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Distinctive Features of the Orange Cane Blotch Disease Cycle on Commercial Blackberry (*Rubus fructicosis*)

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Abstract: The high humidity and short, mild winters of the southeastern United States are conducive to many plant diseases including orange cane blotch (OCB), caused by the algal species *Cephaleuros virescens* (Cv). Since its discovery on blackberry, its presence has been associated with cane cracking, cane girdling, and yield loss. Research detailing the disease cycle on blackberry is limited and is largely inferred from the interactions of Cv with its other hosts. To further detail the disease cycle of OCB, diseased blackberry canes were examined by photography and microscopy. By combining observations made from photography and microscopy, key events in the disease cycle of OCB on blackberry were elucidated as they correspond to blackberry phenology. The alga was observed to be active for a majority of the season, only exhibiting apparent dormancy from December through mid-April, concurrently with blackberry. While it appeared that the presence of algal sexual reproductive structures did not coincide with emerged primocanes, asexual reproductive structures were observed during the period when primocanes emerged. All new infections on newly emerged primocanes appeared around mid-summer, indicating a single infection cycle per year for OCB. These findings provide a foundation for further study and the development of targeted management strategies for OCB.

Keywords: blackberry; orange felt; orange cane blotch; caneberry; disease

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1. Introduction

Orange cane blotch (OCB) of blackberry, also known as orange felt disease, is caused by the filamentous alga *Cephaleuros virescens* (Cv) [1]. This parasitic alga belongs to the Trentepohliaceae family [2]. It thrives in subtropical and tropical climates [3], and it has been identified on 287 plant species and cultivars [4]. OCB was first reported on blackberry in the summer of 1997 in Arkansas [4]. Since then, emerging epidemics of OCB have been recognized throughout the southeastern United States [4]. Its presence on blackberry is associated with cane cracking [5], cane girdling [1], and subsequent yield loss [6].

The disease cycle of OCB on blackberry is not fully understood [7]. Based on diseases caused by Cv on other host plants, infection is believed to occur when thallus fragments, zoospores, or meiozoospores land on susceptible host tissues. These produce disc-like algal thalli, with filaments penetrating the host cuticle and epidermis [3]. The mode of entry into susceptible host tissues is likely via wounds, natural openings such as lenticels or stomata, or direct penetration of the host tissue [8]. Filamentous algae have been seen largely growing intercellularly, but in some cases they may grow intracellularly [2]. This has been confirmed in blackberry, where intracellular growth was observed on occasion. However, it was not known if algal filaments penetrated these cells or entered only after the cell had been compromised [9]. Following infection and expansion, Cv may undergo asexual and sexual reproduction resulting in the production of new propagules [3]. Algal fragments released from the superficial filaments can act as asexual propagules. Asexual, quadriflagellate

zoospores produced in either terminal or lateral zoosporangia travel efficiently through films of water and are thought to serve as the primary inoculum for Cv [3,10,11]. The sexual propagules of Cv, quadriflagellate meiozoospores, are assumed to infect similarly to asexual propagules; however, their role in the disease cycle of OCB is unknown [7–9]. Meiozoospores are produced by meiosporangia which originate from a zygote resulting from fused biflagellate isogametes. The isogametes are formed within a gametangium and fusion may occur either within the gametangium or externally near the parent thallus [12]. On plant hosts, Cv overwinters in the lesions that it causes, either on fallen or dormant plant tissues [3], and it is believed to overwinter as algal filaments on blackberry. Lesions become apparent throughout the summer and continue to expand and coalesce. Other diseases caused by Cv are monocyclic [13], but this has not been verified with OCB on blackberry [7]. The OCB disease cycle is thought to take 8 to 9 months to complete [1], but it has not been studied extensively.

In this study, the objective was to validate and further characterize the disease cycle of OCB induced by Cv in commercial blackberry plantings. To accomplish this objective, photographic images were used to record the emergence and development of symptoms on the surface of primocanes (first-year canes) and floricanes (second-year canes). Microscopic images were then used to characterize the algal structures that were present within blackberry epidermal tissue and when they were present. Host phenology was recorded throughout the study. Information gathered on OCB from both photographic observations and microscopy were combined to elucidate the disease cycle on blackberry.

2. Materials and Methods

2.1. Commercial Field Sites

Observations of season-long progress of OCB on blackberry were conducted in three southern Georgia commercial blackberry plantings from October 2017 through November 2019. Each site was selected because of its high incidence of OCB. One planting was located in Lanier County (cv. "Osage") and two plantings were located in Irwin County (cv. "Ouachita"). For the duration of the observational work, growers were asked not to spray the studied rows or their two neighboring buffer rows with any phosphonates to minimize extraneous impacts of chemical applications on the algal pathogen. Otherwise, management was consistent with commercial blackberry practices observed in southern Georgia.

2.2. Confirmation of Causal Organism

Isolates of Cv were obtained from infected blackberry tissue collected from all three plantings and plated on Bold's basal medium (PhytoTechnology Laboratories, Shawnee Mission, KS) containing 10 ppm of the organic fungicide benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate) (Chem Service Inc., West Chester, PA, USA) and 50 ppm of the antibiotic streptomycin [5,14]. Algal filaments were taken from growing isolates for DNA extraction using 5% Chelex®100 sodium (Sigma-Aldrich, St. Louis, MO, USA). PCR was performed using GRC [15] and PCRB [16] primers based upon a modified PCR protocol [17]. Specifically, each 20 µL reaction contained 10 µM of 1 µL GRC (AGGGCAAGTCTGGTGCCA), 1 µL PCRB (TGATCCTTCTGCAGGTTCACCTAC), 7 µL molecular grade water, and 10 µL 2× GoTaq® Green Master Mix (Promega, Madison, WI, USA). The PCR protocol consisted of an initial denaturing phase at 95 °C for 1 min, followed by 34 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min, and an 8 min final extension at 72 °C. A BioRad S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) was used for amplification, and the PCR product was visualized on a 1% agarose gel stained with GelRed Nucleic Acid Stain (Biotium, Fremont, CA, USA) using a BioRad Molecular Image Gel Doc XR+ with Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA). The PCR product was cleaned using an E.Z.N.A.® Cycle Pure Kit (Omega Bio-tec, Inc., Norcross, GA, USA), checked for quality using a NanoDrop One C spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), and then sent to Eurofins Genomics (Louisville, KY, USA) for sequencing. Sequences were trimmed, aligned, and edited using Geneious v. 2019.1.3

(Geneious, Auckland, New Zealand). Isolates were confirmed to be Cv by comparing processed sequences to known Cv sequences in the Genbank NCBI database (National Center for Biotechnology Information, Bethesda, MD, USA) using the BLASTn function. Sequences with a greater than 99% query coverage, greater than 98% identity, and an E-value of 0 to the best match sequence in Genbank were considered to belong to the same species.

2.3. Photographic Observation of Diseased Canes

Starting in October of 2017, blackberry canes in the three selected plantings exhibiting symptoms of OCB were arbitrarily selected and secured to wooden stakes using cable ties (Figure S1). A 20-cm zone of interest was marked on each wooden stake. A photograph was taken of this 20-cm zone of each cane every 2 weeks and saved for analysis and comparison over time. The corresponding phenology of blackberry at each photography time point was also recorded. This process was repeated for two full seasons from primocane emergence to floricane removal (October 2017 to July 2018, June 2018 to July 2019, and July 2019 to November 2019). From October 2017 through July 2018, photos were taken of 40 marked canes in total with 20 at the Lanier County location and 10 at each Irwin County location. From June 2018 through July 2019 and July 2019 through November 2019 photos, there were a total of 30 marked canes for each time interval, 10 in Lanier and 10 for each Irwin County location. Blackberry canes were photographed with a Nikon Coolpix B700 camera (Nikon, Tokyo, Japan).

2.4. Microscopy of Diseased Tissue

To observe the progression of OCB within the blackberry tissue and on the epidermal surface, microscopy slides and Z-stack microscopy methods were used to examine samples of diseased tissue. To prepare microscopy slides, a razor blade was used to excise sections of OCB blotches. Sections were placed in a fixative in the field and delivered for processing and staining at the Georgia Electron Microscopy (GEM) facility at the University of Georgia. The fixative consisted of 2% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2). At 4 °C, samples were rinsed two times in buffer for 15 min each and post-fixed in 1% osmium tetroxide for 2 h. Samples were then rinsed at room temperature for 15 min two times in deionized water and dehydrated using a graded ethanol series of 25%, 50%, 75%, 95%, 100%, and 100%, followed by two changes in 100% propylene oxide (PO). Samples were infiltrated in PO and Spurr's resin, with 8 h each in 75% PO and 25% Spurr's, 50% PO and 50% Spurr's, 25% PO and 75% Spurr's, 100% Spurr's, and then 100% Spurr's. Samples were polymerized at 60 °C for 24 h. One-micron-thick sections were cut using a Diatome diamond knife and a Reichert Ultracut S ultramicrotome. Sections were placed on Superfrost Plus slides and dried on a hotplate. Slides were stained with 1% toluidine blue staining solution for 1 min and viewed with light microscopy. Microscopy sections were made from samples collected monthly from December 2017 to June 2018. For each month, nine blotch sections were collected for processing, three from each planting in Lanier and Irwin Counties. Processed sections on glass slides were examined and analyzed for each of the sampling periods, and changes over time in algal vegetative and reproductive structures between healthy and diseased tissue were recorded. Z-stack microscopy, an image processing method which combines images of different focal planes into one homogeneous image, was performed periodically throughout the season. This imaging process captures detailed images of three-dimensional surfaces or objects such as the protruding asexual reproductive structures of Cv. Diseased cane samples were collected from the field and surface features were examined using a Leica DVM6 M S Z-stacking Stereoscope (Leica, Wetzlar, Germany). Changes over time in algal vegetative and reproductive structures between healthy and diseased tissue were recorded.

3. Results

3.1. Confirmation of Causal Organism

DNA sequences of seven algal isolates from the field were obtained [accessions: MN637827-MN637833]. Analysis of these sequences indicated > 98.95% similarity, >99.0% query coverage, and an E-value of 0 to the best match sequences among previously identified Cv isolates available in Genbank (accession numbers KM020145, KM020143, DQ399595, or AY220984). This confirmed that the organism isolated from diseased blackberry plants in these commercial fields was Cv, the known causal organism of OCB.

3.2. Photographic Observation of Diseased Canes

Biweekly photography (Figure 1) provided information about the disease progress on blackberry. In late June through early July, small red spots, less than 1 mm in size, were first observed on primocanes which had emerged in May (Figure 1A,B,O,P). Red spots expanded into clearly identifiable blotches, 2–3 mm in diameter, by August (Figure 1C,Q). Observed blotches continued to expand though early December, at which time blotch expansion ceased as canes entered dormancy. Blotches observed by December (Figure 1G,U) could be traced back through photographic evidence as having originated as small red spots that became visible as early as late June (Figure 1A,O) and as late as August (Figure 1C,Q). Concurrent with cane emergence from dormancy in mid-April, algal spot expansion was observed (Figure 1K,Y). Starting in late May (Figure 1L,Z) through the third week of July (Figure 1N,BB), the blotches on these canes (now floricanes) took on a felt-like texture and appearance. In this study, 100 cane sections were photographed in total, and in all cases, no new lesions were observed after the initial disease development in June through August.

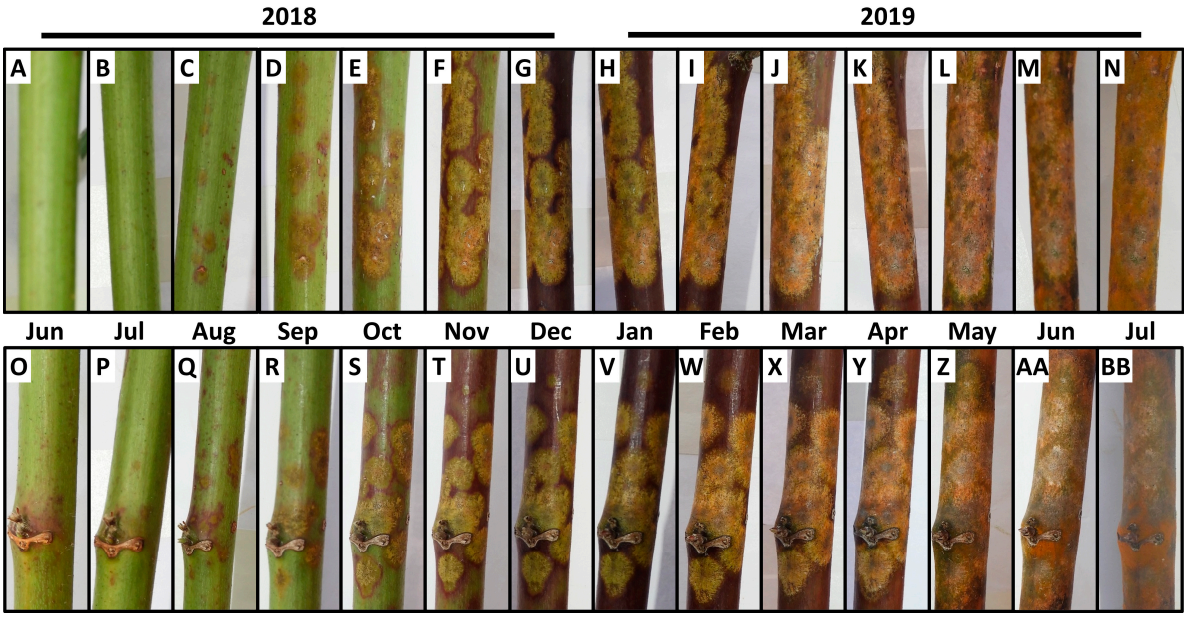

Figure 1. Monthly algal blotch development on blackberry canes that emerged in May 2018. Each row of images (top: (**A**–**N**); bottom: (**O**–**BB**)) corresponds to photographs taken of the same region on a single plant in a commercial blackberry field during the months indicated. All cropped images include ~6 cm of a single infected cane. Photos utilized in panels (**A**–**G**,**O**–**U**) were taken during June through December 2018. Photos utilized in panels (**H**–**N**,**V**–**BB**) were taken during January through July 2019.

3.3. Microscopy of Diseased Tissue

Information gathered from microscopy slides included timing and morphological information about Cv development (Figure 2). Initial observations of algal blotches showed algal filaments separating cane epidermal layers (Figure 2B). Space and filament density

between epidermal cell layers appeared to remain constant during the observed months from December to May. Gametangia were first observed in January (Figure 2C). From January to May, gametes within gametangia became more developed and defined over time. Algal filaments were observed exiting the cane epidermis in April (Figure 2D). Around this time, some gametangia were observed to be collapsed and empty (Figure 2E). In May, nearly all observed gametangia were collapsed and empty. Starting in June, sporangiophores emerged on floricanes and developed sporangia (Figure 2F). Using Z-Stack microscopy, the felt-like material observed in biweekly photography was confirmed to be asexual sporulating structures: sporangiophores bearing zoosporangia. The dense mats of asexual structures (Figure 2G,H) as well as single sporangiophores bearing zoosporangia (Figure 2I) were observed from diseased cane samples.

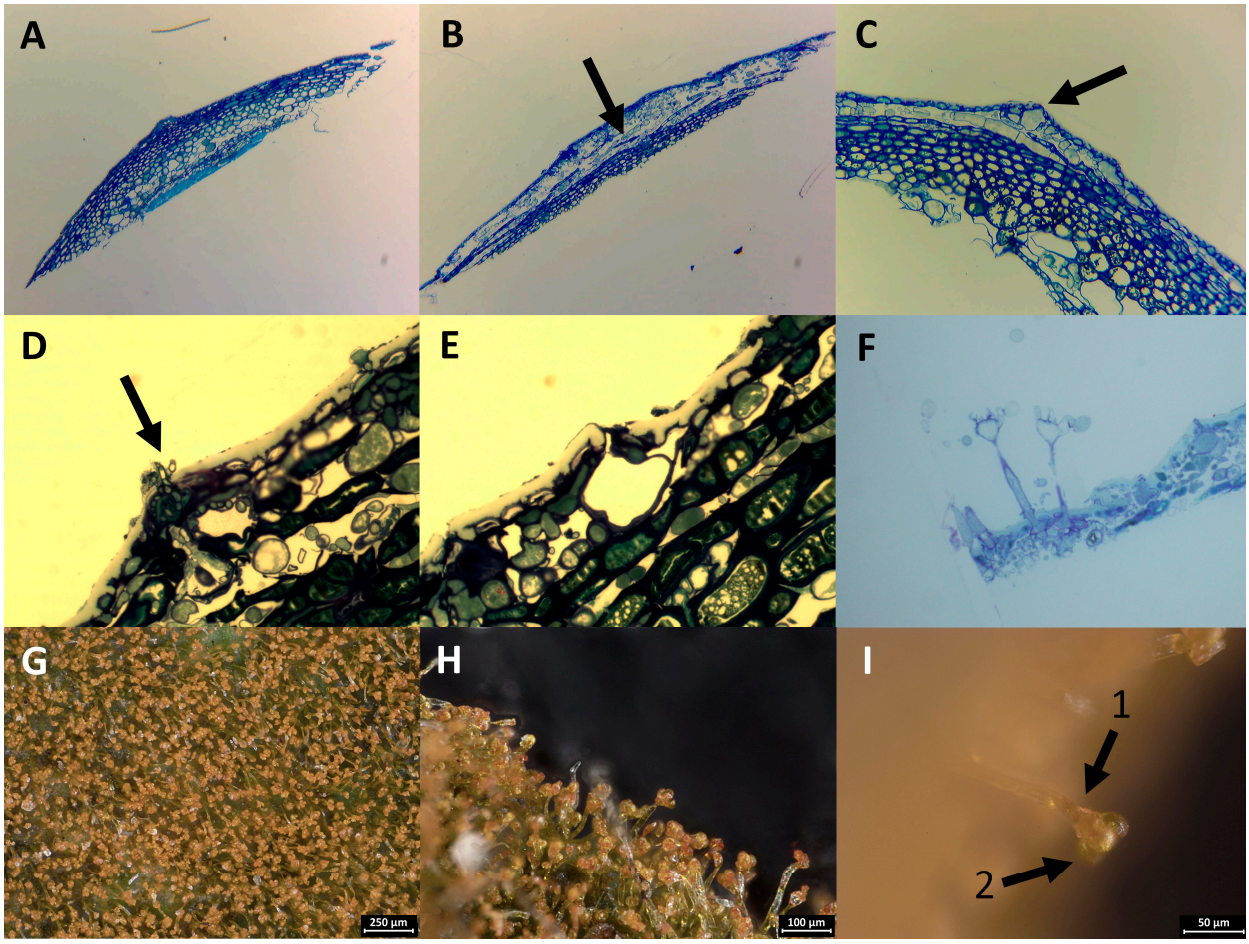

Figure 2. Microscopic imaging of OCB disease on floricanes: (**A**) healthy blackberry epidermal tissue; (**B**) diseased epidermal tissue with algal filaments separating epidermal cells; (**C**) large sexual reproductive cells known as gametangia give rise to isogametes in January; (**D**) algal filaments observed protruding from cane epidermis in April; (**E**) empty gametangia observed in April to late May; (**F**) asexual fruiting structures consisting of sporangiophores bearing zoosporangia containing flagellated zoospores observed in late May to late July; (**G**) dense mats of sporangiophores bearing zoosporangia; (**H**) protruding sporangiophores bearing zoosporangia; (**I**) a single sporangiophore (1) bearing two to four zoosporangia (2). Magnification = 100× for (**A**,**B**), 200× for (**C**), 400× for (**D**–**F**). Scale bar = 250 µm for (**G**), 100 µm for (**H**), and 50 µm for (**I**).

3.4. Observational Timeline

Concurrently with disease monitoring in the field, blackberry phenological stages were recorded. In each year of monitoring, across all three locations, blackberry plants

were observed to break dormancy in April, and primocanes began to emerge in May. On floricanes, fruit maturation was observed to occur from mid-June through mid-July. Following the harvest of mature fruit by the grower (completed by mid-July), blackberry floricanes began to senesce naturally until they were pruned out by the grower during late summer. In December, canes entered dormancy. Blackberry phenological observations, information gathered from bi-weekly photography of diseased canes, and microscopic examination of diseased tissue were combined to create an observational timeline of disease progress as it corresponded to blackberry phenology (Figure 3). After combining the findings on OCB development and the recorded timings of blackberry phenological stages, some associations were noted. For example, concurrently with cane dormancy during December through mid-April, no further visible development and expansion of OCB blotches were observed. Likewise, when the floricanes emerged from dormancy in mid-April, blotches resumed expansion. Of note, gametangia (first observed within blotches in January) were observed to be collapsed and empty largely prior to blackberry primocane emergence in May. By contrast, zoosporangia were present during and after primocane emergence and fruit maturation in June and July. Furthermore, as floricanes began to senesce after harvest was completed in mid-July, the algal blotches present on floricanes likewise began to dry out and presumably die along with their host canes.

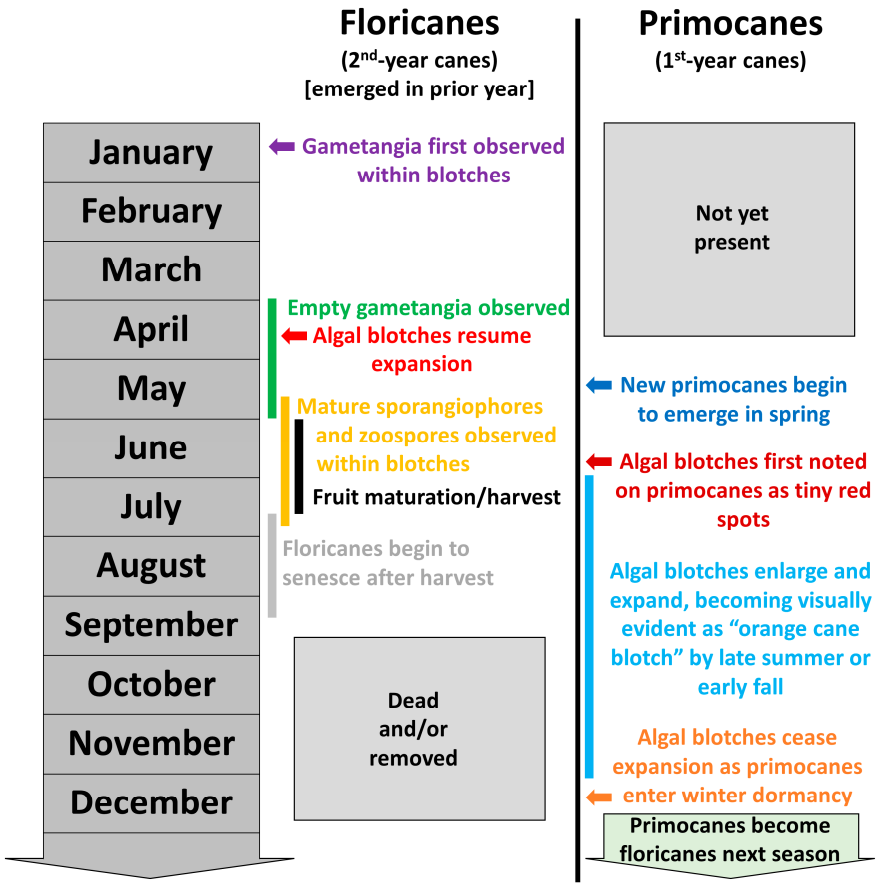

Figure 3. Generalized observational timeline of OCB development and blackberry phenology. Timings of key events in disease development and blackberry phenology are shown relative to a timeline (**left**). Note that primocanes and floricanes exist side-by-side in the field on the same plant; therefore, this timeline enables visualization of concurrent observations on each type of cane present at each time of observation. Observations on mature, symptomatic floricanes (second-year canes) are shown in the center and observations on primocanes (first-year canes) are shown in the (**right**) column. Gray boxes indicate portions of the timeline where each respective type of cane was not yet present or had already been removed from the field (due to death or pruning).

4. Discussion

Based upon our observations, OCB was confirmed to be a monocyclic disease on blackberry in southern Georgia, having one infection cycle per year occurring in the summer. The timing of the appearance of both sexual and asexual reproductive structures of Cv were described in detail. The findings suggested that zoospores likely represent the primary source of initial primocane infection on blackberry, reinforcing a prior suggestion regarding the importance of asexual reproduction [3,10]. During the period in which gametangia were observed to have emptied their contents (presumably biflagellate isogametes), few primocanes had emerged, making it doubtful that sexual propagules play a major role in spread to primocanes. By contrast, primocanes were present for the entirety of the observed asexual reproductive period. In addition, evidence collected from the study suggests that OCB management could potentially be improved by timing control measures to when key algal reproductive structures are present.

Photographic observation of diseased canes revealed many characteristics of the OCB disease cycle. The disease cycle of OCB had not been clarified to date [7]. It was observed that algal development coincided with plant developmental stages. Cv entered and exited dormancy concurrently with blackberry and exhibited the most vigor during the summer months. The OCB disease cycle was theorized to be monocyclic [7,13] and this study revealed several characteristics that support this premise. All blotches observed in December could be traced back through photographic evidence as having originated as small red spots in late June to August. Also, all observed sporulation occurred during the period from late May to early July. Previously, it had been theorized that initial infections may be overlooked considering immature thalli consist of a few cells [5], and this appears to be likely based upon our observations. The hematochrome pigment that gives the algae its orange color is also limited during the early life stages of Cv [8]. We observed that algal blotches start as small red spots, but these are not clearly distinguishable as algal blotches until late August (~2 months after initial symptoms), when their size and pigmentation is much more defined.

Results of microscopic examination validated observations made from field photography and provided more detailed histological evidence of the timing of algal expansion after dormancy and asexual sporulation. Microscopic examination of cross-sections also revealed there may be some algal activity during the dormant season despite the lack of significant blotch expansion during this time. Specifically, gametangia were not evident in samples collected in December, but they were observed in January-collected samples. Based on microscopic observations of diseased tissue, it is theorized that biflagellate isogametes are released from April to late May. Meiosporangia bearing meiozoospores were not observed, suggesting isogametes were the primary pathway of any successful sexual reproduction. However, it should be noted that we did not directly observe isogametes exiting gametangia; rather, an increasing proportion of gametangia were observed to be empty and collapsed throughout this period, suggesting the release of their contents. Structures observed paralleled those detailed in studies of Trentepohliales and *Cephaleuros* spp. [12,13,18,19]; however, in those studies, individual zoospores, isogametes, and/or meiozoospores were observed.

Although both sexual and asexual reproductive structures were observed in our study, our observations suggest that sexual spores play no more than a minor role in infection of new primocanes, as they appear to be released largely before the emergence of primocanes. In other studies of *Cephaleuros* spp., sexual processes have been further characterized. In those studies, the sexual isogametes of Cv never conjugated but germinated directly like asexual zoospores to form a new thallus [13]. This was confirmed first in other species within Trentepohliales, *Printzina* spp. and *Trentepohlia* spp. [19]. They termed these isogamete propagules "biflagellate swarmers" from "presumptive gametangia". Brooks et al. [12] and Suto and Ohtani [13] suggest this same terminology for other *Cephaleuros* spp. Our study was not able to identify these isogamete propagules specifically, and they may have germinated directly. We observed gametangia presumably bearing biflagellate swarmers

(biflagellate isogametes) and empty gametangia, whereas meiosporangia bearing meiozoospores were never observed. Additional work will be needed to further characterize the identity of these propagules within gametangia and to determine what role they are playing in the disease cycle on blackberry. Nonetheless, the fact that observed gametangia had already apparently emptied their contents prior to primocane emergence suggests that these propagules may be of relatively minor importance in the disease cycle on blackberry in Georgia.

The biweekly imaging and microscopy results reported here support previous hypotheses and define the disease cycle of Cv on blackberry. The timing of algal growth, sexual and asexual reproduction, and dispersal have now been detailed on a monthly timeline. Gathered details ultimately expose potential management opportunities. Because a detailed disease cycle was not available previously, developing practical spray programs for this disease has been difficult [5]. This work strongly supports the potential for current chemical management options to be targeted to when they may be more efficacious, such as during production of asexual spores and infection of primocanes. Evidence suggests OCB is a monocyclic disease, meaning there is one infection cycle per season. Targeted management strategies within this period may reduce disease severity on uninfected primocanes by hindering disease development on already diseased canes or provide protection of emerging primocanes from infection. The application of phosphonates has been found to be effective in reducing OCB severity [7]. The current chemical management recommendation is to spray when symptoms on new primocanes are apparent: in late summer until cane dormancy. However, small red spots later determined to be the start of algal blotches were first observed in the middle of the asexual reproductive period in late June. Therefore, it may be advantageous to treat canes with applications of phosphonates prior to and during algal sporulation. Doing so may provide cane protection or inhibit algal sporulation.

Based upon the evidence presented here, future management studies should be carried out. It would be beneficial to further investigate phosphonate application timing and resulting efficacy against OCB development on infected canes and spread to new primocanes. Phosphonates are known to have direct toxic effects on numerous pathogens, but they also induce systemic host resistance by priming plant defenses [20]. Determining the exact effect of phosphonates on algal blotches would aid in maximizing their efficacy in disease management programs targeting OCB. Lastly, given that the asexual sporulation period observed here extended past harvest, studies focusing on after-harvest floricane removal and the resulting impact on OCB dissemination to neighboring uninfected primocanes may be merited. This would help quantify the benefit of recommended cultural management practices and further define the infection period of OCB.

5. Conclusions

While only limited information regarding the disease cycle of OCB was previously available prior to this work, the observational work presented here elucidated several distinctive features of the disease cycle on blackberry. Specifically, though symptoms of the disease had previously been observed in late summer or fall, bi-weekly photography confirmed that disease symptoms first appear on primocanes during late spring and early summer. Furthermore, these photographic observations indicated that all blotches visible by late fall could be traced back to initial infections that occurred during this same period in late spring and early summer, suggesting that OCB has a monocyclic disease cycle on blackberry with a single period of infection per year. In addition, microscopic analysis of diseased plant tissue was able to confirm the presence of asexual reproductive structures (sporangiophores bearing zoospores) during this infection period, suggesting that the infectious propagules of most importance for this disease on blackberry are likely to be zoospores. In addition, microscopic observations indicated the presence of sexual gametangia (assumed to produce biflagellate isogametes) in infected plant tissue; however, these gametangia were largely observed to have emptied their contents prior to the emergence of new primocanes in the spring, suggesting that these may have only a limited role in the disease cycle of

OCB on blackberry. Taken together, these findings should lead to further studies of the biology of OCB disease on blackberry and may suggest opportunities for targeted disease management interventions during the apparently single period of new infections that occur each year.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9050565/s1, Figure S1: Blackberry canes in a commercial blackberry field tied to stakes for bi-weekly imaging of orange cane blotch development.

Author Contributions: Conceptualization: P.M.B. and J.E.O.; methodology: P.M.B., W.H.H., J.E.O. and E.A.R.; software: J.E.O.; validation: J.E.O., W.H.H. and P.M.B.; formal analysis: W.H.H. and J.E.O.; investigation: W.H.H. and E.A.R.; resources: J.E.O.; data curation: W.H.H. and J.E.O.; writing—original draft preparation: W.H.H. and J.E.O.; writing—review and editing: W.H.H., J.E.O. and P.M.B.; visualization: J.E.O. and W.H.H.; supervision: J.E.O. and P.M.B.; project administration: J.E.O. and P.M.B.; funding acquisition: J.E.O. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

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