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Investigating the Etiology, Epidemiology, and Ecology of Blackberry Yellow Vein Disease in South Carolina

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INVESTIGATING THE ETIOLOGY, EPIDEMIOLOGY, AND ECOLOGY OF BLACKBERRY YELLOW VEIN DISEASE IN SOUTH CAROLINA

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by
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ABSTRACT

Blackberry production is growing in popularity in the southeastern U.S. However, blackberry growers in this region report major problems with productivity and longevity due to viruses. First, the state of knowledge on blackberry yellow vein disease (BVYD) complex, associated with mixed infections of a diverse group of viruses, is discussed. Specific viruses associated with symptom types are unknown. Therefore, the etiology of BYVD is not fully understood, which makes BYVD diagnosis challenging. My research focused on improving BYVD diagnosis, etiology, and epidemiology. Second, I developed a farm-level diagnostic protocol to improve virus disease surveillance. High throughput sequencing detected 17 known viruses in the pooled samples, including 11 viruses known to infect blackberry. Third, I investigated the ecology of nine blackberry viruses using a bipartite network analysis approach. More mixed virus infections were detected in the symptomatic and wild plants compared to the asymptomatic plants. Virus accumulation in older plants was significantly higher compared to the virus accumulation observed in younger plantings. This analysis also showed that some cultivars, e.g., 'Navaho', harbored more viruses than others. However, no specific virus or virus combination is associated with specific symptomatology, except for oak leaf pattern, which may be associated with three specific viruses. Fourth, I assessed the ingress of five viruses in two new blackberry plantings to evaluate within-season and across-season spread. Virus incidence was low in both sites during the two years of this study, but secondary spread was observed for two viruses, blackberry line pattern virus (BlaLPV) and blackberry virus E (BVE). This study marks the beginning of a long-term epidemiology study, which

will provide a better understanding of virus spread mechanisms in the field. Lastly, I discuss the progress made based my dissertation research, synthesize conclusions in the context of virus disease management, and provide some potential future research directions.

DEDICATION

To God be the glory

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I would like to thank God for giving me strength, patience and resilience during these last four years. I am grateful for my husband, Richardson Bien-Aime; my son, Oliver Rick Dantes Bien-Aime; my parents; my siblings, especially my sister Nephtalie Dantes; my relatives and all my friends, especially Jean Ribert Francois and Vovener Edmond, for their moral support during my PhD. I am thankful for Dr. Elizabeth Jeannette Cieniewicz, my advisor, who has been an incredible mentor to me. She has equipped me with the necessary tools for my professional journey. I could not have achieved this milestone without her guidance. I am also grateful to the nine participating blackberry farms for allowing access and sample collection of their blackberry plants. I also thank the Extension agents Rob Last, Andy Rollins, Anna Sara Hill, and Bruce McLean for their assistance in coordinating with farms and collecting samples. Special thanks to my advisory committee, Dr. Lucas Boatwright, Dr. Juan Carlos Melgar, Dr. Ksenija Gasic, who provided feedback and comments in many ways to improve data analysis. Thanks also to my current and former labmates, Barbara Blackmon, Brodie Cox, Jordan Withycombe, Christophe Tatgenhorst, Garner Powell, Daniela Moreno, Fabian Rodriguez, Mandeep Tayal, and Zoe Marquez de la Plata for assistance with sample collection and processing. A special thanks to Alexandra Ratay for helping me with data collection, sticky card processing, and aphid identification. Thanks to Elise Schnabel for helping me with PCR testing, sequencing and analysis. I am grateful for the collaboration with Pairwise (Durham, NC) for funding the library preparation and sequencing, Dr. Anna Whitfield and Dr. Cesar Augusto Diniz Xavier (North Carolina

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CHAPTER ONE

INTRODUCTION TO VIRAL DISEASES OF BLACKBERRY IN THE SOUTHEASTERN UNITED STATES

Blackberry production in the southeastern U.S.

Blackberry (*Rubus* subgenus *Rubus*) production acreage has increased in the U.S. during the last two decades due to several factors, including the development of improved varieties, efforts in marketing and fruit availability, and an increase in berry consumption (Fernandez 2021). Blackberry production was estimated at 1,083 hectares in nine U.S. states (Alabama, Arkansas, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, and Virginia), although acreage for each farm is notably small (on average, <1 ha) (Fernandez 2021). Blackberry plantings can be productive for 15-20 years, and some commercial fields can be productive for up to 50 years (Martin et al. 2017). Blackberry plantings have a high cost of establishment, estimated at nearly $25,000 per hectare (Poudel et al. 2018).

In blackberry plants, while the roots and the crown, or the portion of the blackberry plant at the soil line, are perennial, each cane is biennial. Canes grow from the perennial root system, with several canes per plant (Fig. 1-1). Blackberry cultivars are either primocane-fruiting (first-year cane growth with five leaflets) or floricane-fruiting (second-year cane growth with three leaflets). However, most of the production has been based on floricane-fruiting cultivars until 2004, when the University of Arkansas released two primocane-fruiting cultivars (Clark and Finn 2014). In the case of floricane-fruiting cultivars, primocanes, which are first-year canes, do not bear fruit in the first year. In the

second year, these canes will become floricanes that bear fruit and then die (Fig. 1-1A). Primocane-fruiting cultivars, on the other hand, fruit on the first-year canes in the late summer and autumn. This production is localized at the tips of the canes (Fig. 1-1B), then this fruiting part dies. If this cane is not mowed to the ground, its remaining part will set leaf and flower buds the following year and bear fruit in the spring, a process also known as double cropping (Fernandez et al. 2023). Based on cane architecture, blackberry can be classified as erect, semi-erect, or trailing. Blackberry cultivars can also be thornless or thorny.

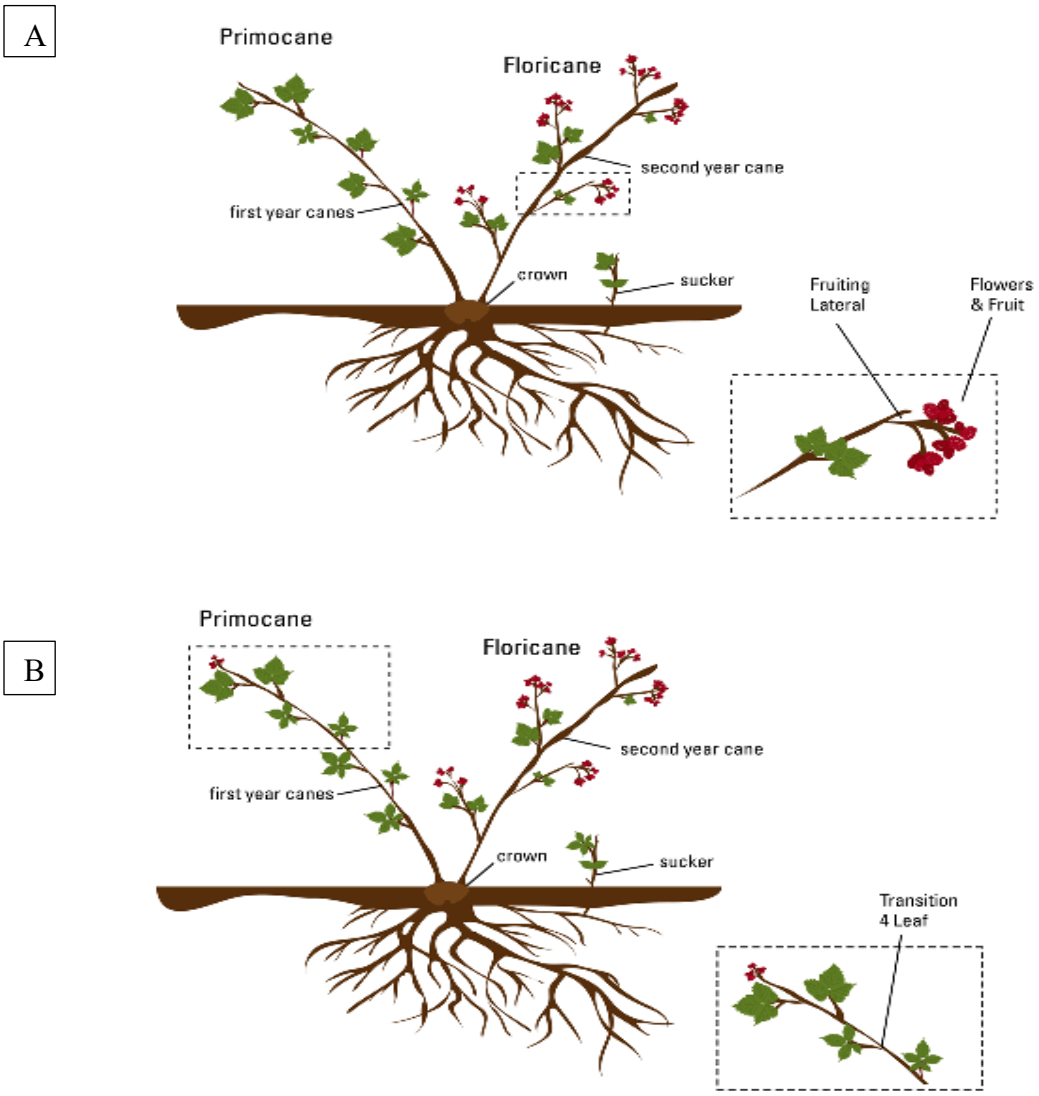

Figure 1-1. Blackberry plant structure or morphology. (A) A floricane-fruiting caneberry with primocane and floricane present. (B) A primocane-fruiting caneberry with both cane types present. The primocane transitions from vegetative to reproductive growth; this occurs when leaves on the canes transition from five leaflets to three leaflets, and the flower bud is apical, not lateral (Fernandez et al. 2023).

Propagation of blackberry plants

Blackberry plants are primarily propagated via root cuttings (Susaimuthu et al. 2007). Micropropagation via tissue culture has also been used to produce virus-negative plants. Excised shoot tips of actively growing blackberry are washed and sterilized for 12-15 minutes in a 0.53% sodium hypochlorite solution. After disinfection, 1 cm of the tissue is placed in the culture media (Anderson 1980). More recently, tissue culture centers have started using less meristematic tissue (Nourse Farms, 2009). About 1 mm of meristem tissue is excised to propagate virus-negative plantlets, which are then grown in tissue culture (Nourse Farms, 2009) and ultimately provided to blackberry growers for planting. Establishing new fields with plants derived from virus-negative sources in tissue culture is one of the main recommendations for managing blackberry virus diseases (Martin et al. 2017, 2013).

Major diseases of blackberry

Blackberries are challenged by various diseases caused by all plant pathogens, including viruses, fungi, oomycetes, bacteria, and even algae (Martin et al. 2017). At least 20 fungal, five bacterial and 16 viral diseases have been reported to cause damage to *Rubus* species worldwide (Martin et al. 2017). These pathogens attack several parts of the plant. Major blackberry diseases that are a high priority in the 2021 Pest Management Strategic Plan for blackberry include anthracnose, cane blight, gray mold, orange rust, and yellow vein disease (Fernandez 2021). Diseases ranked as “emerging” include orange

felt (i.e., orange cane blotch), fire blight, downy mildew, and Armillaria root rot (Fernandez 2021).

Table 1-1. Major diseases that affect blackberry production in the southeastern U.S. compiled from: Schilder et al. 2017; Martin et al. 2017; Fernandez 2021.

Diseases	
Common name	**Causal agents**
Fungal diseases	
Anthracnose	*Elsinoe veneta*
Armillaria root rots	*Armillaria* spp.
Botryosphaeria rots and cankers	*Botryosphaeria* spp.
Cane and leaf rust	*Kuehneola uredinis*
Cane blight	*Leptosphaeria coniothyrium*
Fire blight	*Erwinia amylovora*
Gray mold	*Botrytis cinerea*
Cane botrytis	*Botrytis cinerea*
Double blossom/rosette	*Cercoscoporella rubi*
Septoria leaf spot	*Sphaerulina westendorpii*
Orange rust	*Arthuriomyces peckianus* and *Gymnoconia nitens*
Oomycete diseases	
Downy mildew	*Peronospora sparsa*
Bacterial diseases	
Crown and cane gall	*Agrobacteruim tumefaciens and A. rub*i
Phytoplasma	
Rubus stunt	Rubus stunt phytoplasmas
Parasitic Alga	
Orange cane blotch/ Orange felt	*Cephaleuros virescens*

Virus diseases of blackberry

Viruses and their vectors are classified as the major limiting factor in blackberry production according to a survey conducted in 2020 preceding the 2021 pest management

strategic plan (PMSP) meeting (Fernandez 2021). In fact, *Rubus* spp. can be affected by more than 43 viruses, viroids, or graft-transmissible bacteria (Martin et al. 2017). Blackberry yellow vein disease (BYVD) is a disease complex associated with several viruses. Many viruses are described and found in plants exhibiting BYVD symptoms (Table 1-2), including blackberry yellow vein-associated virus (BYVaV), blackberry virus Y (BVY), beet pseudo yellows virus (BPYV), blackberry chlorotic ringspot virus (BCRV), blackberry leaf mottle virus (BLMV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus S (BIVS), and impatiens necrotic spot virus (INSV). Symptoms of BYVD are similarly diverse and include chlorotic spots, vein banding, leaf distortion, mosaic/mottling, ringspots, line patterns, oak-leaf patterns, irregular chlorosis and field decline and death (Susaimuthu et al. 2006, 2007; Poudel et al. 2013; Hassan et al. 2017). Floricanes can be impacted by virus infection during the fruiting season, sometimes leading to cane dieback. The disease can reduce the productive lifespan of a blackberry planting from 15-20 years to 5-7 years (Poudel et al. 2013).

At first, tobacco ringspot virus (TRSV; *Nepovirus nicotiana*e) was believed to be the causal agent of BYVD, but in one study, TRSV was present in only a small number of infected plants, which led to the discovery of a new virus designated as blackberry yellow vein-associated virus (BYVaV; *Crinivirus rubi*) (Martin et al. 2004). BYVaV was first discovered in blackberry plants in South Carolina in 2004 and was detected in asymptomatic plants as well as plants showing vein yellowing and mosaic patterns (Martin et al. 2004). Because BYVaV infection is latent in several cultivars, further

investigation was done using symptomatic plants to determine the presence of other viruses. Double-stranded RNA (dsRNA) was extracted, cloned, and sequenced, revealing the presence of the potyvirid BVY. Viruses transiently produce dsRNA as a replicative intermediate during infection, so dsRNA was often used for searching for viruses. A one-month-old 'Chickasaw' blackberry plant infected with BYVaV and a one-month-old 'Chester' plant infected with BVY were approach-grafted (a grafting method where both the scion and the rootstock used their own root system), which was evaluated a month later for symptoms development. Plants infected with both viruses exhibited vein clearing and rugosity (Susaimuthu et al. 2008). This study suggested that BYVD resulted from a synergistic interaction in a co-infection between BYVaV and BVY (Susaimuthu et al. 2008). Symptoms similar to those observed on BYVD-infected plants (chlorotic mottling and ringspots) were observed on 'Bedford Giant' blackberry plants in Scotland (Jones et al. 2006), in which BCRV was first detected alongside two other viruses (raspberry leaf spot virus and black raspberry necrosis virus). The symptoms observed on those plants resulted from mixed infection and not BCRV alone. The same year, dsRNA was cloned and sequenced from diseased rose plants suspected of rose rosette disease in the U.S. The virus isolated had a high sequence similarity to the BCRV isolate found in blackberry in Scotland (Tzanetakis et al. 2006). This was the first report of BCRV infecting roses in the U.S.

Blackberry virus E (BVE; *Allexivirus epsilonrubi*) was isolated and characterized from blackberry plants exhibiting BVYD-like symptoms and infected with BVY in Mississippi (Sabanadzovic et al. 2011), and in 2012, a new emaravirus was found in

blackberry plants showing symptoms of mosaic and vein yellowing, which tested negative for any known viruses implicating in BYVD complex. This virus was provisionally named blackberry leaf mottle-associated virus (BLMaV), which was transmitted by eriophyid mites on blackberry 'Natchez' and cleft-grafting (i.e., a grafting technique in which one or two pieces of scion are inserted into a cut made across the rootstock) on black raspberry (Hassan et al. 2017). A year later, another study confirmed the presence of several of these aforementioned viruses (BYVaV, BVY, BCRV, BVE, and BLMaV) in BYVD-affected plants (Poudel et al. 2018).

Other viruses that have been detected in the BYVD-affected plants are BPYV, BlVS, and INSV. BPYV (*Crinivirus pseudobetae*) is another crinivirus of the BYVD complex disease. BPYV is transmitted by the greenhouse whitefly (*Trialeurodes vaporariorum*) (Liu and Duffus 1990) and has a wide host range such as cucurbits, lettuce, beet, strawberry, and blackberry (Martin et al. 2017; Tzanetakis et al. 2013). It was first isolated from a blackberry plant (also infected with BYVaV) sampled in South Carolina using cDNA cloned from dsRNA (Tzanetakis and Martin 2007). BIVS was also found in BYVD plants, always in co-infection with one or more viruses. It was originally detected in native blackberry from the Great Smoky Mountains National Park. Later, BlVS was detected in BYVD-infected plants in Mississippi (Sabanadzovic and Ghanem-Sabanadzovic 2009) and discovered in nectarine plants showing stem-pitting disease symptoms (Villamor et al. 2016). BlVS is a putative new species of the genus *Marafivirus* (no species name has been assigned by the International Committee for the Taxonomy of Viruses (ICTV) (Sabanadzovic and Ghanem-Sabanadzovic 2009).

INSV (*Orthotospovirus impatiensnecromaculae*) can infect more than 300 plant species (Martin et al, 2017). INSV was first reported infecting blackberry plants from South Carolina, North Carolina, and Virginia (Tzanetakis et al. 2009). A survey of 400 blackberry plants revealed that 33% of them were infected by INSV (Tzanetakis et al. 2009). INSV has two main vectors: the western flower thrips (*Frankliniella occidentalis*) and the European flower thrips (*Frankliniella intonsa*) (Wijkamp et al. 1995; Sakurai et al. 2004). They can transmit INSV in a persistent and propagative manner (i.e., the virus can replicate inside the thrips, which is infected for life). In addition to the viruses associated with BYVD, other viruses have been reported from blackberry, but do not have any associations with disease symptoms. Apple mosaic virus, blackberry calico virus, blackberry virus F, and cherry leaf roll virus are among numerous other viruses that have been found in caneberries (Martin et al. 2017). At this point, there is no clear understanding of which viruses are actually associated with BYVD symptoms in blackberry. Multiple infections (i.e., simultaneous infection of several viruses in a plant) appear to be the key to symptom development.

The black raspberry (*Rubus occidentalis*) cultivar commonly named 'Munger' has been used as an indicator host for caneberry viruses (Martin et al. 2016). Indicator hosts are specific plant genotypes that are known to show specific symptomatology when infected with certain viruses, and indicators were used for virus diagnostics before the advent of serological and nucleic-acid-based techniques. Indicators have long been used in regulatory and quarantine facilities as a method to detect novel viruses, but they are now being replaced by high throughput sequencing. Many blackberry viruses associated

with BYVD do not cause symptoms in single infections in 'Munger' (Martin et al. 2013), suggesting that 'Munger' was a sub-optimal indicator host and may have contributed to the unintentional spread of viruses in the U.S. (Martin et al. 2016). For instance, BYVaV and BVY were detected at high incidence in several nursery stocks (Susaimuthu et al. 2007).

Based on the viruses previously associated with BYVD (Martin et al. 2017, 2013) and viruses known to be present in South Carolina (Poudel et al. 2018), this dissertation is focused on eight viruses of blackberry and a novel virus that was discovered in the course of this dissertation research (Table 1-2). Viruses of the families *Closteroviridae*, *Alphaflexiviridae*, *Secoviridae*, *Potyviridae*, *Bromoviridae, Fimoviridae and Mayoviridae* are included in this dissertation (Table 1-2). The governing body of virus taxonomy, the International Committee for the Taxonomy of Viruses (ICTV), has recently completed a major shift in virus taxonomy. Although viruses (the biological entity) and virus species (the taxonomic construct) have been distinguished for many years by the ICTV, the virus species names have all been converted to the Latin binomial nomenclature system (Walker et al. 2022). The virus common names remain unchanged, but species names are now different from the virus common names. At the first mention in the text and in Table 1-2, the species name is included with the corresponding virus common name, but for the purposes of continuity, the virus common names or associated abbreviations will be used throughout the dissertation.

Closteroviridae

Blackberry yellow-vein associated virus (BYVaV: *Crinivirus rubi*; *Closteroviridae*) has a single-stranded, positive-sense RNA genome encapsidated in a flexuous rod-shaped virion. BYVaV has a bipartite (i.e., two-segmented) genome, the smallest of all sequenced members of the genus (Martin et al. 2013b; Tzanetakis et al. 2006). Criniviruses are phloem-limited and have low titers in plants especially in co-infection with potyvirids (Martin et al. 2017). BYVaV is transmitted by whitefly species *Trialeurodes abutilonea* and *T. vaporariorum* (Poudel et al. 2013) and is not mechanically transmissible using plant sap (Tzanetakis et al. 2006), and is only known to infect cultivated and wild blackberry. Although BYVaV has been suggested as the central virus in the BYVD complex, specific symptomatology associated with single infections or specific combinations of BYVaV with other viruses have no ascribed etiology. Additionally, little is known about the spread of BYVaV in blackberry plantings and wild *Rubus*.

In 2013, an ampelovirus was discovered by high throughput sequencing (HTS) in a blackberry plant showing vein banding, ringspots, and chlorotic spots, which was infected with at least two other viruses (Thekke-Veetil et al., 2013). This virus was provisionally named blackberry vein banding-associated virus (BVBaV; *Ampelovirus venarubi; Closteroviridae*) (Thekke-Veetil et al., 2013). No vector has been determined for BVBaV yet, but it may be transmitted by mealybugs or scale insects based on homology with other ampeloviruses. No host other than blackberry has been reported for BVBaV.

Potyviridae

Another virus found in blackberry plants is blackberry virus Y (BVY; *Brambyvirus rubi; Potyviridae*). BVY was first observed from samples from a symptomatic blackberry plant using scanning electron microscopy (Susaimuthu et al. 2008). It has a monopartite genome that has the largest sequenced genome (10.8 Kb) and is the sole member of a novel genus called *Brambyvirus* (Susaimuthu et al. 2008). As with other potyvirids, the BVY genome encodes a large polyprotein, which is proteolytically cleaved into ten functional proteins. There are novel domains in the P1 region, which differ from polyprotein from other potyviruses, and this is one factor that necessitated the creation of a novel genus (Martin et al. 2013; Susaimuthu et al. 2008). Transmission experiments using two aphid species (*Myzus persicae* and *Amphorophora agathonica*) and several eriophyid mites species failed to demonstrate the transmission of BVY, and thus a vector remains elusive (Martin et al. 2017). However, experiments using clean sentinel plants that became infected with BVY in the field suggested the implication of an aerial vector spreading the virus (Poudel et al. 2018). The only known natural host is blackberry.

Bromoviridae

In symptomatic blackberry plants, blackberry chlorotic ringspot virus (BCRV; *Ilarvirus BCRV; Bromoviridae*) is also observed in mixed infections whereas it is symptomless in single infections (Poudel 2011). BCRV is a member of the subgroup 1 of the *Ilarvirus* genus (Tzanetakis et al. 2010). BCRV has a tripartite, single-stranded, positive sense RNA genome, in which each genome segment is separately encapsidated

in icosahedral or quasi-icosahedral particles (Tzanetakis et al. 2010). No vector has been identified for BCRV yet, although other ilarviruses are passively transmitted by thrips (Tayal et al. 2023) and inferred to be pollen-transmitted. However, the virus can be mechanically inoculated into *Chenopodium quinoa*, and propagated by seed (Poudel et al. 2013). BCRV has also been reported as graft-transmissible in the blackberry cultivar Himalaya Giant (Martin et al. 2017). It was detected in 2 of 200 apple trees in an attempt to find its alternative hosts (Poudel et al. 2014).

Fimoviridae

Blackberry leaf mottle virus (BLMV, formerly BLMaV; *Emaravirus rubi; Fimoviridae*) was discovered by HTS in sentinel blackberry plants (Hassan et al. 2012). BLMV is reported as the second most widespread virus in the southeastern U.S. Over 500 samples were tested, and more than 40% were infected with BLMV (Hassan et al. 2017). The BLMV genome is composed of five segments of single stranded, negative-sense RNA (Hassan et al. 2017). BLMV cannot be transmitted mechanically via sap but can be disseminated by grafting to the indicator plant *Rubus occidentalis* 'Munger'. BLMV is transmitted by eriophyid mites (*Phyllocoptes parviflori*) (Hassan et al. 2017; Martin et al. 2017) and causes symptoms such as vein yellowing and chlorotic feathering patterns, ringspots and leaf distortion on blackberry cv. Ouachita in single infections (Druciarek et al. 2024). Blackberry and black raspberry are the only hosts for this virus so far (Druciarek et al. 2024). BLMV is widespread and has been detected recently in California blackberries. So far, BLMV is present in 10 states in the U.S. (Arkansas,

California, Florida, Georgia, North Carolina, Maryland, Mississippi, Oklahoma, Oregon, and South Carolina) (Scheck 2023).

Alphaflexiviridae

Blackberry virus E (BVE; *Allexivirus epsilonrubi*) is an atypical member of the *Alphaflexiviridae* closely related to the allexiviruses, which are known to infect plants in the Alliaceae family. BVE has only been reported to cause infection on blackberry. BVE has a single-stranded RNA, which is 7,718 nt long, excluding the poly-A tail encapsidated in highly flexible filamentous particles (Sabanadzovic et al. 2011a). BVE was discovered in four blackberry plants infected by BVY, exhibiting symptoms described as line patterns and vein yellowing/feathering (Sabanadzovic et al. 2011). No vectors of BVE have been described yet, however, some species of the allexiviruses are vectored by eriophyid mites (Kreuze et al. 2020).

Secoviridae

Tobacco ringspot virus (TRSV; *Nepovirus nicotiana*e) and tomato ringspot virus (ToRSV, *Nepovirus lycopersici*) are members of the family *Secoviridae*. TRSV and ToRSV are mechanically transmissible (sap) and are also transmitted by dagger nematodes (*Xiphinema americanum*), seed, and pollen-borne (Converse 1984; Yang and Hamilton 1974). Nepoviruses have bipartite, positive sense, single-stranded RNA genomes. They have a wide host range; plants from at least 30 different families can be infected by TRSV (Rowhani et al. 2017), while ToRSV can infect hosts in more than 35 plant families (Guzmán-Baeny 2003). TRSV and ToRSV were first detected in blackberry in South and North Carolina in 2002. They were detected in single as well as mixed infections in plants

showing diverse types of symptoms, including mosaic, ringspot, necrosis, chlorotic line patterns, vein chlorosis, leaf distortion, crumbly fruit, necrosis and oak leaf pattern (Guzmán-Baeny 2003). This same study showed that both TRSV and ToRSV prevalence was higher in the roots than in the primocane or floricanes. A few years later, TRSV was detected in 4-year-old blackberry plants, showing stunting of primocane and crumbly berries in Alabama (Coneva et al. 2008).

Phenuiviridae

During the course of this dissertation research, a new virus was discovered by HTS in blackberry plants (*E. Schnabel, data not shown*) and later determined to be the same sequence reported in NCBI GenBank and tentatively named blackberry line pattern virus (BLaLPV) (ON624095). There is no assigned species name at this time because it has not yet been ratified by the ICTV. BlaLPV is a putative member of the genus *Coguvirus* from the family of *Phenuiviridae*. Coguviruses are a recently discovered group of viruses that infect plants in the *Brassicaceae*, *Cucurbitaceae*, and *Rutaceae* families, including citrus concave gum-associated virus (CCGaV), watermelon crinkle leaf-associated virus-1 (WCLaV-1) and citrus virus A (CiVA). Members of this genus are single-stranded, negative sense or ambisense RNA viruses, composed of two or three RNA segments that encode three proteins, RNA-dependent RNA polymerase (RdRp), nucleocapsid (NP) and movement protein (MP). The RdRP is encoded by a negative sense RNA, while NP and MP are encoded by an ambisense RNA (Beris et al. 2021). As this genus is relatively new, information on the biology of its members is lacking. CiVA was grafted onto the indicator host rough lemon, and leaf flecking was observed on the

new growths 18 months post-grafting (Beris et al. 2021). CiVA is also mechanically transmissible to the indicator host 'Madam vinous' sweet orange (*Citrus sinensis*). No vectors have yet been determined for any of the coguviruses.

Wild *Rubus* spp. bordering farms, and their role in disease spread

Wild *Rubus* spp. and roses are often present in close proximity to commercial blackberry fields (Fig 1-2). Most of the viruses implicated in the BYVD complex in commercial blackberries have also been detected in wild *Rubus* species. Wild plants could serve as reservoirs for viruses (Hassan et al. 2017a), and are sources of inoculum for new infections. Virus presence in wild species has been evaluated for several pathosystems in the small fruit industry in which wild plant species constitute virus reservoirs. Grapevine red blotch virus (GRBV), a geminivirus whose primary host is grapevine, was detected in wild vines near commercial vineyards (Perry et al. 2018). Prior to this study, in an attempt to find alternative hosts of GRBV, 13 plant species were tested. The results confirmed that free-living *Vitis* spp. could be alternative hosts to GRBV and did not find additional hosts of GRBV (Bahder et al. 2016). Grapevine Pinot gris virus (mite-vectored) and grapevine vein-clearing virus (aphid-vectored) were also detected in wild species in the *Vitaceae* family (Petersen et al. 2019; Diaz-Lara et al. 2021). Prunus necrotic ringspot virus was detected in wild *Prunus* spp. near peach orchards in the southeastern U.S. (Bonilla and Cieniewicz 2022). In each case, the putative role of wild hosts in the epidemiology of the associated diseases in susceptible crops is not well understood.

In the case of BYVD complex, BYVaV has been detected in wild *Rubus* spp. in 11 states (Arkansas, Florida, Georgia, Kentucky, Illinois, Tennessee, Mississippi, California, West Virginia, North and South Carolina) (Poudel et al. 2013; Martin et al. 2013). BCRV was detected in wild *Rubus* and rose in Arkansas, Illinois, West Virginia, Missouri, and South Carolina (Poudel et al. 2013). Hassan et al. (2017) detected BLMV in wild blackberries in Arkansas. BVBaV was found in both blackberry plants and wild *Rubus* from at least five states (Arkansas, Georgia, Mississippi, North Carolina, and South Carolina) (Thekke-Veetil et al. 2013). BVY was also detected in wild *Rubus* species in two counties in Arkansas (Susaimuthu et al. 2008). TRSV and ToRSV can infect a wide range of host plants, from herbaceous hosts to perennial crops like blackberry (Converse 1984).

Figure 1-2. Examples of wild *Rubus* spp. in the proximity of commercial blackberry fields in South Carolina.

Use of high throughput sequencing (HTS) in virus detection and discovery

Discovery and characterization of viruses in plants have evolved with technological advances. Initially, indicator hosts were used for both virus discovery and diagnostics. Many viruses were characterized first by the identification of symptoms in indicator hosts, followed by electron microscopy and virion purification. Most of the known blackberry viruses were discovered using genome sequencing techniques (Susaimuthu et al. 2006, 2008; Tzanetakis et al. 2006; Sabanadzovic et al. 2011; Thekke-Veetil et al. 2013; Hassan et al. 2017). Characterization of BYVaV, BVY and BCRV was done via Sanger sequencing of dsRNAs which had been cloned into recombinant plasmids (Susaimuthu et al. 2006, 2008; Tzanetakis et al. 2006).

More recently, high throughput sequencing (HTS) has resulted in a boom of virus discovery. HTS techniques allow for detection of virus genome sequences from all known and novel viruses, which makes HTS poised to replace all previous detection assays (Villamor et al. 2022). Various library preparation techniques and sequencing chemistries have been used to study plant viruses. Several HTS platforms, such as Illumina and Oxford Nanopore Technology (ONT), have been used for plant virus diagnostics and discovery. Most studies have utilized dsRNA or total RNA sequencing and the Illumina short-read platform for virome characterization (Diaz-Lara et al. 2019; Bester et al. 2021). However, HTS detection comes with several caveats, with the major limitation being the lack of knowledge on biological relevance of novel viruses (Villamor et al. 2019). BLMV was first detected by high throughput sequencing using the

Illumina platform (Hassan et al. 2017), which was also the case for BVBaV (Thekke-Veetil et al. 2013).

Recent studies have either discovered or detected plant viruses in the last few years using ONT such as southern tomato virus, dioscorea bacilliform virus, yam mild mosaic virus and yam chlorotic necrosis virus (Gaafar et al. 2019; Filloux et al. 2018). Early detection of cassava mosaic begomoviruses was done in Tanzania, Uganda and Kenya (Boykin et al. 2019) using ONT. By 2020, only two peer-reviewed papers were published using ONT to investigate plant virus detection (Liefting et al. 2021). Although HTS can be useful and practical to screen for the presence of viruses, all detection should be confirmed by PCR (Liefting et al. 2021).

Analysis of HTS data for virome characterization is challenging. Although some virus enrichment protocols have been published (Fitzpatrick et al. 2021), most virome studies are using total RNA sequencing to capture the widest array of viruses (Villamor et al., 2019). Enrichment procedures such as poly-A enrichment are not optimal for virome sequencing because many virus genomes lack poly-A tails. Therefore, in HTS datasets there are large amounts of sequencing data produced, but only a small portion of the sequence reads are associated with virus sequences. Trained personnel are needed to analyze these types of data. Several bioinformatic pipelines have been developed for virus detection in HTS datasets, including Virtool (Boyes et al. 2020), VirFind (Ho and Tzanetakis 2014), and others (Villamor et al., 2019).

Virus diagnostics and detection assays available

HTS is already being routinely used in research and regulatory (i.e., quarantine) purposes (Villamor et al. 2019, 2022; Gaafar et al. 2021; Soltani et al. 2021). However, this technology has yet to be made practical for diagnostic clinic settings, primarily due to high costs and bioinformatics expertise required. Currently, most of the blackberry virus diagnostics conducted in clean plant centers, commercially, or for research purposes, are using PCR-based technologies (Thekke-Veetil and Tzanetakis 2017; Poudel et al. 2018). Very few blackberry viruses have available antisera, so serological assays like immunostrips and ELISA are not available for most blackberry viruses. However, PCR assays are limited by effective primer design, which is based on the knowledge of sequence diversity for the various viruses. Unfortunately, most of the blackberry viruses have only one or very few sequences available. Therefore, it is likely that many of the PCR assays designed are missing variants of these viruses (Villamor et al. 2022). Therefore, It would be a great benefit for both research and disease management purposes if HTS could be optimized for blackberry virus detection and diagnostics.

Network analysis

Network analysis is an approach with promise to resolve some of the complexity of the blackberry virome and uncertainty about disease etiology. Network analysis describes the relationships between a set of objects. Relationships are represented by links, and nodes are specific entities. An example of a network is host-vector-pathogen, in which the nodes are represented by host, vector and pathogens species and the links are

associations between them (Garrett et al. 2018). A network could be mono-, bi- or tri-partite. An example of a monopartite network could be an informal trade network of seed and planting materials between farmers (Buddenhagen et al. 2017). This type of network possesses one type of node, and the link between the nodes is represented by the informal exchange (Buddenhagen et al., 2017). On the other hand, a bipartite and tripartite network implicates two and three functional groups, respectively. A bipartite network could represent a plant virome in one or more agroecological regions where viruses and their hosts/regions are the nodes (Alcalá-Briseño et al. 2020). Network analysis has been used extensively to decipher co-occurrence in microbial systems (Matchado et al. 2021).

Virus disease management in blackberry

No blackberry cultivars have been reported as being either tolerant or resistant to viruses implicated in the BYVD complex. The design and implementation of effective management strategies is difficult because of the plurality of viruses and vectors implicated in the disease. Additionally, once a plant is infected with a virus, it is generally infected for the duration of its life. Thus, preventive measures such as planting and replanting of planting stocks negative for targeted viruses (Martin et al. 2013, 2017), roguing and sanitation measures such as cleaning tools when pruning or cutting back of canes, could prevent the spread of viruses in the field. Vector management is not currently feasible because vectors are mostly unknown, and for the few vectors identified, their phenology and behavior in blackberry plantings are unknown. Plants derived from virus-negative mother plants and propagated in tissue culture should be used to establish

new plantings (Martin et al. 2017, 2013; Poudel et al. 2018). Another complicating factor is that single infections of most of these viruses are symptomless; thus, they are more likely to be transmitted without intervention. Relying solely on the visual assessment of symptoms exacerbates the spread of the viruses.

Many growers report that although they plant tissue culture-derived "clean" plants, the plantings always develop virus symptoms within five years (*E. Cieniewicz, personal communication with growers*). This trend highlights the likelihood that blackberry viruses are circulating in weeds or wild host reservoirs, older neighboring blackberry plantings, or potentially in viruliferous vectors in the environment. The constant pressure of virus ingress into new plantings begs the question of whether blackberry production will ever be truly sustainable in the southeastern United States. There are many gaps in the ecology and biology of blackberry viruses. Until we fill knowledge gaps on disease etiology, vectors, and the role of wild hosts, disease management options will remain limited.

Table 1-2. Blackberry viruses detected in Rubus and associated with the blackberry yellow vein disease complex. Relevant biological knowledge is also included. This table was adapted from Martin and Tzanetakis (2015) and Martin et al. (2017).

Virus common name	Virus species	Transmission Mode	Implicated in disease symptoms?	Geographic Distribution in U.S.	Natural host range
Blackberry chlorotic ringspot virus (BCRV)	*Ilarvirus BCRV*	Pollen/seed [a]	Yes, in co-infections	SC, NC, GA, FL, MS, WV, WA, AR, MO, TN, IL	*Rosacea*e
Blackberry yellow vein associated virus (BYVaV)	*Crinivirus rubi*	Whiteflies/semi-persistent	Yes, in co-infections	AR, SC, NC, GA, CA, MS, OK, FL	*Rubus*
Blackberry virus Y (BVY)	*Brambyvirus rubi*	Unknown	Yes, in co-infections	SC, AR	*Rubus*
Blackberry virus E (BVE)	*Allexivirus epsilonrubi*	Unknown	Yes, in co-infections	SC, AR, MS	*Rubus*
Blackberry leaf mottle virus (BLMV)	*Emaravirus rubi*	*Phyllocoptes parviflora* mite	Yes, in single and mixed infections	SC, AR, FL, GA, NC, MS, OK, OR	*Rubus*
Tobacco ringspot virus (TRSV)	*Nepovirus nicotianae*	*Xiphinema* spp. nematodes	Yes, in co-infections	Widespread	Many families
Tomato ringspot virus (ToRSV)	*Nepovirus lycopersici*	*Xiphinema* spp. nematodes	Yes, in co-infections	Widespread	Many families
Blackberry vein banding associated virus (BVBaV)	*Ampelovirus venarubi*	Unknown	Yes, in co-infections	SC, AR, GA, MS, NC	*Rubus*
Blackberry virus F (BVF)	*Badnavirus phirubi*	Unknown	Yes, in co-infections	Widespread	*Rubus*
Beet pseudo yellow virus (BPYV)	*Crinivirus pseudobetae*	Whiteflies/semi-persistent	Yes, in co-infections	Widespread	Many families
Raspberry bushy dwarf virus (RBDV)	*Idaeovirus rubi*	Pollen/seed	Yes, in co-infections	Widespread	*Rubus*
Blackberry virus S (BlVS)	NA	Unknown	Yes, in co-infections	TN, MS	*Rosaceae*
Impatiens necrotic spot virus (INSV)	*Orthotospovirus impatiensnecromaculae*	Thrips/persistent	Yes, in co-infections	Widespread	Many families
Blackberry line pattern virus (BlaLPV)	NA	Unknown	Yes, in co-infections	SC, OR	Rubus

a: BCRV can be spread indirectly by pollen carriers such as thrips and bees.

Abbreviation of U.S. state names: AR: Arkansas, WA: Washington, WV: West Virginia, NC: North Carolina, SC: South Carolina, GA: Georgia, FL: Florida, MS: Mississippi, OK: Oklahoma, OR: Oregon, MO: Missouri, TN: Tennessee, IL: Illinois

NA: No species names have been assigned yet by ICTV

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CHAPTER TWO

COMPARING RT-PCR OF INDIVIDUAL SAMPLES WITH HIGH THROUGHPUT SEQUENCING OF POOLED PLANT SAMPLES FOR FIELD-LEVEL SURVEILLANCE OF VIRUSES IN BLACKBERRY AND WILD RUBUS

Abstract

Blackberry production is increasing in the southeastern U.S. with the availability of new cultivars. In addition to high production costs, growers are challenged by virus diseases. Blackberry yellow vein disease (BVYD) significantly limits blackberry production. BYVD is associated with the crinivirus blackberry yellow vein-associated virus (BYVaV) in mixed infections with other viruses. The specific disease etiology and ecological factors underlying BYVD are not well understood and rely on the effective diagnosis of several viruses involved in the complex. In 2021, we collected samples from blackberry plants showing BYVD symptoms, asymptomatic blackberry plants, and wild Rosaceae species from nine farms across South Carolina, for a total of 372 individual plant samples. RNA from individual samples was isolated and pooled into sample groups (i.e., symptomatic, asymptomatic, and wild) from each farm for a total of 24 pooled samples. We sequenced the pooled RNA using Illumina and analyzed sequence profiles using the Virtool bioinformatics application. We also tested each plant for six viruses by RT-PCR or RT-qPCR and compared plant (PCR)-level and field (high throughput sequencing (HTS))-level data. Virtool detected 17 known viruses in the pooled samples, including 11 blackberry viruses. PCR testing was mostly consistent with HTS, with some notable disagreements for specific viruses. Our study demonstrates that HTS could be

used as an efficient tool to detect viruses in bulked samples in blackberry fields, though limitations to using HTS for field-level surveillance exist and are also discussed here.

Dantes, W., Boatwright, L., & Cieniewicz, E. J. (2024). Comparing RT-PCR of individual samples with high throughput sequencing of pooled plant samples for field-level surveillance of viruses in blackberry and wild Rubus. Plant Disease (In “First Look”).

Introduction

Blackberries (*Rubus* subgenus *Rubus* Watson) are increasing in production in the southeastern United States. In 2017, blackberry acreage was estimated at 1,083 hectares accounting for 2,318 farms in nine southern U.S. states (Alabama, Arkansas, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, and Virginia). The production value ranges from $100-250 million USD (IPM centers, 2021). About 20 fungal, five bacterial and 16 viral diseases have been reported to cause damage to Rubus species worldwide (Martin et al. 2017). Viruses were recently ranked as one of the top concerns by blackberry growers in the Southeast U.S. in a survey conducted in parallel to the development of a Pest Management Strategic Plan for blackberry (IPM Centers, 2021). Viruses reduce crop productivity and shorten the lifespan of blackberry plants from an estimated 15-20 years to 5-7 years or less (Poudel et al. 2018). Blackberry viruses are sometimes latent (i.e., asymptomatic) in single infections, and thus can go unmanaged and spread rapidly across farms (Martin et al. 2013). Blackberry virus symptoms (e.g., ringspots, mosaics, vein-banding) may only manifest in mixed infections and become more severe as more viruses accumulate in the plant (Martin et al. 2017). Complicated symptomatology and disease etiology interfere with accurate diagnosis and, thus, effective management of virus diseases. In fact, *Rubus* spp. can be affected by 43 viruses, which can be disseminated through vegetative propagation and by various biotic vectors and abiotic transmission modes (Martin et al. 2017).

Prior to the discovery of blackberry yellow vein associated virus (BYVaV), the nepovirus tobacco ringspot virus (TRSV) was thought to be the causal agent of BYVD,

but TRSV was present in only a small percentage of symptomatic plants, and BYVaV was eventually determined as the major virus associated with the BYVD complex (Martin et al. 2004). BYVaV is a whitefly-transmitted crinivirus, which may be latent in single infections but is consistently associated with BYVD symptoms in mixed infections with other viruses (Martin et al. 2004). In addition to BYVaV, at least nine viruses have been associated with BYVD symptoms, including blackberry virus Y (BVY), beet pseudo-yellows virus (BPYV), blackberry chlorotic ringspot virus (BCRV), blackberry leaf mottle-associated virus (BLMaV), blackberry vein banding-associated virus (BVBaV), blackberry virus E (BVE), blackberry virus S (BIVS), and impatiens necrotic spot virus (INSV) (Martin et al. 2013). Symptoms associated with BYVD are diverse and include vein-clearing of primocane leaves, mosaic, mottling, oak leaf pattern, and irregular chlorosis (Poudel et al. 2013b).

Effective disease management is predicated on early and accurate detection of viruses associated with BYVD. High throughput sequencing (HTS) is an important tool for plant virus detection and discovery (Villamor et al. 2019; Soltani et al. 2021). HTS technologies have revolutionized genomics, allowing the simultaneous detection of known viruses and the discovery of novel ones (Villamor et al. 2019). While PCR-based detection methods target specific sequences and require prior sequence knowledge to design primers, HTS provides a non-biased method of virus detection (Gaafar et al. 2021). Use of HTS comes at a higher cost per sample than PCR but has the advantage of minimizing false negative results (Soltani et al. 2021). HTS is efficient at screening for viruses on individual samples and is already being used in plant regulatory programs (Al

Rwahnih et al. 2015; Villamor et al. 2022) and for research purposes to characterize viromes (Soltani et al. 2021). HTS for routine diagnostic purposes is still limited by high cost per sample, but it may be useful and cost-effective for farm-level detection of viruses using pooled samples (Bester et al. 2021).

In this study, we assess HTS and PCR as methods for farm-level virus detection. The objectives of this study were to (1) identify the prevalent viruses in blackberry (*Rubus* subgenus *Rubus* Watson) plants and wild *Rubus* spp. and roses in South Carolina using Illumina sequencing and the Virtool bioinformatic application (http://www.virtool.ca; Boyes et al. 2020); and (2) compare the detection of viruses in pooled samples by HTS with the ability to detect the viruses in individual plant samples by RT-PCR.

Methods

Sampling strategy.

Locations for blackberry and wild host sampling were identified and selected by working with Clemson Cooperative Extension county agents based on diverse locations and types of production. Samples were collected from nine farms in South Carolina (Figure 2-1) in April and May of 2021. At each farm, 20-60 samples were collected from three types of plants (Figure 2-2): 1) blackberry (*Rubus*. spp.) plants showing virus-like symptoms (i.e., vein banding, chlorotic mottling, ringspots), 2) asymptomatic blackberry (*Rubus* spp.) plants, and 3) wild *Rubu*s and roses surrounding each farm (Figure 2-2). Sample groups will hereafter be referred to with the farm number followed by the sample

type, in which “A” refers to asymptomatic blackberry, “S” refers to symptomatic blackberry, and “W” refers to wild hosts. Of the nine farms visited, two lacked symptomatic plants, and one lacked wild Rubus spp., resulting in 24 groups of samples and 372 total plant samples (Table 2-1). Two 6-inch shoot tips were collected from each plant, one from a primocane and one from a floricane. In collection of wild hosts from farm 2, samples of pokeweed showing mottling symptoms were also collected, and inadvertently included in the pooled 2W group. Samples were bagged and stored on ice until they reached the lab. Leaves and petioles from individual plant samples were cut into small (~2mm^2) pieces using a razor blade, which was cleaned in 10% bleach for 30 s, rinsed with distilled water, and dipped in RNase-Away between samples. For each sample, 25 mg of tissue was stored in 2 mL tubes with a sterile metal bead at -80°C for RNA extraction. Extra tissue for each sample was also stored at -80°C.

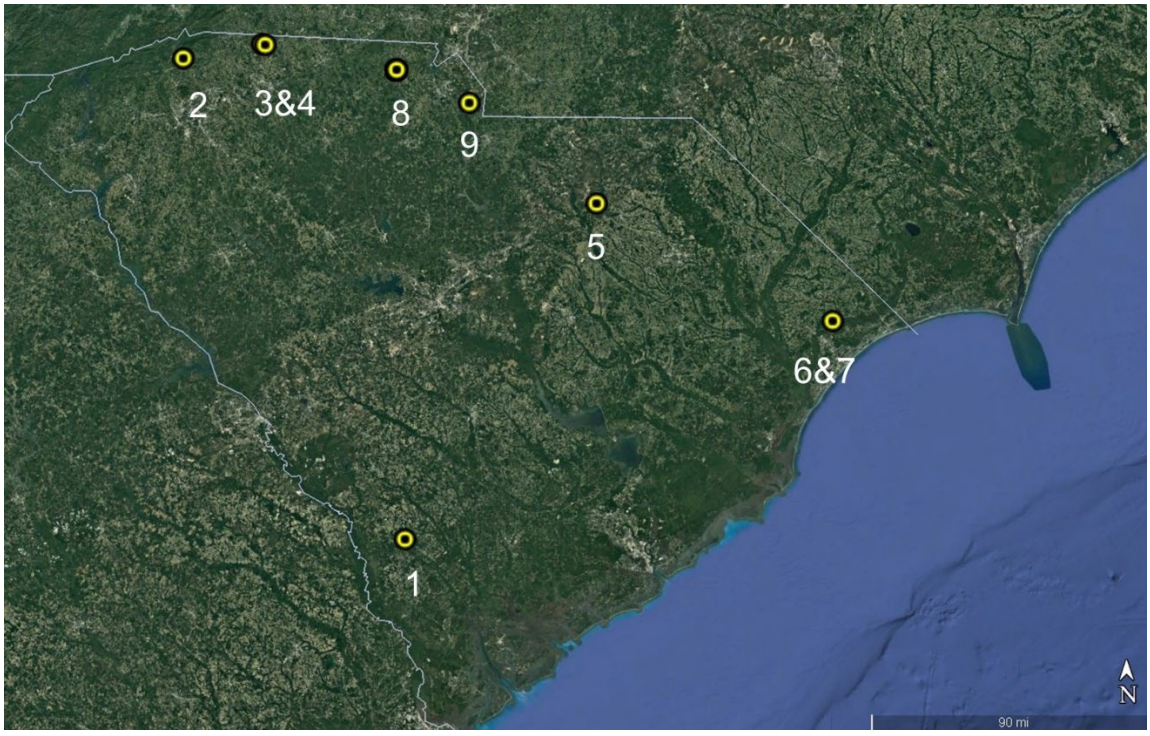

Figure 2-1. Approximate locations of the nine farms sampled in 2021 in South Carolina. Eight farms have open-field production systems except for farm 1, which has a high tunnel production system. Three of nine farms are close to the coast while the others are situated in the upstate.

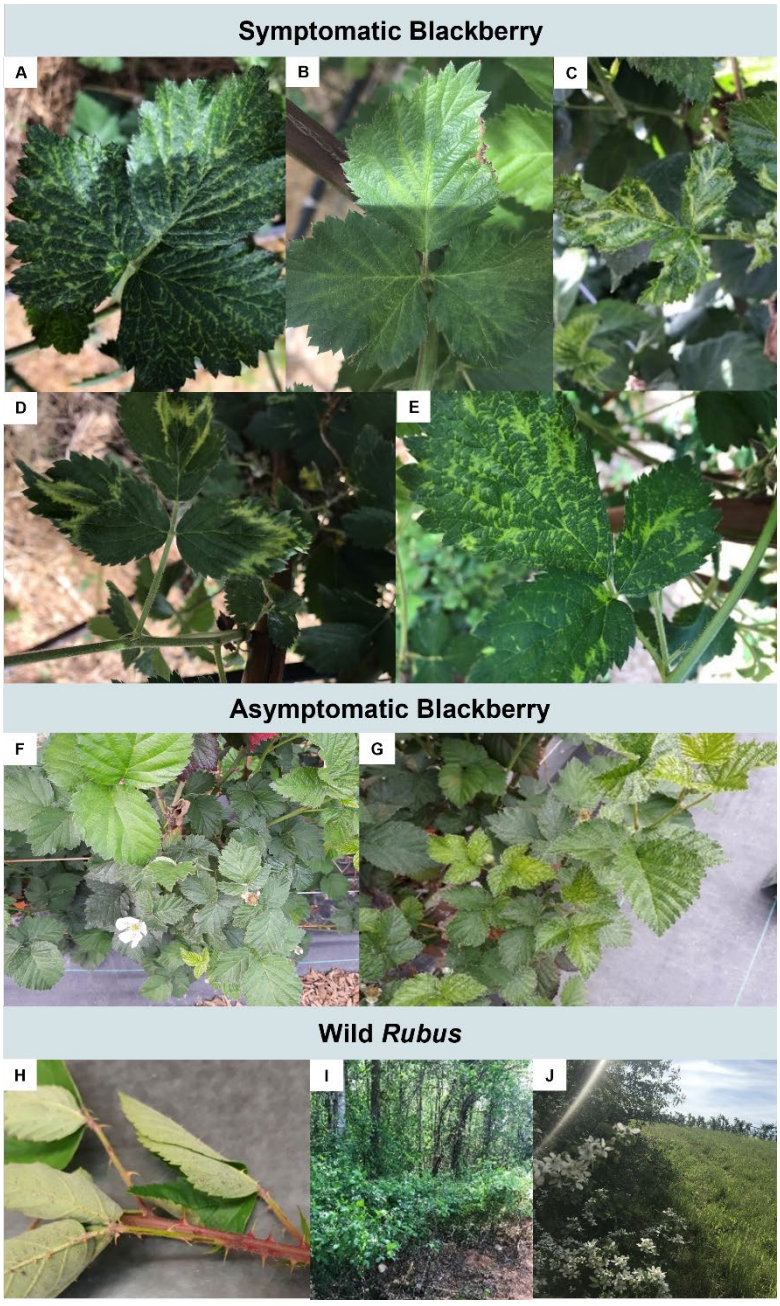

Figure 2-2. Representative symptomatology included in the 2021 sample collection in blackberry and wild Rubus. A and B illustrate veinal chlorosis, C and D illustrate oak leaf pattern, E illustrates irregular chlorosis. Asymptomatic blackberry plants are illustrated in F and G. H-J show wild *Rubus* spp. in proximity to blackberry production.

Table 2-1. Information about the pooled plant samples used in this study, including sample name, number of individual plant samples included in each pooled sample, cultivars associated with each sample group, and the planting age.

Pooled Sample name[a]	GenBank Sequence Read Archive Accession numbers	Number of individual samples	Blackberry cultivar or wild plant type	Age of planting (years old)
1A	SAMN37734627	20	'Caddo' and 'Ouachita'	1
1S	SAMN37734628	20	'Caddo' and 'Ouachita'	1
2A	SAMN37734632	12	'Natchez'	4
2S	SAMN37734633	12	'Natchez' and 'Ouachita'	1 and 6
2W	SAMN37734634	15	Wild *Rubus* and pokeweed	NA[b]
3A	SAMN37734629	14	'Navaho'	4
3S	SAMN37734630	14	'Navaho'	4
3W	SAMN37734631	10	Wild *Rubus*	NA
4A	SAMN37734635	14	'Navaho' and 'Ouachita'	1 and 5
4S	SAMN37734636	13	'Navaho' and 'Ouachita'	5
4W	SAMN37734637	16	Wild *Rubus* and wild rose	NA
5A	SAMN37734638	20	'Prime Ark 45', 'Navaho', 'Ouachita'	NR[c]
5S	SAMN37734639	20	'Prime Ark 45', 'Navaho', 'Ouachita'	NR
5W	SAMN37734640	10	Wild *Rubus* and wild rose	NA
6A	SAMN37734641	10	'Arapaho', 'Apache', 'Osage'	1
6W	SAMN37734642	11	Wild *Rubus*	NA
7A	SAMN37734643	30	'Brazos'	5-8
7W	SAMN37734644	10	Wild *Rubus*	NA
8A	SAMN37734645	30	'Natchez', 'Von', and 'Prime-Ark 45'	< 1, and 4-5
8S	SAMN37734646	20	'Natchez', 'Von', and 'Prime-Ark 45'	3-5
8W	SAMN37734647	10	Wild Rubus	NA
9A	SAMN37734648	16	'Caddo', 'Ponca', and 'Osage'	< 1, 2, and 4
9S	SAMN37734649	15	'Osage', 'Natchez', 'Ouachita', and 'Von'	2, and 4
9W	SAMN37734650	10	Wild *Rubus*	NA

[a]Sample names describe the farm number and "A" refers to asymptomatic blackberry samples, "S" refers to symptomatic blackberry samples, and "W" refers to wild plants collected at the edge of the farm.

[b]NA: Not applicable for wild plant samples.

[c]NR: Not recorded.

RNA Extraction.

RNA extractions were performed using the RNeasy Plant Mini Kit (Qiagen) with some modifications. Briefly, tubes containing 25 mg of plant tissue were flash-frozen in liquid nitrogen and disrupted into powder using a Retsch mixer mill at 30 Hz for two minutes. Tubes were returned to liquid nitrogen until the addition of the lysis buffer. Then, 450 µl of 'Buffer RLC' (supplemented with 2% PVP and 1:100 (vol:vol) β-mercaptoethanol) was added to the homogenized tissue. The remainder of the protocol was d according to manufacturer recommendations. To elute the purified RNA, the RNeasy spin column was placed in a new 1.5 mL tube, and 30 µl RNAse-free water was added directly to the spin column membrane, which was left at room temperature for 1 min and centrifuged for 1 min at 10,000 x g. The elution step was repeated twice for a total elution volume of 60 µl. Individual plant sample RNA was stored at -80°C.

RNA Sample Pooling

For HTS, individual plant RNA was pooled into groups corresponding to their sample type (i.e. symptomatic blackberry, asymptomatic blackberry, and wild plant hosts) for each farm. The pooled samples included RNA from 10 to 30 individual plant samples (Table 2-1). To prepare the pooled samples, 20 µl was taken from each individual RNA sample and combined into a composite sample. This yielded a total of 24 pooled samples with RNA concentrations ranging from 81.1 to 478.4 ng/µl. The RNA quantity and quality were assessed using the Nanodrop (Thermofisher) and the Qubit 4.0 using the broad-spectrum RNA kit (Thermofisher). RNA quality and quantity was also assessed using the Bioanalyzer at Eremid (Kannapolis, NC) prior to library preparation.

Illumina library preparation, sequencing, and data analysis

Library preparation and sequencing was performed at Eremid (Kannapolis, NC). RNA libraries (N=24) were prepared using the Plant RNA kit TruSeq® Stranded Ribo-Zero RNA kit (Illumina®) following the manufacturer's instructions. After library quality check on the Bioanalyzer, all 24 libraries were mixed proportionally in equimolar concentrations into one pool and loaded, along with 5% PhiX as spike-in, onto an S1 flow cell and sequenced on an Illumina NovaSeq 6000, which yielded 150 bp paired-end reads. Resulting FASTQ files (accession number PRJNA1026119) were trimmed and cleaned by FASTP (Chen et al. 2018) and FASTQC (Andrews 2010) respectively.

Bioinformatic analyses were completed using the Virtool application (Boyes et al. 2020). In PathoScope (Hong et al. 2014), read files are mapped to a reference virus sequence for each operational taxonomic unit (OTU) using Bowtie2 (Langmead and Salzburg 2012). PathoScope also assigned the following metrics to each virus isolate detected in each library: weight (i.e., the proportion of reads that match a specific virus isolate), depth (i.e., the number of times that the mapped reads covered a virus isolate genome), and coverage (i.e., how well the mapped reads cover the full length of each virus reference genome). A report file detailing the weight, coverage, and depth of each identified virus OTU in each library was generated (Appendix B). Cutoff values recommended by Virtool for a positive detection of a virus are a coverage greater than 0.5 and a weight greater than or equal to 0.001. The median depth (i.e., rate of redundancy) is calculated for each virus isolate detected in the sample. Because our study used pooled RNA samples, we reduced the cutoff values (weight ≥ 0.0001 and coverage

≥ 0.2) to determine the presence of a virus in a pooled sample. Since PathoScope maps read to specific virus isolates in the reference database, a virus (i.e., OTU) may be represented by several isolates in the reference database.

RT-qPCR for detection of BYVaV, RBDV and TRSV

Due to the apparently widespread nature of BYVaV and RBDV according to the HTS dataset, we tested every individual plant sample for these viruses (N=372). A multiplex probe-based RT-qPCR was used for BYVaV and RBDV, coupled with primers and probe amplifying the plant NADH dehydrogenase ND2 subunit (ndhB) gene as a reference (Thompson et al. 2003). RT-qPCRs were performed in a BIORAD thermocycler using the qScript™ XLT One-Step RT-qPCR ToughMix (Quantabio) in 12.5 µl reactions (6.25 µl of ToughMix, 0.5 µl of each primer [10 µM] for BYVaV, 0.3 µl of each primer [10 µM] for RBDV, 0.3 µl of each primer [10 µM] for the ndhB gene, 0.1 µl of each probe [10 µM], 2.75 µl of nuclease-free water, and 1 µl of RNA). The RT-qPCR program for the detection of BYVaV, RBDV, and ndhB consisted of a reverse transcription step for 10 min at 50°C followed by RT inactivation/ Taq activation for 5 min at 95°C, and 40 cycles of 10 s of denaturation at 95°C and 45 s of annealing and extension at 58°C. The same protocol was used to test 347 samples for TRSV using a duplex probe-based RT-qPCR coupled with the same reference gene (ndhB). Amplification resulting in Cq values of less than 37 were considered a positive detection. Positive controls for each virus were verified by PCR and Sanger sequencing.

RT-qPCR SYBR assay for detection of BCRV

A 10 µl SYBR green reaction (Biorad) was used to screen 67 individual samples for BCRV. The 67 samples (13 asymptomatic blackberry, 27 symptomatic blackberry, and 26 wild Rubus) were selected for BCRV screening because BCRV was detected at farms 3 and 4 in the pooled samples by HTS. In each RT-qPCR, 5 µl of 2X iTaq Universal SYBR® Green 1-Step Buffer, 0.125 µl of iScript Reverse Transcriptase, 0.5 µl of each primer (10 µM), 2.875 µl of nuclease-free water, and 1 µl of RNA were added. Each RT-qPCR run was performed in a Biorad thermocycler. The cycling consisted of an RT step for 10 min at 50°C followed by an initial denaturation for 1 min at 95°C, and 40 cycles of 10 s of denaturation at 95°C and 30 s of annealing at 55°C. Reactions with the ndhB gene primers were run in parallel to ensure the RNA quality. A melt curve analysis was included after each PCR run (65°C to 95°C in 5°C increments) to determine any off-target amplification and eliminate false positives. Amplification resulting in Cq values lower than 37 and with a melt profile consistent with the positive control was considered a positive detection. The positive control for BCRV was verified by Sanger sequencing of the PCR product followed by a NCBI BLASTn search. All primer sequences used for virus detection are listed in Table 2-2.

End-point RT-PCR for detection of BVY and BVE

Based on HTS data from pooled samples, 95 samples from farms 3, 4, 5, and 8, and 157 individual plant samples across all farms were also screened for the presence of BVY and BVE for which the expected amplicons were 246 bp and 384 bp respectively (Table 2-2). RT-PCRs were carried out using the qScript™ XLT One-Step RT-qPCR

ToughMix (Quantabio) according to manufacturer recommendations (5 µl of qScript™ XLT One-Step RT-qPCR ToughMix, 0.5 µl of each primer and 3 µl of nuclease-free water). Each RT-PCR run was performed in an Eppendorf thermocycler. Cycling conditions for the detection of BVY and BVE consisted of a RT step for 10 min at 48°C followed by an initial denaturation for 3 min at 94°C, and 40 cycles of 20 s of denaturation at 94°C, 40 s of annealing at 60°C (for BVY)/63°C (for BVE) and 60 s of extension at 70°C. RT-PCR products were resolved on a 1.5% agarose gel followed by post-staining with GelRED (Biotium) and imaging on a Bio-Rad gel documentation system. Positive controls for each virus were confirmed by Sanger sequencing of the respective RT-PCR products and verified using NCBI BLASTn.

Table 2-2. Primers and probes used for the detection of viruses of blackberry via RT-PCR or RT-qPCR.

Virus/ target	Primer and Probe Sequences	Amplicon size (bp)	Reference
BYVaV	**Sense:** 5' ATAGAAGCGAGGTTAARACCTG 3' **Antisense:** 5' CACRTYGTTACCTCTAAGCTCG 3' **Probe:** 5' Cy5/TTGAAAAGA/TAO/TGGGTYGGHGTGGACA/3IAbRQSp 3'	131	Poudel et al. 2013
RBDV	**Sense:** 5' TGGGAGATCCAATGTTCATAGT 3' **Antisense:** 5' CATCAGACTCTCAGTCATCGT 3' **Probe:** 5' FAM/ACGATGAGT/ZEN/ATGTCGTTCATTGTCCCT/3IABkFQ 3'	94	Quito-Avila and Martin 2012
TRSV	**Sense:** 5' CCTGGGCACAAGTGAAATGTTG 3' **Antisense:** 5' GCTACCAGAAACAACGGTCTAAC 3' **Probe:** 5' FAM/ TCGTGCGCTTCACTATGCAACG /3IABkFQ 3'	68	Beaver-Kanuya unpublished
BCRV	**Sense:** 5' AGGTTGAAATGGCTTTGACCC 3' **Antisense:** 5' AAGCAGCRCATCGCCTTATAC 3'	137	Poudel et al. 2014
BVY	**Sense:** 5' CTGTGGGGAGATTTGGAGAA 3' **Antisense:** 5' TCATTCCATGGGTGTGTC 3'	384	Susaimuthu et al. 2008
BVE	**Sense:** 5' TGTGGACGATGCACGCCAGATCCC 3' **Antisense:** 5' GCTCCACTGGAGGAGATTCTGGTG 3'	246	Sabanadzovic et al. 2011
ndhB	**Sense:** 5' AAGCAAAAGTTCCTAGATTCATGG 3' **Antisense:** 5' TTGCGTATTCGTCCATAGGTC 3' **Probe:** 5' Hex/TGCTTGCATATCCACCATTTGAGTCTCC 3'	132	Thompson et al. 2003

Virus and target name abbreviations are as follows: blackberry yellow vein associated virus (BYVaV), raspberry bushy dwarf virus (RBDV), tobacco ringspot virus (TRSV), blackberry chlorotic ringspot virus (BCRV), blackberry virus Y (BVY), blackberry virus E (BVE), and NADH dehydrogenase ND2 subunit (ndhB).

Results

Viruses detected in pooled samples

A minimum of 63 million paired-end reads was obtained for each sample. Illumina sequencing and PathoScope analysis of samples collected from nine farms detected one RNA satellite (RNA satellite of tobacco ringspot virus) and 17 viruses, including 11 characterized blackberry viruses in the pooled samples (Table 2-3). The analysis detected several other viruses that have not previously been detected in *Rubus* spp., such as lilac leaf chlorosis virus (LLCV), white clover mosaic virus (WClMV), red clover vein mosaic virus (RCVMV), clover yellow mosaic virus (ClYMV), peanut stunt virus (PSV), and pokeweed mosaic virus (PkMV). PathoScope resulted in the detection of blackberry viruses associated with BYVD from several genera, including two nepoviruses (TRSV and tomato ringspot virus [ToRSV]), a crinivirus (BYVaV), an allexivirus (BVE), an ilarvirus (BCRV), an ampelovirus (blackberry vein banding associated virus [BVBaV]), and a potyvirus (BVY). It also detected two viruses from different genera which are associated with the raspberry mosaic disease complex, an idaeovirus (RBDV) and a closterovirus (raspberry leaf mottle virus [RLMV]) (Table 2-3).

Based on our cutoff values, RBDV was detected in all 24 pooled samples and BYVaV was detected in 19/24 pooled samples. However, there were high levels of variability in Virtool data metrics (i.e., weight, coverage, and median depth) among the samples. TRSV was detected in 11 pooled samples, but only at high levels in four: farm 2 symptomatic blackberry, farm 2 wild hosts, farm 6 asymptomatic blackberry, and farm 9 asymptomatic blackberry (Table 2-3). BVE was detected in 14 pooled samples from

seven of the nine farms. BCRV and BVY were detected in three and five sample groups, respectively (Table 2-3, Figures 2-3 and 2-4). Two isolates of RBDV were used as references to map sequenced reads; reads corresponding to the first isolate (KJ007639) were found at a higher weight and coverage than the second isolate (KJ007640.1), which was only found at two farms (farms 3 and 4) (Figure 2-5). A single isolate was used as reference for the other five viruses, BYVaV, BVY, BVE, BCRV, and TRSV, which were the focus of this study.

Table 2-3. Summary of virus detection in each sample group of wild hosts and symptomatic and asymptomatic blackberry samples from nine farms in South Carolina.

		Previously reported in *Rubus* spp.											Not previously reported in *Rubus* spp.					
		RBDV[C]	BCRV	BVBaV	BVE	BVY	BYVaV	ToRSV	TRSV	BVF	RLMV	ApMV	RCVMV	PSV	TRSV-sat	WClMV	ClYMV	LLCV	PkMV
1A[A]	W[B]	0.7646			0.0004														0.0041
	MD	42			0														5
	C	0.993			0.328														0.907
1S	W	0.7620					0.0003												0.0066
	MD	43					0												6
	C	0.99					0.311												0.922
2A	W	0.4160					0.4315		0.0005							0.0004	0.0001		0.0148
	MD	47					52		7							0	0		7
	C	0.993					0.998		0.863							0.463	0.371		0.934
2S	W	0.6610					0.0883	0.0006	0.0424	0.0287							0.0002		0.0014
	MD	56					4	0	40817	0							0		7
	C	0.993					0.957	0.43	0.951	0.209							0.232		0.939

[A]Farm number followed by "A" for asymptomatic blackberry, "S" for symptomatic blackberry, or "W" for wild hosts.

[B]This column indicates the metric from the high throughput sequencing and Virtool PathoScope analysis where "W" stands for weight, "MD" is median depth, and "C" is coverage as defined by Virtool (Boyes et al. 2020).

[C]Virus name abbreviations across the top are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), tomato ringspot virus (ToRSV), tobacco ringspot virus (TRSV), blackberry virus F (BVF), raspberry leaf mottle virus (RLMV), apple mosaic virus (ApMV), red clover vein mosaic virus (RClVMV), peanut stunt virus (PSV), tobacco ringspot virus satellite (TRSV-sat), white clover mosaic virus (WClMV), clover yellow mosaic virus (CYMV), lilac leaf chlorosis virus (LLCV), and pokeweed mosaic virus (PkMV).

Table 2-3. Continued

		Previously reported in *Rubus* spp.											Not previously reported in Rubus spp.						
		RBDV[C]	BCRV	BVBaV	BVE	BVY	BYVaV	ToRSV	TRSV	BVF	RLMV	ApMV	RCVMV	PSV	TRSV-sat	WClMV	ClYMV	LLCV	PkMV
2W	W	0.0104			0.0012			0.1515	0.0006										0.8159
	MD	49			369			4299	13078										98655
	C	0.991			0.911			0.96	0.989										0.995
3A	W	0.1530					0.7322	0.0011	0.0001										0.0004
	MD	48208					453	2	8										8
	C	0.993					1	0.717	0.858										0.964
3S	W	0.3040			0.0011		0.5556	0.0012				0.0006							0.0020
	MD	30521			300		150	1				0							10
	C	0.993			0.929		0.998	0.506				0.308							0.934
3W	W	0.7760	0.0200	0.0006		0.0072	0.0007		0.0014						0.0001				0.0072
	MD	62	26	9		3	1		6						1				9
	C	0.99	0.972	0.632		0.909	0.706		0.852						0.554				0.953
4A	W	0.3840	0.0004		0.0270		0.4110	0.0013					0.0026						0.0017
	MD	57	5		290		23	1					0						8
	C	0.993	0.853		0.877		0.997	0.551					0.384						0.942

Table 2-3. Continued

		Previously reported in *Rubus* spp.											Not previously reported in *Rubus* spp.						
		RBDV[C]	BCRV	BVBaV	BVE	BVY	BYVaV	ToRSV	TRSV	BVF	RLMV	ApMV	RCVMV	PSV	TRSV-sat	WClMV	ClYMV	LLCV	PkMV
4S	W	0.5440			0.2826			0.0030		0.0324									0.0033
	MD	2178			3153			1		0									8
	C	0.993			0.919			0.582		0.217									0.943
4W	W	0.0096	0.0007		0.0008		0.0331	0.9198	0.0002	0.0005									0.0001
	MD	1082	3067		1764		128	7302	7	0									6
	C	0.993	0.988		0.923		0.998	0.978	0.968	0.336									0.92
5S	W	0.3840			0.0245	0.1288	0.2831	0.0022											0.0115
	MD	48			41	183	14	0											7
	C	0.991			0.895	0.914	0.998	0.209											0.94
5W	W	0.4380			0.0013		0.1514			0.0076								0.1706	0.0022
	MD	72			28		377			0								16	8
	C	0.991			0.672		0.999			0.372								0.99	0.935
6A	W	0.0042					0.0448		0.0005	0.9361									
	MD	57					308		13535	1300									
	C	0.99					1		0.941	0.834									
6W	W	0.6180					0.1391			0.0266									0.0039
	MD	53					41			0									8

Table 2-3. Continued

		Previously reported in *Rubus* spp.																
														Not previously reported in *Rubus* spp.				
		RBDV[C]	BCRV	BVBaV	BVE	BVY	BYVaV	ToRSV	TRSV	BVF	RLMV	ApMV	RCVMV	PSV	TRSV-cat	WClMV	ClYMV	LLCV	PkMV
7A	W	0.6530			0.0002			0.0002	0.0034										0.0341
	MD	55			0			1	5										6
	C	0.992			0.444			0.539	0.818										0.931
7W	W	0.7280			0.0010				0.0050										0.0358
	MD	44			0				4										7
	C	0.989			0.48				0.881										0.924
8A	W	0.6880					0.0837	0.0008											0.0065
	MD	54					8	1											6
	C	0.995					0.983	0.545											0.93
8S	W	0.6560				0.2108	0.0001	0.0062											0.0041
	MD	50				125	0	0											7
	C	0.992				0.962	0.249	0.474											0.941
8W	W	0.1140					0.8256			0.0007				0.0006					0.0034
	MD	57					1090			0				0					7
	C	0.991					1			0.241				0.204					0.926
9A	W	0.0034					0.0082		0.0002	0.9852									
	MD	43					49		17980	1228									
	C	0.995					0.998		0.973	0.838									
9S	W	0.4230			0.0005		0.3844	0.0002		0.0238	0.0019								0.0032
	MD	71			1		42	1		0	0								9
	C	0.996			0.421		0.998	0.246		0.801	0.56								0.642
9W	W	0.4280			0.0013	0.0123	0.3751	0.0001		0.0101									0.0008
	MD	60			28	170	201	1		0									9
	C	0.992			0.72	0.959	0.999	0.685		0.496									0.943

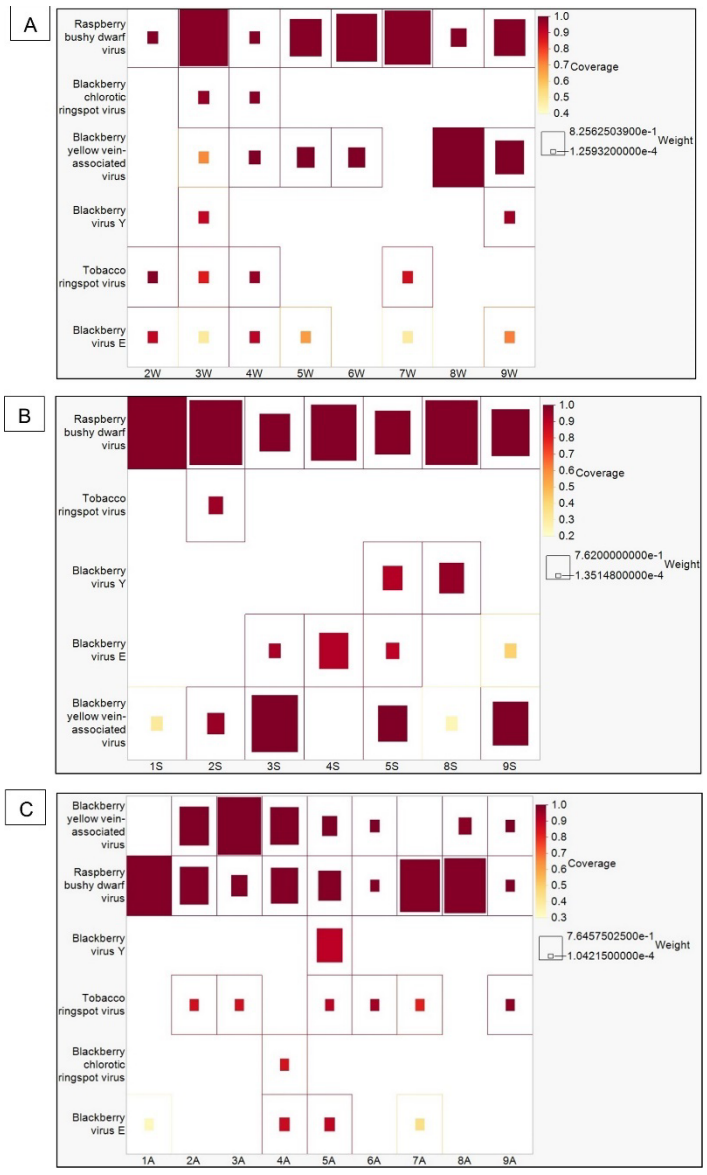

Figure 2-3. Six viruses detected by high throughput sequencing and Virtool analysis in pooled samples from (A) wild *Rubus* spp, (B) symptomatic blackberry, and (C) asymptomatic blackberry at each of nine farms in South Carolina. The color gradient represents the virus genome coverage as defined by Virtool. The rectangle size represents the weight calculated for each virus isolate.

BYVaV detection by HTS compared to RT-qPCR

BYVaV was detected by RT-qPCR in 19/92 wild *Rubus* spp. samples distributed across six farms, in 16/113 symptomatic blackberry samples from five farms, and 24/166 asymptomatic blackberry samples across seven farms. BYVaV was detected in 19/24 pooled samples by HTS from eight farms and by RT-qPCR from 18/24 of the pooled samples from seven farms. HTS and RT-qPCR results agreed for the asymptomatic blackberry and wild *Rubus* sample groups but disagreed for 3/7 symptomatic blackberry groups. BYVaV was detected in pooled samples 1S and 8S by HTS but not RT-qPCR and by RT-qPCR in 4S but not HTS (Table 2-4). It should be noted that BYVaV was detected with low coverage in 1S and 8S by HTS (Table 2-3).

RBDV detection by HTS compared to RT-qPCR

The multiplex TaqMan PCR screening assay detected RBDV in 38/372 individual samples found in six different pooled samples representing two farms in the upstate. RBDV was detected by RT-qPCR in six wild, 16 symptomatic, and 16 asymptomatic individual samples, only from farms 3 and 4 in the upstate region. RBDV was detected in all pooled samples by HTS (Table 2-3) but was only detect by RT-PCR in samples from the pools from only two farms, 3A, 3S, 3W, 4A, 4S and 4W (Table 2-4). In HTS, RBDV was detected in two sample groups (3A and 3S) with median read depths of greater than 30,000 (Table 2-3) which corresponded to 100% of individual samples testing positive by RT-qPCR in the respective groups (Table 2-4). In pooled samples 4S and 4W, RBDV was detected with a median read depth of 1,082 and 2,178, which corresponded to 16% and 25% of individual plants testing positive by RT-qPCR. In the remaining pooled

samples, RBDV was detected at low levels (i.e., median read depth of 42-72) (Table 2-3). Two isolates of RBDV were detected in these two farms by HTS and PathoScope analysis, with weights ranging from 2.24E-06 to 0.78 and coverage ranging from 0.2 to 0.99 (Figure 2-5). However, only reads mapping to a single RBDV isolate (corresponding to NCBI GenBank accessions KJ007639 and KJ007640.1 for RNA1 and RNA2, respectively) were detected in the remaining 18 pooled samples, with weights ranging from 0.01 to 0.76. Overall, agreement between methods was relatively poor for RBDV since it met the threshold for detection in all samples by HTS but was detected in only six by RT-qPCR.

TRSV detection by HTS compared to RT-qPCR

TRSV was found in 20 individual samples (4/92 wild *Rubus*, 11/98 symptomatic blackberry, and 5/166 asymptomatic blackberry) in 12 of the sample groups by RT-qPCR (Figure 2-4). TRSV reached the threshold of detection in eleven of the pooled samples (Table 2-3). Of the 21 sample groups tested by both HTS and RT-qPCR for TRSV, 11 showed congruent results and six groups with conflicting results (Table 2-4). Of those which disagreed, TRSV was detected in samples 3S, 5S, 8A, 8S, and 9W by RT-qPCR in individual plants but did not meet the threshold of detection in HTS, whereas sample 5A was detected by HTS but not by RT-qPCR (Table 2-4).

BCRV detection by HTS compared to RT-qPCR

Overall, the two methods showed some agreement in detecting BCRV, but only five of the sample groups were tested by RT-qPCR. BCRV was detected in 12/26 wild *Rubus,* 13/27 symptomatic blackberry, and 5/14 asymptomatic blackberry individual

samples from farms 3 and 4 by RT-qPCR (Figure 2-4). BCRV was detected in three sample groups (3W, 4A, and 4W) by both methods but only by RT-qPCR (and not HTS) in 3S and 4S (Table 2-4). Sample 3S did not meet the HTS threshold requirement (low weight and low coverage) while sample 4S had low weight but high coverage. In group 4S, 38% of these samples were positive for BCRV by RT-qPCR. This is likely a false negative by HTS due to the cutoff values established.

BVY detection by HTS compared to RT-PCR

BVY was detected in 22/96 individual samples tested by RT-PCR; of these, BVY was found in 9/36 wild *Rubus*, 4/40 symptomatic blackberry, and 9/20 asymptomatic blackberry) found at farms 3, 4, 5, 8, and 9 (Figure 2-4). BVY reached the threshold of detection in five of the pooled samples by HTS, 3W, 5A, 5S, 8S, and 9W, all with high coverage of 0.9 (Table 2-3). BVY was detected in pooled sample 4W at a lower weight (2.41E-05), which did not meet the cutoff for detection, but six individual samples of the 4W group tested positive for BVY by RT-PCR. Overall, with the exception of pooled sample 4W, the two methods showed agreement in detecting BVY (Table 2-4). However, it should be noted that only six sample groups were tested individually by RT-PCR.

BVE detection by HTS compared to RT-PCR

BVE was detected by RT-PCR in 22/92 wild hosts, 20/62 symptomatic blackberry, and 5/94 asymptomatic individual blackberry samples tested at six farms (Figure 2-4). BVE was detected by HTS in 14 sample groups across seven farms (Table 2-3) although pooled samples 1A, 7A, 7W, and 9S had relatively low coverage and weight for BVE. We tested 12 sample groups using both methods, and they provided

congruent results for eight of the groups (Table 2-4). BVE was detected in sample groups 1S and 8W by RT-PCR but did not meet the threshold for detection by HTS in these groups. It nearly met the cutoff in sample 8W, with a weight of 0.000095, a coverage of 0.849, and a median read depth of 349. Considering that 40% of individual samples in 8W tested positive for BVE by RT-PCR, this is likely a false negative by HTS due to the cutoff values established. RT-PCR failed to detect BVE in sample groups 4W and 9S, even though it met the cutoff in the pooled samples in HTS (Table 2-4).

Table 2-4. Comparison of the weight (i.e., loosely equivalent to virus titer in Virtool application) as determined by high throughput sequencing of pooled blackberry samples, compared to the proportion of individual plant samples in the corresponding sample group testing positive for each virus by RT-PCR or RT-qPCR. Bolded values indicate agreement in detection between the two methods for each specific sample group.

Pooled sample	**Weight detected by Pathoscope (if detection cutoff was met)**						**Proportion of positives by PCR**					
	BYVaV[a]	**RBDV**	**TRSV**	**BCRV**	**BVY**	**BVE**	**BYVaV**	**RBDV**	**TRSV**	**BCRV**	**BVY**	**BVE**
1A	ND[b]	1.70E-01	ND	ND	ND	3.96E-04	0	0	0	NT[c]	NT	NT
1S	**3.32E-04**	1.90E-01	ND	ND	ND	ND	**0.05**	0	0	NT	NT	**0.25**
2A	**4.32E-01**	1.20E-01	**5.46E-04**	ND	ND	ND	**0.16**	0	**0.25**	NT	NT	NT
2S	**8.83E-02**	1.60E-01	**4.28E-02**	ND	ND	ND	**0.41**	0	**0.67**	NT	NT	NT
2W	ND	2.52E-03	**1.75E-02**	ND	ND	**1.23E-03**	0	0	**0.33**	NT	NT	**0.33**
3A	**7.32E-01**	**6.00E-02**	**1.04E-04**	ND	ND	ND	**0.71**	**1**	**0.14**	NT	NT	NT
3S	**5.56E-01**	**9.00E-02**	ND	ND	ND	**1.09E-03**	**0.42**	**1**	**0.21**	**0.43**	NT	**0.071**
3W	**7.22E-03**	**1.90E-01**	1.43E-04	**5.67E-04**	**7.30E-04**	ND	**0.1**	**0.2**	0	**0.7**	**0.30**	NT
4A	**4.11E-01**	**1.20E-01**	ND	**4.32E-04**	ND	**2.70E-02**	**0.2**	**0.2**	NT	**0.35**	NT	**0.21**
4S	ND	**1.30E-01**	ND	ND	ND	**2.83E-01**	0.08	**0.16**	0	**0.38**	NT	**0.71**
4W	**3.31E-02**	**2.80E-03**	**2.20E-04**	**6.73E-04**	ND	8.33E-04	**0.13**	**0.25**	**0.31**	**0.31**	**0.38**	**0.25**

[a]Virus name abbreviations: BYVaV is blackberry yellow vein associated virus, RBDV is raspberry bushy dwarf virus, TRSV is tobacco ringspot virus, BCRV is blackberry chlorotic ringspot virus, BVY is blackberry virus Y, and BVE is blackberry virus E. This table reports the weight corresponding to BYVaV RNA 2, RBDV RNA2, TRSV RNA 2, and BCRV RNA3. Bolded number indicates agreement between the two methods.
[b]ND: not detected.
[c]NT: not tested.

Table 2-4: Continued

Pooled sample	Weight detected by Pathoscope (if detection cutoff was met)						Proportion of positives by PCR					
5A	**1.29E-01**	1.00E-01	2.13E-04	ND	**3.55E-01**	**3.02E-02**	**0.1**	0	0	NT	**0.45**	**0.1**
5S	**2.83E-01**	1.10E-01	ND	ND	**1.29E-01**	**2.45E-02**	**0.05**	0	**0.1**	NT	**0.2**	**0.3**
5W	**1.51E-01**	1.30E-01	ND	ND	ND	**1.35E-03**	**0.6**	0	0	NT	NT	**0.7**
6A	**4.49E-02**	3.08E-01	**5.36E-04**	ND	ND	ND	**0.20**	0	**0.1**	NT	NT	NT
6W	**1.39E-01**	2.10E-01	ND	ND	ND	ND	**0.18**	0	NT	NT	NT	NT
7A	ND	3.10E-01	3.42E-03	ND	ND	ND	0	0	0	NT	NT	NT
7W	ND	2.30E-01	4.98E-03	ND	ND	1.04E-03	0	0	0	NT	NT	NT
8A	**8.37E-02**	2.10E-01	ND	ND	ND	ND	**0.06**	0	**0.06**	NT	NT	NT
8S	1.35E-04	1.20E-01	ND	ND	**2.11E-01**	ND	0	0	**0.052**	NT	**0.26**	NT
8W	**8.26E-01**	4.00E-02	ND	ND	ND	ND	**0.10**	0	0	NT	NT	**0.4**
9A	**8.21E-03**	1.32E-03	**2.33E-04**	ND	ND	ND	**0.25**	0	**0.19**	NT	NT	NT
9S	**3.84E-01**	1.60E-01	ND	ND	ND	4.52E-04	**0.21**	0	NT	NT	NT	0
9W	**3.75E-01**	1.20E-01	ND	ND	**1.23E-02**	**1.28E-03**	**0.7**	0	**0.7**	NT	**0.1**	**0.6**

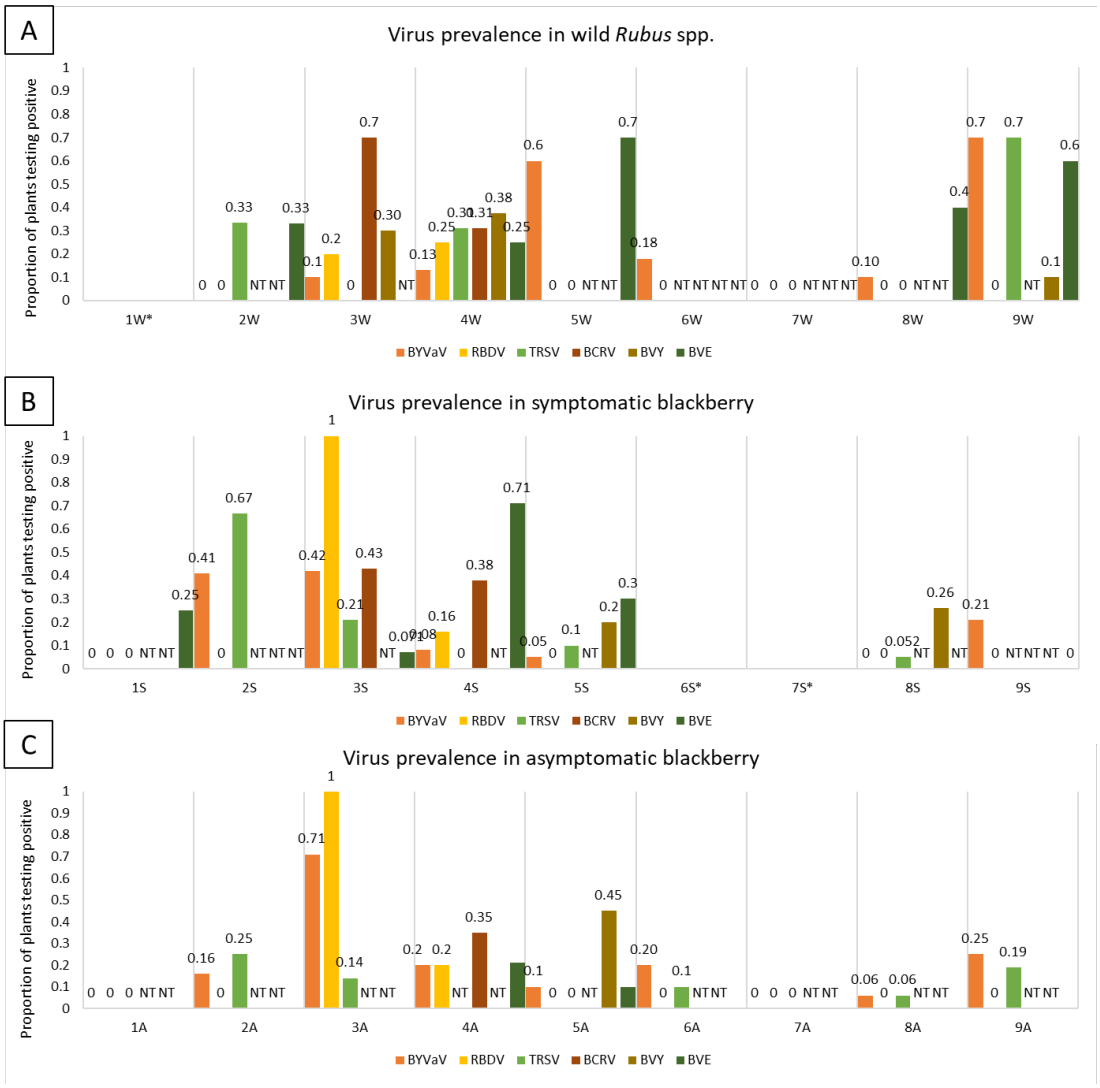

Figure 2-4. Individual blackberry and wild *Rubus* spp. samples tested positive by RT-qPCR or RT-PCR for the presence of blackberry yellow vein associated virus (BYVaV), raspberry bushy dwarf virus (RBDV), tobacco ringspot virus (TRSV), blackberry chlorotic ringspot virus (BCRV), blackberry virus Y (BVY), and blackberry virus E (BVE) in **(A)** wild hosts surrounding (within 15 meters) of blackberry farms, **(B)** symptomatic blackberry samples, and **(C)** asymptomatic blackberry samples. An asterisk close to the farm name means that that group was not sampled.

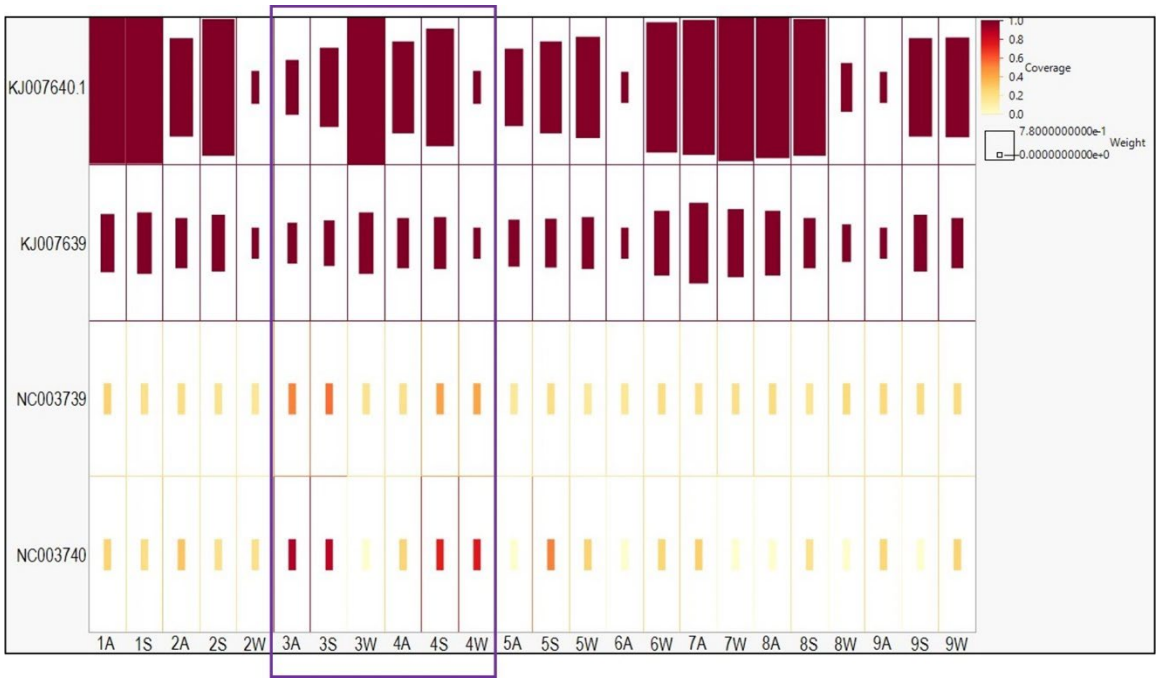

Figure 2-5: The coverage and weight of two raspberry bushy dwarf virus (RBDV) isolates that were used as reference genomes in Pathoscope analysis of pooled samples. The purple box indicates the six sample groups that tested positive by both HTS and RT-PCR.

Discussion

In this study, we aimed to determine whether HTS and the Virtool application could be used as a time-effective, cost-effective, and reliable diagnostic tool for farm-level virus surveillance that can detect all known and novel viruses. Our analysis confirmed that Illumina sequencing is a sensitive diagnostic tool for pooled samples composed of 10-30 individual samples, with some limitations. For example, with higher sensitivity comes the challenge of distinguishing false positives, and thus setting appropriate cut-off values is important, particularly for diagnostics using HTS (Rott et al. 2017). On the other hand, PCR-based detection is target-specific, dependent on optimal primer design, and thus can potentially miss some variants of a virus. Nonetheless, RT-PCR was useful to confirm HTS results in detecting viruses in the pooled samples. Comparisons in sensitivity cannot be addressed by our study, because individual plant samples were used for PCR-based detection, and pooled samples were used for HTS. Testing by PCR for six viruses in 372 individual samples would have been arguably more cumbersome, time-consuming, and possibly more costly than HTS. The RNA extractions in this study were completed on individual plant samples for both methods (later pooled in groups of 10-30 for HTS), but it would be interesting to compare these results with a different pooling strategy, e.g., pooling and homogenizing plant tissue prior to extraction (Gaafar et al. 2021). Pooling tissue for composite RNA extraction would also have the benefit of saving on RNA extraction materials and time. To more accurately determine detection thresholds for specific viruses in blackberry, it would be useful to use a dilution

approach with known virus-positive samples as demonstrated in grapevine by Soltani et al. (2021).

Overall, our data suggest that Virtool is potentially useful for applying HTS to virus diagnostics as an effective, user-friendly bioinformatic approach, as previously demonstrated (Gaafar et al. 2021), but with some notable limitations. Generally, we found that the weight as defined by Virtool of RBDV RNA2 is higher than RNA1 across the 24 pooled samples as a result of more reads mapped to RNA2 compared to RNA1 (Figure 5), which is to be expected because RNA2 is expressed at a higher level than RNA1. The primer sets and Taqman probe used in RT-qPCR partially amplify the RBDV polymerase gene (Quito-Avila and Martin 2012), which is encoded by RNA1. In addition, these primers and probe were designed based on sequences of RBDV isolates from Oregon and Washington (Quito-Avila and Martin 2012), and thus may miss RBDV variants present in South Carolina. On the other hand, RBDV found at low levels across HTS datasets could also be a result of cross-contamination during the library preparation phase or during sequencing, so resolving the discrepancies between HTS and PCR for RBDV detection here is challenging.

RBDV was detected in two sample groups with median read depths of greater than 30,000, in two sample groups with median read depth of 1,000-2,000, and in all other samples at low levels (i.e., median read depth of 42-72), and thus we suspect cross-contamination in the samples with low levels of RBDV. Virtool, and specifically the "weight" measurement in Virtool, cannot be effectively compared across datasets, and may be better when analyzing single plant samples rather than pooled samples.

Interestingly, in a related study, HTS and PCR nearly congruently detected several berry viruses in individual samples (Villamor et al. 2022). However, RT-PCR failed to detect RBDV in three *Rubus* samples due to the presence of a novel variant, and the PCR primers used could not capture the sequence diversity (Villamor et al. 2022). A novel RBDV variant is a possibility in our samples, but the even distribution of RBDV at low levels across all datasets more strongly supports the cross-contamination hypothesis. The presence of RBDV across our libraries could be due to liquid cross-contamination during RNA extraction or library preparation, which is sometimes observed in HTS datasets (Rott et al. 2017). There could also be index-hopping during sequencing, leading to false positives (Illumina 2018). Index-hopping is usually managed by cleaning up the library from free sequencing adapters or primers, but it may not be entirely efficient. Contamination may also explain the observation of pokeweed mosaic virus (PKMV) presence across most of the libraries, even though pokeweed RNA was only inadvertently included in sample group 2W. Follow-up work determined that PkMV was not detected in wild *Rubus* in follow-up sample collection and RT-PCR testing, so blackberry is unlikely to be a systemic host for PkMV (data not shown).

A contrasting situation was observed with BYVaV; the RT-qPCR primers and probe that amplify part of the heat shock protein 70 homolog gene of BYVaV (Poudel et al. 2013b) are mapped in RNA2, the most abundant transcript found in the PathoScope analysis. Consequently, BYVaV detection by RT-qPCR was somewhat consistent with HTS-based detection. Reads mapping to BCRV RNA3, which encodes the movement and coat proteins genes, were found at higher proportion than RNA1 and 2. The RT-

qPCR primers for BCRV amplify a 137 nt region in the movement protein encoded by RNA3 resulting in consistency between HTS and RT-PCR for BCRV. Additionally, detection primers used in this work for BYVaV and BCRV were designed using virus sequences isolated from different states, including some from South Carolina (Poudel et al. 2013a, 2013b). The BVE RT-PCR primers amplify a 247 nt region in the genes that encode a serine-rich p40 protein. RT-PCR did not detect BVE in samples from farms 6 and 7, but BVE was detected with low weight (8.28E-11 and 6.06E-06) and coverage (0.285 and 0.48) by HTS. BVY primers amplify a 384 nt region of the polyprotein that codes the P1 protein (Poudel et al. 2018). These primers detect individual positive samples in all the sample groups in which HTS was able to detect BVY, demonstrating consistency between diagnostic methods.

We observed partial agreement between HTS detection in pooled samples and RT-PCR in individual samples for the six viruses selected. HTS and PCR detected BYVaV congruently except RT-qPCR did not detect BYVaV in the individual samples of group 8S, which had low weight and coverage, possibly because BYVaV is phloem-limited. HTS is known for its ability to detect phloem-limited viruses present in low titer in grapevine (Al Rwahnih et al. 2015). Moreover, our sampling was conducted in the spring and although we did include both petioles and leaf tissue, it is possible that spring is not the optimal sampling time for BYVaV, as has been observed in other systems (Fiore et al. 2009; Setiono et al. 2018). Nonetheless, it is not possible with our current data to conclusively determine whether HTS or RT-qPCR yielded the correct result.

RT-PCR detected BYVaV in 145/234 (62%) cultivated and wild symptomatic *Rubus* samples during a 4-year survey across nine states (Poudel et al. 2013b). In contrast, our multiplex RT-qPCR assay detected it in 16/113 (less than 15%), with no apparent association with symptomatology. This difference could suggest that BYVaV might not be the main virus driving symptom development of BYVD in South Carolina, or that perhaps disease etiology can be more refined. Another possibility which has been suggested before is that symptoms simply are a result of more viruses accumulating in the plant (Martin et al. 2013). Blackberry is susceptible to numerous viruses, and thus it was not surprising to detect so many viruses in our study. A notable exception to this phenomenon was observed in Mexico, where only TRSV was detected by HTS, despite the researchers specifically targeting symptomatic plants (Diaz-Lara et al. 2019).

One of the bottlenecks of using HTS for routine diagnostics has been the lack of accessible bioinformatics for data analysis (Villamor et al. 2019). Effective interpretation of HTS data is dependent on using optimal bioinformatics approaches. In this study, we used Virtool, which uses a curated virus reference database from NCBI GenBank for mapping virus reads. The Virtool database must therefore be updated regularly (Gaafar et al. 2021), which could result in unexpected virus absence, as observed in the case of BLMV. Virtool-PathoScope failed to detect BLMV in any of the 24 pooled samples, likely because it was not included in the Virtool reference database at the time of our analysis. Further, we expect that BLMV is present at some of the farms because it was previously detected at some of these sites (Poudel et al. 2018). We did not complete the NuVs workflow of the Virtool application, but presumably any viruses not included in

the curated database, including BLMV, would have been identified by NuVs (Gaafar et al. 2021).

Some viruses which met our thresholds for a positive detection by HTS were unexpected. For example, LLCV, an ilarvirus closely related to apple mosaic virus (ApMV) (James et al. 2010), was detected by HTS in farm 5 in the wild *Rubus* and asymptomatic blackberry, although it is unknown to infect *Rubus* spp. The farm 5 wild pooled sample contained RNA from wild *Rubus* and wild roses. It was found with high coverage and weight in samples 5W and 5A, which warrants follow-up research. ApMV and raspberry leaf mottle virus (RLMV) have been previously detected in *Rubus* spp. ApMV is reported to affect several plants in the Rosaceae family, including blackberry. RLMV is associated with the other complex disease affecting blackberry, raspberry mosaic disease (Martin et al. 2017). During sample collection, clover plants were commonly observed in the ground cover. Several clover viruses were detected in sample groups of all three categories at various farms, albeit at a low weight and coverage (Table 3). Blackberry and/or wild *Rubus* could be an alternative host to these clover viruses, but a more plausible explanation for their detection is that it is a result of environmental contamination on the leaves via pollen, insect excrement, or other plant matter on the surface of blackberry or wild *Rubus* leaves analyzed in our study. In a HTS survey of peach in Tennessee, turnip vein clearing virus and WClMV were detected with low read counts, and authors suggested environmental contamination as well (Dias et al. 2022). Environmental contamination through insect excrement has also been suggested to explain the inconsistent detection of grapevine red blotch virus on wild *Rubus* spp.

(Bahder et al. 2016). Regardless, to be certain of true infections, we would need to test individual plants for these viruses.

This study demonstrates that HTS can be used as a time- and cost-efficient tool to detect viruses in pooled samples from blackberry farms. HTS can be particularly useful for crops like blackberry, in which viruses are abundant and cause important disease problems. Farm-level surveillance of viruses can ultimately be used to inform specific management strategies, which will benefit growers. HTS is useful for diagnostics for clean plant and regulatory programs but still has limitations for routine diagnostics (Bester et al. 2021). Virtool, despite some limitations for pooled samples, is a user-friendly bioinformatics approach that may help to bridge the technology gap for HTS use in diagnostics settings (Boyes et al. 2020, Gaafar et al. 2021). This study provides insights into the use of HTS for more routine diagnostics, albeit at the field/farm level. This farm-level detection method could be used in monitoring and surveillance-based programs for small fruit crops.

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CHAPTER THREE

NETWORK ANALYSIS TO INVESTIGATE THE ECOLOGY OF BLACKBERRY VIRUSES AND ETIOLOGY OF BLACKBERRY YELLOW VEIN DISEASE

Abstract

Blackberry producers in the southeastern United States face problems with orchard productivity and longevity due to virus diseases. The blackberry yellow vein disease (BVYD) complex limits blackberry production in the mid-southern and southeastern U.S. BYVD is associated with mixed infections of viruses including blackberry yellow vein-associated virus (BYVaV), tobacco ringspot virus (TRSV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry vein banding associated virus (BVBaV), blackberry chlorotic ringspot virus (BCRV), blackberry leaf mottle virus (BLMV), and more. Single infections are often latent, and specific viruses associated with symptom types (e.g., vein banding, ringspots, mottling) are not known. Therefore, the etiology of BYVD is not fully understood, which makes BYVD diagnosis more challenging. In this study, we apply network analysis in an attempt to elucidate the ecological factors impacting BYVD distribution in South Carolina. More mixed virus infections were detected in the symptomatic and wild groups compared to the asymptomatic plants. In cultivated blackberry, virus accumulation in older plants was significantly higher compared to the virus accumulation observed in younger plantings, suggesting that blackberry plantings are mostly clean at planting and virus ingress happens over time. The bipartite network analysis showed that some cultivars, e.g., cv. Navaho, harbored more viruses than others. The analysis did not clarify whether any

specific virus or virus combination is associated with specific symptomatology, except for oak leaf pattern, which may be associated with BYVaV, RBDV, and BLMV. This work provided insights into ecological factors that could influence BYVD spread in the field, such as the age of plantings and cultivars.

Introduction

Viruses cause significant damage to perennial crops such as blackberry, resulting in reduced crop yields and quality, and economic losses. Blackberry yellow vein disease (BYVD) is associated with reduced yield and, in many cases, reduces the productive lifespan of blackberry fields from 20 years to 5-7 years (Martin et al., 2017). Numerous viruses have been detected in blackberry plants showing symptoms associated with blackberry yellow vein disease (BYVD), including blackberry yellow vein-associated virus (BYVaV), blackberry chlorotic ringspot virus (BCRV), blackberry virus Y (BVY), blackberry virus E (BVE), blackberry leaf mottle virus (BLMV; formerly blackberry leaf mottle-associated virus), tobacco ringspot virus (TRSV) and more (Hassan et al., 2012; Martin et al., 2004; Sabanadzovic et al., 2011; Susaimuthu et al., 2006, 2008). Raspberry bushy dwarf virus (RBDV) is not reported to be associated with BYVD but rather has been associated with raspberry mosaic disease (RMD) (Martin et al., 2017), although RBDV was associated with reduced yield of blackberry cv Marion in Oregon (Strik and Martin, 2003). It is also unclear whether tomato ringspot virus (ToRSV) is associated or not with BYVD as it has not been formally reported as a component of the disease. However, both RBDV and ToRSV have been detected in blackberry plants exhibiting symptoms similar to BYVD (Chapter 2, Dantes et al. 2024).

These viruses are typically latent in single infections (Martin et al. 2013). Specific viruses associated with the diverse symptoms of BYVD (e.g., vein banding, ringspots, mottling) are not known. Diverse chlorotic patterns are observed on blackberry plants,

including oak leaf pattern, mosaic, mottling, ringspots, vein banding, chlorosis, vein yellowing, and chlorotic feathering patterns (Martin et al., 2013; Poudel et al., 2013), and symptoms are described inconsistently in different studies. Seven viruses implicated in the BYVD complex have been previously detected in South Carolina: BYVaV, BVE, BVY, BLMV, BCRV, BVBaV, TRSV, and ToRSV (Poudel et al., 2018, Chapter 2, this dissertation).

The viruses implicated in the BYVD complex are diverse, and vectors are unknown for many of them. BYVaV is a crinivirus transmitted by whiteflies, likely in a semi-persistent manner. BLMV is an emaravirus transmitted by eriophyid mites (Druciarek et al. 2024; Poudel et al., 2013) and TRSV is a nepovirus transmitted by nematodes (Converse, 1984). No vectors have been determined for BVY, BVE, BVBaV, BCRV, and RBDV, although RBDV is transmitted through pollen. Virus accumulation in field-grown plants results in more severe symptom expression and plant decline, as observed in blackberry fields (Martin et al. 2017; 2013). In other crops, visual scouting and observation of symptoms in the field can be a reliable first step to addressing virus disease problems (Gonsalves et al., 2010; Qazi, 2016). However, most blackberry viruses are latent in single infections. Thus, several of the recently discovered blackberry viruses were discovered in symptomatic plants in mixed infections with other viruses (Hassan, 2012; Sabanadzovic et al., 2011; Thekke-Veetil & Tzanetakis, 2017), which complicates our ability to decipher the disease etiology of specific viruses. Additionally, latent virus infections in blackberry fields could delay timely management strategies due to the lack of knowledge of their presence (Martin et al., 2013). In addition to mixed infections,

symptom development could be affected by other factors such as host genotype and plant age (Naidu et al., 2014; Ogbe et al., 2003; Qazi, 2016).

Virus populations can be considered at different scales, e.g., within a plant or insect vector, at the farm level, at the regional level, or in an environmental sample such as soil or water (Alcalá Briseño et al., 2023). In studies of plant virus populations, network analysis can be applied at various levels, ranging from local interactions within a single plant to complex ecological networks encompassing multiple plant species and environments (Alcalá-Briseño et al., 2020; Alcala-Briseño et al., 2021). Bipartite network analysis can be used to understand complex interactions within a plant virome such as host-virus interactions (Alcalá-Briseño et al., 2020; Garrett et al., 2018). Bipartite networks have been used to evaluate interactions in plant-pollinator systems, which share some ecological traits with vector-borne pathosystems (Garrett et al., 2018; Alcalá-Briseño et al., 2020). By representing viruses as nodes and their relationships to their hosts as edges, bipartite network analysis can reveal co-occurrence patterns of virus populations within a group of samples. This approach may reveal drivers of ecological dynamics among virus populations, such as their hosts (crop plants and alternative hosts) and other factors such as plant age and geographic location.

Here we apply a bipartite network analysis approach to understand the ecological factors impacting BYVD symptom expression and distribution of several viruses in blackberry and wild *Rubus* spp. in South Carolina. The objectives of this study are (1) to determine the most prevalent virus combinations in each sample type (asymptomatic blackberry, symptomatic blackberry, and wild *Rubus*), and (2) to assess if blackberry

cultivar, plant age, and location are potential factors that can affect BYVD expression in the field.

Materials And Methods

In this study, we used 369 samples collected from blackberry and wild Rubus spp. in South Carolina, and some of the virus testing results of samples collected from the field in 2021 (Chapter 2, Dantes et al., 2024). In previous work, a subset of the individual plant samples was tested for BVE, BCRV and BVY to confirm virus detection by high throughput sequencing in pooled samples. To achieve individual plant-level resolution for this study, all 369 samples were tested for BVE, BCRV, BVY, BLMV, BVBaV, and a novel virus, blackberry line pattern virus (BlaLPV).

Sampling strategy and symptom classification

Locations for sampling were identified and selected by working with Clemson Cooperative Extension county agents based on diverse locations and types of production. Samples were collected from nine farms in South Carolina (Figure 1) in April and May of 2021, as described in Chapter 2, (Dantes et al. 2024). At each farm, a total of 20-60 samples were collected from three types of samples: 1) blackberry plants showing virus-like symptoms (if present), 2) asymptomatic blackberry plants, and 3) wild *Rubus* surrounding each farm (if present) adding to a total of 369 plant samples. During sample collections, a photograph was taken of each symptomatic blackberry plant. Symptoms were recorded and categorized based on symptomatology associated with BYVD in the literature (Martin et al., 2017; Martin et al., 2013; Poudel et al., 2013) and our own

assessments (Figure 3-1). The blackberry plant sampling included 11 different cultivars ranging from less than a year to seven years old (Table 3-1). Two 6-inch shoot tips were collected from each plant, one from a primocane and one from a floricane. Samples were stored on ice for transport to the Plant Virology lab at Clemson University. Leaves and petioles from individual plant samples were cut into small (~2mm^2) pieces using a razor blade, which was cleaned in 10% bleach for 30 s, rinsed with distilled water, and dipped in RNase-Away between samples to avoid cross-contamination. For each sample, 25 mg of tissue was stored in 2 mL tubes with a sterile metal bead at -80^{o}C for RNA extraction. Extra tissue for each sample was also stored at -80^{o}C.

Photos of symptomatic plants from six farms were included in the analysis, the full collection of photos and associated viruses detected in each sample have been deposited at Dryad (https://doi.org/10.5061/dryad.9kd51c5rm). After assessment of each picture taken from the field, we classified 12 types of symptoms: vein banding, necrotic and chlorotic spots, interveinal chlorosis, chlorotic feathering pattern, marginal necrosis, leaf distortion, oak leaf pattern, irregular chlorosis, transverse zigzag chlorosis, vein yellowing, and rugosity (Figure 3-1). Some of these symptoms appeared in combinations in single plants. For network analysis, a matrix was prepared to include binary (0, 1) presence or absence of the viruses with symptom classes for each symptom type observed on a plant.

Table 3-1. Inventory of blackberry samples collected in commercial farms in each cultivar and the age of fields (year old) found at each sampling site.

Farm number	**Age range (years)**	**Number of blackberry samples collected in each cultivar**										
		'Navaho'	'Ouachita'	'Natchez'	'Prime-ark 45'	'Von'	'Caddo'	'Ponca'	'Osage'	'Brazos'	'Arapaho/ Apache/ Osage'	'Ouachita/ Caddo'
1	1	0	0	0	0	0	0	0	0	0	0	**40**
2	1, 4, 6	0	**10**	**14**	0	0	0	0	0	0	0	0
3	4	**28**	0	0	0	0	0	0	0	0	0	0
4	5	**20**	**7**	0	0	0	0	0	0	0	0	0
5	unknown	**10**	**10**	0	**20**	0	0	0	0	0	0	0
6	1	0	0	0	0	0	0	0	0	0	**10**	0
7	5-8	0	0	0	0	0	0	0	0	**29**	0	0
8	<1, 3, 6	0	0	**9**	**30**	**10**	0	0	0	0	0	0
9	<1, 2, 4		**3**	**3**	0	**2**	**4**	**6**	**12**	0	0	0

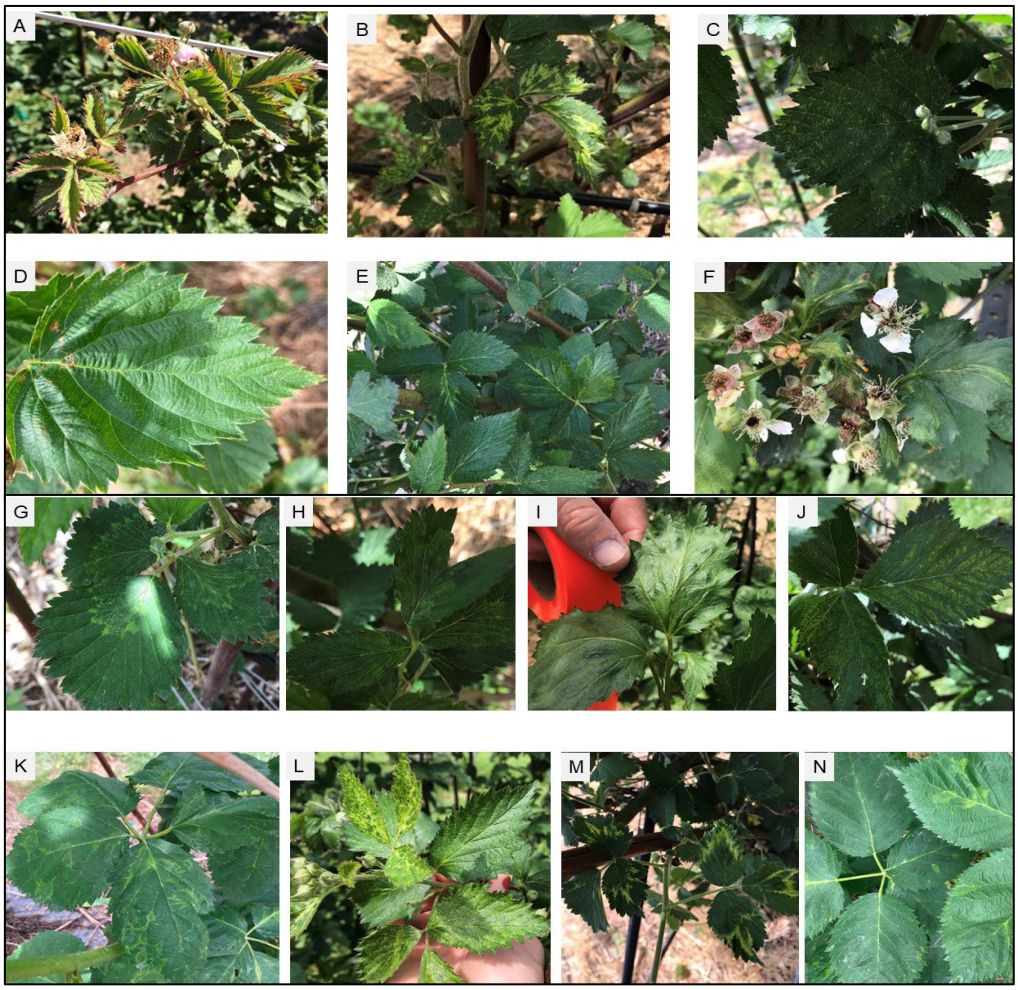

Figure 3-1: Symptoms associated with blackberry yellow vein disease observed on different cultivars in blackberry fields in South Carolina. A: marginal necrosis on cv. Ouachita, B: vein banding on cv. Navaho, C: chlorotic spots on cv. Navaho, D: necrotic spots on cv. Prime-Ark 45, E: chlorotic feathering pattern on cv. Ouachita, F: vein banding and leaf distortion on cv. Navaho, G: oak leaf pattern on cv. Navaho, H: irregular chlorosis on cv. Navaho, I: rugosity on cv. Navaho, J: interveinal chlorosis on cv. Ouachita, K: line patterns on cv. Natchez, L: chlorotic mottling on cv. Ouachita, M: transverse zigzag chlorosis on cv. Navaho, and N: ringspots on cv. Von.

RNA Extraction

RNA extractions were performed using the RNeasy Plant Mini Kit (Qiagen) with some modifications, as described previously (Chapter 2, Dantes et al., 2024). Because the RNeasy kit provided limited quantities of RNA and most were used for a previous study (Chapter 2, Dantes et al., 2024), a modified CTAB (cetyltrimethylammonium bromide) protocol was used to extract total nucleic acid from the extra tissue stored at -80°C. Briefly, 2-ml tubes containing leaf and petioles tissues were flash-frozen in liquid nitrogen and disrupted into a fine powder using a mixer mill at 30 Hz for two minutes. A second quick flash-freeze was given to each sample before adding the lysis buffer. Then, 900 µl of CTAB buffer as described previously (Chen et al., 2019) and 18 µl of β-mercaptoethanol were added to each tube, and then vortexed to homogenize the samples. The samples were heated at 65°C for 20 min. and 600 µl of chloroform: isoamyl alcohol (24:1) was added to each tube and the samples were vortexed and incubated at room temperature for 5 min. The samples were centrifuged at 22,000 RCF for 7 minutes at room temperature. The supernatant was pipetted and transferred into a new 1.5-ml tube and 500-µl of chloroform:isoamyl (24:1) was added to the supernatant. The solution was vortexed and centrifuged at 22,000 RCF for 7 minutes at room temperature. The supernatant was carefully pipetted into a new 1.5-ml tube and 1/10 volume of 3M sodium acetate (pH 5.2) and 2/3 volume of cold isopropanol were added into each tube. The solution was well-mixed and centrifuged at 22,000 RCF for 15 min at 4°C to precipitate nucleic acids. The pellet was washed with 300 µl of 70% ethanol. The ethanol was discarded, the samples were quick-spinned and the remaining ethanol was pipetted out

without disturbing the pellet. The samples were air-dried in a laminar flow hood for 30 min and nucleic acids were reconstituted with 100 µl of nuclease-free water.

RT-qPCR for detection of BYVaV, RBDV and TRSV

A multiplex probe-based RT-qPCR was used for the detection of BYVaV and RBDV, coupled with primers and probe amplifying the plant NADH dehydrogenase ND2 subunit (*ndhB*) gene as a reference (Thompson et al., 2003). RT-qPCRs were performed as described previously (Chapter 2, Dantes et al., 2024). The same protocol was used to test the samples for TRSV using a duplex probe-based RT-qPCR coupled with the same reference gene (ndhB). Amplification resulting in Cq values of less than 38 was considered a positive detection. Positive controls for each virus were verified by RT-PCR and Sanger sequencing.

RT-PCR for detection of BVY and BVE

RT-PCR was used to test for BVY in 95/369 samples from farms 3, 4, 5, and 8. We also tested 157/369 plant samples across all farms for BVE using the qScript™ XLT One-Step RT-qPCR ToughMix (Quantabio) according to manufacturer recommendations. Cycling conditions for the detection of BVY and BVE consisted of reverse transcription for 10 min at 48°C followed by an initial denaturation for 3 min at 94°C, and 40 cycles of 20 s of denaturation at 94°C, 40 s of annealing at 60°C (for BVY) or 63°C (for BVE) and 60 s of extension at 70°C. RT-PCR products were resolved on a 1.5% agarose gel followed by post-staining with GelRED (Biotium) and imaging on a Bio-Rad gel documentation system. Positive controls were included in each set of reactions, for each

virus, and were confirmed by Sanger sequencing of the respective RT-PCR products and verified using NCBI BLASTn.

RT-qPCR SYBR assay for detection of BVY, BlaLPV, BVBaV, BVE, BCRV and BLMV.

The remaining 277 samples were tested for BVY using a Luna® Universal One-Step RT-qPCR SYBR kit. All the samples were tested for BlaLPV and BVBaV using this same kit. A 10- µl SYBR green reaction (Biorad) was used to screen the 372 samples for BCRV and BLMV, and the remaining 215 samples for BVE. The manufacturer's instructions were followed to prepare the reaction mix and for cycling conditions. The annealing temperature was standardized for each virus (Table 3-2). A melt curve analysis was included after each RT-qPCR run (65°C to 95°C in 5°C increments) to determine any off-target amplification and eliminate false positives. Amplification resulting in Cq values lower than 38 and with a melt profile consistent with the positive control was considered a positive detection. The positive control for each virus was verified by Sanger sequencing of the PCR product followed by a NCBI BLASTn search. All primer sequences used for virus detection are listed in Table .

Table 3-2. Primers used to screen the samples for the viruses.

Virus/target	Forward	RT-PCR/RT-qPCR kit	Annealing temperature	Amplicon size	Reference
BYVaV	**Sense:** 5' ATAGAAGCGAGGTTAARACCTG 3' **Antisense:** 5' CACRTYGTTACCTCTAAGCTCG 3' Probe: 5'Cy5/TTGAAAAGA/TAO/TGGGTYGGHGTGGACA/3IAbRQSp 3'	Quantabio	58°C	131	Poudel et al. 2013
RBDV	**Sense:** 5' TGGGAGATCCAATGTTCATAGT 3' **Antisense:** 5' CATCAGACTCTCAGTCATCGT 3' probe	Quantabio	58°C	94	Quito-Avila and Martin 2012
TRSV	**Sense:** 5' CCTGGGCACAAGTGAAATGTTG 3' **Antisense:** 5' GCTACCAGAAACAACGGTCTAAC 3' probe	Quantabio	58°C	68	Beaver-Kanuya unpublished
BCRV	**Sense:** 5' AGGTTGAAATGGCTTTGACCC 3' **Antisense:** 5' AAGCAGCRCATCGCCTTATAC 3'	Biorad	55°C	137	Poudel et al. 2014
BVEq	**Sense**: TCAGCAAAGCCCTGAACACA **Antisense**: AGCABAGYTCGGAAYAAGCYG	Biorad	60°C	105	This study
BVE	**Sense:** 5' TGTGGACGATGCACGCCAGATCCC 3' **Antisense:** 5' GCTCCACTGGAGGAGATTCTGGTG 3'	qScript™ XLT One-Step RT-qPCR ToughMix (Quantabio)	63°C	246	Sabanadzovic et al. 2011
BVY	**Sense:** 5' CTGTGGGGAGATTTGGAGAA 3' **Antisense:** 5' TCATTCCATGGGTGTGTC 3'	qScript™ XLT One-Step RT-qPCR ToughMix (Quantabio)	60°C	384	Susaimuthu et al. 2008
BVY2	**Sense**: GAATTTGATGCAGAGGTYATA **Antisense**: TGCTTRAAGTGRGCSTTTCCA	Luna® Universal One-Step RT-qPCR SYBR	60°C	186	This study
BLMV	**Sense**: CATAAAGGAATTCATACCCAGGAAC **Antisense**: AGTTGCATCTTACCTTTCGCG	Biorad	60°C	349	(Poudel et al., 2018)
BlaLPV	**Sense**: GCTAGCTGACGGGAAAGTGT **Antisense**: CGAACCTGTTCAGGCGGATA	Luna® Universal One-Step RT-qPCR SYBR	60°C	285	Elise et al, 2024, in preparation
BVBaV	**Sense**: CTGCTRTAYAGCACRGTTAAACA **Antisense**: RTACGTGCGGACTTTGTTAGT	Luna® Universal One-Step RT-qPCR SYBR	60°C	157	(Thekke-Veetil & Tzanetakis, 2017)

Virus name abbreviations are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), BLMV (blackberry leaf mottle virus), BlaLPV (blackberry line pattern virus), and TRSV (tobacco ringspot virus)

Bipartite network analysis

We conducted a bipartite network analysis where one node represents a virus and the other represents an individual sample. Node degree is the number of links between a sample and a virus species, in the network. In our case, the node degree of a sample represents the number of viruses detected in that sample. We evaluated the node degree to understand virus prevalence within sample groups. This analysis was done in the R programming language using several packages: dplyr (Wickham et al., 2023), igraph (Csárdi et al., 2024), and bipartite (Dormann et al., 2009). Code was deposited in the GitHub repository (https://github.com/ricardoi/blackberrysc_virome).

Statistical analysis.

We used Fisher's exact test (Fisher, 1922) to evaluate if the age of planting or cultivars was related to virus accumulation in blackberry fields. Age was divided into two categories (0-3 or 4-7 years). Due to sample size variability, only data for six cultivars were statistically analyzed. Cultivars that had a sample size equal to or greater than 26 were retained. In each age category or for each cultivar, we calculated the number of uninfected samples and those infected by one or more viruses. Statistical tests were performed in R.

Results

Single and mixed infections detected in blackberry and wild Rubus samples

Our testing revealed that eight of the nine viruses, all except for BLMV, were detected in the asymptomatic blackberry group, while all nine viruses were detected in symptomatic blackberry and wild Rubus plants. We detected only one virus in 44/165 asymptomatic blackberry plants, 35/112 symptomatic blackberry plants, and 22/92 wild *Rubus* plants (Figure 3-2). The number of samples infected with two viruses was similar across sample groups (23/165 asymptomatic, 22/112 symptomatic and 21/92 wild samples). However, more samples with three and four viruses detected were in the symptomatic and wild groups compared to the asymptomatic group (Figure 3-3). In one sample from a symptomatic plant, five viruses were detected (Figure 3-2B). In total, we detected 19 double and 19 triple virus combinations in the symptomatic group and nine double and three triple virus combinations in the asymptomatic group. The wild *Rubus* group had nine double and nine triple virus combinations detected (Figure 3-4). Overall, more mixed-infected plants were found in the symptomatic blackberry and wild *Rubus* groups compared to the asymptomatic blackberry group. Single infections were detected more frequently in asymptomatic and symptomatic blackberry plants compared to the wild *Rubus* (Figure 3-3).

In the asymptomatic group, eight plants were co-infected with BYVaV and RBDV, the most prevalent double virus infection in this group (Figure 3-2A). BYVaV and TRSV were found in double infections in four symptomatic blackberry and five wild *Rubus* samples (Figure 3-2 A and B). This virus combination (BVE, BVBaV and BCRV)

was detected in three symptomatic samples and one asymptomatic sample. Three symptomatic plants and one wild plant were co-infected with BCRV, RBDV and BlaLPV (Figure 3-2 A and B). RBDV, BLMV and BYVaV co-infected two symptomatic samples and RBDV, BLMV and TRSV also co-infected two symptomatic samples from cultivar cv. Navaho (Figure 3-2 A and B).

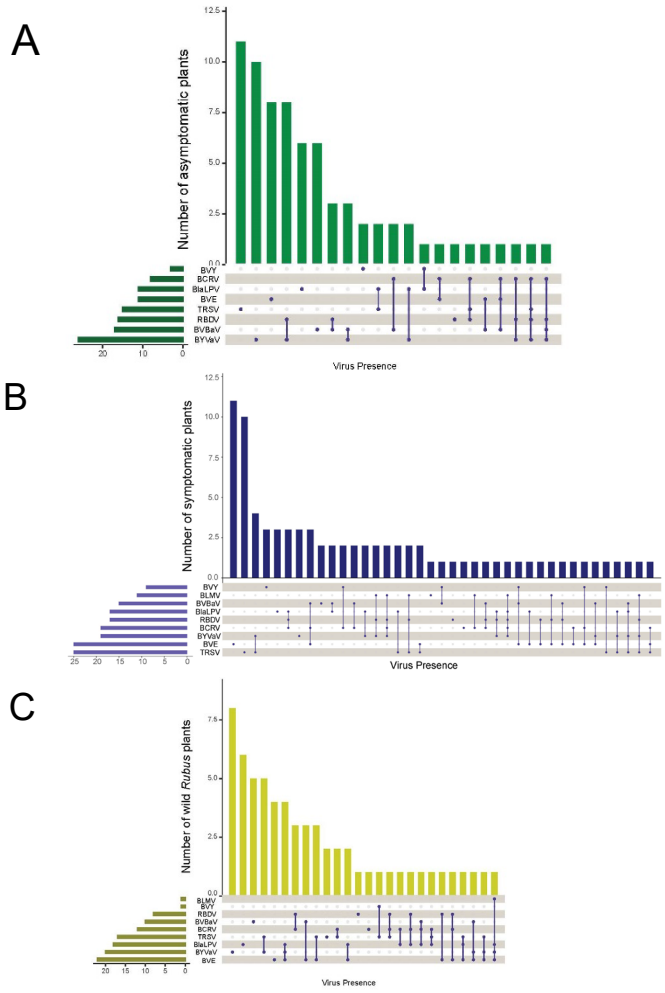

Figure 3-2. Upset plots of specific single, double, triple and quadruple infection combinations detected in blackberry plants and wild *Rubus* plants collected from the crop borders. (A): Asymptomatic plants, (B) symptomatic plants and (C) wild plants.

Virus name abbreviations are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), tobacco ringspot virus (TRSV), blackberry leaf mottle virus (BLMV), blackberry line pattern virus (BlaLPV).

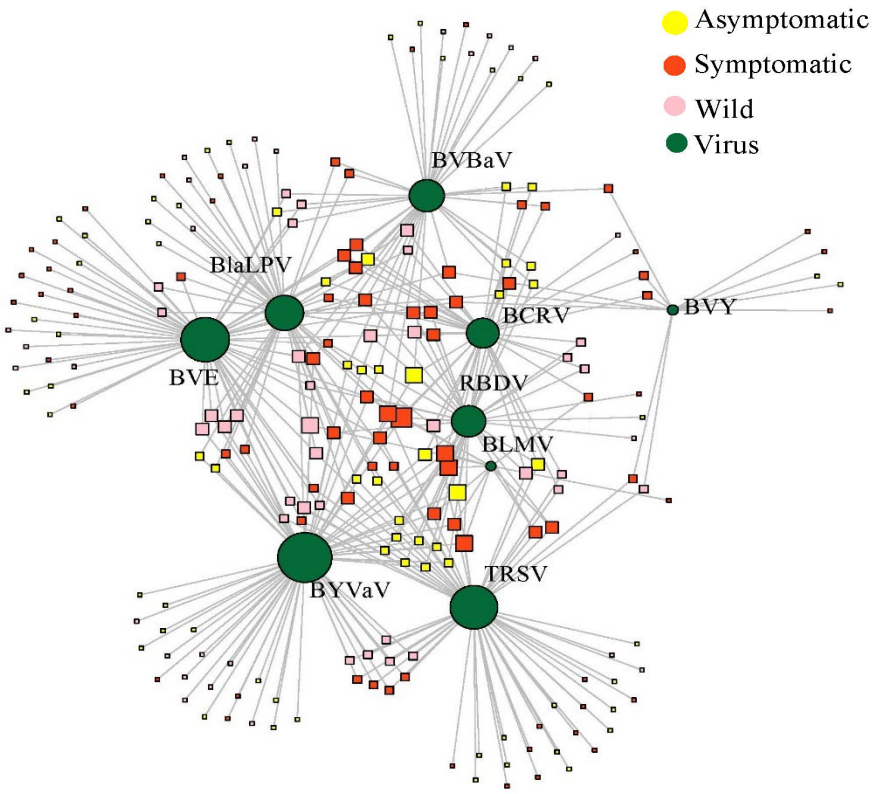

Figure 3-3: Virus prevalence and mixed infections in blackberry farms in South Carolina (green nodes represent the viruses, yellow nodes are asymptomatic blackberry plants, red nodes are symptomatic blackberry plants, and pink nodes are wild plants (*Rubus* plants, and roses). Node size is proportional to the node degree.

Virus name abbreviations are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), BLMV (blackberry leaf mottle virus), BlaLPV (blackberry line pattern virus), and TRSV (tobacco ringspot virus).

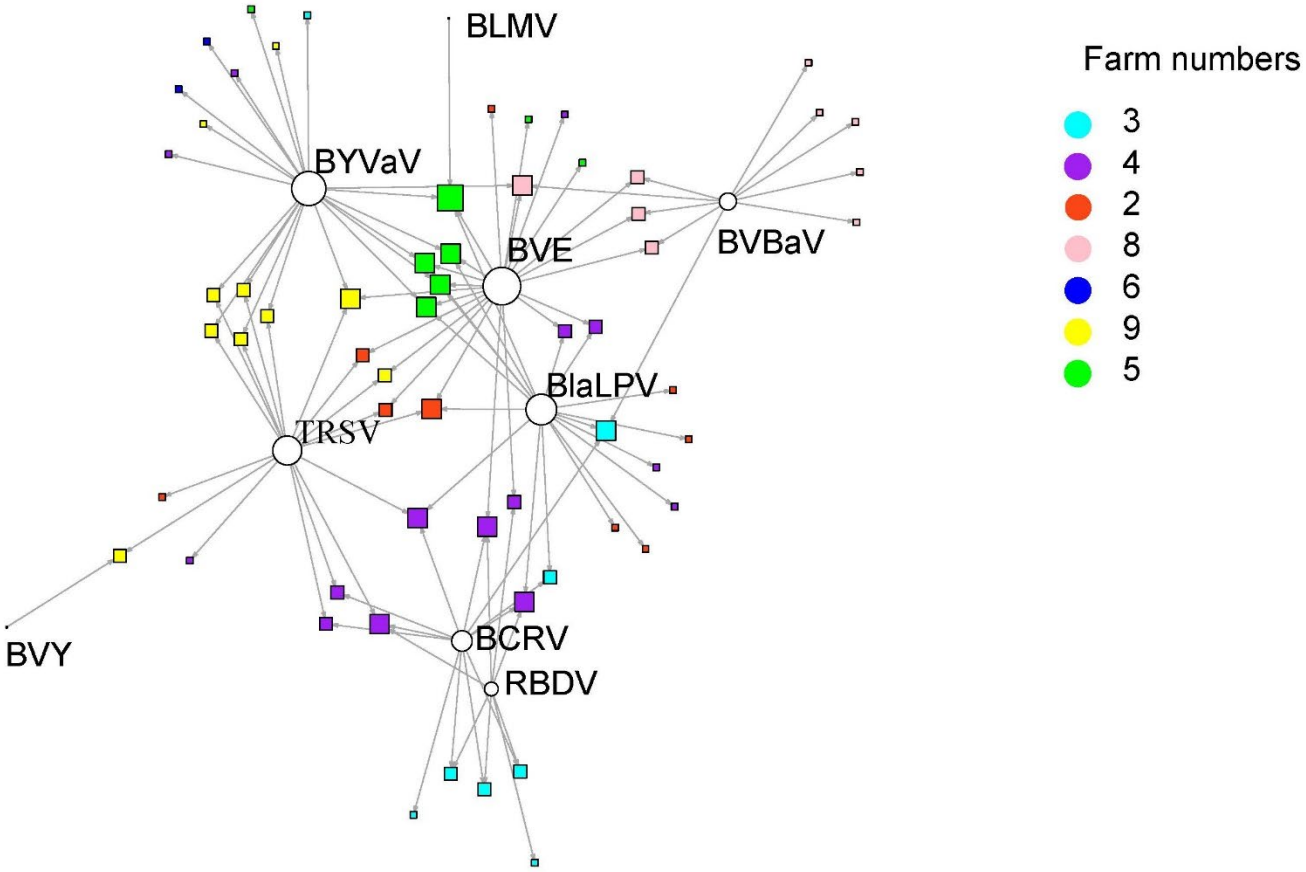

Figure 3-4. Virus prevalence and mixed infections in wild plants surrounding blackberry farms in South Carolina (circular nodes are viruses and squared nodes are wild plant samples). Node colors represent the farm numbers. Node size is proportional to the number of viruses detected in each sample.

Virus name abbreviations are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), tobacco ringspot virus (TRSV), blackberry leaf mottle virus (BLMV), blackberry line pattern virus (BlaLPV).

Blackberry cultivar and plant age as factors in virus accumulation

'Navaho' harbored more viruses than the cultivars 'Ouachita', Prime-Ark 45', 'Natchez', and 'Brazos'. During sample collection, 58 plants from cultivar 'Navaho' were collected; 86% (50/58) of those plants tested positive for one or more viruses (Figure 3-5). These plants were collected from 4–5-year-old plantings in the upstate and midstate from three different farms (Figure 3-6). We collected samples of 'Ouachita' blackberry from four farms, which were 4-6 years old. Of the samples from 'Ouachita', 67% (20/30) were infected by one or more viruses (Figure 3-5). Fewer viruses were detected in'Prime-Ark 45' (48%; 24/50) and 'Natchez' (58%; 17/26). Samples of 'Prime-Ark 45' blackberry plants were collected from two farms, but age was not recorded for 20 of them, 10 plants were one-month post-planting, and 20 were six years old (Figure 3-5). Of the 50 'Prime-Ark 45' plants sampled, 24 tested positive for at least one virus. None of the 'Prime-Ark 45' plants tested positive for RBDV, BLMV and BlaLPV. Of the 26 'Natchez' plants (1-4 years old), 17 were infected by one or two viruses. We detected at least one virus in 75% (9/12) and 67% (8/12) of the samples collected from cultivars 'Osage' (4 years old) and 'Von' (the age was not recorded for 10 plants and two plants were 4 years old), respectively (Figures 3-5 and 3-6).

Only single infections were detected in mixed cultivars (cv. Arapaho/Apache/Osage and cv. Ouachita/Caddo) and cultivars Ponca, Caddo and Brazos. Plants were infected by one virus from the mixed cultivars Arapaho/Apache/Osage (7/10) and Ouachita/Caddo (8/40), respectively. These plants were one year old. The cultivar Brazos had the lowest percentage of virus-infected samples; 14% of virus infection

accounted for 4/29 samples tested, even though these samples were collected from an older planting (seven years old). ‘Caddo’ and ‘Ponca’ had small sample sizes (4 and 6 plants, respectively) and 50% of those were infected by one virus (Figure 3-5). ‘Ponca’ plants were one month old, while those from ‘Caddo’ were two years old. With the exception of the 7-year-old Brazos planting, the plants in which only a single virus was detected were new plantings (Figure 3-6).

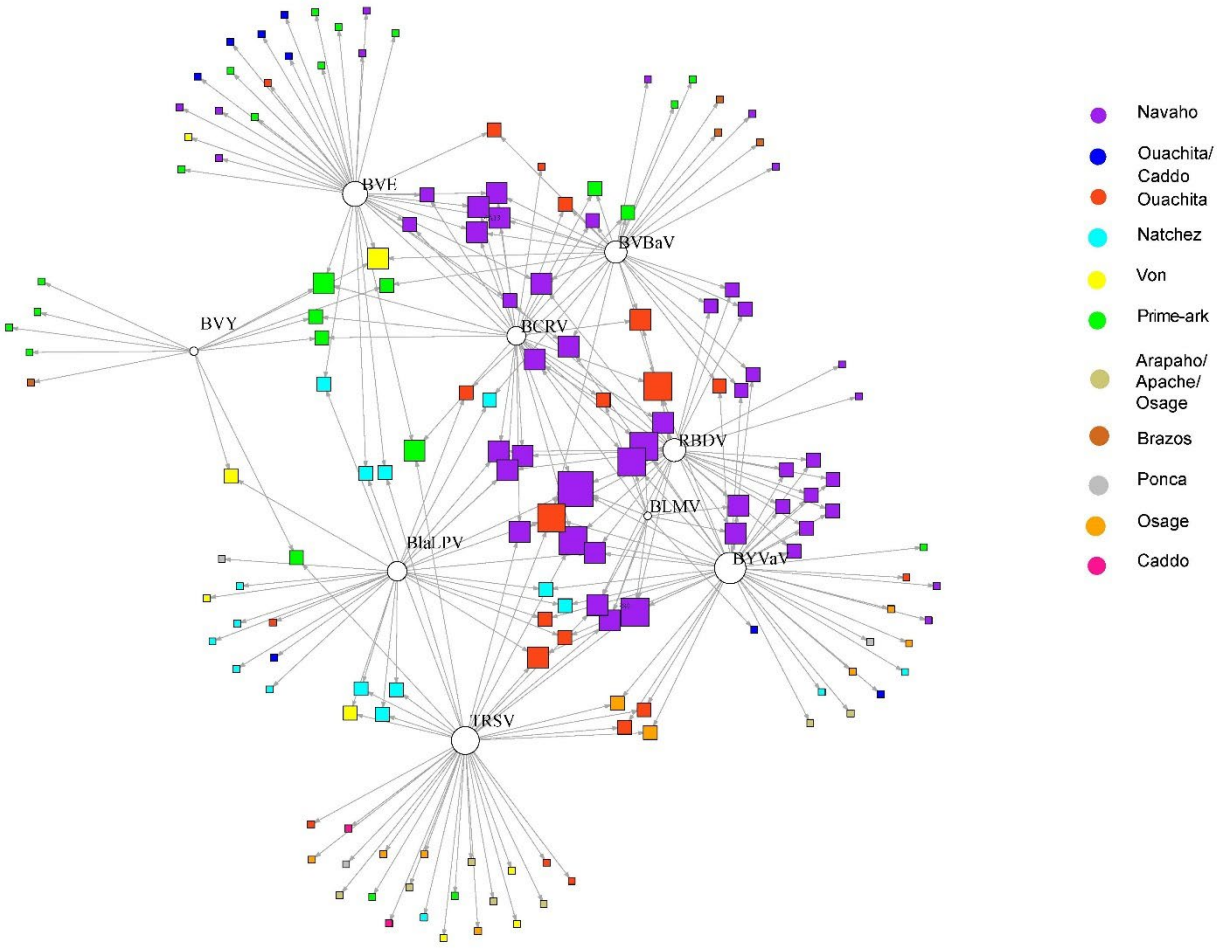

Figure 3-5: Virus prevalence and mixed infections in blackberry farms in South Carolina (circular nodes are viruses and squared nodes are plant samples). Node size is proportional to the node degree. Node colors represent the cultivars.

Virus name abbreviations are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), tobacco ringspot virus (TRSV), blackberry leaf mottle virus (BLMV), blackberry line pattern virus (BlaLPV).

Blackberry fields that are 4-7 years old tended to harbor more viruses than younger fields (Fisher's exact test, $P < 00001$). Samples collected from one-month-old to three-year-old plants (32%, 32/101) tested positive for one or two viruses (Figure 4). However, 67% (98/145) of the samples collected from 4-7-year-old blackberry plants tested positive for one or more viruses. No viruses were detected from 56%, 28% and 34% of the asymptomatic, symptomatic, and wild plants respectively (Table 3-3).

Table 3-3. Blackberry plants and wild Rosaceae plants that tested negative for all the viruses tested.

Number of negative plants in each group (number of negatives/total)		
Asymptomatic	Symptomatic	Wild
93/165	31/112	31/92

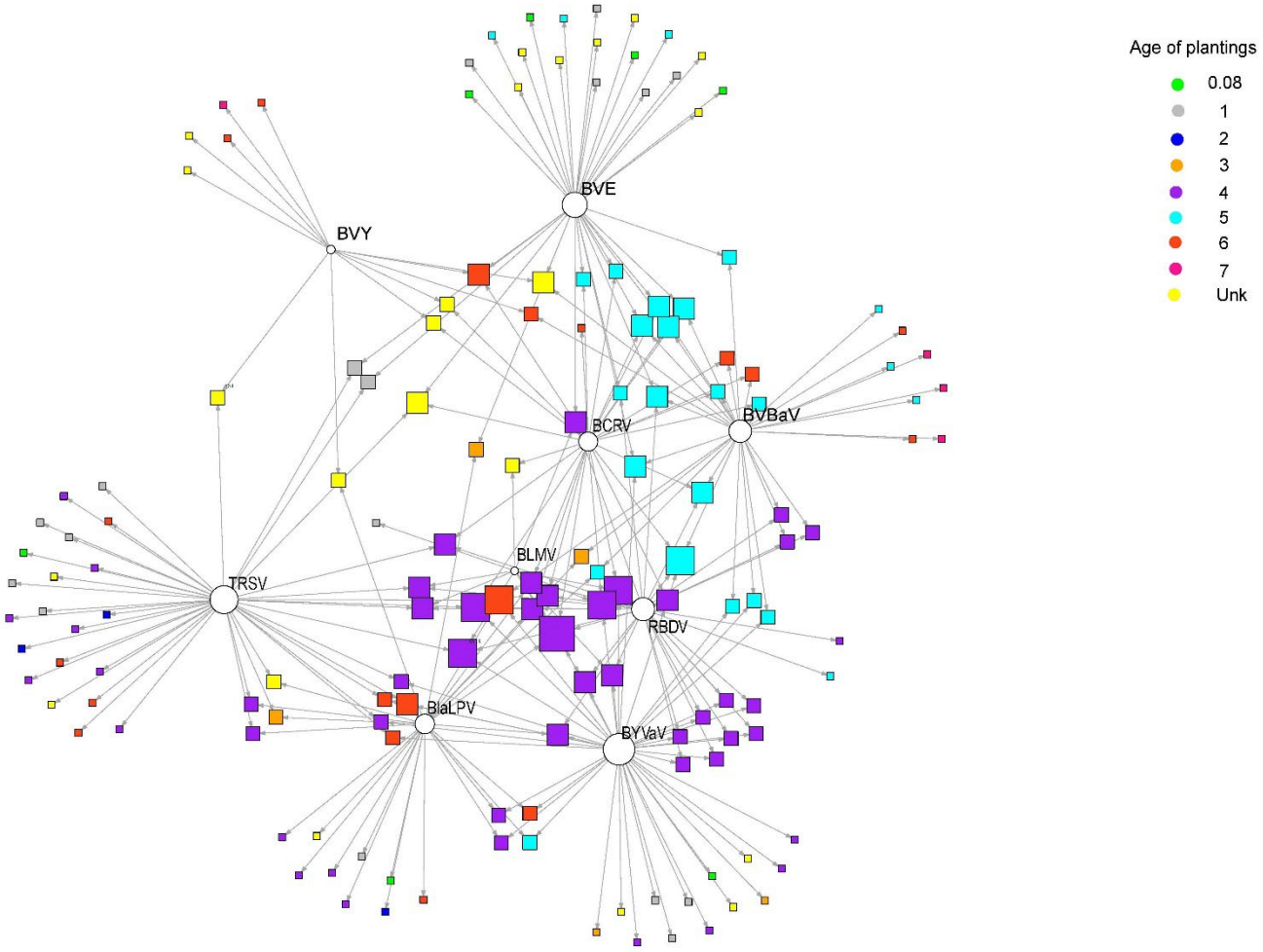

Figure 3-6. Virus prevalence and mixed infections in blackberry farms in South Carolina (Circular nodes are viruses and squared nodes are plant samples). Node colors represent the age of blackberry plantings.

Virus name abbreviations are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), tobacco ringspot virus (TRSV), blackberry leaf mottle virus (BLMV), blackberry line pattern virus (BlaLPV). The “Unk” category means that the age of some plants was not recorded.

Virus combinations linked to blackberry symptom types observed in the field In single infections, TRSV, BVE, BVY, BlaLPV, and BVBaV were all detected in multiple plants displaying symptoms such as necrotic and chlorotic spots, vein banding, and rugosity. Three plants showing leaf distortion were infected with BVE. Leaf distortion was also associated with a plant infected only by RBDV. Vein banding was also observed on plants infected with BYVaV or BCRV (Figure 3-7). In 'Caddo', 'Von', and 'Osage', TRSV was detected in plants displaying chlorotic and necrotic spots in single infections, however, in the cultivar 'Ouachita', TRSV was detected in plants showing vein banding, rugosity and interveinal chlorosis (Figures 3-7 and 3-8). Similarly, BVE was detected in plants displaying symptoms such as leaf distortion, vein banding and rugosity in the cultivar 'Navaho' but was also detected in plants showing chlorotic and necrotic spots, and rugosity in 'Prime-Ark 45'. BVE was detected in an 'Ouachita' plant showing vein banding and necrotic spots (Figure 3-8). In addition, BlaLPV was detected in single infections in three different cultivars. In cv. Natchez, plants displayed rugosity and vein banding; in 'Ouachita', chlorotic and necrotic spots; and in 'Von', vein banding and ringspots. On the other hand, BVY and BVBaV were only detected in plants displaying chlorotic and necrotic spots, vein banding and rugosity in 'Prime-Ark 45' (Figures 3-7 and 3-8). Overall, symptoms such as vein-banding, chlorotic and necrotic spots, rugosity and leaf distortion were not associated with any of the specific viruses included in this study.

In general, plants co-infected with two viruses exhibited the same symptom types as those infected with one virus. For instance, symptomatic plants co-infected with

BYVaV and TRSV displayed chlorotic, necrotic spots, vein banding and rugosity. Similarly, plants infected with TRSV showed chlorotic, necrotic spots, vein banding and rugosity. TRSV might be the main driver of these symptom expressions. A virus combination tends to induce different symptoms in different genotypes (cultivars). As an example, BlaLPV and TRSV co-infected two samples; one showed line patterns (cv. Natchez), and the other showed chlorotic and necrotic spots (cv. Von). The most common symptom types observed in single and double infections were chlorotic and necrotic spots, rugosity, and vein banding (Figure 3-7).

Three plants from cultivar 'Navaho' were triple-infected with BCRV, BVE, and BVBaV. Two of them displayed leaf distortion and vein banding whereas only rugosity was observed on the other plant. Oak leaf pattern was observed on two plants in the field (cv. Navaho). Both were triple-infected with BYVaV, BLMV and RBDV. This same combination of three viruses and BCRV was associated with chlorotic feathering patterns in one plant in the same cultivar. However, BCRV, BlaLPV and RBDV were also found in another 'Navaho' plant displaying chlorotic feathering pattern and marginal necrosis. This same virus combination was found in two other plants (one showing vein banding and the other, chlorotic spots). Additionally, BYVaV, TRSV, BCRV and BlaLPV co-infected another plant showing chlorotic feathering pattern from 'Ouachita'. A new symptom was observed in one plant in the field. It was described as transverse zigzag chlorosis, which was associated with TRSV, RBDV and BLMV. This combination differs by one virus (TRSV) from the one detected in plants displaying oak leaf patterns. Overall, the network analysis showed almost no clear grouping in terms of virus

combination and symptom types. Symptoms such as vein banding, necrotic spots, chlorotic spots, rugosity, and chlorotic feathering pattern were associated with different virus combinations. Oak leaf pattern, on the other hand, is consistently associated with one virus combination (BYVaV, BLMV and RBDV) (Figure 3-7). Plants co-infected with BYVaV and RDBV were asymptomatic. However, in the presence of a third virus, BLMV, symptoms of oak leaf pattern were observed in both plants, suggesting that BLMV might be driving symptom development in this mixed infection. When BYVaV is replaced by TRSV, the symptoms observed on the plants are different (irregular chlorosis on one plant and transversal zigzag chlorosis on the other).

Common viruses found in different combinations inducing the same symptom type

Our analysis showed that one symptom type could result from different virus combinations. However, there are also cases of one or two viruses being common across the combinations of mixed infections. For example, leaf distortion was associated with four different virus combinations (Figure 3-7), with BVE present in all the samples except for one. Line pattern symptoms were associated with three different combinations of mixed infections, with BlaLPV shared among two of them. RBDV and BCRV are two common viruses found in coinfection with other viruses in two samples that showed chlorotic feathering pattern symptoms in cv. Navaho. In ‘Ouachita’, BYVaV and TRSV were the common denominators of chlorotic feathering patterns. These viruses might play a crucial role in symptom development.

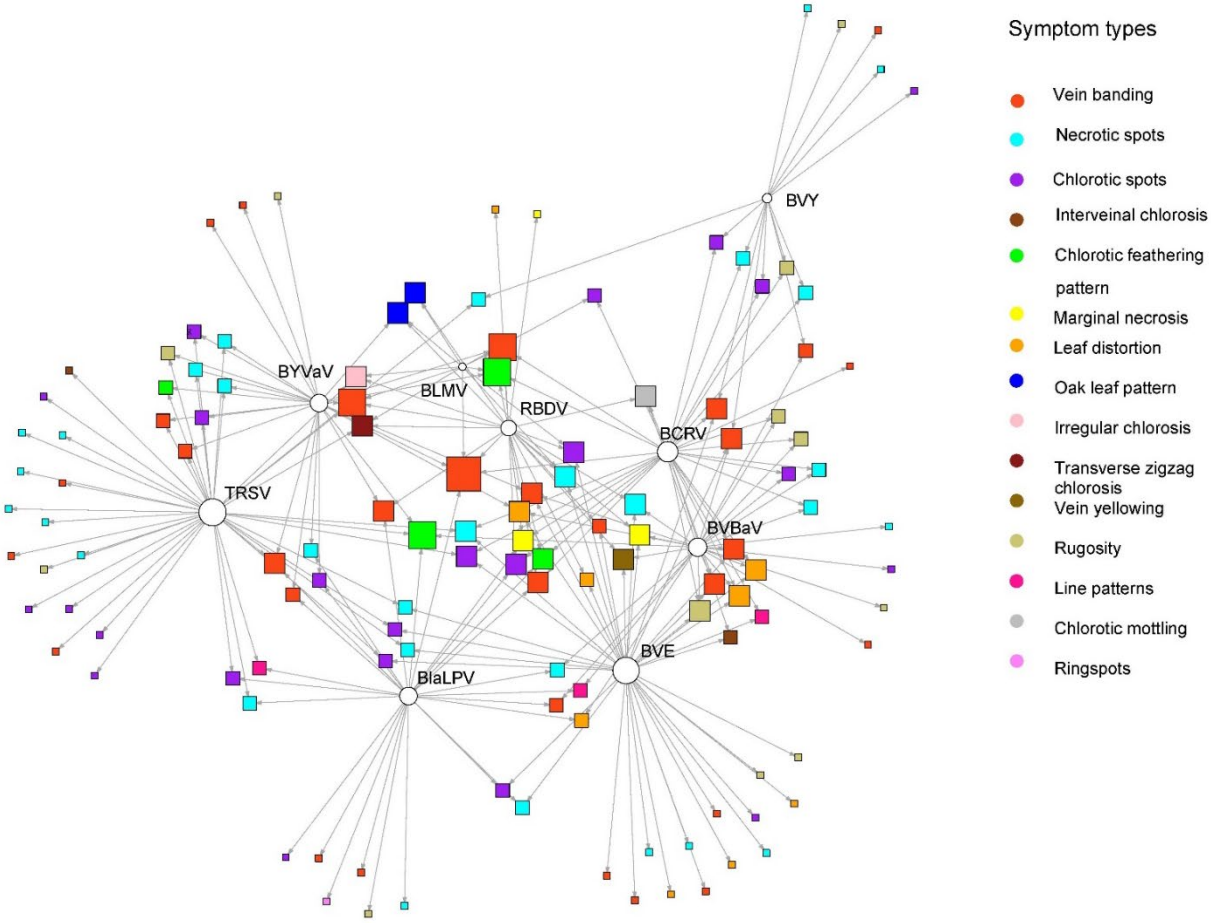

Figure 3-7. Symptomatology network showing single and mixed virus infections associated with different symptom types in blackberry farms in South Carolina. Circular nodes are viruses and squared nodes are blackberry plants. Node colors represent the symptom types observed in the field.

Virus name abbreviations are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), tobacco ringspot virus (TRSV), blackberry leaf mottle virus (BLMV), blackberry line pattern virus (BlaLPV).

Links between symptoms and blackberry cultivars

Within the cultivar Prime Ark 45, plants infected with at least one virus displayed one or more of these four symptom types: chlorotic spots, necrotic spots, rugosity, and vein banding. Single and mixed infections detected in the plants within the cultivars 'Von' and 'Osage' were all associated with chlorotic spots, necrotic spots, vein-banding, and ringspots, except for no ringspots being observed on 'Osage' plants. 'Natchez' plants also displayed chlorotic and necrotic spots and/or line patterns in the presence of different virus combinations except for one plant in which only BlaLPV was detected, which showed vein banding and rugosity (Figure 3-8). 'Ouachita' plants displayed one or more symptoms of these seven symptom types (chlorotic mottling, chlorotic spots, necrotic spots, leaf distortion, rugosity, vein banding, and chlorotic feathering pattern), whereas all 12 symptom types were observed on plants within the 'Navaho' cultivar (Figure 3-8). Cultivar may be an important factor driving symptom expression. Overall, more mixed infections were found in 'Navaho' plants than in any other cultivars. 'Navaho' plants also exhibited a more diverse range of symptoms. On the other hand, 'Prime-Ark 45' plants, in which we mostly detected single or double infections, showed a lower range of symptoms. This same trend was observed for the 'Natchez' and 'Osage' plants.

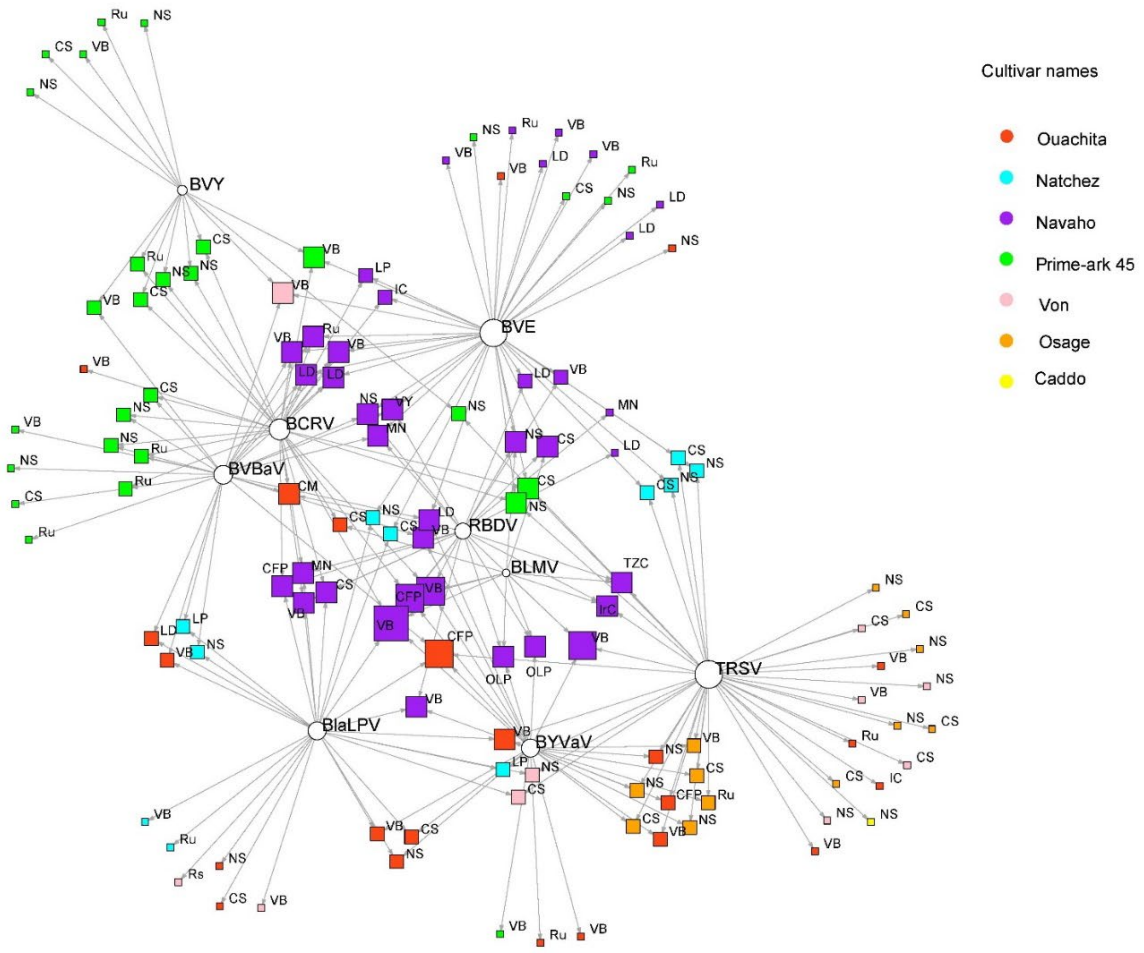

Figure 3-8. Symptomatology network showing virus single and mixed infections associated with different symptom types in blackberry farms in South Carolina (Circular nodes are viruses and squared nodes are blackberry plants. Node colors represent the symptom types observed in the field. Node size is proportional to the node degree. Node labels are different symptom types observed in the field.

Symptom name abbreviations are as follows: Vein banding (VB), necrotic spots (NS), chlorotic spots (CS), interveinal chlorosis (IC), chlorotic feathering pattern (CFP), marginal necrosis (MN), leaf distortion (LD), oak leaf patterns (OLP), irregular chlorosis (IrC), Transverse zigzag chlorosis (TZC), vein yellowing (VY), Rugosity (Ru), Line patterns (LP), Chlorotic mottling (CM), ringspots (Rs).

Virus name abbreviations are as follows: raspberry bushy dwarf virus (RBDV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV), blackberry virus E (BVE), blackberry virus Y (BVY), blackberry yellow vein associated virus (BYVaV), tobacco ringspot virus (TRSV), blackberry leaf mottle virus (BLMV), blackberry line pattern virus (BlaLPV).

Discussion

In this study, we were able to resolve a large number (369) of individual plants for several viruses, but the large number of plants made full virome characterization (e.g., by using high throughput sequencing) impractical. Our study illustrates the importance of, and some constraints of, sampling strategy. There is a tradeoff between sample size and method practicality, and here, we sacrificed the ability to detect all viruses in order to achieve single-plant resolution. Samples from 35 plants that exhibited virus disease symptoms had only one virus detected. One major caveat of these results is that although we tested for the major viruses known to be associated with BYVD (Martin et al., 2017) and present in South Carolina (Chapter 2, Dantes et al., 2024; Poudel et al., 2018), it is possible that other viruses not included in our testing panel were present in these plants and contributed to symptomatology. Other factors may also contribute to symptomatology, and we cannot be sure that symptoms are attributed to virus infection at all. Insect vector feeding could induce leaf distortion, chlorosis, and russeting as has been observed in the case of eriophyid mites (Oldfield, 1970). Mixed infections can change symptom types, resulting in mild to severe symptoms (Alcalá-Briseño et al., 2020; Moreno & López-Moya, 2020). Our results suggest that RBDV and BYVaV may be latent even in co-infection while some other double infections, such as TRSV and BYVaV, were associated with symptoms. Ultimately, network analysis is a correlative approach that has the potential to highlight possible etiologies, but conclusively resolving specific etiologies requires pathogenicity tests. Getting blackberry viruses into single infections in plants is challenging (Susaimuthu et al., 2008), so the development of

infectious virus clones and controlled inoculations would be a better approach (Yepes et al., 2018).

It is uncommon for blackberry plants to show symptoms when infected with just one virus, and generally, symptoms have been thought to appear mostly in mixed infections (Martin et al, 2013). However, some viruses are associated with symptoms in single infections. In Mexico, a single infection of TRSV was associated with a blackberry plant exhibiting ringspots and yellowing (Diaz-Lara et al., 2019). In our case, TRSV was associated with chlorotic and necrotic spots, vein banding, rugosity, and interveinal chlorosis in single infections. A recent study showed that BLMVwas associated with ringspots, vein yellowing, and chlorotic feathering patterns in single infections in the cultivar 'Ouachita' (Druciarek et al., 2024). In our samples, BLMV was not detected in asymptomatic plants and was always detected in co-infection with one or more viruses in the symptomatic and wild groups, except for one cv. Ouachita symptomatic plant, which displayed chlorotic feathering patterns symptom, in which we only detected BLMV. Our results on single infections of BLMV complements the work done on single infection of BLMV (Drucariek et al., 2024) because we are providing in-field context of symptoms associated with presumed single infections of BLMV.

BYVD can be severe and spread rapidly in blackberry plantings, even resulting in total loss of plants within two years (Susaimuthu et al., 2008). When BYVaV was discovered, it was initially proposed as the causal agent of BYVD (Martin et al., 2004), but later studies suggested that BYVD was the result of synergistic interactions in mixed virus infections, with BYVaV as the central virus necessary for disease development

(Susaimuthu et al., 2008). In our study, BYVaV was detected in only 19 of 112 symptomatic plants and 18 of these exhibited yellow-vein symptoms (vein banding only or vein banding coupled with either chlorotic and necrotic spots, leaf distortion, and/or rugosity, chlorotic feathering patterns, or oak leaf patterns). The plants in our study showed some symptoms that are similar to the plants in which BYVaV was first characterized (Martin et al., 2004; Susaimuthu et al., 2006). BYVaV was later found in samples exhibiting chlorotic feathering pattern, ringspots, vein banding, and mosaic (Poudel et al., 2013). However, BYVaV was not detected in the remaining symptomatic samples that showed ringspots, chlorotic mottling, vein banding, vein yellowing, line patterns, implying that other viruses can cause similar symptoms in the absence of BYVaV. Our recent study with these same samples, in which we conducted HTS and Virtool analysis, suggested based on pooled samples that BYVaV might not be crucial to symptom expression in the BYVD complex in South Carolina (Chapter, Dantes et al., 2024), and this finding was further supported by the lack of association of BYVaV with symptoms here.

Most symptom types were not specifically associated with one virus or one virus combination, supporting previous observations that symptom types in the field are independent of the virus combination (Martin et al. 2013). Symptom types such as oak leaf patterns, chlorotic feathering patterns, marginal necrosis, transverse zigzag chlorosis, and line patterns resulted from mixed infections by two or more viruses. On the other hand, vein banding, chlorotic and necrotic spots, leaf distortion and rugosity were associated with either a single virus infection or several viruses in mixed infections.

Martin et al. (2013) stated that symptom severity is linked to the accumulation of viruses in mixed infections, which is supported by the trend revealed by our network.

In our samples, 'Navaho' and 'Ouachita' harbored more viruses in mixed infections than any other cultivars whereas 'Prime-Ark 45', 'Natchez', and 'Von' mostly had single and double infections. 'Osage' and 'Caddo' had a few single-infected samples, which is not surprising as these plants were recently planted. Cultivar as a driving factor could not be accurately assessed in our study because we did not have equal numbers of each cultivar with equivalent distributions of ages. Older blackberry plantings tend to accumulate more viruses (Figure 3-6; Susaimuthu et al. 2008). There was one exception to this: the seven-year-old Brazos planting, a small plot located in the coastal region, where blackberry production is sparse compared to the upstate farms, and therefore, regional virus inoculum levels may be overall lower near the coast. These trends suggest that plantings are generally clean at the time of planting, and then viruses are coming into the plantings from external sources. This idea was further supported by the high rates of virus detection in wild *Rubus* at the borders of the blackberry farms, which seem to group by farm (Figure 3-4). An alternative possibility is that 'Navaho' and 'Ouachita' are more susceptible to virus accumulation than other cultivars, but we cannot speculate on cultivar susceptibility differences based on our sampling strategy. More research is needed to assess blackberry cultivar susceptibility to virus accumulation.

The National Clean Plant Program helps to mitigate virus spread by identifying known virus-negative starting material and eliminating viruses from infected stocks (Martin et al. 2012). However, the virus pressure in blackberry fields is still high and

leads to the decline of older plantings after virus accumulation. Overall, our network analysis approach showed that more viruses are accumulating in blackberry plants over time, some specific combinations of viruses are associated with specific types of symptoms, but using this approach we were unable to fully resolve the complexity of virus disease etiology in blackberry.

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CHAPTER FOUR

VIRUS INGRESS AND POTENTIAL VECTOR DYNAMICS IN NEW PLANTINGS OF BLACKBERRY IN SOUTH CAROLINA

Abstract

Blackberry production in the southeastern U.S. is threatened by a devastating disease complex, blackberry yellow vein disease (BYVD). BYVD affects plant yield and fruit quality and reduces the crop lifetime from 15-20 years to 5-7 years. The epidemiology of the numerous viruses implicated in BYVD is not well understood. There is a need for the identification and movement dynamics of natural field vectors for all the viruses associated with BYVD. Assessing the rate of spread of these viruses in the field is crucial for devising disease management strategies. In this chapter, I evaluated the abundance of potential insect vectors (aphids, whiteflies) of important blackberry viruses in two new plantings at two different farms. I also evaluated the ingress of five viruses in those two new plantings both within the season (spring and late summer) and between two seasons (2023 and 2024). The population of whiteflies peaked between mid-May and early June at both sites, while the aphid population peaked from early May to early June in the open field site. At the high tunnel site, the potential vector population was higher between mid-May and early June; however, it was high until the end of June in the open-field site. Blackberry yellow vein-associated virus (BYVaV) and blackberry virus Y (BVY) were not detected at either site. However, in 2023, the incidence of blackberry line pattern virus (BlaLPV) varied from 1% (spring) to 4.8% (summer) and dropped to 4% in the following season (spring 2024). We observed the same trend for blackberry

virus E (BVE) incidence in the field, which was 1% during both testing time points in 2023 and increased to 4% in spring 2024. On the other hand, blackberry leaf mottle virus (BLMV) was detected in one plant in spring 2023, which tested negative in subsequent testing. This study revealed that at least some of the viruses associated with BYVD are spreading into new plantings, and this work will continue in future years to evaluate the stability of virus vector movement and virus incidence overtime.

Introduction

Blackberry yellow vein disease (BYVD) complex is a devastating disease that affects blackberry production in the major growing regions. BYVD may reduce plant productivity from 20 to 5-7 years (Martin et al., 2013). BYVD epidemiology is complex due to the number of viruses associated with the disease, as well as high diversity in their modes of transmission and ecology. This complex disease is associated with more than ten viruses, including blackberry yellow vein-associated virus (BYVaV), blackberry virus Y (BVY), blackberry virus E (BVE) and blackberry leaf mottle virus (BLMV), previously named blackberry leaf mottle-associated virus (BLMaV), blackberry vein banding-associated virus (BVBaV), blackberry chlorotic ringspot virus (BCRV) and others (Martin et al. 2017; Martin et al. 2013). Insect vectors for only two viruses in the BYVD complex have been confirmed experimentally (BYVaV and BLMV) (Druciarek et al. 2024; Poudel et al. 2013). However, their movement dynamics, feeding behaviors, and interaction with these viruses in the field are still unknown.

The epidemiology of blackberry viruses in field settings is still in its infancy. Sentinel plants have been used to assess how some of these viruses spread in blackberry fields, and this work was done in South Carolina. Virus-negative, potted plants were placed in the field near BYVD-infected blackberry plants. They became infected a month later with one or more viruses, suggesting the implication of aerial insect vectors in spreading five viruses (BYVaV, BVY, BVE, BCRV and BLMV) in the field (Poudel et al. 2018; Susaimuthu et al. 2007). Although most vectors of blackberry viruses are not

characterized, some speculations have been made based on the homology of related viruses. For example, BVBaV is an ampelovirus related to grapevine leafroll-associated virus 3, which is vectored by several species of mealybugs (Cabaleiro & Segura, 1997; Engelbrecht & Kasdorf, 1990; Golino et al. 2002; Manuel de Borbón et al. 2004; Tsai et al. 2008), so it is reasonable to suspect that BVBaV is also mealybug-transmitted, but this remains to be tested. Viruses associated with BYVD may be transmitted by numerous other arthropod vector groups, including whiteflies, aphids, and eriophyid mites.

BYVaV (*Crinivirus rubi*) is transmitted by whiteflies of the species *Trialeurodes abutilonea* and *T. vaporariorum* (Poudel et al., 2013). *Trialeurodes* species are likely to transmit in a semi-persistent manner virus species that belong to the genus Crinivirus group I, such as BYVaV and beet pseudo-yellows virus (BPYV) (Tzanetakis et al. 2013). It is expected that other *Trialeurodes* species might transmit BYVaV in the field. BLMV (*Emaravirus rubi*) is transmitted experimentally by eriophyid mites (*Phyllocoptes parviflori*) (Druciarek et al. 2024). BYVaV and BLMV transmission under field conditions has not been confirmed. No vectors have yet been reported for BVY, BVE, blackberry line pattern virus (BlaLPV), or others in the BYVD complex. Most of these viruses have a narrow host range that includes cultivated blackberry, black raspberry and wild *Rubus* spp, commonly found in the vicinity of commercial fields, potentially serving as inoculum sources for new infections (Poudel et al. 2012). The availability of alternative hosts in close proximity to commercial farms and the growing number of blackberry viruses pose a threat to deploying effective management strategies for BYVD to mitigate virus spread.

It can be assumed that each virus associated with BYVD has a distinct spread mechanism depending on its vector. For instance, a stylet-borne virus might spread differently than a foregut-borne virus (Thresh, 1974). Polycyclic epidemics can result from non-persistent viruses due to the short acquisition and latency period, whereas semi-persistent or persistent viruses tend to cause monocyclic epidemics (Thresh, 1974). It is important to note that in epidemic development, the host phenology and virus latency in the host play a crucial role. Thus, BYVD progress in the field can develop as either monocyclic and/or polycyclic epidemics within a season (Figure 4-1). Potato virus Y (PVY), a potyvirid like BVY, is transmitted in a stylet-borne manner by more than 50 species of aphids. Aphids acquire and transmit PVY in less than one minute (Gray et al. 2010). This phenomenon is different for a virus that is transmitted in a circulative manner in which the retention time is longer, like grapevine red blotch virus (GRBV, Grablovirus) (Flasco et al. 2021) or wheat streak mosaic virus (WSMV, *Tritimovirus),* transmitted by wheat curl mite (*Aceria tosichella*; Keifer). Once the mite acquires the virus, it remains viruliferous for up to 9 days at 20-25 ºC (Singh et al., 2018). Another mite species in this same genus (*Aceria tulipae*) transmits viruses in the genus *Allexivirus* (Kreuze et al. 2020). BVE (*Allexivirus epsilonrubi*) therefore may be transmitted by a mite vector. BlaLPV is a putative member of a new genus of plant viruses (*Coguvirus*) in the *Phenuiviridae* (ON624095; Navarro et al. 2017, 2018). Although coguviruses are known to be graft-transmitted, there are no reported vectors yet for any viruses in this genus. The *Phenuiviridae* family contains genera that infect humans and livestock animals, birds, crustaceans, plants and fungi (Sasaya et al., 2023). Other plant-infecting

viruses in the *Phenuiviridae* (genus *Tenuivirus*) are transmitted by leafhoppers and planthoppers, but inter-genus vectors may differ widely as observed in other plant virus families. For this reason, it is difficult to infer potential vectors of coguviruses, and therefore an alternative approach is to search for potential vectors in areas where secondary spread of these viruses is occurring.

The objectives of this study were to 1) assess within- and between-season spread of four known blackberry viruses, BYVaV, BVY, BVE, and BLMV, and a novel virus BlaLPV based on previously determined presence of these viruses near our study sites (Chapter 2, Dantes et al. 2024) and 2) determine spatiotemporal dynamics of potential insect vector species at these sites, with a focus on aphids and whiteflies.

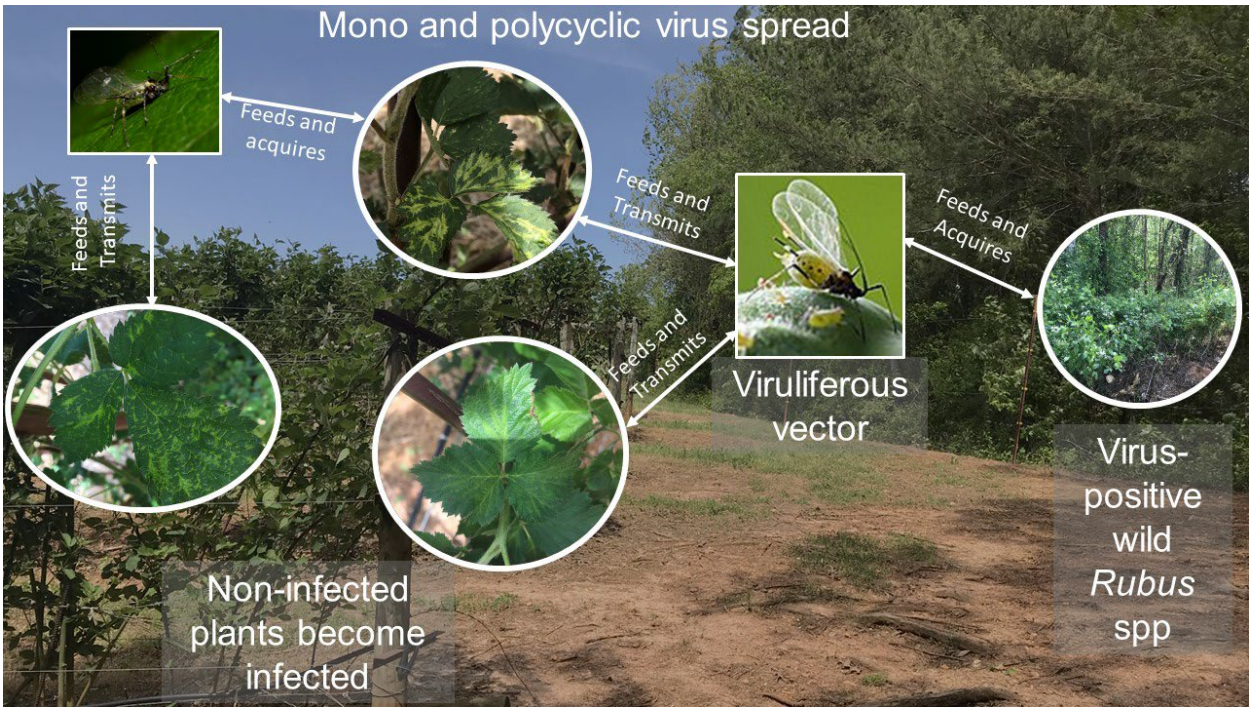

Figure 4-1. Schematic representation of potential virus spread within or across growing seasons in new blackberry plantings when the wild hosts or nearby blackberry fields serve as the primary source of inoculum.

METHODS

Leaf sample collection and processing.

Leaf samples were collected from blackberry plants in two newly established plantings, an open-field site planted in 2021 and a high tunnel site planted in 2020. The open-field site is located in the upstate in York County, while the high-tunnel site is in the coastal region in Fairfax County of South Carolina. In 2023, 384 samples were collected at the two sites (i.e., 200 in a high tunnel production and 184 in an open-field production). Each plant was sampled in spring and summer to assess within-season spread of viruses. Samples were collected from three cultivars in the open-field site (‘Galaxy’, ‘Prime-Ark 45’, and ‘Von’) and two cultivars from the high-tunnel field (‘Ouachita’ and ‘Caddo’) (Figure 4-2). The same plants were sampled in spring 2024 to assess the spread of viruses across two seasons. Approximately 200 mg of young leaf and petiole of each sample were put in pre-labeled tissue grinding bags (Bioreba) and stored at -80°C until RNA extraction.

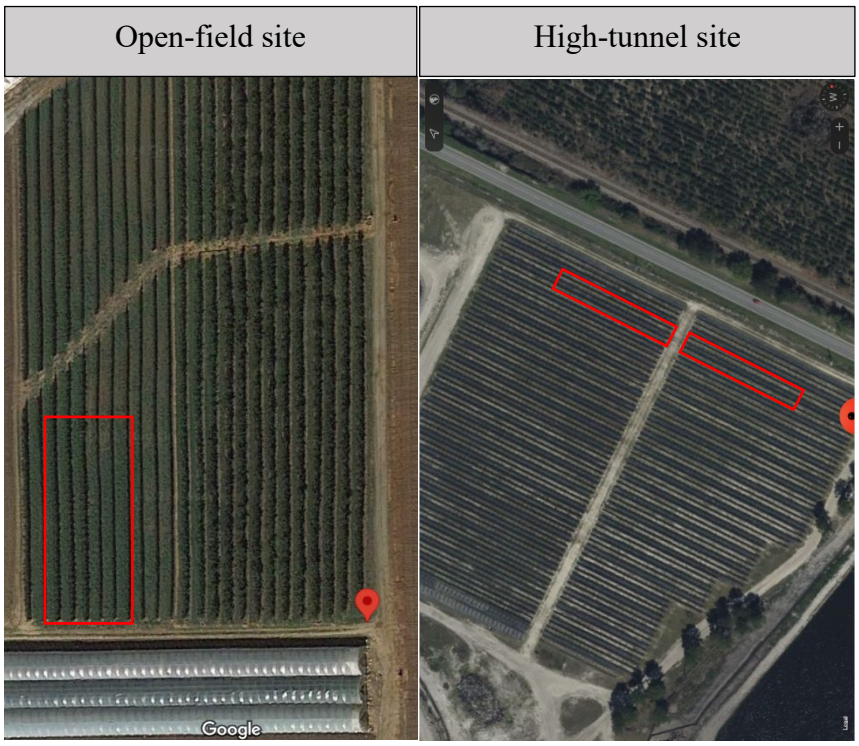

Figure 4-2. Sampling schemes of the fields. The red box in the open field is a delimitation of the plot in which 100 plants were sampled twice in the season, while the red boxes in the high-tunnel site delimitate the plots in which 50 plants were sampled.

Insect collection and processing

Three yellow sticky traps (8 inches × 4 inches) were hung along the trellis in each row, with one on the edge close to the first plant, one in the middle close to the 8th or 9th plant, and the other close to the 17th plant. A fourth trap was placed 10 cm from the ground below the middle trap (Figure 4-3A). The traps were collected and replaced every other week starting in April through July. The cards were collected and immediately placed in plastic bags covered with ice on-site until they reached the lab, where they were kept at 4°C until processing. Each card was screened under a stereoscope for the presence of aphids and whiteflies. Each insect type was counted and logged into an Excel

spreadsheet, including the date the trap was collected, the processing date, and the blackberry cultivar. A single aphid or whitefly was retrieved from the card using “Goo-Gone” citrus oil solvent, placed in a 2-ml tube, and stored at -80°C until RNA extraction.

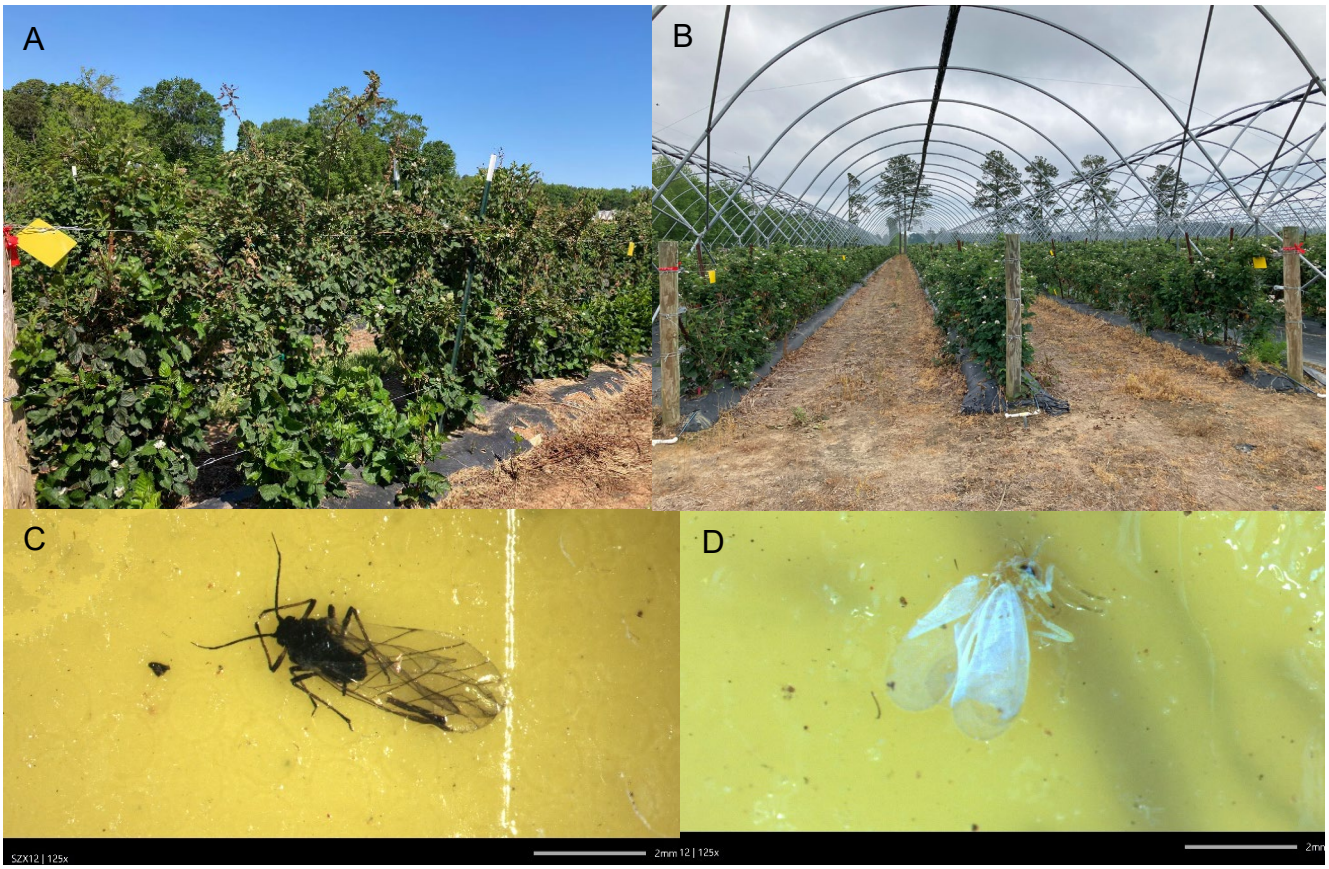

Figure 4-3. Sticky trap placement for potential virus vector monitoring: (A) an open-field blackberry production, (B) a high-tunnel blackberry production. (C) & (D) Aphid and whitefly specimens on sticky cards collected in the blackberry field.

Nucleic acid extraction from leaf samples

Guanidine isothiocyanate buffer (pH 5.0) was prepared using 4M guanidine isothiocyanate, 0.2M sodium acetate, 25 nm EDTA and 2.5% (W/V) PVP-40. The frozen plant tissue was disrupted in 2 ml of buffer using a Homex 6 (Bioreba) grinder. The lysate was incubated at room temperature for 20 min and pipetted into a well of a 96-well plate, which was centrifuged at 1,000 RCF or 5 min to precipitate any leaf residue. Five plates of buffer for the Kingfisher Flex automated extraction system were prepared (one wash buffer 1, one wash buffer 2, two 100% ethanol, and one plate of elution buffer) and loaded into the Kingfisher instrument alongside the sample plate containing 10 µl of magnetic RNA-binding beads (OMEGA Biotek), 80 µl of cold isopropanol and 150 µl of lysate. The protocol for each run consisted of a lysis step for 6 min followed by a bead collection of 1 min. The magnetic RNA-binding beads were washed in 250 µl wash buffer 1 and 250 µl wash buffer 2. These steps were followed by an ethanol wash (250µl). This step was repeated twice. The beads were dried and eluted in 200 µl of nuclease-free water, followed by a bead collection step on the instrument. The plate containing the eluted RNA was then placed on a 96-well magnet for 10 min to isolate excess beads, and then the purified RNA was pipetted into a new 96-well plate and stored at -80°C.

RNA extraction from insects

RNA was extracted from 100 aphids using the E.Z.N.A.® MicroElute® Total RNA Kit (OMEGA Biotek) with modifications. A single insect was crushed in the tube using a sterile pipette tip in 350 ml of TRK lysis buffer amended with β -

mercaptoethanol. Each tube was vortexed for 30 seconds and incubated at 65°C for 10 minutes. The remainder of the protocol was followed according to manufacturer recommendations. To elute the purified RNA, the MicroElute® LE RNA column was placed in a new 1.5 mL tube, and 15 µl of RNAse-free water was added directly to the spin column membrane and centrifuged for one minute at maximum speed. The elution step was repeated twice for a total elution volume of 30 µl. The insect RNA was stored at -80°C.

RT-qPCR SYBR assay for detection of BYVaV, BLaLPV, and BVY in blackberry plants

A 10- µl SYBR green reaction (Luna) was used to screen 384, 184, and 184 individual samples for BYVaV, BLaLPV, and BVY, respectively. The samples were screened for those viruses because they were detected in another set of samples collected at the farms in 2021 by HTS and PCR (Chapter 2, Dantes et al. 2024). In each RT-qPCR, 5 µl of Luna® Universal One-Step Reaction Mix Buffer, 0.5 µl of Luna® WarmStart® RT Enzyme Mix, 0.4 µl of each primer (10 µM), 2.7 µl of nuclease-free water, and 1 µl of RNA were added. The cycling consisted of an RT step for 10 min at 55°C followed by an initial denaturation for 1 min at 95°C, and 40 cycles of 10 s of denaturation at 95°C and 30 s of annealing at 60°C. After each PCR run (65°C to 95°C in 5°C increments), a melt curve analysis was included to determine any off-target amplification and eliminate false positives. Amplification resulting in Cq values lower than 37 and a melting profile consistent with the positive control was considered a positive detection. The positive controls for BYVaV, BLaLPV, and BVY were verified by Sanger sequencing of the PCR

product followed by an NCBI BLASTn search. All primer sequences used for virus detection are listed in Table 4-1.

RT-qPCR SYBR assay for detection of BLMV and BVE

A 10- µl SYBR green reaction (Biorad) was used to test 384 individual plant samples for BLMV and BVE. The samples were tested for BLMV and BVE due to their presence at the two farms based on previous testing done in 2021. In each RT-qPCR, 5 µl of 2X iTaq Universal SYBR® Green 1-Step Buffer, 0.125 µl of iScript Reverse Transcriptase, 0.5 µl of each primer (10 µM), 2.875 µl of nuclease-free water, and 1 µl of RNA were added. The cycling consisted of an RT step for 10 min at 50°C followed by an initial denaturation for 1 min at 95°C, and 40 cycles of 10 s of denaturation at 95°C and 30 s of annealing at 60°C. After each PCR run (65°C to 95°C in 5°C increments), a melt curve analysis was included to determine any off-target amplification and eliminate false positives. Amplification resulting in Cq values lower than 37 and a melting profile consistent with the positive control was considered a positive detection. In 2024, we used an RT-PCR protocol to screen the samples for BVE. It was carried out using the qScript™ XLT One-Step RT-qPCR ToughMix (Quantabio) according to manufacturer recommendations (5 µl of qScript™ XLT One-Step RT-qPCR ToughMix, 0.5 µl of each primer and 3 µl of nuclease-free water). Cycling conditions for the detection of BVE consisted of a reverse transcription step for 10 min at 48°C followed by an initial denaturation for 3 min at 94°C, and 40 cycles of 20 s of denaturation at 94°C, 40 s of annealing at 63°C and 60 s of extension at 70°C. RT-PCR products were resolved on a 1.5% agarose gel followed by post-staining with GelRED (Biotium) and imaging on a

Bio-Rad gel documentation system. The positive control for BLMV and BVE were verified by Sanger sequencing of the PCR product followed by an NCBI BLASTn search.

Duplex RT-PCR for detection of BVY from aphids

A 12- µl endpoint duplex RT-PCR (New England Biolabs) was used to screen 100 individual aphid samples for BVY and an aphid mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene fragment, which ensured nucleic acid presence. The aphids were tested for BVY because this virus was detected in different plants in the vicinity, collected at the farms in 2021. In each RT-PCR, 6 µl of Luna® Universal One-Step Reaction Mix Buffer, 0.5 µl of Luna® WarmStart® RT Enzyme Mix, 0.5 µl of each primer (10 µM), 1.5 µl of nuclease-free water, and 2 µl of RNA were added. The cycling consisted of an RT step for 10 min at 55°C followed by an initial denaturation for 1 min at 95°C, 40 cycles of 10 s of denaturation at 95°C, 30 s of annealing at 58°C and 1 min of extension at 70°C. The COI gene fragment was sequenced (Sanger sequencing), followed by an NCBI blast search.

Table 4-1. Primers used for PCR-based virus detection in this study.

Target	Primers	Amplicon size	Reference
BYVaV	**Sense:** 5' ATAGAAGCGAGGTTAARACCTG 3'	131	(Poudel et al.
	Antisense: 5' CACRTYGTTACCTCTAAGCTCG 3'		2013)
BVEq	**Sense**: 5' TCAGCAAAGCCCTGAACACA 3'	105	This study
	Antisense: 5' AGCABAGYTCGGAAYAAGCYG 3'		
BVE	**Sense:** 5' TGTGGACGATGCACGCCAGATCCC 3'	246	(Poudel et al.
	Antisense: 5' GCTCCACTGGAGGAGATTCTGGTG 3'		2018)
BVY2	**Sense**: 5' GAATTTGATGCAGAGGTYATA 3'	186	(Susaimuthu et al.
	Antisense: 5' TGCTTRAAGTGRGCSTTTCCA 3'		2008) With small modifications
BLMV	**Sense**: 5' CATAAAGGAATTCATACCCAGGAAC 3'	349	(Poudel et al.
	Antisense: 5' AGTTGCATCTTACCTTTCGCG 3'		2018)
BlaLPV	**Sense**: 5' GCTAGCTGACGGGAAAGTGT 3'	285	Elise Schnabel,
	Antisense: 5' CGAACCTGTTCAGGCGGATA 3'		unpublished
Aphid	**Sense: 5'**TCATCAATTTTAGGAGCAATTAA3'	707	(He et al. 2006)
COI	**Antisense: 5'**GCAATAATTGCAAATACAATTCCTAT3'		

Target abbreviations. - BYVaV: blackberry yellow vein-associated virus, BVE: blackberry virus E, BLMV: blackberry leaf mottle virus, BVY: blackberry virus Y, BlaLPV: blackberry line pattern virus, and COI: Cytochrome oxidase.

Results

Virus ingress into the 2-year-old open-field site

At the open-field site, among the 100 plants tested for BYVaV, BVY, BlaLPV and BVE, none of the samples tested positive for BYVaV and BVE at any time point. BlaLPV was detected in 1% (1/100) of the samples (cv. Galaxy) in the spring of 2023. For the summer testing, 4.8% (4/84) of the samples tested positive for BlaLPV (one was cv. Prime-Ark 45 and three were cv. Von). The plants from cv. Galaxy were cut down in the summer of 2023 and, thus, could not be sampled. The following spring, the 100 plants were tested for only two viruses (BVY and BlaLPV) because BYVaV and BVE were not detected in the previous year. However, we retained the RNA so BYVaV and BVE can be included in later testing. It is important to note that two false positives were registered for BVY in 2023. The RT-PCR amplicons melted at the same temperature as the positive control and the expected band size was visualized on an agarose gel as a second confirmation method. However, these plants tested negative for BVY in 2024, which prompted retesting of the two samples from 2023 with a different primer pair, and no BVY was detected. None of the 100 plants tested positive for BVY in 2024, while 4% (4/100) tested positive for BlaLPV, including two new plants (all from cv. Von). Three BlaLPV-positive plants in 2023 tested negative for BlaLPV in spring 2024. These plants are the positive from cvs. Galaxy, from Prime-Ark 45, and Von. The first two plants were cut down in the summer of 2023. A 285-bp fragment of the coat protein of BlaLPV was Sanger-sequenced, and BLAST searched for all the positives in spring 2024 as a second confirmation. Overall, I observed both within-season and between-season spread of

BlaLPV at this site. Positives plants for BVY could not be confirmed in 2024. Thus, I cannot speculate on the spread of BVY at this site. Further testing of our existing sample sets and future sampling should help decipher whether or not these other viruses are spreading at this site.

Virus ingress into the 3-year-old high-tunnel site

Among the 100 plants tested for BYVaV, BVE, and BLMV at the high-tunnel site, none of them tested positive for BYVaV. BLMV was detected in 1% (1/100) of the samples in the spring of 2023 (cv. Ouachita) but tested negative during the summer 2023 and spring 2024 testing. Symptoms of chlorotic mottling and rugosity were observed on this plant (Figure 4-4A). BVE was detected in one plant of cv. Caddo, which tested positive for BVE at both time points in 2023. BVE incidence increased to 4% (4/100) in spring 2024, and the positive plant from 2023 was confirmed in 2024 by sequencing the PCR fragment. Symptoms of chlorotic flecking were observed on these plants (Figure 4-4B). Overall, low virus incidence was observed in the first year of testing, indicating that the plants were generally virus-negative during planting site establishment. Few symptomatic plants were observed at this site, which is consistent with the low incidence of viruses. This study will be continued in the future to monitor virus spread over time.

Table 4-2. Virus spread in the two new blackberry plantings.

Site	**Virus**	**# of plants tested positive/total plants sampled**		
		Spring 2023	Summer 2023	Spring 2024
High-tunnel	BYVaV	0/100	0/100	NT
	BVE	1/100	1/100	4/100
	BLMV	0/100	1/100	0/100
Open-field	BYVaV	0/100	0/84	NT
	BVE	0/100	0/84	NT
	BlaLPV	1/100	4/84	4/100
	BVY	0/100	0/100	0/100

Virus abbreviations. - BYVaV: blackberry yellow vein-associated virus, BVE: blackberry virus E, BLMV: blackberry leaf mottle virus, BVY: blackberry virus Y, and BlaLPV: blackberry line pattern virus.

NT: means not tested.

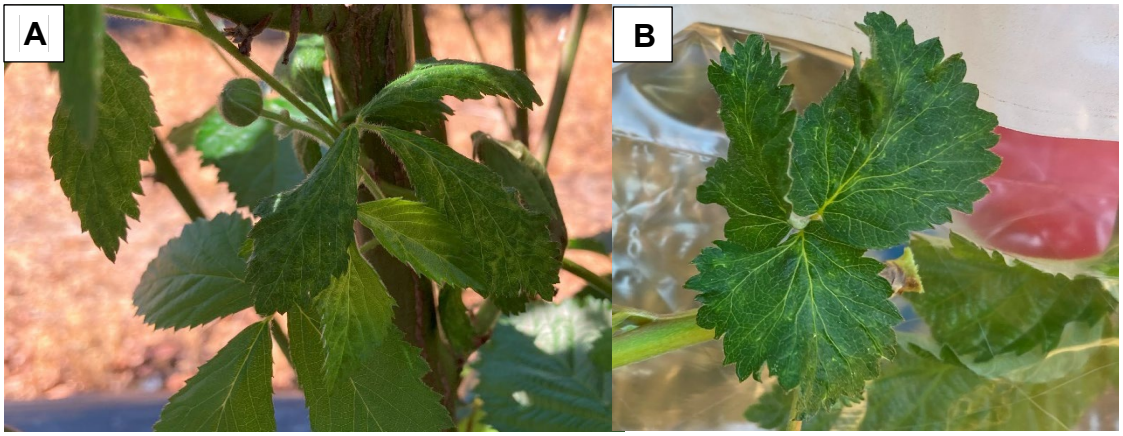

Figure 4-4. Symptoms observed on several plants at the high-tunnel site. (A) Chlorotic mottling was observed on one blackberry (cv. Ouachita) plant that tested positive for blackberry leaf mottle virus. (B) Chlorotic flecking and leaf distortion were observed on multiple blackberry plants (cv. Caddo) that tested positive for blackberry virus E.

Aphid and whitefly population dynamics in the field

The population peak for whiteflies recorded on the sticky traps was in early June, which started to decline in mid-June at the open-field site. However, the whitefly population peak at the high-tunnel site was in late May (Figure 4-5). At the open-field site, the number of aphids recorded on the traps was similar from early May to early June (81-105), which dropped in late June. However, the aphid population peak at the high tunnel site occurred in late June. There were 956 whiteflies and 356 aphids on the sticky cards at the open-field site, which is different from the high-tunnel site, where more aphids (323) were collected than whiteflies (122) (Figure 4-5). Overall, the aphid and whitefly populations were higher in the open-field site than in the high tunnel. This study is still ongoing to assess the stability of these dynamics over several years.

Duplex PCR testing for BVY in aphids

None of the aphids tested positive for BVY. BLASTn search of the COI gene fragment of 10 aphids revealed a diverse population (seven different genera) of aphids in the field (Table 4-3). The COI gene fragment did not amplify for 25% (25/100) of the samples.

Table 4-3. Results of a blast search of the mRNA COI fragment from 10 aphids.

Trap number	**GenBank sequence**	**Accession number**	**Query coverage (%)**	**Percent identity (%)**
T5	*Macrosiphum* sp.	AF077771.1	97	95.14
T15	*Macrosiphum* sp.	AF077771	96	94.97
T48	*Aulacorthum solani*	JF969253.1	96	99.15
T51	*Aphis craccivora*	KX447142.1	99	99.28
T55	*Aphis thaspii*	KC905706.1	96	89.14
T60	*Fibriaphis fimbriata*	AF077768.1	98	97.72
T63	*Greenidea psidii*	NC_041198.1	99	91.85
T168	*Melanaphis sacchari*	MW811104.1	97	99.27
T169	*Rhopalosiphum maidis*	OR148359.3	97	98.71
T182	*Melanaphis sacchari*	MW811104.1	97	99

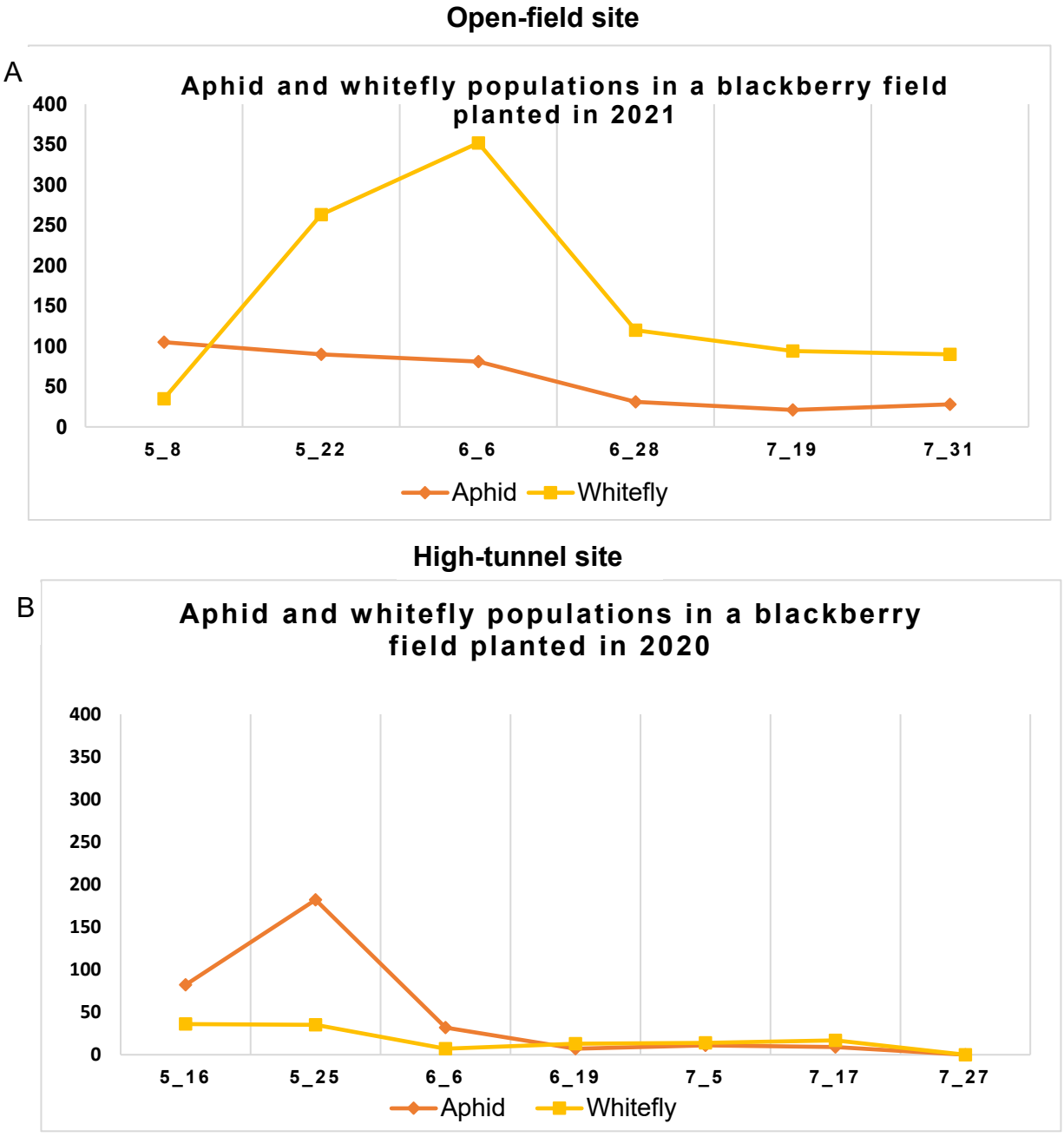

Figure 4-5. Abundance of aphids and whiteflies collected on yellow sticky cards in two new blackberry plantings, with specimen counts plotted over dates. (A) Aphid and whitefly populations in the open field site. (B) Aphid and whitefly populations in the high tunnel site.

Discussion

In this study, the spread of five viruses was assessed in two new blackberry plantings with different modes of production, open-field and high-tunnel. Surprisingly, BYVaV was not detected at either site in 2023, even though BYVaV was detected in several samples collected in 2021 at both sites (Chapter 2, Dantes et al., 2024). BYVaV might spread slowly in the field, especially if its vector is absent, present but in low abundance, present but inefficient, or BYVaV inoculum load is low. Poudel et al., (2013) investigated the ability of two whitefly species to transmit BYVaV in a greenhouse setting, which transmitted the virus at a lower rate (less than 50%). Poudel et al., (2013) also stated that blackberry is not the preferred host of *T. abultionea* and *T. vaporariorum*. However, BYVaV can be spread naturally from infected plants to new plants in a 30-day window (Poudel et al. 2018). It is also important to note that we did not identify whiteflies further than to the family (Aleyrodidae) level. Therefore, it is not guaranteed that the whiteflies captured in this study were even vectors of BYVaV. Prior to that, transmission studies with *T. ruborum* and *T. Packardii* collected from a blackberry field in Arkansas failed to transmit BYVaV (Susaimuthu et al. 2007). The population of these populations peak was observed in July and August (Susaimuthu et al. 2007). However, the whitefly population peak was observed in May and June here in South Carolina in 2023. Future work should focus on testing the whiteflies collected in the field for BYVaV and identifying which species are present in these blackberry fields where viruses are likely to spread. Ideally, surveying for vector candidates would coincide with

the confirmed secondary spread of the associated viruses in the same site (Cieniewicz et al. 2019), but we did not detect BYVaV in any of the plants in the two years of this study.

In spring 2024, BlaLPV incidence dropped to 4% due to the removal of four rows in the experimental block in summer 2023, consequently removing three plants that previously tested positive. Testing the new growth in the following season (spring 2024) revealed negative results for these plants that previously tested positive for BlaLPV. In blackberry plants, carbohydrates and nutrients move to the roots in the fall (Fernandez et al., 2023). Viruses move to the roots alongside the carbohydrates and nutrients (Navarro et al. 2019). One plausible explanation for inconsistent detection between years is that the canes of these plants may have been mowed before the virus moved into the roots, resulting in the plant testing BlaLPV-negative the following year. Another possibility is that spring is not the optimal sampling time for BlaLPV, as observed for several viruses of grapevines (Fiore et al. 2009; Setiono et al. 2018). However, BlaLPV is a newly discovered virus, in a new genus, and much remains to be learned about its biology and ecology.

BlaLPV was discovered recently in blackberry plants, which showed symptoms associated with BYVD (Mollov et al., *unpublished*). Little is known about its biology and epidemiology. At the open-field site, the increase in BlaLPV incidence coincides with the increase in the whitefly (Figure 4A) and thrips (*data not shown*) populations, but this could be coincidental. The approach of monitoring BlaLPV spread and empirically testing potential vectors for transmission competency would likely answer the question of

BlaLPV transmission modes, but as of now, there are no viable leads on coguvirus transmission mechanisms.

BVE incidence increased at the high-tunnel site. Some other members of the genus *Allexivirus* are transmitted by eriophyid mites (de Lillo & Skoracka, 2010; R. Martin et al., 2017). Sampling for eriophyid mites in the field can be challenging due to their small size; however, it would be worthwhile to determine whether eriophyid mites can transmit BVE to better understand BVE epidemiology.

BLMV was detected in one plant in spring 2023 but tested negative in the two later time points, although the plant developed symptoms similar to those observed on plants where BLMV was first detected and described (Figure 4-4A; Hassan et al., 2017). My first hypothesis for this inconsistency was that the virus titer dropped in late July and RT-qPCR might not be able to detect it, as was the case for impatiens necrotic spot orthotospovirus during summer months in blackberry (Martin et al., 2017). This could explain the inconsistency for 2023, but we expected to find the BLMV in the spring of 2024. A fragment of the nucleocapsid gene (P3) (349 bp) of BLMV for the positive sample was sequenced, and a BLASTn search revealed a 96.33% nucleotide similarity, confirming the virus presence in that sample. An alternative explanation is that uneven distribution of BLMV in the blackberry plant could lead to false negatives in the two last tests, as observed for other emaraviruses (Rehanek et al. 2022). Due to the growth habit of blackberry, different canes may have been sampled.

Although BVY was detected in several samples from an older planting from the open-field site (Chapter 2, Dantes et al. 2024), it was not detected in any plants tested in the younger planting, even though these plantings are only a few meters apart. It is, therefore, not surprising that BVY was also not detected in any of the aphids. Although other members of the *Potyviridae* are transmitted by aphids, BVY might not be transmitted by aphids at all as it is the sole member of the genus *Brambyvirus* (Susaimuthu et al. 2008). An attempt at aphid-mediated transmission where *Myzus persicae* and *Amphorophora agathonica* and several eriophyid mites were used failed to transmit BVY (Martin et al. 2017; Susaimuthu et al. 2008). Another possible insect vector are whiteflies because some members of the genus *Ipomovirus* of the *Potyviridae* family, such as sweet potato mild mottle virus and cucumber vein yellowing virus, are transmitted semi-persistently by whiteflies (*Bemisia tabaci*) (Dombrovsky et al. 2014). More research is needed to investigate BVY transmission in both controlled settings and also in the field to determine which aerial vector is involved in transmitting the virus in the field since Poudel et al. (2018) detected BVY in sentinel plants placed near infected plants in two fields in South Carolina. Although we collected whiteflies in the field, they have yet to be tested for BVY as this project is ongoing.

Overall, BVE and BlaLPV appear to be spreading in the new plantings within and across seasons, however, their modes of secondary spread are still unknown. At the same time, it is plausible to suspect that BVE could be transmitted by eriophyid mites based on homology to other members in the genus. No assumption can be made for BlaLPV as it is a newly discovered virus and vectors of other coguviruses are still unknown. Other

members of the genus *Coguvirus* are graft-transmitted (Sasaya et al., 2023) and watermelon crinkle leaf-associated viruses 1 and 2 (WCLaV-1 and -2) are sap-transmitted (Xin et al. 2017). These sites should be monitored closely for BYVD symptom development. The plants infected by BLMV and BVE showed symptoms typical of BYVD. Further virus testing is needed to investigate the presence of other viruses in those plants because BYVD symptom development is often associated with mixed infections. However, BVE was detected in symptomatic plants exhibiting chlorotic and necrotic spots, vein banding, leaf distortion and rugosity in previous testing (see Chapter 3) and BLMV is known to induce symptoms of chlorotic feathering pattern, chlorotic mottling and vein yellowing in single infections in blackberry (Druciarek et al. 2024). Comprehensive understanding of transmission modes is critical for this crop since the approach of planting with virus-negative plants is apparently not effective. Developing management strategies to reduce spread of viruses requires a deeper understanding of the ecological factors driving spread of these viruses.

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CHAPTER FIVE

CONCLUSION AND FUTURE DIRECTIONS

Perspectives Related to Chapter 2: Future work to improve diagnostics

Management of blackberry yellow vein disease (BYVD) is crucial for improving blackberry crop longevity and reducing crop losses. Due to the number of viruses associated with BYVD and the diversity of symptoms observed in the field (Martin et al. 2013), there is a need to improve diagnostics at multiple levels, including in individual plants and at the field-level. Viruses associated with BYVD include blackberry yellow vein associated virus (BYVaV), blackberry virus Y (BVY), blackberry virus E (BVE), blackberry leaf mottle virus (BLMV), blackberry chlorotic ringspot virus (BCRV), blackberry vein banding associated virus (BVBaV) and more. Most detection methods for blackberry viruses rely on molecular techniques such as PCR (RT-PCR for RNA viruses) (Thekke-Veetil and Tzanetakis 2017; Poudel et al. 2018). With PCR, only one or a few viruses could be detected simultaneously, and designing primers for PCRs requires prior information about the virus genome (Ward et al. 2004). This is impossible in the case of novel viruses or a new strain of a virus, unless primers are designed to recognize viruses at the genus-level.

In some scenarios, discovery of novel viruses is necessary. For example, in regulatory settings, biological indexing was previously used to identify novel viruses, because the presence of symptoms in the indicator plants did not require prior knowledge of the virus in the same way that PCR or ELISA does. High throughput sequencing

(HTS) has replaced biological indexing as the method to detect novel pathogens in some import and quarantine programs, e.g. for grapevine, pome, and stone fruits (FPS, 2021). HTS is able to detect both known and novel viruses by sequencing all genomic material in a given sample (Gaafar et al. 2021).

In my dissertation research, HTS was used to determine the virome of blackberry and wild plant hosts at farms across South Carolina, with one of the major goals being to assess feasibility for farm-level detection of viruses. As a standard to which HTS could be compared, I also used RT-PCR and RT-qPCR to test individual plant samples for six known viruses and compared the two methods. To analyze HTS data, I used the bioinformatic pipeline Virtool, which provides a user-friendly interface for plant virus detection. My study showed that HTS and Virtool could be used as an efficient tool to detect viruses in pooled samples with some notable limitations. Although the use of HTS in regulatory settings (i.e., APHIS labs and clean plant centers) is well documented (Malapi-Wight et al. 2021; Soltani et al. 2021; Villamor et al. 2022, 2016, 2019), HTS has not been routinely adopted in diagnostic clinics. Although HTS and Virtool did allow us to accomplish the goal of determining the viruses present in pooled samples, there are still limitations in applying this technology in diagnostic settings, including cost and specialized training in bioinformatics.

RNA was extracted from individual plants and then pooled from 10 to 30 individual samples to create composite samples for HTS (Dantes et al., 2024; Chapter 2). However, it would be interesting to instead try pooling plant tissue from 10 to 20 blackberry plants and then extract RNA for farm-level virome analysis via HTS. This

approach would be cost and time-effective because more samples could be included, but the number of RNA extractions would not be so large. A similar approach was adopted to test if HTS could be used as a diagnostic tool in laboratory settings (Gaafar et al. 2021). To confirm the HTS results, I would use RT-PCR or RT-qPCR using the pooled RNA, and Sanger-sequence the resulting amplicons as was done by Dias et al. (2022). Additionally, the same groupings of plants could be tested side-by-side with the two different pooling approaches (i.e., pooling RNA vs. pooling tissue).

Prior to this work done in Chapter 2, I sequenced six pooled samples using the Oxford Nanopore Technology (ONT), specifically the MinION sequencer, to assess its ability to detect blackberry viruses in pooled samples. The pooling strategy was similar to the one described in Chapter 2 and the library preparation was done using the PCR-cDNA barcoding kit. I used the Epi2me platform, which is a cloud-based, built-in bioinformatic pipeline offered by ONT, to analyze the reads generated during the sequencing process. This analysis revealed the presence of eight characterized blackberry viruses in the pooled samples. However, the read counts were low, suggesting sub-optimal sensitivity for handling pooled samples (Appendix C). The ONT is not optimal for detecting viruses in pooled samples for farm-level detection. However, it does not require much bioinformatic skills as it provides the results in real-time for known viruses. Thus, it has the potential for diagnostic clinics.

Another approach to consider would be to simultaneously detect multiple viruses in BYVD-blackberry plants via HTS using amplicon-based sequencing. Viruses from different genera infect blackberry plants including a crinivirus, a brambyvirus, an

allexivirus, an emaravirus, and more (Martin et al. 2017). First, specific primers will be designed to amplify a conserved gene for a species or across variants of a species. Up to 20 primer pairs will be developed per virus, targeting a virus or multiple variants of a species associated with BYVD, and will be used in multiplex PCRs, to amplify the different viruses present in the pooled samples during the PCR step. A similar approach was adopted by Costa et al. (2022, 2024) for pome and stone fruit viruses. The pre-amplified product will be used for library preparation (removal of primers, ligation of adapters, PCR to amplify sequencing primers) and sequencing of the amplicons using the Illumina platform. This protocol could be adapted as an alternative for sequencing from pooled samples. This approach increases virus detection sensitivity by reducing background amplification of the host genome and other organisms that might be in the samples as is the case of whole genome sequencing. This approach will also simplify the bioinformatic analysis as it will not require filtering of the reads generated by the host genome or other organisms present in the sample.

Perspectives related to Chapter 3: Future work to improve BYVD etiology

BYVD is a complex virus disorder, and etiology is not well characterized. In Chapter 3, I applied network analysis to virus testing results (nine viruses) in the context of cultivar, location, plant age, symptoms, and combinations of viruses in mixed infections in attempt to progress knowledge on the etiology of BYVD. Virus prevalence was higher in symptomatic blackberry and wild *Rubus* samples compared to asymptomatic blackberry. The analysis showed that older plantings (4 years or older)

tend to accumulate more viruses than younger plantings. In other crop systems, visual assessment of symptoms in the field is the first step toward diagnosis (Gonsalves et al. 2010; Qazi 2016). This approach can be challenging in blackberry due to the plurality of viruses and symptom types associated with BYVD. In the field, having an educated guess of a specific group of viruses associated with a specific symptom type is difficult. In an attempt to resolve etiology, we used a bipartite network approach to couple virus presence and symptom types. The network showed little to no grouping in terms of symptom types and virus presence. One virus could induce different symptom types in blackberry, as was also recently demonstrated for single infections of BLMV (Druciarek et al. 2024). Therefore, any combination of one of these viruses with another virus could result in the same symptom types, making it difficult to associate a symptom with a virus or a virus combination. Vein banding, chlorotic and necrotic spots, rugosity, chlorotic feathering pattern and leaf distortion were observed on plants infected by multiple virus combinations. However, oak leaf pattern was coupled twice with mixed infection of BYVaV, RBDV and BLMV. This approach provided some insights into the symptomatology of BYVD. However, Koch's postulates should be fulfilled to study the symptom expression of BYVD using the same virus combinations found in those plants. We only tested for nine viruses in this study, but more viruses might be present in the plants. Using HTS to sequence individual samples would be costly due to the high number of samples. However, HTS on individual samples coupled with the network analysis would be a better approach than using the network alone to improve etiology.

Proving causation requires fulfilling Koch's postulates, which is the best approach to resolving the etiology of BYVD.

To address the remaining questions of BYVD etiology, I would consider designing a greenhouse experiment to study the interactions in mixed infection of specific combinations of viruses using virus-negative blackberry plants from tissue culture. Mechanical inoculation, such as leaf-rubbing, is not feasible for phloem-limited viruses like BYVaV. In addition, finding plants naturally infected with only BYVaV for graft transmission is not practical due to the frequency of mixed infections, and the latency of most viruses in single infections. Vector transmission studies are challenging, with low infection rates (Poudel et al. 2013) and with most blackberry viruses having no proven vectors. In this study, I would (1) engineer full-length infectious cDNA clones of BYVaV, RBDV, BCRV, BVE, BVBaV and BLMV, and (2) inoculate potted blackberry plantlets in specific combinations of single infections, in mixed infections of 2, 3, and more viruses in growth chambers via agro-inoculation, and (3) describe symptomatology associated with each virus or virus combination. Oak leaf pattern is associated with BYVaV, RBDV and BLMV (Chapter 3). This combination will be inoculated into 'Navaho' plants for symptom assessment because it was the cultivar in which this combination was detected. The second combination will be BVE, BCRV, and BVBaV, which will also be inoculated into 'Navaho' plantlets to evaluate symptom expression. Expected symptoms for this combination are vein banding and leaf distortion, which were the symptoms observed on the plants infected with this virus combination. The controls for this experiment will be non-inoculated plants (inoculated with buffer only),

plants inoculated with a single virus and a combination of two viruses. Eventually, this work would help us fulfill Koch's postulates for two prevalent symptom types associated with BYVD.

Perspectives on Chapter 4: Future work to better understand the epidemiology of blackberry viruses

Virus ingress over time was assessed in two new blackberry fields. I also monitored the abundance of aphids and whiteflies in those same fields. I began a multi-year study to monitor the spread of five viruses (BYVaV, BVE, BVY, BLMV, and BlaLPV) in the field in 2023 and 2024. BlaLPV and BVE incidence increased from 1% to 4% on average in the new plantings, suggesting the secondary spread of these viruses. Aphids and whiteflies population peaked mid-May to early June.

Gut content analysis is being used to assess the plants and plant pathogens within insect vector guts as a way of determining feeding history and landscape-level movement of vectors (Cooper et al. 2019, 2016). However, here, I propose a different approach. It consists of conducting a gut content analysis on potential insect vectors collected from blackberry fields where BYVD-associated viruses are spreading to identify which viruses they carry or vector. This could help in identifying potential vectors for viruses with no known insect vectors, but with apparent spread occurring. Even for known vectors, gut content analysis can help to inform virus transmission ecology. This work could be approached in two ways using HTS: 1) a total RNA sequencing approach in which all RNA inside a given insect is sequenced, or 2) an amplicon-based approach where only

blackberry viruses that may be present in the insect are targeted. I opt for the second option because it is a more direct approach. Whole genome sequencing would complicate data analysis as it would generate more sequencing reads (in which the viruses' reads might represent a small fraction) compared to the amplicon-based approach, which will only amplify the genes targeted. This approach could also increase the detection rate for viruses that are present in low titer in the insect. Since the objective of this work would be to identify blackberry viruses carried by these insects, it makes sense to use the amplicon sequencing approach. I would sample insects of interest in the field where high virus inoculum is present, via sweep netting twice a month during the growing season (April to August). Insects of interest would be sorted and flash-frozen in the field to optimize RNA integrity. The insects of interest would be aphids, whiteflies, thrips, leafhoppers, and planthoppers. Viruses associated with BYVD can be vectored by at least one of these insects. Insects would be pooled based on species and size (i.e., smaller insects like thrips would contain more specimens in a pool compared to leafhoppers). Virus-specific primers will be designed to amplify specific virus genes and used in a multiplex RT-PCR or RT-qPCR assay for this experiment. The multiplex assay will be carried out using RNA from each pooled sample. Individual barcodes will be assigned to each pooled sample during library preparation to allow demultiplexing after the sequencing run. This work will potentially help identify insects that are spreading viruses in the field, which will help in designing and implementing effective management strategies.

In Chapter 4, some samples that tested positive in the summer of 2023 for BlaLPV tested negative in the spring of 2024. I speculated that this inconsistency was due to the fact that these plants had been mowed before the virus moved into the roots, resulting in the plant testing BlaLPV-negative the following year. Viruses move to the roots alongside the carbohydrates and nutrients (Navarro et al. 2019). It would be interesting to track the virus movement of BlaLPV in the plant after inoculation. Little is known about the biology of BlaLPV, and no vector has been identified yet. I would take some cuttings from these newly infected plants in the field and try to have this virus in culture by grafting as was done for citrus virus A (Navarro et al. 2018). I would also try mechanical inoculation in blackberry because other coguviruses such as watermelon crinkle leaf associated virus -1 can be transmitted mechanically (Xin et al. 2017). I would test different parts of the newly infected plants (roots, stems, flowers) at different time points during the growing season to assess virus movement in the plant. Another approach to monitor virus movement in the plant is to use the BlaLPV infectious clone tagged to the green fluorescent protein (GFP).

Future work to inform disease management

Currently, one management strategy to keep viruses at bay in blackberry fields is to start any new plantings with virus-negative planting stocks (Martin et al. 2013, 2017). Doing so might guarantee a few years of productive plantings. My research confirmed the notion that most new blackberry plantings are negative for important viruses at planting. However, virus ingress in the field can start as early as a month old when the inoculum

load is present nearby (e.g., in wild *Rubus* species and older blackberry plantings). As such, another effective management action is to clear and uproot the wild Rubus in the surroundings, if possible. In addition, diversifying the cultivars when establishing a new planting may be warranted in case a cultivar is more susceptible to virus accumulation than others. To my knowledge, no resistant cultivars have been identified yet, however, some might be more susceptible than others to virus accumulation. More research needs to be done in that area to identify resistant blackberry genotypes to for use in breeding programs.

Identifying and characterizing insect vectors of these viruses should be one of the research priorities for blackberry in the southeastern states. In the meantime, managing insect vectors such as whiteflies, aphids, eriophyid mites, and nematodes in the soil might reduce the spread of viruses in the field. However, determining viruses that might be present in a specific region is important to narrow down the number of insect populations that require management. For example, a soil test is useful to assess *Xiphinema americanum* presence before planting, knowing that this nematode transmits nepoviruses such as tobacco ringspot virus (TRSV). Regular scouting for virus symptoms and uprooting diseased plants showing these symptoms might help reduce the inoculum load in the field. It is important to note most of the viruses are latent in single infections while some are latent in double infections (Chapter 3). So, scouting and uprooting symptomatic plants might not be an effective management strategy for the viruses implicated in BYVD as it is in other fruit crops, such as fruit trees and grapevines.

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APPENDICES

Appendix A

This is a Plant Disease Note published as part of my internship at the Plant Pest and Diagnostic Clinic in 2022.

First report of *Alternaria cinerariae* causing leaf blight on *Farfugium japonicum* in South Carolina, U.S.A.

Wanita Dantes, G. Curtis Colburn, Margaret Williamson, and Xiao Yang

Farfugium japonicum, commonly known as leopard plant, is a popular perennial used in landscapes in the Southeastern United States. In March 2022, leaf blight was observed on 20 leopard plants at a landscape site in Georgetown County, SC. Almost all leaves were infected. Symptoms included purple to brown necrotic leaf spots and blighted petioles. Large spots had concentric circles and coalesced, causing entire leaves to blight. Leaf pieces surrounding necrotic spots were excised, sterilized in 10% bleach for 1 min, rinsed in sterile water, placed onto potato dextrose agar (PDA), and incubated at 25°C. Three Alternaria isolates, 22-094-A, 22-094-B, and 22-094-C, were obtained by transferring hyphal tips to new plates. All isolates had identical morphological traits. Colonies on PDA were blackish at the center and brownish at the edge. Conidia were produced using a technique described by Shahin and Shepard (1979). Conidiophores were mostly short and unbranched. They were characterized by solitary conidia or short chains of two to three conidia. Conidia (n = 30) were obpyriform to obclavate and averaged 88.5 ± 26.1 µm in body length, 118.4 ± 36.3 µm in total length, and 23.9 ± 5.9 µm in width. They had

three to seven transverse septa and zero to four longitudinal septa. Beaks were broadly tapered. The sequence of the internal transcript spacer (ITS) region of isolate 22-094-A (GenBank accession no. OP481973) had 100% homology to that of CBS 116495 (KC584190), a representative strain of *A. cinerariae (*Woudenberg et al. 2013). Based on the morphological and sequence characters, the casual fungus was identified as A. cinerariae. Pathogenicity confirmation was done in two separate assays. In a detached-leaf assay, mature leaves were collected from 5-year-old *F. japonicum* 'Gigantea' plants. Five leaves (abaxial surface) were sprayed with a mixture of conidial suspensions of the three isolates at 300 conidia per ml and 1.5 ml per leaf, while sterile water was used for a noninoculated control leaf. Leaves were placed in a plastic tray with wet paper towels. The tray was placed at 22°C for an 8-h photoperiod and covered for 3 days to maintain moisture. Small purple to brown spots were visible on inoculated leaves 2 days after inoculation (DAI). More than 90% of inoculated leaf areas were blighted 10 DAI, whereas the control leaf remained asymptomatic. In a whole-plant assay, three *F. japonicum* 'Argenteo Marginata' plants grown in 10-inch pots were placed in a plastic tray and sprayed with a conidial suspension of 22-094-A onto both the abaxial and adaxial surfaces at 300 conidia per ml and 40 ml per plant. The tray was maintained as described above. Sterile water was used for a non inoculated control plant. Small leaf spots appeared on the inoculated plants 2 DAI. Large necrotic areas developed on leaves and girdled petioles causing aboveground tissues to collapse 4 DAI. All inoculated leaves were blighted 7 to 10 DAI, while the non inoculated control plant remained healthy. Each assay was repeated once. *Alternaria cinerariae*, identified by distinct morphological traits

(Nishikawa and Nakashima 2015), was consistently reisolated from inoculated leaves in both assays. Leaf spot on *F. japonicum* caused by *A. cinerariae* has been reported in CA, U.S.A. (Woudenberg et al. 2013), and Japan (Sakoda et al. 2010). This is the first report in SC, U.S.A. This fungus also infects at least 25 other hosts (Farr and Rossman 2022). This disease may pose a threat to leopard plants in nurseries and landscapes under conducive conditions. Disease management strategies are warranted.

Reference

Dantes, W., Colburn, G. C., Williamson, M., & Yang, X. (2023). First report of *Alternaria cinerariae* causing leaf blight on *Farfugium japonicum* in South Carolina, USA. Plant Disease, 107(6), 1953.

Appendix B

Table 2-1. Report file detailing the weight, coverage, and depth of each identified virus OTU in each library.

Sample_ name	Virus_presence	Isolate	Sequence	Length	Weight	Median Depth	Coverage
1A	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.825625	1090	1
1A	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.113961	57	0.991
1A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.003351	7	0.926
2S	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.042444	40817	0.951
4S	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.282603	3153	0.919
1A	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.000659	0	0.241
1A	Peanut stunt virus	Strain ER	NC_002040	2188	0.000598	0	0.204
1S	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.762122	43	0.99
1S	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.00662	6	0.922
7W	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.004976	4	0.881
1S	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.000332	0	0.311
7A	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.65254	55	0.992
7A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.034061	6	0.931
7A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.003418	5	0.818
7A	Tomato ringspot virus	Isolate Rasp1-2014	KM083894.1	8224	0.000185	1	0.539
5A	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.030246	106	0.898
7W	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.727535	44	0.989
7W	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.035847	7	0.924
3W	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.002791	6	0.852
4A	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.027015	290	0.877
7W	Tomato ringspot virus	Isolate Rasp-CL	KR911669.1	8209	9.86E-05	0	0.427
6A	Blackberry yellow vein-associated virus	Unknown unknown	NC_006962	7800	0.013039	371	1
6A	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.004201	57	0.99
2W	Tobacco ringspot virus	Unknown unknown	NC_005097	7514	0.000593	13078.5	0.989
6A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	1.95E-05	9	0.941
6A	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.936148	1300	0.834
6A	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	2.90E-05	1	0.525
5S	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.024522	41	0.895
6W	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.13907	41	0.999

6W	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.618302	53	0.99
6W	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.003887	8	0.949
2A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.000546	7	0.863
5W	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.00135	28	0.672
6W	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	9.01E-05	0	0.406
6W	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.026612	0	0.262
2A	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.43152	52	0.998
2A	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.415684	47	0.993
2A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.014848	7	0.934
6A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.000536	13535	0.941
2A	White clover mosaic virus	Unknown unknown	NC_003820	5845	0.000385	0	0.463
2A	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	2.68E-06	0	0.389
2A	Clover yellow mosaic virus	Unknown unknown	NC_001753	7015	0.000128	0	0.371
2S	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007639	5449	0.160437	42	0.994
2S	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.088319	4	0.957
9A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.000233	17980	0.973
2S	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.001387	7	0.939
2S	Turnip vein-clearing virus	Strain OSU	NC_001873	6311	2.54E-06	1	0.512
2S	Tomato ringspot virus	Isolate Rasp1-2014	KM083894.1	8224	0.000598	0	0.43
2S	Clover yellow mosaic virus	Unknown unknown	NC_001753	7015	0.000173	0	0.232
2S	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.028685	0	0.209
2W	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.815931	98655.5	0.995
2W	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.010406	49	0.991
4W	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.00022	7	0.968
2W	Tomato ringspot virus	Isolate Rasp1-2014	KM083894.1	8224	0.151543	4299	0.96
9W	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.001279	28	0.72
3A	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.732175	453	1
3A	Raspberry bushy dwarf virus	Isolate Ec_Az		2231	0.153332	48208	0.993
3A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.000362	8	0.964
5A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.000213	6	0.904
3A	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	0.001146	2	0.717
3S	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.55565	150	0.998
3S	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.303901	30521	0.993
3S	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.001951	10	0.934
2W	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.001232	369	0.911

3A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	0.000104	8	0.858
3S	Tomato ringspot virus	Isolate Rasp-CL	KR911669.1	8209	0.001155	1	0.506
3S	Apple mosaic virus	Unknown unknown	NC_003480	2056	0.000586	0	0.308
3W	Blackberry chlorotic ringspot virus	Unknown unknown	NC_011555	2290	0.02005	26	0.972
3W	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.775668	62	0.99
4W	Blackberry chlorotic ringspot virus	Unknown unknown	NC_011555	2290	0.000673	3067.5	0.988
3W	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.007217	9	0.953
5A	Blackberry virus Y	Isolate 3	NC_008558	10851	0.354715	880	0.914
4A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	3.67E-05	5	0.825
3W	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.00073	1	0.706
3W	Blackberry vein banding associated virus	Isolate Mississippi1	NC_022072	18643	0.000567	9	0.632
3W	Tobacco ringspot virus satellite RNA	Unknown unknown	NC_003889	359	0.000143	1	0.554
3S	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.001093	300	0.929
3W	Tomato ringspot virus	Isolate Rasp-CL	KR911669.1	8209	1.03E-06	0	0.42
4A	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.41101	23	0.997
4A	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.383625	57	0.993
4A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.001669	8	0.942
7W	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	1.04E-03	0	0.48
4A	Blackberry chlorotic ringspot virus	Unknown unknown	NC_011555	2290	0.000432	5	0.853
4S	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	3.42E-05	5	0.87
4A	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	0.001327	1	0.551
4A	Blackberry vein banding associated virus	Isolate Mississippi1	NC_022072	18643	3.37E-08	1	0.507
4A	Red clover vein mosaic virus	Isolate Washington	NC_012210	8604	0.002624	0	0.384
4S	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.544421	2178	0.993
4S	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.003347	8	0.943
4W	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	8.33E-04	1764	0.923
8A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	1.26E-05	5	0.82
3S	Blackberry chlorotic ringspot virus	Unknown unknown	NC_011555	2290	1.14E-05	0	0.256
4S	Blackberry vein banding associated virus	Isolate Mississippi1	NC_022072	18643	1.13E-05	23	0.661
4S	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	0.002981	1	0.582
4S	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	6.44E-05	0	0.263
4S	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.032362	0	0.217
4W	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.03315	128	0.998

4W	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.009627	1082	0.993
4S	Blackberry chlorotic ringspot virus	Unknown unknown	NC_011555	2290	3.98E-06	3	0.79
4W	Tomato ringspot virus	Isolate Rasp-CL	KR911669.1	8209	0.919837	7302	0.978
8S	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	1.11E-05	6	0.867
8S	Blackberry virus Y	Isolate 3	NC_008558	10851	0.210806	125	0.962
9S	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.000452	1	0.421
4W	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.000133	6	0.92
4W	Blackberry vein banding associated virus	Isolate Mississippi1	NC_022072	18643	5.41E-07	52	0.676
4W	Strawberry necrotic shock virus	Unknown unknown	NC_008707	2876	1.75E-10	3	0.526
4W	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.000548	0	0.336
5A	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.129487	46	0.999
5A	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.291404	48	0.991
5A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.006214	9	0.945
5S	Blackberry virus Y	Isolate 3	NC_008558	10851	0.128761	183	0.914
3S	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	8.18E-06	6	0.925
1A	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	3.96E-04	0	0.328
5A	Lilac leaf chlorosis virus	Unknown unknown	NC_025481	2117	0.016004	1	0.683
5S	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.28308	14	0.998
5S	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.383616	48	0.991
5S	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.011547	7	0.94
9W	Blackberry virus Y	Isolate 3	NC_008558	10851	0.012296	170	0.959
7A	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.000171	0	0.444
6W	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	5.47E-06	5	0.839
5S	Tomato ringspot virus	Isolate Rasp1-2014	KM083895.1	7563	0.002154	0	0.209
5W	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.151409	377	0.999
5W	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.437925	72	0.991
5W	Lilac leaf chlorosis virus	Unknown unknown	NC_025481	2117	0.170567	16	0.99
5W	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.002203	8	0.935
5W	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	4.88E-06	7	0.875
3W	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	0.000126	0	0.486
5W	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	2.52E-05	1	0.558
5W	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.007558	0	0.372
8A	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.687869	54	0.995
8A	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.08373	8	0.983
8A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.00654	6	0.93

5S	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	4.42E-06	5	0.883
8A	Tomato ringspot virus	Isolate Rasp-CL	KR911669.1	8209	0.000767	1	0.545
8S	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.655668	50	0.992
3W	Blackberry virus Y	Isolate 3	NC_008558	10851	0.007198	3	0.909
8S	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.004147	7	0.941
1S	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	2.67E-06	5	0.865
8S	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	0.006178	0	0.474
8S	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.000135	0	0.249
8W	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.825625	1090	1
8W	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.113961	57	0.991
8W	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.003351	7	0.926
1A	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	2.66E-06	6	0.875
8W	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	9.52E-05	349	0.849
8W	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.000659	0	0.241
8W	Peanut stunt virus	Strain ER	NC_002040	2188	0.000598	0	0.204
9A	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.008215	49	0.998
9A	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.003443	43	0.995
8W	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	2.66E-06	6	0.875
9A	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	6.77E-06	6	0.932
9A	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.985248	1228	0.838
9S	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.422523	71	0.996
9S	Blackberry yellow vein-associated virus	Unknown unknown	NC_006962	7800	0.008133	25	0.959
9S	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.02384	0	0.801
9S	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.003216	9	0.642
9S	Raspberry leaf mottle virus	Isolate HCRL Glen Clova	NC_008585	17481	0.001873	0	0.56
6W	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	6.06E-06	0	0.422
9S	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	0.000181	1	0.246
9W	Blackberry yellow vein-associated virus	Unknown unknown	NC_006963	7916	0.375084	201	0.999
9W	Raspberry bushy dwarf virus	Isolate Ec_Az	KJ007640.1	2231	0.428323	60	0.992
4W	Blackberry virus Y	Isolate 3	NC_008558	10851	2.41E-05	3	0.954
9W	Pokeweed mosaic virus	Isolate PkMV-PA	NC_018872	9512	0.000798	9	0.943
9W	Tobacco ringspot virus	Unknown unknown	NC_005096	3929	1.17E-06	6	0.811
1S	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	3.71E-07	0	0.336
9W	Tomato ringspot virus	Isolate Rasp-CL	KR911672.1	7325	0.000146	1	0.685
9W	Blackberry virus F	Isolate BBV-3X	NC_029303	7663	0.010103	0	0.496
6A	Blackberry virus E	Isolate BB_Ellis-1	NC_015706	7718	8.28E-11	0	0.285

Appendix C

Table 5-1. Blackberry viruses detected by HTS in pooled samples using the minION sequencer (Oxford Nanopore Technology)

Sample name	Virus Presence/Number of Reads							
	ToRSV	TRSV	BYVaV	BVE	RBDV	BVY	BCRV	BVF
3A	NT	NT	17	NT	16	NT	NT	NT
3S	NT	NT	15	8	15	NT	NT	NT
3W	1	NT	2	NT	NT	1	NT	12
6A	NT	171	8	NT	NT	NT	NT	NT
6W	1	NT	1	NT	NT	NT	NT	NT
4W	1423	1	27	68	NT	3	1	NT

Virus name abbreviations are as follows: Tomato ringspot virus (ToRSV), tobacco ringspot virus (TRSV), blackberry yellow vein associated virus (BYVaV), blackberry virus E (BE), raspberry bushy dwarf virus (RBDV), blackberry virus Y (BVY), blackberry chlorotic ringspot virus (BCRV), blackberry virus F (BVF).
NT means not detected.