry, *Rubus*, orange cane blotch, orange felt, *Cephaleuros virescens*, chemical control

INTRODUCTION

Recent releases of novel cultivars with desirable horticultural traits have led to an expansion of the blackberry industry into the southeastern United States. In particular, thornless blackberry varieties provide easier maintenance and harvestability. They are less cold-hardy and therefore better suited for warmer climates, allowing the bramble industry to experience a 45% production increase between 1995 and 2005 (Strik et al. 2007). Unfortunately, high humidity and warm temperatures associated with southern climes create a conducive environment for yield-limiting cane diseases such as orange cane blotch (OCB), caused by the filamentous alga *Cephaleuros virescens*. This unusual plant pathogen interaction has created considerable concern among producers in the Coastal Plains of the southeastern United States.

Parasitic green algae are considered economically important (Joubert and Rijkenberg, 1971), but are relatively understudied and very little is known about the epidemiology of OCB. Many algal diseases are monocyclic (Suto and Ohtani, 2013). With this said, algal lesions on blackberry canes have been observed to sporulate for long periods of time (2 to 6 months) (Chapman and Good, 1983), resulting in a long period of dispersal and potential infection. Cortex damage has been consistently reported on OCB-infected blackberry plants (Holcomb et al., 1998), and it is thought that the alga can girdle canes at the base of the plant. Intercellular growth in the epidermal layers of plant tissue is common among *Cephaleuros* species (Suto et al., 2014), possibly resulting in the symptomatic cracks associated with OCB infection. These cracks provide wounds where secondary pathogens could gain entry, a combination that could result in further decline and dieback of plants (Brooks, 2004).

Early algal infections may be easily overlooked, as immature thalli may only consist of a few cells. Also, the haematochrome pigment that causes the bright coloration of algal lesions is

only present in small amounts and may make detection of disease difficult in the early stages (Chapman and Good, 1983). Older infections are easily recognized as large, green to yellow-orange, oval blotches on canes. Colonization of large areas of plant tissue is the result of coalescence of algal thalli.

Mature thalli on floricanes produce sporangiophores, which are responsible for the velvet-like appearance of algal lesions (Suto and Ohtani, 2009). Each sporangiophore bears multiple sporangia, each containing several quadriflagellate zoospores, the infective propagule. In addition to the asexual reproduction described above, *Cephaleuros* species can also reproduce sexually through dissemination of biflagellate zoospores from gametangia (Chapman and Good, 1983; Thomas and Wujek, 1997).

The biennial life cycle typical of floricane-fruiting blackberry varieties complicates disease management. In the case of OCB, it is thought that infected floricanes spread inoculum to newly emerging primocanes in the late spring or early summer, possibly as a result of splashing water. Because infected floricanes are the primary source of inoculum, pruning and destroying of floricanes immediately after harvest is recommended to reduce inoculum levels and to prevent further infection. Other management options focus on reducing humidity and free water to decrease zoospore release, movement, and infection, processes that are dependent on water. Such recommendations include: (1) sufficient drainage and adequate bedding for planting areas, (2) selective pruning and cane selection to allow for air movement and rapid drying throughout the canopy, (3) weed control, and (4) use of plastic mulch in combination with drip irrigation. Plant vigor must also be maintained, as stressed plants appear to be more easily infected by algal pathogens (Aliferi, 1969; La Rue, 1923; Schubert, 1981). Excess crop load, plant injury, and other plant diseases can all predispose host plants to severe algal infections (Huq et al., 2010; Ramaya

et al., 2013; Suto and Ohtani, 2011). However, the management options mentioned previously have not provided producers in the southeastern United States with adequate control of OCB, and chemical-management options are needed.

Although application of algicides, specifically copper products, has been recommended, these recommendations have been made in the absence of research specifically on blackberry. Copper hydroxide has been the traditional chemical used for control of algal diseases on several other crops. For example, copper is efficacious for management of *Cephaleuoros* spp. affecting tea, effectively reducing disease severity and resulting in increased yields (Huq et al. 2010); however, the *Cephaleuros* species that infects blackberry stems invades the space underneath a thick waxy cuticle, which may render contact fungicides such as copper less effective. Hence, the objective of this study was to evaluate a range of algicides, fungicides, and disinfectants with varying modes of action (and select combinations of these) in field trials in order to identify the most efficacious products for OCB suppression.

MATERIALS AND METHODS

Algicides, disinfectants, and fungicides were evaluated over a period of 3 years in commercial plantings of the thornless blackberry cultivar ‘Ouachita’. In each trial, treatments were applied to a randomized complete block with five replications. Each replicate consisted of a single blackberry plant, and one plant was skipped between treated plants to minimize spray drift. A CO_2 backpack sprayer (R & D Sprayers, Opelousas, LA) was utilized for applications, and treatments were sprayed until runoff. Treatments, active ingredients, rates, and manufacturer information are listed in Table 3.1. Treatments in 2013/2014 in Lanier County consisted of chemicals commonly used in the food industry to minimize microbial growth, and fungicides/algicides commonly used

on turfgrass for management of algae. Treatments included: chlorothalonil, sodium hypochlorite, ethylene bisdithiocarbamate, mancozeb, copper hydroxide, calcium polysulfide, fluazinam, hydrogen peroxide + peroxyacetic acid, benzoic acid, sorbic acid, potassium phosphite, and an untreated control. Applications were made on 9 September, 23 September, 9 October, and 18 October 2013. Field plots were assessed visually for percent cane coverage by algal blotches on 29 October and 20 November 2013 and on 10 March 2014.

Due to some similarities in life cycles of oomycetes and algae and the fact that the oomycete-active potassium phosphite was the only efficacious material in the first trial, treatments in 2014/15 included several oomycete-active materials in addition to agrichemicals with purported activity against algae. Several products were applied solo and in combination. Field efficacy trials that year were conducted in Lanier and Berrien counties. Treatments included: captan, ethylene bisdithiocarbamate, copper hydroxide, oxathiapiprolin, fluopicolide, potassium phosphite, potassium phosphite + captan, potassium phosphite + copper hydroxide, mandipropamid, mefenoxam + copper hydroxide, mefenoxam, mefenoxam + ethylene bisdithiocarbamante, ametoctradin + dimethomorph + oxo-2-porpenylmorpholine, and an untreated control. Treatments were applied on 25 May, 2 July, 7 August, 22 October, and 19 November 2014. Field plots in Berrien County were assessed on 7 November and 11 December 2014 and on 19 March 2015. Field plots in Lanier County were evaluated on 10 November and 15 December 2014 and on 20 March 2015.

Treatments in 2015/16 again consisted of several products applied solo and in combination. Field trials were again located on commercial farms in Berrien and Lanier counties. Chemicals tested included: ethylene bisdithiocarbamate, ethylene bisdithiocarbamate + copper hydroxide, ethylene bisdithiocarbamate + potassium phosphite, copper hydroxide, potassium phosphite,

potassium phosphite + copper hydroxide, hydrogen dioxide + peroxyacetic acid, hydrogen dioxide + peroxyacetic acid + copper hydroxide, hydrogen dioxide + peroxyacetic acid + potassium phosphite, and an untreated control. Treatments were applied on 7 August, 28 August, 25 September, 16 October, 30 October, and 20 November 2015. Berrien County field plots were assessed for percent cane coverage by algal blotches on 19 October and 9 November 2015 and on 13 March, 29 April, and 18 May 2016. Field plots in Lanier County were evaluated on 16 October and 6 November 2015 and on 11 March, 29 April, and 16 May 2016.

Disease severity (percent cane coverage) was obtained from visually evaluating both sides of the plant and averaging results. Disease severity values were used to create an area under the disease progress curve (AUDPC) in units of percent-days for each treatment. AUDPC values were used for an analysis of variance using the GLIMMIX procedure in SAS (SAS 9.4, Cary, NC) and least squares means were used for comparisons. In cases where potassium phosphite treatments were used solo and in combination, factorial analyses using Fisher's protected LSD test were used to further analyze main effects and interactions of potassium phosphite and the combination treatment.

RESULTS

Relatively high disease severity was observed in Lanier County in the 2013/14 trial (Table 3.2). Potassium phosphite was the only treatment that was significantly different from the untreated control, and no other chemicals suppressed OCB. Of interest, copper hydroxide, the traditional chemical used for control of algal diseases, did not show activity in this trial.

The two sites in Berrien and Lanier counties showed variable disease levels in 2014/15 (Table 3.3). In Berrien County, potassium phosphite and potassium phosphite + copper hydroxide

were the only treatments which provided significant disease suppression. Again, copper hydroxide alone did not suppress OCB, and the other oomycete-active products included in this trial had no effect on the disease. In the companion trial, Lanier County had lower disease levels, and no treatments were significantly different from the untreated control; however, disease levels were numerically decreased by nearly 50% with applications of potassium phosphite + captan and potassium phosphite + copper hydroxide. Captan, copper hydroxide, and potassium phosphite treatments, applied alone and in combinations, were further analyzed with a 2 X 3 factorial for both locations. Berrien County results showed a significant potassium phosphite main effect ($P < 0.0001$), whereas neither the main effect of the companion product ($P = 0.3793$) nor the interaction ($P = 0.2620$) were statistically significant (Table 3.4). The factorial analysis conducted with the data from Lanier County, where disease severity was lower, showed a similar general pattern (Table 3.5), but no statistically significant effects were observed ($P = 0.2376$, 0.4011, and 0.7919 for the potassium phosphite main effect, companion product main effect, and interaction, respectively).

As in the previous year, field trial locations in Berrien and Lanier counties experienced very different disease levels in 2015/16. Berrien County data showed abnormally low disease, but some treatments still showed efficacy in that potassium phosphite, potassium phosphite + copper hydroxide, and potassium phosphite + hydrogen dioxide + peroxyacetic acid were significantly better than the untreated control (Table 3.6). Ethylene bisdithiocarbamate + hydrogen dioxide + peroxyacetic acide also provided significant efficacy, but this was not observed in the companion trial. Indeed, other than potassium phosphite, no other solo chemicals tested in Berrien County showed efficacy against OCB. Lanier County experienced much higher levels of disease than Berrien County, and ethylene bisdithiocarbamate, potassium phosphite, potassium phosphite +

ethylene bisdithiocarbamate, and potassium phosphite + copper hydroxide treatments significantly suppressed disease at this site. A 2 X 4 factorial analysis containing copper hydroxide, ethylene bisdithiocarbamate , hydrogen dioxide + peroxyacetic acid, and potassium phosphite as solo and combination treatments (Tables 3.7 and 3.8) demonstrated significant main effects of potassium phosphite (P = 0.0001 for both Berrien and Lanier counties), whereas neither the main effect of the companion product nor the interaction were statistically significant (P > 0.0930).

DISCUSSION

OCB is an increasing problem for blackberry producers in the southeastern United States. The disease threatens production, as it is associated with reduced yields (Chapter 2) and may exacerbate secondary diseases. While management strategies have been proposed, they do not effectively control the disease. Some agrichemicals were recommended in the past due to their utility in other crops; however, algal disease management on blackberry may be further complicated by the anatomy of the plant, whereby the waxy cuticle of canes may give the alga protection from the traditionally used contact chemicals such as copper.

Disease levels differed among locations and years, likely due to variable weather and general crop management. However, the different disease levels did not appear to have any major effect on the results observed. While many of the chemicals evaluated in this study were contact chemicals, one systemic fungicide, potassium phosphite, significantly suppressed disease in four out of five trials, and the same pattern of suppression was observed in the fifth trial. All field trials demonstrated the ability of potassium phosphite to decrease OCB disease severity by nearly 50% (Tables 3.2, 3.3, and 3.6). Potassium phosphite has two modes of action, direct toxic action against pathogens and activation of plant defense mechanisms (Spolti 2015). Algal blotches on blackberry

canes treated with potassium phosphite are still visible but are onsiderably reduced in size, in turn affecting disease severity. Potassium phosphite is also active against major oomycete pathogens. For this reason, products active against oomycetes were selected for additional trials; however, none of the other oomycete-active materials suppress OCB.

Coppers were recommended as a chemical control for OCB in the past, because copper has been shown to successfully suppress algal disease on other important crops such as tea (Huq et al. 2010). These recommendations were originally made without research data on blackberry, and copper applications may also cause a certain degree of phytotoxicity. Copper hydroxide did not suppress OCB in any of our trials. This lack of activity of copper may be due to the barrier provided by the protective blackberry cuticle. In addition to copper, all other materials tested with purported activity against algae, such as ethylene bisdithiocarbamate and hydrogen dioxide + peroxyacetic acid, generally did not decrease OCB severity. Ethylene bisdithiocarbamate only suppressed disease in one trial at one location.

CONCLUSIONS

In summary, potassium phosphite was the only treatment that successfully suppressed OCB, regardless of the amount of disease present at a particular test site. Additional epidemiological research should be conducted to determine the correct timing of chemical applications against OCB. Potassium phosphite applications at the time of infection, possibly in early summer, may allow for more efficient control.

Blackberyy producers are encouraged to follow recommended cultural practices to decrease humidity and free water in combination with chemical control. Control of OCB will be difficult when considering the potentially long infection period of OCB combined with a limited

number of applications of potassium phosphite allowed on the label. Additional materials may be required in order to prevent resistance development of algal pathogens. Highly systemic chemicals have shown some usefulness in other crops (Ramaya et al. 2013) and combinations could be effective. Selective breeding for OCB resistant cultivars may also prove useful in the future.

Acknowledgments

We thank the commercial blackberry producers who dedicated acreage of production to these trials. All agrichemicals were donated by their respective manufacturers, and their support is greatly appreciated.

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Table 3.1

Products used in efficacy trials against orange cane blotch of blackberry.

Commercial name	Active ingredient	Rate/ha	Company name	Company location
Bleach	Sodium hypochlorite	21.7 L	Clorox	Oakland, CA
Bravo Weatherstik	Chlorothalonil	4.7 L	Syngenta	Greensboro, NC
Captec 4L	Captan	4.7 L	Arysta LifeScience	Cary, NC
Dithane F45	Ethylene bis(dithiocarbamate)	11.2 L	Dow Agrosciences	Indianapolis, IN
Junction	Mancozeb	3.9 L	SePRO	Carmel, IN
Kocide 3000	Copper hydroxide	2.0 kg	DuPont	Wilmington, DE
Lime Sulfur	Calcium polysulfide	49.4 L	Ag Formulators	Fresno, CA
Omega 500	Fluazinam	1.5 L	Syngenta	Greensboro, NC
Orondis	Oxathiapiprolin	365 mL	Syngenta	Greensboro, NC
Oxidate 2.0	Hydrogen dioxide + peroxyacetic acid	8.2 L	Biosafe Systems	East Hartford, CT
Potassium benzoate	Benzoic acid	151.6 L	Emerald Kalama Chemical	Kalama, WA
Potassium sorbate	Sorbic acid	151.6 L	Emerald Kalama Chemical	Kalama, WA
ProPhyt	Potassium phosphite	4.7 L	Helena	Collierville, TN
Presideo	Fluopicolide	292 mL	Valent	Walnut Creek, CA
Revus	Mandipropamid	584 mL	Syngenta	Greensboro, NC
Ridomil Cu	Mefenoxam + copper hydroxide	2.2 kg	Syngenta	Greensboro, NC
Ridomil Gold SL	Mefenoxam	4.2 L	Syngenta	Greensboro, NC
Ridomil MZ	Mefenoxam + ethylene bis(dithiocarbamate)	2.8 kg	Syngenta	Greensboro, NC
Sulforix	Calcium polysulfide	2.1 L	Ag Formulators	Fresno, CA
Zampro	Ametoctradin + dimethomorph + oxo-2-porpenylmorpholine	1.0 L	BASF	Research Triangle Park, NC

Table 3.2

Efficacy of products applied for management of orange cane blotch of blackberry in 2013/14 in Lanier County, GA.

Treatment	**Rate/ha**[a]	**AUDPC (%-days)**[b]
Untreated control	-------	5478.0 bcd
Bravo Weatherstik (chlorothalonil)	4.7 L	6582.4 ab
Diluted household bleach (sodium hypochlorite)	21.7 L	5630.9 bcd
Dithane F45 (ethylene bisdithiocarbamate)	11.2 L	5065.5 dc
Junction (mancozeb)	3.9 kg	6094.0 abc
Kocide 3000 (copper hydroxide)	2.0 kg	5717.8 bcd
Lime sulfur (calcium polysulfide)	49.4 L	6102.8 abc
Omega 500 (fluazinam)	1.5 L	6050.0 abc
Oxidate (hydrogen dioxide + peroxyacetic acid)	8.2 L	5468.1 bcd
Potassium benzoate (benzoic acid)	151.6 L	6965.2 a
Potassium sorbate (sorbic acid)	151.6 L	4606.8 d
ProPhyt (potassium phosphite)	4.7 L	1464.1 e
Sulforix (calcium polysulfide)	2.1 L	5100.7 dc
LSD		1157.7

[a]Application dates: 9 September, 23 September, 9 October, and 18 October 2013.

[b]Area under the disease progress curve developed from disease assessments on 29 October and 20 November 2013 and 10 March 2014. Means followed by same letter do not differ significantly based on a mixed model analysis of variance of a randomized complete block (P = 0.05).

Table 3.3

Efficacy of products applied for management of orange cane blotch of blackberry in 2014/15 in Berrien and Lanier counties, GA.

Treatment	Rate/ha[a]	AUDPC Berrien County (%-days)[b]	AUDPC Lanier County (%-days)[c]
Untreated control	------	728.2 abcd	491.3 abc
Captec 4L (captan)	4.7 L	834.5 abc	234.8 bc
Dithane F45 (ethylene bisdithiocarbamate)	11.2 L	511.7 cde	262.8 abc
Kocide 3000 (copper hydroxide)	2.0 kg	947.6 ab	436.3 abc
Orondis (oxathiapiproline)	365 mL	853.9 abc	720.3 a
Presideo (fluopicolide)	292 mL	1074.5 a	643.0 abc
ProPhyt (potassium phosphite)	4.7 L	317.3 e	346.0 abc
ProPhyt + Captec 4L (potassium phosphite + captan)	4.7 L + 4.7 L	405.7 de	186.3 c
ProPhyt + Kocide 3000 (potassium phosphite + copper hydroxide)	4.7 L + 2.0 kg	295.5 e	181.0 c
Revus (mandipropamid)	584 mL	627.9 bcde	638.5 abc
Ridomil Cu (mefenoxam + copper hydroxide)	2.2 kg	906.4 ab	399.3 abc
Ridomil Gold SL (mefenoxam)	4.2 L	1039.4 a	668.8 ab
Ridomil MZ (mefenoxam + ethylene bisdithiocarbamate)	2.8 kg	747.2 abcd	478.5 abc
Zampro (ametoctradin + dimethomorph + oxo-2-porpenylmorpholine)	1.0 L	621.4 bcde	602.5 abc
LSD		383.5	456.8

[a]Application dates: 25 May, 2 July, 7 August, 22 October, and 19 November 2014.

[b]Areas under the disease progress curve developed from disease assessments on 7 November and 11 December 2014 and 19 March 2015 in Berrien County. Means followed by same letter do not differ significantly based on mixed model analysis of variance of a randomized complete block ($P = 0.05$).

[c]Areas under the disease progress curve developed from disease assessments on 10 November and 15 December 2014 and 20 March 2015 in Lanier County. Means followed by same letter do not differ significantly based on a mixed model analysis of variance of a randomized complete block ($P = 0.05$).

Table 3.4

Orange cane blotch severity on blackberry following application of ProPhyt (potassium phosphite) with or without companion materials subjected to factorial analysis (Berrien County, 2014/15).

	AUDPC (%-days)[c]		
Other algicide	No ProPhyt (potassium phosphite)	ProPhyt (potassium phosphite)	Average[a,b]
None	728	317	523 a
Kocide (copper hydroxide)	948	295	622 a
Captec (captan)	835	406	620 a
Average[x]	837 a	340 b	

[a]Each value is the mean of five replications. Algicide applications made on 22 May, 25 May, 2 July, 7August, 22 October, and 19 November 2014. Three disease assessments were conducted in the fall of 2014 and spring of 2015 (7 November, 11 December, and 19 March) to develop the disease progress curves.

[b]Means followed by the same letter are not significantly different based on a mixed model analysis of variance of a randomized complete block ($P = 0.05$).

[c]No significant interactions were observed ($P = 0.2620$).

Table 3.5

Orange cane blotch severity on blackberry following application of ProPhyt (potassium phosphite) with or without companion materials subjected to factorial analysis (Lanier County, 2014/15).

	AUDPC (%-days)[c]		
Other algicide	No ProPhyt (potassium phosphite)	ProPhyt (potassium phosphite)	Average[a,b]
None	491	346	419 a
Kocide (copper hydroxide)	436	181	309 a
Captec (captan)	235	186	211 a
Average[x]	387 a	238 a	

[a]Each value is the mean of five replications. Algicide applications made on 25 May, 2 July, 7 August, 22 October, and 19 November 2014. Three disease assessments were conducted in the fall of 2014 and spring of 2015 (10 November, 15 December, and 20 March) to develop the disease progress curves.

[b]Means followed by the same letter are not significantly different based on a mixed model analysis of variance of a randomized complete block ($P = 0.05$).

[c]No significant interactions were observed ($P = 0.7919$).

Table 3.6

Efficacy of products applied for management of orange cane blotch of blackberry in 2015/16 in Berrien and Lanier counties, GA.

Treatment	Rate/ha[a]	AUDPC Berrien County (%-days)[b]	AUDPC Lanier County (%-days)[c]
Untreated control	-------	1010.2 a	5761.7 a
Dithane F45 (ethylene bisdithiocarbamate)	11.2 L	916.2 ab	4502.0 bc
Kocide 3000 (copper hydroxide)	2.0 kg	871.6 ab	4701.3 abc
ProPhyt (potassium phosphite)	4.7 L	329.7 c	3178.3 d
Dithane + Kocide 3000 (ethylene bisdithiocarbamate + copper hydroxide)	11.2 L + 2.0 kg	535.2 bc	4813.0 abc
ProPhyt + Dithane F45 (potassium phosphite + ethylene bisdithiocarbamate)	4.7 L + 11.2 L	652.6 abc	3904.0 dc
ProPhyt + Kocide 3000 (potassium phosphite + copper hydroxide)	4.7 L + 11.2 L	470.8 c	3199.9 d
Oxidate (hydrogen dioxide + peroxyacetic acid)	8.2 L	952.3 a	5258.1 ab
Oxidate + Kocide 3000 (hydrogen dioxide + peroxyacetic acid + copper hydroxide)	8.2 L + 2.0 kg	909.8 ab	5210.8 ab
Oxidate + ProPhyt (hydrogen dioxide + peroxy acetic acid + potassium phosphite)	8.2 L + 4.7 L	540.5 bc	4627.4 abc
LSD		445.6	1413.8

[a]Application dates: 7 August, 28 August, 25 September, 16 October, 30 October, and 20 November 2015.

[b]Areas under the disease progress curve developed from disease assessments on 19 October and 9 November 2015 and 13 March, 29 April, and 18 May 2016 in Berrien County. Means followed by the same letter do not differ significantly based on a mixed model analysis of variance of a randomized complete block ($P = 0.05$).

[c] Areas under the disease progress curve developed from disease assessments on 16 October and 6 November 2015 and 11 March, 29 April, and 16 May 2016 in Lanier County. Means followed by the same letter do not differ significantly based on a mixed model analysis of variance of a randomized complete block ($P = 0.05$).

Table 3.7

Orange cane blotch severity on blackberry following application of ProPhyt (potassium phosphite) with or without companion materials subjected to factorial analysis (Berrien County, 2015/16).

	AUDPC (%-days)[c]		
Other algicide	No ProPhyt (potassium phosphite)	ProPhyt (potassium phosphite)	Average[a,b]
None	1010	330	670 a
Kocide (copper hydroxide)	872	471	671 a
Dithane (mancozeb)	916	652	784 a
Oxidate (hydrogen dioxide)	952	541	746 a
Average[x]	938 a	498 b	

[a]Each value is the mean of six replications. Algicide applications made on 7 August, 28 August, 25 September, 16 October, 30 October, and 20 November 2015. Five disease assessments were conducted in the fall of 2015 and spring of 2016 (19 October, 9 November, 13 March, 29 April, and 18 May) to develop the disease progress curves.

[b]Means followed by the same letter are not significantly different based on a mixed model analysis of a randomized complete block ($P = 0.05$).

[c]No significant interactions were observed ($P = 0.5288$).

Table 3.8

Orange cane blotch severity on blackberry following application of ProPhyt (potassium phosphite) with or without companion materials subjected to factorial analysis (Lanier County, 2015/2016).

	AUDPC (%-days)[c]		
Other algicides	No ProPhyt (potassium phosphite)	ProPhyt (potassium phosphite)	Average[a,b]
None	5762	3178	4470 ab
Kocide (copper hydroxide)	4701	3200	3951 b
Dithane (mancozeb)	4502	3904	4203 ab
Oxidate (hydrogen dioxide)	5258	4627	4943 a
Average[x]	5056 a	3727 b	

[a]Each value is the mean of six replications. Algicide applications were made on 7 August, 28 August, 25 September, 16 October, 30 October, and 20 November 2015. Five disease assessments were conducted in the fall of 2015 and spring of 2016 (16 October, 6 November, 11 March, 29 April, 16 May) to develop the disease progress curves.

[b]Means followed by the same letter are not significantly different based on a mixed model analysis of variance of a randomized complete block ($P = 0.05$).

[c]No significant interactions were observed ($P = 0.0930$).

CHAPTER 4

ORANGE CANE BLOTCH OF COMMERCIAL BLACKBERRIES IN THE SOUTHEASTERN UNITED STATES

[3]Frances B. Browne, Phillip M. Brannen, Harald Scherm, Marin Brewer, Susan Wilde and Elizabeth Richardson. To be submitted to: *Plant Health Progress.*

ABSTRACT

Recent expansion of the commercial blackberry industry into warmer climates has led to exposure of blackberry plants to several limiting diseases. Among these, orange cane blotch (OCB), caused by the alga *Cephaleuros virescens*, is prevalent on blackberry canes in the southeastern United States. Symptoms and signs produced on canes include brightly colored green to yellow-orange blotches that often result in cracks in the bark. Decreased fruit yield associated with OCB has been documented in commercial blackberries in Georgia. Disease progress curves showed that symptoms appear in late summer to early fall, and lesions continue to expand throughout the winter and spring of the following year. Preliminary phylogenetic studies revealed that algal isolates from blackberry clustered separately from those isolated from blueberry. Cultural and chemical control measures are being recommended to suppress OCB, with potassium phosphite currently being the most effective chemical control.

Keywords: Orange felt, parasitic alga, yield loss, chemical control.

PLANT HEALTH BRIEF

Orange cane blotch (OCB) is a cane disease affecting commercial production of blackberries (*Rubus fruticosus*) in the southeastern United States, mainly in the Coastal Plains region. The causal agent is a parasitic alga, *Cephaleuros virescens* (Holcomb 1986). The bramble industry has experienced a dramatic expansion due to release of blackberry cultivars capable of substantial production in warmer climates (Strik et al. 2008). While these cultivars have several attractive qualities, such as thornless stems and fruit with great consumer appeal, they are also susceptible to algal infection. OCB was first reported from Texarkana, AR, on the popular blackberry variety 'Navajo' (Holcomb 1986), with a subsequent report on blackberries at a research station in north-central Louisiana (Holcomb 1998).

Algal colonies on blackberry canes present themselves as oval blotches that range in color from green to yellow or bright orange (Fig. 4.1A). Some colonies and underlying plant tissue have been observed to desiccate over time, leading to cracking on blackberry canes (Fig. 4.1B). The cracks associated with OCB may provide entry points for infective propagules of secondary pathogens (Brooks 2004), although this has not been confirmed experimentally. Colonies are composed of branching algal filaments that grow beneath the host cuticle (Fig. 4.2). Although the algal filaments only colonize the epidermal layers near the surface of the cane, colonies coalesce and can cover the entire cane.

While the life cycle of *Cephaleuros* algae has been reported in general terms, the biennial growth pattern of blackberries is critical for understanding the disease cycle. Primocanes (initial vegetative canes) emerge in late spring, as floricanes (flower and fruit-bearing canes) are actively producing fruit. As soon as the harvest is over, old floricanes are removed (mid-summer) as a cultural disease management practice, and primocanes remain to continue the biennial cycle.

Primocane emergence coincides with sporulation of algal lesions on floricanes; these sporulating lesions consist of masses of sporangiophores having a velvet-like, yellow to orange appearance (Fig. 4.3), and OCB is often confused with rust diseases as a result. Sporangiophores with multiple sporangia (Fig. 4.4) are produced on mature floricanes for 2 to 6 months during the spring and summer (Chapman and Good 1983). Rain splash and berry harvest are responsible for the spread of zoospores from mature floricanes to the new primocanes, with heavier infection occurring at the cane base. OCB is assumed to be monocyclic, based on what has been reported for similar algal diseases in other tropical and subtropical crops (Suto and Ohtani 2013).

In order to better understand OCB development, disease progress curves were derived in commercial blueberry plantings in three Georgia locations (Berrien, Clinch, and Lanier counties) in 2015/16. One-hundred untreated 'Ouachita' canes were assessed 5 to 6 times from October 2015 through May 2016. Percent of algal cane coverage was assessed visually at each assessment date, and disease severity was plotted over time to create disease progress curves. At all three sites, symptoms appeared in the early fall, and algal lesions continued to expand throughout the winter, spring, and early summer (Fig. 4.5). Disease pressure varied across locations, possibly due to variation in weather patterns and differing management strategies in previous years.

Parasitic algae deplete host plants of water and nutrients (Wolf 1930), secrete harmful secondary metabolites (Joubert and Rijkenberg 1971), and cause significant necrosis of green tissue (Safeeulla and Govindu 1948). Although the majority of necrosis occurs in the upper layers of the epidermis (Suto et al. 2014), complete or partial girdling of canes may result. In our previous work, *C. virescens* has been shown to cause significant blackberry cane damage, resulting in decreased fruit yields with increasing disease severity (Chapter 2). Reducing disease severity over

time is therefore critical to maximizing yields. For a disease that develops over a period of 1 year, effective disease management is difficult to achieve, particularly once infections are established.

Several management strategies have been recommended to manage OCB. Many recommendations are focused on reducing air humidity and free water and removal of inoculum. Management options include the following: 1) proper water drainage and bedding, 2) herbicide use to reduce weed density, 3) strategic pruning to allow for air flow throughout the canopy, 4) removal and destruction of infected floricanes following harvest, and 5) maintenance of stress-free plants, since stressed plants may be more susceptible to disease (Chapman and Good 1983). Potassium phosphite (a fungicide with both direct and host-induced modes of action), has been shown in our studies to successfully suppress OCB progression in multi-year trials in commercial blackberry plantings (Chapter 3). Hence, a combination of chemical and cultural controls should provide substantial disease reduction. Nevertheless, control of OCB will be difficult due to the presumed long infection period and a limited number of applications allowed for potassium phosphite on product labels. Additional research is needed to determine exact timing of infection to allow for more effective timing of chemical control.

Cephaleuros virescens is also a pathogen of blueberry, particulary in Florida, where it is reported to cause plant death in some cultivars (Schilder 2017). In order to determine relatedness of blueberry and blackberry isolates, algal filaments from lesions on blackberry canes and blueberry twigs were cultured on fungicide amended Bold's basal medium (Bischoff and Bold 1963). The fungicide benzimidazole (benomyl, metyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate) was added at a concentration of 10 ppm (Benlate 50WP, Dupont, Wilmington, DE), which does not inhibit algal growth (Suto and Ohtani 2010). Algal lawns appeared bright green, produced raised thallus, and developed in a roughly circular growth pattern

characteristic of *C. virescens* (Fig. 4.6). Colonies did not produce sporangiophores in culture, because *C. virescens* may require host plant hormones for reproduction (Chapman and Good 1983).

DNA-based phylogenies generated over the past decade revealed that many of the morphological characteristics commonly used to classify filamentous algae have no phylogenetic significance (Rindi et al. 2009). When we compared 18S rDNA sequences of algal isolates (Table 4.1) from blueberry (5 isolates) and blackberry (4 isolates) in the southeastern United States using maximum likelihood phylogeny (Fig. 4.7), blackberry isolates from different geographical locations clustered together and appeared to be genetically similar to each other and different from the blueberry isolates. Surprisingly, not all reference strains of *C. virescens* and *C. parasiticus* form distinct clades. Hence, there are inconsistencies among available sequence data and species assignment, and more work is needed to conclude unequivocally the species responsible for algal diseases in the southeastern United States.

Blackberry producers in the Coastal Plains region of southeastern United States are encouraged to use proper measures to manage OCB, given the documented negative impact of the disease on blackberry yield. Control of OCB will be difficult in light of the presumed long infection period of the pathogen. However, potassium phosphite has been found to consistently decrease OCB severity in commercial blackberry plantings when applied during summer and fall. Additional investigations on proper timing of chemical applications could result in more efficacious control. Molecular phylogenetic analyses conducted in this study could not distinguish whether the causal agent of OCB is *C. virescens* or *C. parasiticus*. Limited available sequence data restricts molecular investigations; therefore, additional multi-gene phylogenies are needed to validate the species responsible for OCB. A taxonomic revision may be required.

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Figure 4.1. Infected blackberry canes displaying oval blotches associated with orange cane blotch (A). Desiccation occurs in centers of lesions producing symptomatic cracks (B). Images courtesy of Patrick Willis and Tim Flanders, UGA Extension.

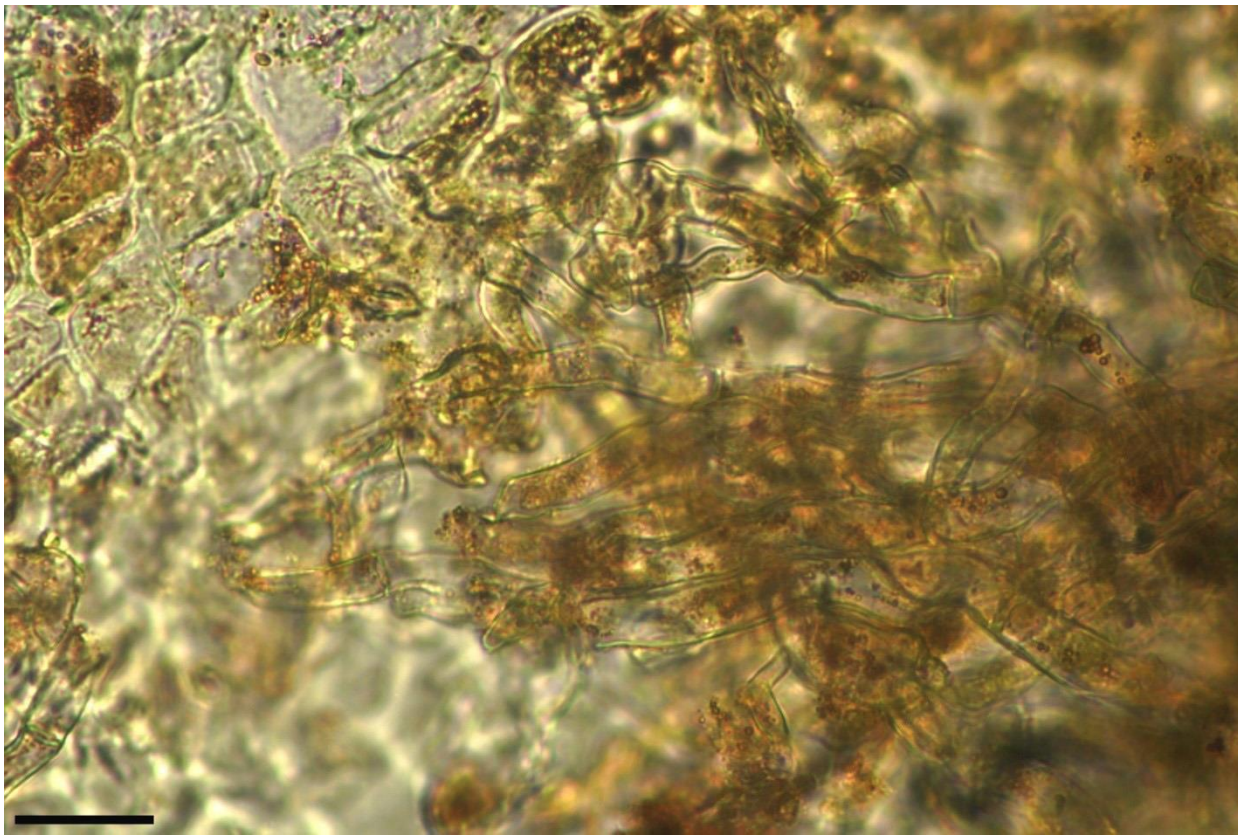

Figure 4.2. Light microscopic image of algal filaments (toward right) growing beneath blackberry host cuticle. Healthy plant cells can be observed in the top left corner. Magnification = 400X; scale bar = 20 µm.

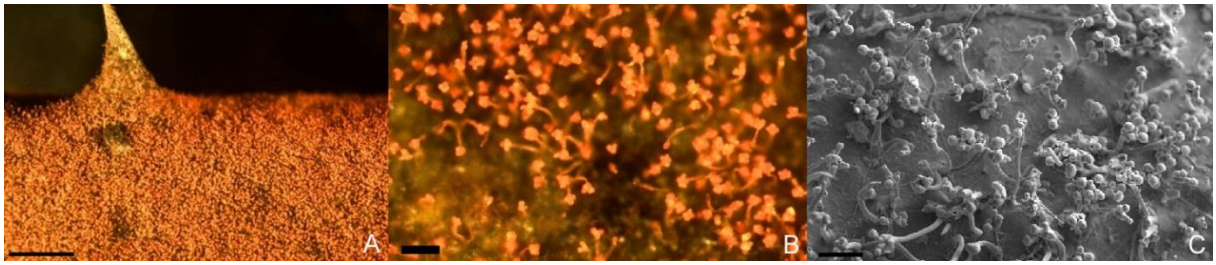

Figure 4.3. Light microscopic images showing sporulation of *Cephaleuros virescens* on infected blackberry cane surface. Macroscopic imagine (A) and close-up (B) of sprangiophores; magnification = 10X and 40X, respectively. SEM micrograph of infected cane surface (C) showing large numbers of sporangiophores arising from algal thallus located beneath the cane surface; scale bar = 0.5 cm, 100 µm, and 100 µm for A, B and C, respectively.

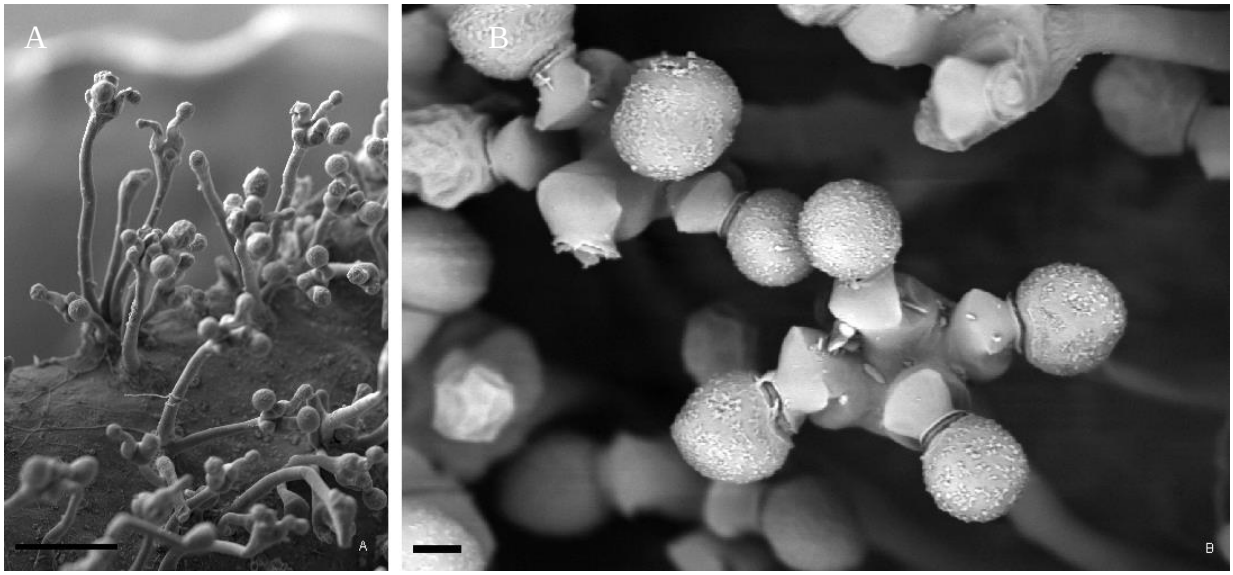

Figure 4.4. SEM micrograph of several sporangiophores of *Cephaleuros viresecens* breaking through infected blackberry cane cuticle (A); scale bar = 100 µm. Each sporangiophore bears multiple sporangia (B); scale bar = 10 µm.

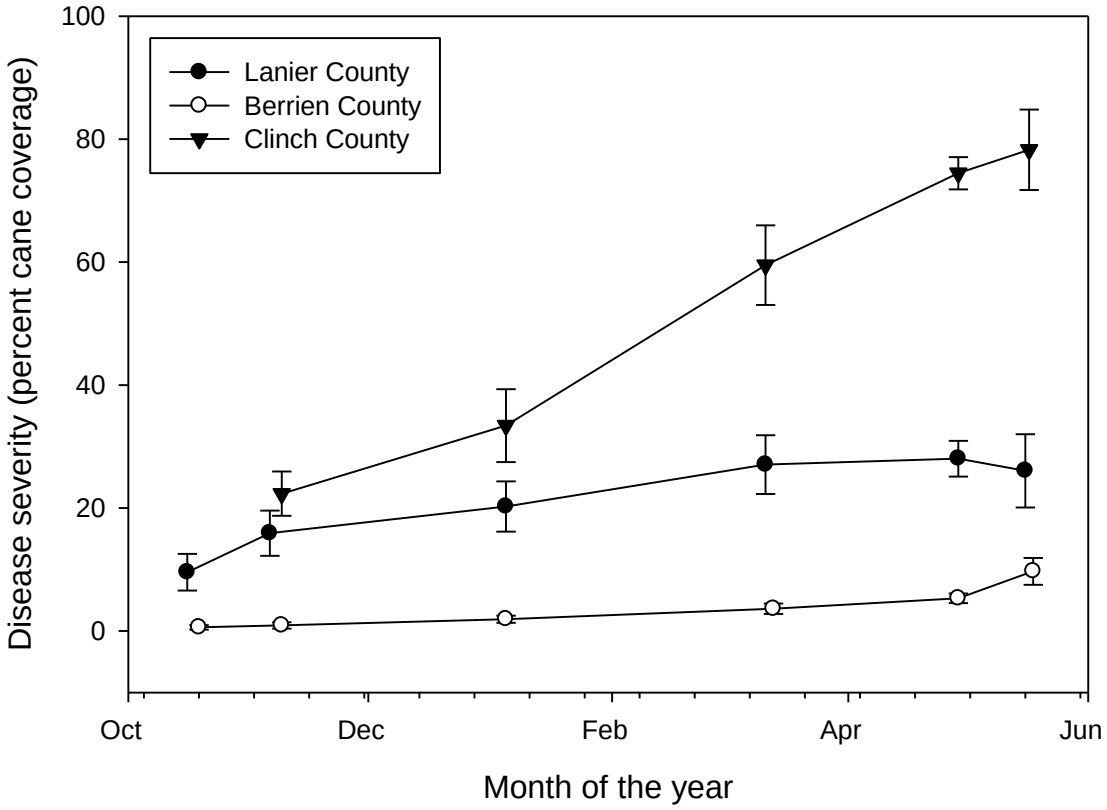

Figure 4.5. Disease progress curves of orange cane blotch in untreated blackberry plots in Berrien, Clinch, and Lanier counties in 2015/16. Data points are means and standard errors of 100 canes per plot on each assessment date.

Figure 4.6. Growth of *Cephaleuros virescens* in culture on Bold's basal medium in a 100-mm petri dish for a period of 6 months. Individual colonies produce raised thallus without sporulation.

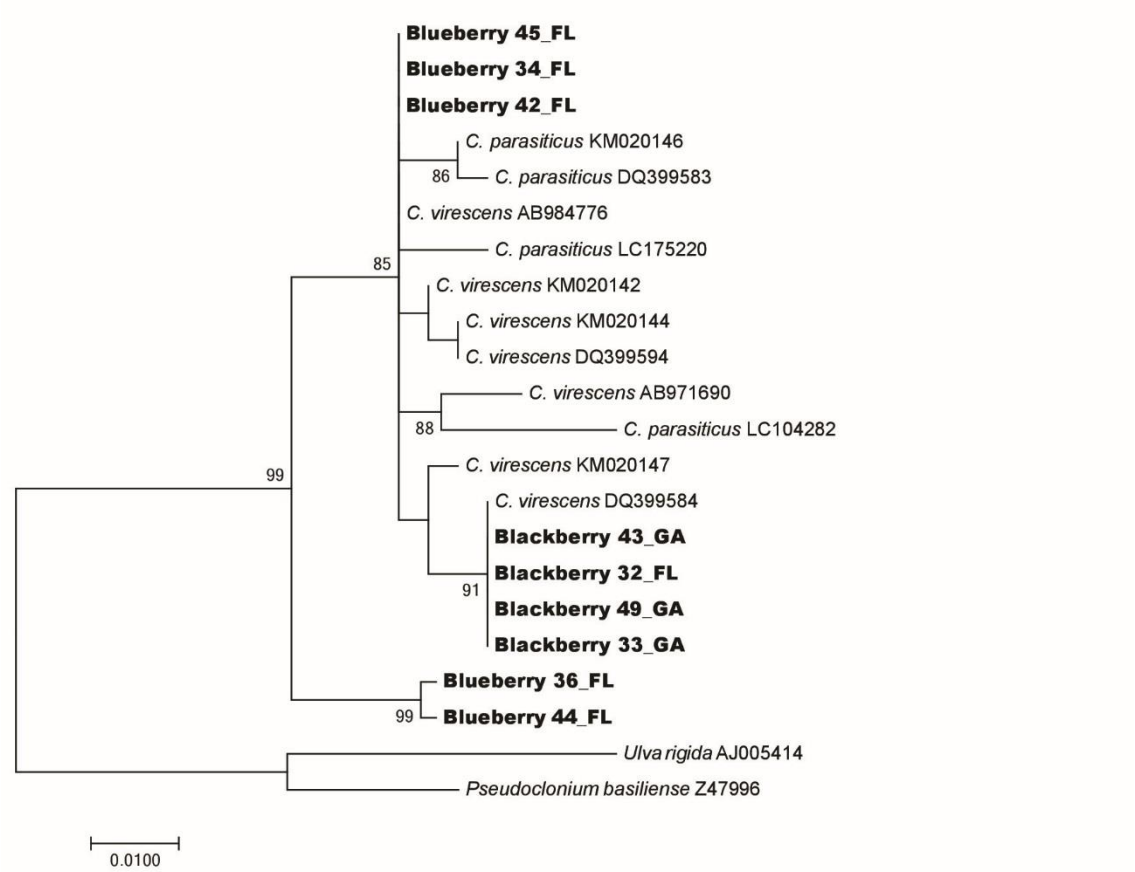

Figure 4.7. Maximum likelihood phylogeny of *Cephaleuros* spp. based on 18S rDNA sequence data. *Ulva rigida* and *Pseudoclonium basiliense* were used to root the tree. Isolates collected in this study are listed in bold with host and location from where the isolate was obtained. Bootstrap support values > 75% are shown below branches. Reference sequences of *C. virescens* and *C. parasiticus* were included (GenBank accession numbers included).

Table 4.1
Algal isolates collected from infected blackberry canes and blueberry shoots.

Isolate number[a]	Host (Common and scientific name)	Location (County, State)
32	Blackberry (*Rubus fruticosus*)	Hernando County, FL
33	Blackberry (*Rubus fruticosus*)	Lanier County, GA
34	Blueberry (*Vaccinium corymbosum*)	Hernando County, FL
36	Blueberry (*Vaccinium corymbosum*)	Hernando County, FL
42	Blueberry (*Vaccinium corymbosum*)	Pasco County, FL
43	Blackberry (*Rubus fruticosus*)	Berrien County, GA
44	Blueberry (*Vaccinium corymbosum*)	Polk County, FL
45	Blueberry (*Vaccinium corymbosum*)	Polk County, FL
49	Blackberry (*Rubus fruticosus*)	Clinch County, GA

[a]Isolate number.
[b]Host of algal pathogen.
[c]Location where isolates were collected (County and State).

CHAPTER 5

CONCLUSIONS

Orange cane blotch (OCB), a prevalent disease affecting blackberry production in the southeastern United States, is capable of causing significant damage to blackberry canes. Algal filaments were observed to grow between epidermal host tissue layers. Sporangiophores emerge from these subcuticular filaments, causing breaks in the cane surface. After relatively long periods of growth, the lesions desiccate and produce symptomatic cracks, creating wounds that could allow for secondary infections. Heavily infected canes may result in partial or complete girdling, interfering with translocation of nutrients to main and lateral shoots from which leaves, flowers, and fruit emerge.

Infection by the OCB pathogen on blackberry canes resulted in decreased fruit yield as disease severity increased. Decreased total fruit weight was strongly correlated with fruit number. This finding has implications for blackberry production in high-risk areas. Individual fruit weight and cane size were generally unaffected by infection. Mature algal colonies, present on floricanes, sporulate and provide inoculum in the form of zoospores for infection of emerging primocanes in the late spring and early summer. Primocanes will produce the blackberry crop for the following season; therefore, OCB may become more severe in subsequent years if not adequately controlled from season to season.

While certain management strategies have been recommended to mitigate OCB in the past, they have not provided adequate control. Copper, the traditionally recommended chemical for control of algal diseases, did not have any effect on OCB development in the field. *Cephaleuros virescens*, the causal agent of OCB, grows beneath the host cuticle and may be protected from

contact chemicals. After testing nearly 20 agrichemicals individually and in combination, the only active ingredient that suppressed OCB when applied during a summer-fall application window was the systemic fungicide and host resistance inducer, potassium phosphite. This particular chemical has direct action against several important plant-pathogenic fungi and oomycetes, and also activates plant host defenses. Applications of potassium phosphite tended to increase yield due to a reduction in OCB severity. Additional work is needed to target the most effective timing for chemical applications.

While the causal agent of OCB has been reported as *C. virescens* based on morphology, molecular phylogenetic analyses in this study indicated that the genetic relationships of the algal pathogens on blueberry and blackberry is complex. Recent papers indicate that many morphological characteristics commonly used to identify filamentous algae are phylogenetically insignificant. According to 18S rDNA analysis, all blackberry isolates collected from Georgia and Florida in this study were similar to each other and different from blueberry algal isolates collected from Florida. Blackberry isolates clustered with a single reference strain of *C. virescens*; however, relationships and taxonomy of *C. virescens* and *C. parasiticus* are unclear based on molecular phylogeny of 18S rDNA. Amplification of the *rbc*L locus resulted in additional complexity in that maximum likelihood phylogeny of blueberry and blackberry sequences were not in agreement with results of the 18S rDNA analysis. In the case of the *rbc*L sequences, not all blackberry isolates clustered together. The results of this study did not confirm the species of the causal agent, and additional research is needed to elucidate the genetic relationships and taxonomy of the alga observed on blackberry and blueberry.

OCB will continue to be problematic in commercial blackberry fields where environmental conditions are favorable. Additional research is needed on the epidemiology of the disease and

proper timing of chemical applications for its control. There is a substantial lack of clarity in classification of filamentous plant-parasitic algae. The genus *Cephaleuros* may require a complete reassessment involving a combination of multigene phylogeny and phenotypic observations in order to correctly identify the causal agents of algal diseases. Blackberry producers should continue to follow recommended management strategies to reduce free moisture and humidity, and in addition, they should apply potassium phosphite to decrease disease, which will result in higher blackberry yields.

APPENDIX A

UTILITY OF DORMANT APPLICATIONS OF HERBICIDES AND OTHER COMPOUNDS FOR CONTROL OF ORANGE CANE BLOTCH

Orange cane blotch (OCB), caused by the parasitic alga, *Cephaleuros virescens*, is recognized by the brightly-colored lesions on blackberry canes. Parasitic algae belong to the kingdom Viridiplantae, along with green land plants. Controlling OCB will be difficult in light of the physiological similarities between the pathogen and host. Many algicides and fungicides have no effect on OCB (Chapter 3); therefore, the efficacy of several herbicides and other chemicals was tested for control of OCB in 2016 in three commercial blackberry plantings in Berrien, Clinch, and Lanier counties in Georgia The thornless cultivar 'Ouachita' was used for this study. Treatments were applied to a randomized complete block with five replications, and each replicate consisted of a single blackberry plant. One plant was skipped between treated plants to minimize spray drift. Treatments, applied at the dormant stage (5 January 2016) included mineral oil,potassium bicarbonate, diquat bromide, hydrogen cyanamide, ammonium sulfate, carfentrazone-ethyl, pelargonic acid, and an untreated control. Treatments, rates, and manufacturer information are listed in Table 6.1. Treatments were applied to runoff using a CO_2 backpack sprayer.

Treatments of diquat bromide resulted in severe phytotoxicity, and host plant death occurred in some cases. Host plants that did survive diquat bromide applications produced large quantities of malformed berries that ripened simultaneously during the spring. No treatments applied consistently suppressed OCB at any of the three locations (Table 6.2). Although no treatment significantly suppressed disease in Berrien County, applications of potassium bicarbonate and mineral oil significantly increased disease at this location when compared with the untreated control. Results in Clinch County showed that treatments of ammonium sulfate and diquat bromide significantly decreased disease compared with the untreated control; however, these treatments did not prove useful in trials at Berrien and Lanier counties. Mineral oil was the only chemical that significantly suppressed OCB in Lanier County, but it did not suppress disease at any other location. The inconsistent results in these trials were likely due to high variability of disease levels and unexplained variation.

Table 6.1

Herbicides and other agrichemicals used in dormant spray trials against orange cane blotch of blackberry.

Commercial name	Active ingredient	Rate/ha	Company name	Company location
Diquat SPC 2 L	Diquat bromide	2.3 L	Nufarm	Burr Ridge, IL
Dormex	Hydrogen cyanamide	12.3 L	Degussa Ag	Trostberg, Germany
Milstop	Potassium bicarbonate	5.6 kg	Bio Works	Victor, NY
QuickSilver	Carfentrazone-ethyl	0.47 L	FMC	Philadelphia, PA
Scythe	Pelargonic acid	41.2 L	Mycogen Coporation	San Diego, CA
Spray-grade ammonium sulfate	Ammonium sulfate	41.2 L	Wilbur-Ellis	Yakima, WA
Superior Spray Oil	Mineral oil	12.3 L	Wilbur-Ellis	Fresno, CA

Table 6.2

Efficacy of herbicides and other agrichemicals used in dormant spray trials against orange cane blotch of blackberry.in Berrien, Clinch, and Lanier counties, 2016.

Treatment	Rate/ha[a]	AUDPC Berrien County (%-days)[b]	AUDPC Clinch County (%-days)[b]	AUDPC Lanier County (%-days)[b]
Diquat SPC 2 L (diquat bromide)	2.3 L	479.1 c	6539.5 bc	3626.3 ab
Dormex (hydrogen cyanamide)	12.3 L	642.0 abc	7219.5 ab	4059.0 a
Milstop (potassium bicarbonate)	5.6 kg	899.3 a	6862.3 abc	3943.5 a
QuickSilver (carfentrazone-ethyl)	0.47 L	705.8 abc	7273.0 ab	3622.5 ab
Scythe (pelargonic acid)	41.2 L	806.7 ab	7572.0 ab	3940.8 a
Spray-grade ammonium sulfate (ammonium sulfate)	41.2 L	814.3 ab	5790.3 c	3781.8 ab
Superior spray oil (mineral oil)	12.3 L	860.4 a	7623.5 ab	2974.8 b
Untreated control	----	568.7 bc	7894.8 a	4059.0 a

[a]Application date: 5 January 2016.

[b]Areas under the disease progress curve developed from disease assessments on 5 January, 11 March, 29 April, and 16 May 2016. Means followed by same letter are not significantly different based on Fisher's protected LSD (P = 0.05)

APPENDIX B

PHYLOGENY OF PARASITIC ALGAE ISOLATED FROM BLACKBERRY AND BLUEBERRY IN THE SOUTHEASTERN U.S. BASED ON 18S rDNA AND CHLOROPLAST-ENCODED LARGE SUBUNIT RUBISCO SEQUENCES

ABSTRACT

Parasitic algae are becoming more prevalent in the southeastern United States. The causal agent of algal diseases on both blackberry and blueberry has been consistently reported as *Cephaleuros virescens* based on morphological characteristics. Nine algal isolates from blackberry in Georgia and Florida and from blueberry in Florida were collected for phylogenetic analyses of 18S rDNA and chloroplast-encoded large subunit RuBisCO (*rbc*L) gene sequences. Maximum likelihood phylogenies could not resolve the species of the causal agent of algal disease of blackberry and blueberry based on existing sequence information, indicating the need for more research on algal phylogeny.

INTRODUCTION

The causal agent of orange cane blotch (OCB) of blackberry has been consistently reported as *Cephaleuros virescens* based on morphological characteristics; however, taxonomy of parasitic subaerial algae has recently been associated with considerable controversy. Historically, morphological characters alone were used for classification (Smith 1950; Ettl and Gartner 1995). DNA sequence data of these algae has been slowly produced over the past 15 years allowing some clarity in the evolution and classification of filamentous algae. Terrestrial green algae are a highly polyphyletic group and are thought to have evolved from both freshwater and marine aquatic algae (Huss et al. 1999; Lewis and McCourt 2004; Lewis and Lewis 2005; Watanabe et al. 2006; Lewis 2007; Cardon et al. 2008). Alternate methods may need to be adopted to correctly classify groups of subaerial algae at the genus and species levels (Pröschold and Leliaert 2007). The lack of DNA sequences available has presented some difficulty in the classification of these algae.

Several parasitic *Cephaleuros* species are classified in the order Trentepohliales. Although algae found in this order are typically associated with tropical regions, they can also occur on bark, leaves, fruit and stems of vascular plants in temperate zones (Thompson and Wujek 1997; López-Bautista et al. 2002; Rindi and López-Bautista 2007). These algae are most often associated with red, orange, or yellow pigments caused by an abundance of carotenoids inside cells. In the past, alga in the order Trentepohliales were classified through use of a morphological scheme developed by Printz (1939). *Cephaleuros* was among one of five genera recognized by this system. The genus included 17 species of filamentous algae. Colonies are composed of irregularly branched, free or coalescent filaments and grow to form irregular shaped discs. These colonies grow under the cuticle or epidermis of vascular plants.

A reassessment of taxonomy may be necessary for filamentous green algae. The following morphological characters have been used for classification in the past: size and shape of vegetative cells, development of thallus, branching pattern, presence of hair-like protrusions, arrangement of sporangiate laterals, shape and size of zoosporangia and suffultory cell, arrangement of gametangia, shape of apical cells, color and presence of corrugations in cell wall, and substrate colonized (Hariot 1890; De Wildeman 1900; Printz 1921, 1939; Sarma 1986; Ettl and Gartnet 1995). These character states can often vary among the same species and at times, the same specimen. Morphological characters of algae in the order Trentepohlia can be influenced by environmental factors (Howland 1929; Nakano and Handa 1984; Thompson and Wujek 1997; Rindi and Guiry 2002). Misidentification of these algae is a common occurrence due the lack of correspondence with morphology and DNA sequences. For this reason, some species in the order Trentepohlia are not always identified to species level, at times only to genus, family, or order.

Few studies have been conducted where phylogeny was examined using molecular data, specifically 18S rDNA sequences. A study by Lopez-Bautista et al. (2006) revealed lack of agreement between the molecular phylogeny and classification of organisms based on morphology. Limited by the use of sequences from a single gene, conclusions suggested the only morphological characters with phylogenetic significance were the subcuticular habitat, heteromorphic life history, and clustering of zoosporangia. Sequence data for Trentepohlialean algae are limited to few 18S rDNA and phragmoplastin loci, restricting species confirmation data (López-Bautista and Chapman 2003; López-Bautista et al. 2003; López-Bautista et al. 2006). According to Rindi et al. (2009), it can be expected that chloroplast-encoded large subunit RuBisCO (*rbc*L) sequences will show improved resolution for classifying specimens at the genus and species levels. Even when partial sequences are used, the *rbc*L locus has been a useful tool

when classifying algae at different taxonomic levels. However, it was reported that *rbc*L has such a high substitution rate in some species that it may not work adequately on all Trentepohlialean algae (Rindi et al. 2009). The goal of this study was to confirm the species of parasitic algae isolated from blackberry and blueberry using *rbc*L and 18S rDNA sequence data.

MATERIALS AND METHODS

Isolation. Isolates of subaerial green algae were collected from Florida commercial blueberry sites in Hernando, Pasco, and Polk counties and from commercial blackberry sites in Berrien, Clinch, and Lanier counties in Georgia and Hernando County in Florida. Samples of algae-infected blackberry canes and blueberry branches were selected in the field. Samples were collected in plastic bags and transported to the lab where algae were cultured on specialized media. Algal filaments were isolated from thalli growing on blueberry branches and blackberry canes. Agarized Bold's basal medium (BBM, Bischoff and Bold 1963) was used for isolations. An organic fungicide, benzimidazole (benomyl, metyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate; Benlate SP, Wilimington, DE), was added to inhibit fungal contaminants at 10 ppm, a concentration reported not to inhibit algal growth (Suto and Ohtani, 2010). Streptomycin was also added at 50 ppm to suppress bacterial growth.

Thalli were isolated by first washing stems under running water for 1 h. Next, sterile cotton wool was dipped in 70% ethanol and used to wipe the surface of stems before rinsing with sterile distilled water three times. A razor blade was used to remove portions of thalli which were placed on agarized BBM (Suto and Ohtani 2010). Algal isolates were then allowed to grow for a minimum of 2 months under constant cool white fluorescent light at 25ºC.

DNA extraction, PCR and sequencing. DNA was extracted from algae isolated from four blackberry isolates, five blueberry isolates, one reference *Cephaleuros virescens* isolate (UTEX, Austin, TX), and one reference *Cephaleuros parasiticus* isolate (UTEX, Austin, TX) using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) in combination with a bead beater apparatus (BIOSPEC, Bartlesville, OK). A GeneAMP PCR 2700 thermal cycler (Applied Biosystems, Foster City, CA) was used for PCR amplification. Overlapping primer sets (Tables 7.1 and 7.2) were used to amplify loci of interest, 18S rDNA and *rbc*L. PCR was performed using these loci as overlapping fragments with multiple primer combinations obtained from literature (Rindi et al. 2009). Each 25-µL reaction contained 0.5 µL of each primer (forward and reverse), 0.5 µL of genomic DNA, 23.5 µL water, and a PuReTaq Ready-To-Go PCR Bead (GE Healthcare Life Sciences, Pittsburg, PA). The same protocol was used for both 18S rDNA and *rbc*L primers and consisted of a denaturation phase at 96°C for 10 s, 40 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 8 min (Rindi et al. 2009). PCR products were examined on an 1% agarose gel stained with ethidium bromide under UV light for correct yield, length, and purity. A Qiagen MinElute Gel Extraction Kit was used to purify the PCR products and a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to quantify the amount of DNA in PCR products before sequencing in both directions using capillary sequencing with Big Dye version 3.1 (Applied Biosystems). Sequence data were obtained with an ABI 3100 automated sequencer (Applied Biosystems).

Geneious 8.0 (Geneious, Auckland, New Zealand) was used to align sequences. Alignments were exported to MEGA7 (Mega Software, Tempe, AZ) to construct maximum likelihood phylogenies. Initially, a model test was used in MEGA7 to select the most appropriate evolutionary model. The K + G (Kimura 2-parameter, gamma distributed) model was selected for

the 18S rDNA sequence alignment and the GTR + G + I (general time reversal, gamma distributed with invariant sites) model was selected for the *rbc*L sequence alignment. Bootstrap analyses were performed with 1000 repetitions. A subset of previously published 18S rDNA sequences, 4 from *C. parasiticus* and 7 from *C. virescens*, along with the outgroups *Ulva rigida* (Tan and Sluiman 1998) and *Pseudoclonium basiliense*. were selected for phylogenetic analyses.

RESULTS

Amplification of *rbc*L (Fig. 7.1) showed a higher substitution rate than that of 18S rDNA (Fig. 7.2). Algal isolates can be found in Table 4.1 (see Chapter 4). The blackberry isolate from Hernando County, FL (isolate 32) did not amplify using *rbc*L primers. Maximum likelihood analysis of the *rbc*L locus in the remaining isolates revealed that most blueberry isolates clustered together with the exception of one isolate from Florida (Fig 7.1). Two blackberry isolates clustered together with a strongly supported branch (100%), and one Georgia blackberry isolate was shown to be more similar to several blueberry isolates, also strongly supported (100%). Bootstrap values with low support (< 75%) were removed from the maximum likelihood tree. Not all *C. virescens* reference strains clustered together, indicating some variability among species or poor taxonomy.

Results from the maximum likelihood tree constructed based on *rbc*L sequence data was not congruent with the tree constructed from 18S rDNA sequence data. The 18S rDNA tree revealed that all isolates from blackberry canes clustered together with a strongly supported branch (91%) (Fig. 7.2). Blackberry isolates clustered separately from algae isolated from blueberry. Furthermore, blackberry isolates clustered with a single *C. virescens* reference strain (DQ399584). Not all blueberry algal isolates clustered together indicating some variation among sequences. Interestingly, neither the *C. virescens* nor the *C. parasiticus* reference strains consistently clustered

together, indicating some diversity among sequences identified as the same species and the need for improved taxonomic treatment of this group.

DISCUSSION

The results of this study have important implications for the phylogenetic relationships among parasitic algae found on blackberry and blueberry in the southeastern United States. Filamentous algae are relatively understudied, and there is a lack of clarity in the classification of *Cephaleuros* species. It appears there is some diversity the same species in published sequence data. 18S rDNA sequences reveal that blackberry isolates are somewhat genetically different from blueberry algal isolates and most blackberry isolates are genetically similar to each other. Parasitic thalli on blackberry only exists for the lifespan of the blackberry cane (2 years); therefore, each year results in new infections. However, in the case of blueberries, the host plant life span may be longer than 10 years in commercial production, meaning infections can persist for long periods of time. This may explain the variability observed in sequence data generated in this study. Plant damage may result in secondary colonization of multiple algal species, both epiphytic and parasitic, complicating species relationships.

Phylogenetic analyses of 18S rDNA and *rbc*L sequences in this study did not show congruent results; therefore, the causal agent of algal disease of blackberry and blueberry cannot be confirmed with certainty, and additional research is needed. *rbc*L gene sequences showed that only two blackberry isolates clustered together whereas most blueberry isolates were similar. The *rbc*L locus is known to have a high rate of evolution, and caution is urged among researchers when using *rbc*L sequence data to estimate phylogenies (Bousquet et al. 1992). This locus is thought to have evolved more rapidly in some modern lineages. Taxa with small population sizes may

speciate more readily, possibly explaining differences among isolates collected from the same host at the same location. 18S rDNA, on the other hand, is more conserved.

A complete reassessment of the order Trentepohliales has been suggested in the past due to a lack of agreement between classification based on morphological characteristics and new sequence data. Additional genes may be useful for species confirmation. Identification of species may have some implications in terms of management for different commercial crops. Because this data cannot confirm the species of the causal agent of OCB, it will continue to be referred to as *C. virescens*.

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Table 7.1. Overlapping primer sets used for amplification of chloroplast-encoded large RuBisCO subunit (*rbc*L) of *Cephaleuros* species.

Primer	Sequence	Orientation	Annealing positions	Reference
FT122	CAGAAGAAGCAGGAGCAGCA	Forward	122-141	Rindi et al. (2009)
FT147	GGCAGAATCATCAACAGGAA	Forward	147-166	Rindi et al. (2009)
*rbc*L 320	TATTCGAAGAAGGTTCAGTAAC	Forward	320-341	Nozaki et al. (1995)
RT994	TCTATCTCCTTCTAATTTTCCTAC	Reverse	971-994	Rindi et al. (2009)
RT1134	CATGTGCCAAATGTGAATACC	Reverse	1113-1134	Rindi et al. (2008b)
R1220	GGTGCATTACCCCATGGGTGTCCTA	Reverse	1195-1220	Rindi et al. (2008b)

Table 7.2. Overlapping primer sets used for amplification of 18S rDNA locus of *Cephaleuros* species.

Primer	Sequence	Orientation	Annealing positions	Reference
PCRA	AACCTGGTTGATCCTGCCAGT	Forward	5' end	Medlin et al., 1988
18B	TGCATGGCTTAATCTTTGAGACAAGC ATATG	Forward	25-55	Hamby et al., 1988
GRC	AGGGCAAGTCTGGTGCCA	Forward	554-571	Hamby et al., 1988
18G	TGGCACCAGACTTGCCCT	Reverse	554-571	Hamby et al., 1988
PCRB	TGATCCTTCTGCAGGTTCACCTAC	Reverse	3' end	Medlin et al., 1988

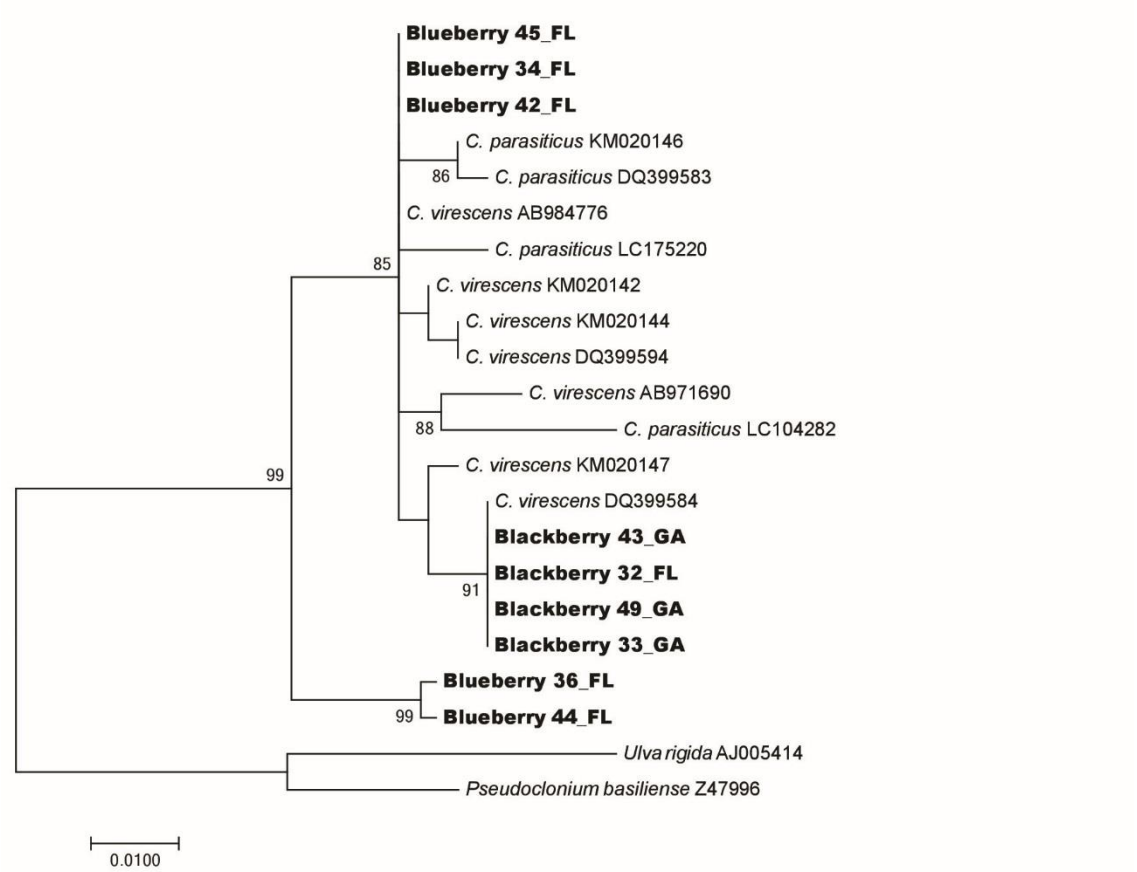

Fig. 7.1. Maximum likelihood phylogeny of blackberry isolates (GA and FL) and blueberry isolates (FL) of *Cephaleuros* spp. based on *rbc*L sequence data. *Ulva rigida* was used to root the tree. Weak bootstrap values (≤ 75%) were removed from branching. Reference sequences of *C. virescens* and *C. parasiticus* were used for comparison (accession numbers included).

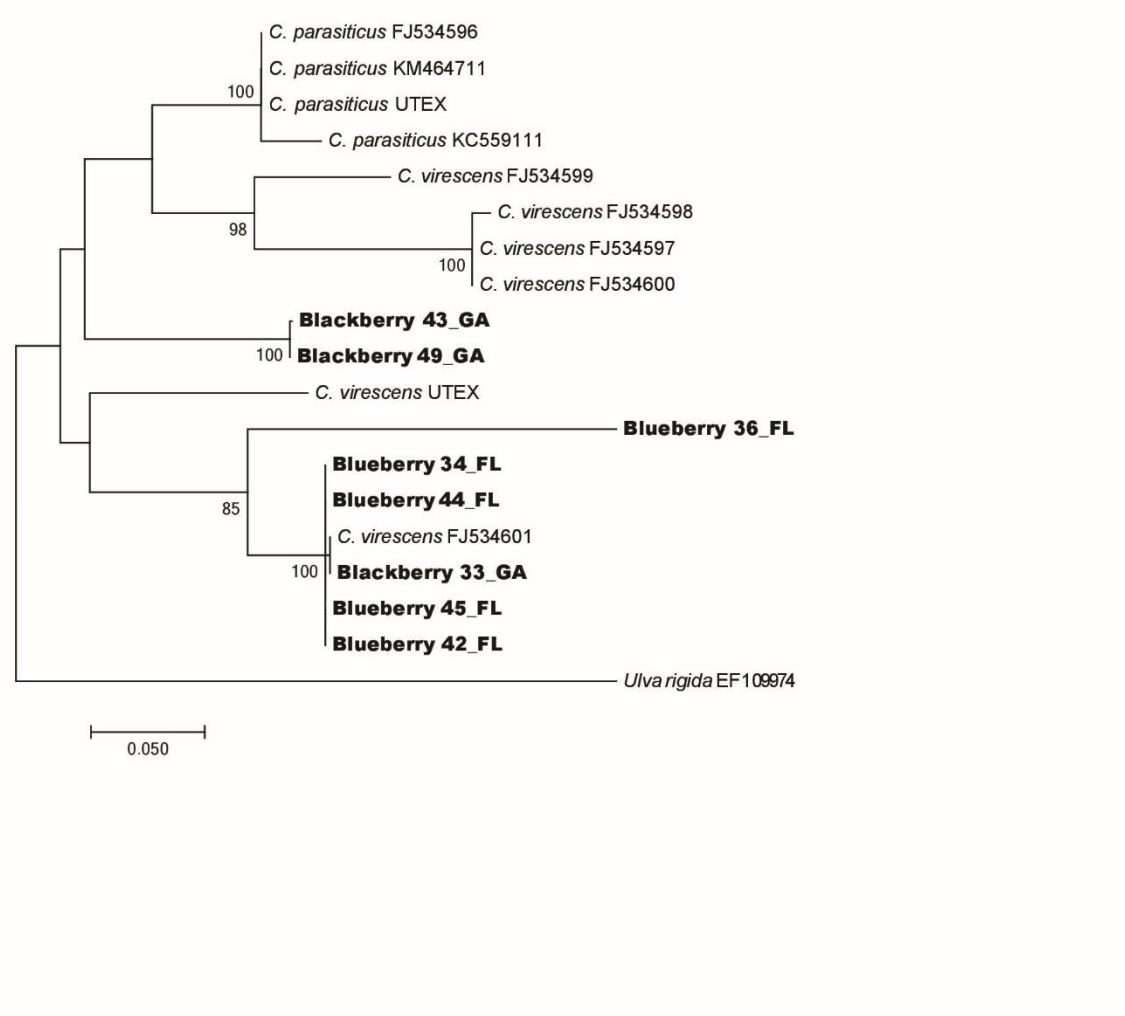

Fig. 7.2, Maximum likelihood phylogeny of blackberry isolates (GA and FL) and blueberry isolates (FL) of *Cephaleuros* spp. based on 18S rDNA sequence data. *Ulva rigida* and *Pseudoclonium basiliense* were used to root the tree. Weak bootstrap values (≤ 75%) were removed from branching. Reference sequences of *C. virescens* and *C. parasiticus* were used for comparison (accession numbers included).