EPIDEMIOLOGY OF *BLUEBERRY NECROTIC RING BLOTCH VIRUS* OF SOUTHERN HIGHBUSH BLUEBERRY IN GEORGIA

by

TANISHA SHEREE ROBINSON

(Under the Direction of Carl M. Deom)

ABSTRACT

Blueberry necrotic ring blotch is a new viral disease of southern highbush blueberries (SHB; *Vaccinium corymbosum* interspecific hybrids) in the southeastern United States. Epidemiological studies were conducted to fill critical knowledge gaps related to disease development, and spatio-temporal spread of necrotic ring blotch in the individual plant and the entire field. Field surveys demonstrated that symptom spread within rows appears to be more rapid than spread across rows, suggesting that a slow-moving vector may be involved in transmission. The surveys also showed that symptoms appear earlier and are more severe in the interior of the bush. Symptomatic and asymptomatic foliage of susceptible 'Star' blueberry plants was collected to confirm the presence or absence of the virus via RT-PCR. Results suggest that *Blueberry necrotic ring blotch virus* causes local infections within the host plant and likely does not overwinter in SHB plants.

INDEX WORDS: Blueberry necrotic ring blotch, Epidemiology, RT-PCR, Spatio-temporal progress, *Vaccinium corymbosum*, Virus disease

EPIDEMIOLOGY OF *BLUEBERRY NECROTIC RING BLOTCH VIRUS* OF SOUTHERN HIGHBUSH BLUEBERRY IN GEORGIA

by

TANISHA SHEREE ROBINSON

B.S., Norfolk State University, 2010

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

EPIDEMIOLOGY OF *BLUEBERRY NECROTIC RING BLOTCH VIRUS* OF SOUTHERN HIGHBUSH BLUEBERRY IN GEORGIA

by

TANISHA SHEREE ROBINSON

Major Professor: Carl M. Deom

Committee: Phillip M. Brannen
Harald Scherm

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2013

DEDICATION

First, I would like to thank my Lord and Savior Jesus Christ. Without my faith in him, I would not have accomplished so many great things in my young adult life. This journey has been wonderful, but the road was not always straight and narrow. When I felt like giving up and quitting, I had to remind myself that he did not bring me this far to leave me.

"I can do all things through Christ who strengthens me".

- *Philippians* 4:13

I dedicate this work to my amazing family and friends for being very supportive throughout my tenure here at the University of Georgia. You all are always there to see me through the next chapters in my life.

There is nothing impossible with God (*Luke* 1:37) and through him and his strength, there is nothing impossible for us to achieve on this earth.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to those individuals who have helped and supported me during my time here at The University of Georgia. You all have made this journey worthwhile and quite interesting to say the least. First, I would like to thank my major professor, Mike Deom, for his excellent guidance and advice, for allowing me to be independent, for his trust in me, and giving me the extra push that I needed to get me to the finish line. He has helped keep me grounded and focused, not to mention that he has spoiled me to no end. I thank him for his wisdom and tough love. Although maybe not always asked for, his wisdom and tough love has helped me to effectively think in ways I may not have on my own. I would also like to thank my co-advisor, Phillip Brannen, for his patience and faith in me, and for guiding me in the right direction when I fell off course. He has provided invaluable advice and knowledge of techniques, methodologies, and design elements that were very helpful to my thesis project. He has also challenged me to become a better field pathologist and I am forever grateful. Because of him, I most definitely have a newfound respect for the agricultural community. Lastly, I would like to thank the final member of my committee, Harald Scherm, for keeping his door open anytime I needed help with my project, especially for the endless meetings of data analysis. I thank him for his invaluable knowledge of field epidemiology and willingness to provide insight and feedback. Thanks for the support and encouragement. I have enjoyed working with each of you, and you all have made this milestone in my life worth the blood, sweat, and tears.

This work would not be possible without the knowledge, assistance, and support of several individuals. I would like to thank to our project collaborators Dr. Robert Martin and Dr.

Philip Harmon, and the members of their labs for development of diagnostic tools, and information concerning *Blueberry necrotic ring blotch virus*. This project would not have been possible without their helpful contributions. I thank the Department of Plant Pathology faculty and staff for their help and support during my time here. In particular, I am appreciative of Shavannor Smith, Ronald Walcott, and Jean Williams-Woodward for their "open door policy," advice, and share of equipment. A special thank you to Mary Ard and Beth Richardson for their patience, time, and invaluable knowledge in microscopy for this project. I also would like to thank lab technicians Dave Andrews, Judy Gray, Onofre Paguio, and Ashley Turner for their help in technical support and training. To our undergraduate interns John Morgan, Patrick Savelle, Andrew Shirley, Benjamin Shirley, Mason Westmoreland, and Laura Williford, thank you for your hard work and dedication throughout this project.

While here at the University of Georgia, I have developed many positive relationships. First and foremost, I would like to express my sincere appreciation to my dear friend Laura Williford. She has been very supportive, and I couldn't be more grateful to have her in my life. I would like to thank Mrs. Amy Savelle for her moral support and pep talks throughout my time here. Lastly, it would not be fair if I did not thank my wonderful friends Michele Burnham, Mavis Finger, Ansuya Jogi, Renee Holland, and Tom Ingram for their support, lunchtime talks, and all the great memories we've all created together.

We sometimes lose track of purpose on the unwinding roads of our journey in life, but then there are always those special people to remind us to keep "trotting" on. To the most important people in my life, my family, close friends, and my fiancé, I give you all my utmost gratitude for your patience, and supporting my endeavors; you are the wind beneath my wings.

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Importance and types of blueberries in Georgia. Within Georgia's fruit and nut industry, blueberries are a leading commodity. Currently, Georgia is ranked as the third-largest blueberry producer in the nation (7). While Georgia is still "The Peach State," blueberries have surpassed peaches in value and have become the number one fruit commodity in the state. In 2011, the blueberry farm gate value exceeded $254 million (3). More than 9,300 hectares of land in Georgia is devoted to blueberry production, as compared with 3,237 hectares 10 years ago (7, 17). Production is concentrated in the southeastern parts of the state, with Appling, Bacon, Clinch, and Ware Counties accounting for more than 75% of the total production (9). The demand for blueberries has increased in the past decade, primarily because of their widely recognized health benefits. Blueberries have high levels of polyphenols, which have antioxidant and anti-inflammatory properties. In addition, blueberries are low in fat and are an excellent source of vitamin C and manganese (2).

There are two types of blueberry species grown commercially in Georgia. The majority of blueberry land (90%) is devoted to rabbiteye blueberry (*Vaccinium virgatum = V. ashei*), which are native to the southeastern United States and is well adapted to the regions warm and humid climate (22). The remaining 10% is planted to various southern highbush blueberry cultivars (SHB; *Vaccinium corymbosum* interspecific hybrids) (21). Although no formal surveys have been conducted recently, southern highbush blueberries have increased in production, possibly accounting for 20-30% of current production (P.M. Brannen, *personal communication*).

Compared with rabbiteye blueberries, SHB have more stringent soil requirements. As such, they are grown successfully in well-drained soils with naturally high organic matter, soils highly amended with pine bark, or in high-density pine park beds (9). Rabbiteye cultivars are more tolerant of low organic matter soils and are less management-intensive than SHB cultivars (9). SHB cultivars have been developed by crossing northern highbush blueberry (*V. corymbosum*) with native southern species such as *V. darrowii* (13), resulting in tetraploid interspecific hybrids. SHB are earlier-maturing than rabbiteye (late April to mid-May), and are currently bred and selected for superior fruit quality, soil adaptability, heat tolerance, and low winter chilling (12, 13). Approximately 65% of Georgia's SHB fruit ships fresh (17).

SHB are generally more susceptible to diseases and pests than rabbiteye blueberries. Over the years, SHB have suffered from many leaf spot diseases, predominately Septoria and anthracnose leaf spots (23). These leaf spot diseases impact plant health and subsequent yield of SHBs through inhibition of photosynthesis and premature defoliation during summer and fall (15). In addition, SHB are affected by systemic diseases such as bacterial leaf scorch (caused by *Xylella fastidiosa*) and red ringspot (caused by *Blueberry red ringspot virus*, BRRV) (15, 24). More recently, a new viral disease has been observed on SHB in Georgia, Florida, and North Carolina, designated as necrotic ring blotch, which is caused by *Blueberry necrotic ring blotch virus* (BNRBV) (19). In severe cases, the disease leads to premature defoliation of affected bushes, and it is presumed that it would lead to losses in return yield, similar to the processes incurred by fungal leaf spot diseases (5). At present, the etiology of the disease has not been clarified, so attempts to manage the disease have not been developed. Although the disease does not appear to be lethal to the plants, it is likely that it reduces yields and causes plant stress that could lead to other problems such as, winter kill or Botryoshaeria stem diseases (5).

Overall importance of viruses in blueberry. Over the past 15 years, blueberry production has expanded significantly in North America and worldwide (15). Although this significant change has been very beneficial to the industry, it comes with a high cost of introduction and spread of viral diseases (15). Plant viruses can cause significant economic yield losses in most agricultural crops, including small fruits such as blueberry, strawberry, and raspberry (25). Plant viruses are typically spread through infected plant material via propagation, vectors such as insects and nematodes, and sometimes pollen. Viruses are dependent on the host plant for both replication and protein-synthesizing machinery, interfering with the plants ability to function normally (11). Viral diseases, depending on the virus and the host range, can range in symptomatology from asymptomatic to severe, even resulting in plant death.

Furthermore, like most plants, *Vaccinium* spp. are known to harbor viruses belonging to several different families such as *Rhabdoviridae, Caulimoviridae*, and *Bromoviridae* (15). Most blueberries are grown in North America and that is where most of the plant viruses have been described (15). Blueberries have become a prime candidate of diseases caused by new and reemerging viruses for several reasons: (a) the rapid expansion of the blueberry industry; (b) plantings established in new areas of production where they may be exposed to new viruses and vectors; (c) the introduction of new cultivars that may lack important information on their susceptibility to viruses and other pathogens; and lastly, (d) the lack of grower awareness of diseases that can emerge when vegetatively propagating non-certified material (14, 15). Due to the above considerations, scientists are working on new novel disease management strategies to better control plant viruses that affect blueberry which will in turn help safeguard the blueberry industry.

Occurrence and symptoms of BNRBV in Georgia. In Georgia, BNRBV was first observed in 2006 in Bacon, County (15). The initial distribution of the disease in Georgia was limited to very sporadic reports and observations in 2006 and 2007, but in 2008 it was found in multiple locations throughout the major blueberry production counties (5). Since 2008, the disease has been reported throughout the southeastern United States, including the states of Florida, Mississippi, North Carolina, and South Carolina. To date, the disease has only been observed in SHB, but not in the native, more widely grown rabbiteye.

Symptoms of BNRBV are observed as irregularly-shaped rings or blotches with or without green centers on the adaxial and abaxial leaf surfaces (Figure 1.1). Symptoms are distinct from those of BRRV, which produces ring spots on leaves and stems that are more reddish in color, have thinner margins around the ring spots, and are less blotchy in appearance (5). With BNRBV, ring spots are generally more prevalent on older tissue (i.e. the lower leaves of plants), but they can inundate a bush from bottom to top. The ring colors can initially vary from dark brown to purplish-black, but the ring spots often coalesce to form solid brown to black necrotic ring spots or blotches (Figure 1.2), which may be similar to Septoria leaf spot or other fungal leaf spots (5). However, Septoria leaf spots do not have green centers, and fungal structures (pycnidia and cirrhi) are usually observed in spots caused by *Septoria* (5). No symptoms develop on blueberry stems or fruit. Plants infected severely with BNRBV will defoliate prematurely (15), but the impact on crop yield is currently unknown. In Georgia, the symptoms of BNRBV are initially observed in late May or early June, but they are much more apparent after harvest during the summer and autumn timeframe.

Identification and characterization of BNRBV. Investigations into the causal agent of the disease began with microscopic examinations and isolation attempts that failed to identify

fungal or bacterial pathogens associated with symptomatic tissue (15, 19). Although symptoms resembled those of viral diseases, enzyme-linked immunosorbent assay and polymerase chain reaction (PCR) diagnostic tests were negative for known blueberry viruses (19). However, double-stranded RNA (dsRNA) was isolated from symptomatic leaves suggesting the presence of a viral pathogen (15). Specifically, six dsRNA segments were observed consistently from more than 10 sources of diseased tissue (19). Four of the dsRNA fragments were specific to blueberries showing disease symptoms. Partial sequences were obtained from three of the four dsRNAs and were used to develop diagnostic primers for reverse-transcription PCR (RT-PCR) (19). The four genomic RNAs were sequenced and the new virus was designated *Blueberry necrotic ring blotch virus* (BNRBV) (19). Presently, BNRBV particles have not yet been detected or visualized via electron microscopy.

BNRBV possesses a 14 kb nucleotide genome divided into four ssRNA segments (19). RNA 1 (5.9 kb) has a single open reading frame (ORF), which encodes for a putative 215 kDa protein having an N-terminal methyltransferase (MT) domain, a central cysteine protease (C-prot) domain and a C-terminal helicase domain (HEL-1). RNA 2 (3.9 kb) encodes for a putative 130 kDa protein having a N-terminal helicase domain (HEL-2) and a C-terminal RNA-dependent RNA-polymerase domain (RdRp). RNA 3 (2.5 kb) has 5 ORFs encoding for putative proteins of 7, 9, 22, 28, and 31 kDa. RNA 4 (1.7 kb) encodes for a putative movement protein. Phylogenetic analysis revealed that BNRBV clustered with members of the family *Bromoviridae* based on the MTR domain. An analysis of the RdRp domain grouped BNRBV with members of *Virgaviridae*, specifically with *Citrus leprosis virus* (CiLV, genus *Cilevirus*) and the newly characterized *Hibiscus green spot virus* (HGSV: proposed genus *Higrevirus*). BNRBV possesses two helicases, which is a unique genetic feature among all known viruses. It is

interesting that the two HEL domains do not cluster in the same clade. HEL-1 is grouped with members of CiLV and HGSV sharing a common ancestor with the tobamoviruses (*Virgaviridae)*, whereas HEL-2 showed a closer relationship with members of the genus *Ilarvirus* (*Bromoviridae*). The diverse lineages of the helicases suggest that BNRBV is a result of a recombination event between members of the *Bromoviridae* and *Virgaviridae* family. The analysis of the MP suggests that BNRBV is most closely related to CiLV, which will be discussed in more detail later in this chapter. Although there is a strong genetic relationship between BNRBV and CiLV and HGSV, there are features that distinguish BNRBV from its relatives. CiLV and HGSV possess a poly (A) tailed bipartite and tripartite genome, respectively. BNRBV has a quadra-partite genome lacking a poly (A) tail. It was also found that BNRBV has conserved nucleotide elements at the 3' non-coding regions for each RNA segment. The conserved sequence 5' –CACAAAT was found at the 5' terminus of all segments. In addition, nucleotide sequence CG-3' was highly conserved at the 3' terminus, with RNA 1 and RNA 2 sharing two additional bases at the 3' end (-**AT**CG-3'). The findings suggest that BNRBV represents a new genus; *Blunervirus* is the proposed genus.

Susceptible blueberry cultivars. Unfortunately, there are many southern highbush blueberry varieties that are affected by this disease, including 'Star' (most susceptible), 'Rebel', 'O'Neal', and 'FL 86-19' (also known as 'V1') (4). However, it has been observed that some SHB cultivars remain symptomless, even when planted among cultivars exhibiting severe disease symptoms. For example, SHB cultivars 'Emerald' and 'Millennia' seem to be more resistant to the disease than other SHB varieties (4).

Disease cycle and epidemiology. Currently, there is little known about the epidemiology of BNRBV, including the means of transmission/spread or alternate hosts.

However, there is research being conducted in Georgia and Florida to further develop this necessary information. Anecdotally, the disease seems to be more prevalent in dry years, such as those observed in 2008 and 2011 (5). In 2009 and 2012, the disease was absent at sites that had been heavily affected the year before; this could be due to the heavy rainfall during the growing season (5), possibly suggesting a vector that is tied to rainfall or other local environmental conditions (Refer to Appendix B). The fact that disease symptoms vary considerably from year to year in affected plantings also suggests a limited systemic nature of the disease, whereby plants are partly or completely cured from the virus as a result of natural defoliation during fall and winter

Most plant viruses are transmitted by vectors from one host plant to another, although they are efficiently disseminated by human activities such as vegetative plant propagation, grafting, global exchange of infected material, changes in cropping systems, and the introduction of novel crops in existing or new agricultural areas (1). Presently, the vector of BNRBV is unknown. Rapid progression of symptom development in the field suggests an aerial vector (25). CiLV and HGSV are the closest relatives of BNRBV and are both vectored by false spider mites in the genus *Brevipalpus* (Acari: Tenuipalpidae) (20). Based on phylogenetic relatedness of BNRBV and CiLV, it is possible that BNRBV may also be transmitted by a mite, and field and greenhouse observations in Florida and Georgia have in fact suggested that an eriophyid mite may vector BNRBV (6, 15). Significant populations of an eriophyid mite were found associated with SHB showing symptoms of BNRBV in commercial fields in Georgia and Florida (P. M. Brannen and P.F. Harmon*, personal communication*). In addition, introduction of tissue-cultured, virus-free 'Star' plants into a University of Florida greenhouse containing both BNRBV-infected plants and eriophyid mites has twice resulted in rapid transfer of both mites

and disease symptoms to the disease-free plants. Based on morphological characteristics, the mite, a vagrant foliar feeder, is a potential new species in the genus *Calacarus* (6). Additional research must be conducted to confirm the presumptive vector is in fact transmitting the virus.

Other plant viruses similar to BNRBV. BNRBV is related to *Citrus leprosis virus* (CiLV). While little is known about BNRBV at this time, CiLV can initially be used as a guide to better understand the potential epidemiology of the newly emerging BNRBV. CiLV is a highly destructive disease that affects mainly sweet orange (*Citrus sinensis*) and mandarins (*C. reticulate, C. reshni,* and *C. deliciosa*) (18). It is endemic in Brazil and is rapidly spreading throughout Central America (18), causing premature fruit drop, severe defoliation, and death of twigs and branches, all of which can lead to a reduction of fruit quality and yield (16, 20).

CiLV moves locally, but does not move systemically in the host plant. CiLV has been shown to move for short distances along the mid-vein or secondary veinlets in leaves, but its movement is rather limited (20). Citrus leprosis is mainly spread to host plants by movement and feeding of viruliferous mites. Unlike some plant virus/vector interactions, CiLV is not passed to vector offspring transovarially. When mites acquire the virus, they are able to transmit the virus for their entire lifespan. Furthermore, experiments have revealed that CiLV can be transmitted mechanically between susceptible citrus hosts. Graft transmission, on the other hand, has been difficult. Grafting is successful only when symptomatic areas are in direct contact with receptor tissue on healthy plants (tip grafting) (8).

BNRBV management. No current control measures are currently recommended for BNRBV. Based on initial studies, BNRBV does not appear to be readily transmitted through propagation (10). The primary management method for virus control will likely be host resistance, even though it takes many years to generate resistant varieties. Producers should

always utilize plants free of BNRBV and other known viruses and diseases, allowing for field longevity and grower profitability (15). As mentioned earlier, there is observational evidence that an eriophyid mite is the likely vector. If confirmed, vector management with acaricides (pesticides specifically for mites and ticks) would be recommended for SHB plants susceptible to the disease. However, until a vector is identified, control measures cannot be recommended.

Goals and objectives. Presently, there is no information available on the epidemiology of BNRBV. Studies have been designed in this thesis to determine the spread of disease within plants and across fields; this will be important for understanding the dynamics of the disease and possible mechanisms of disease progress and spread. BNRBV is widespread in Georgia, and it has the potential to significantly impact production. Once the disease etiology is better understood, novel disease management strategies can be developed to control the virus.

Based on the above considerations, the overall goal of this thesis is to fill critical knowledge gaps regarding the epidemiology of BNRBV. The specific objectives were to:

1. Determine how BNRBV spreads within individual blueberry plant.
2. Quantify how BNRBV spreads within an established field.
3. Determine the distribution of the virus in the plant and the degree to which it is systemic in plant tissues.

LITERATURE CITED

1. Andret-Link, P., and Fuchs, M. 2005. Transmission specificity of plant viruses by vectors. J. Plant Pathol. 87(3):153-165.
2. Anonymous. 2013. Blueberries: A handful of health. U.S. Highbush Blueberry Council, Folsom, CA. Online.

3. Boatright, S. and McKissick, J. 2012. Georgia Farm Gate Value Report 2011. AR12-01, University of Georgia, Center for Agribusiness and Economic Development, Athens.
4. Brannen, P.M. and Scherm, H. 2009. Blueberry necrotic ring blotch. Small Fruits News. 9(2):1-18.
5. Brannen, P.M., Scherm, H. Deom, C.M., Srinivasan, B. and Harmon, P. 2011. Blueberry necrotic ring blotch disorder widespread and severe in 2011. Dixie Blueberry News 11(6):21-25.
6. Burkle, C., Olmstead, J.W., and Harmon, P.F. 2012. A Potential vector of *Blueberry necrotic ring blotch virus* and symptoms on various host genotypes. (Abstr.). Phytopathology. 102, S4.17.
7. Chapman, D. Georgia's peachy image turns blue. The Atlanta Journal-Constitution. 25 June 2012 main ed.: A1. Online. 16 Oct. 2012.
8. Chung, K.R. and Brlanksy, R. 4H. 2006. Citrus diseases exotic to Florida: Citrus Leprosis. Fact Sheet PP-226. Department of Plant Pathology, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, Gainesville, FL.
9. Fonsah, E.G., Harrison, K., and Bruorton, M. 2006. Economic analysis of producing southern highbush blueberries in Georgia. Bulletin 1303, College of Agricultural and Environmental Sciences, University of Georgia, Athens.
10. Holland, R.M. 2013. Location, transmission, and impact of *Xylella fastidiosa* in southern highbush blueberries. M.S. thesis. Department of Plant Pathology, University of Georgia, Athens.
11. Hull, Roger. 2009. Comparative Plant Virology. 2nd ed. Elsevier, Amsterdam.

12. Kole, C. 2007. Genome Mapping and Molecular Breeding in Plants Vol. 4. Fruits and Nuts. Springer, Berlin.

13. Lang, G.A. 1993. Southern highbush blueberries: Physiological and cultural factors important for optimal cropping of these complex hybrids. Acta Hort 346:72-80.

14. Martin, R.R., Tzanetkis, I.E., Caruso, F.L., and Polashock, J.J. 2009. Emerging and reemerging virus diseases in blueberry and cranberry. Acta Hort. 810:299-304.

15. Martin, R. R., Polashock, J. J., and Tzanetakis, I. E. 2012. New and emerging viruses of blueberry and cranberry. Viruses 4:2831-2852.

16. Nunes, M.A, de Oliveira, C.A., de Oliveria, M.L, Kitajima, E.W., Hilf, M.E., Gottwald, T.R., and Freitas-Astúa, J. 2012. Transmission of *Citrus leprosis virus* C by *Brevipalpus phoenicis* (Geijskes) to alternative host plants found in citrus orchards. Plant Dis. 96(7):968-972.

17. Ohlemeier, D. Georgia blueberry production goes big. The Packer. 2 Apr. 2012: B1 and B3. Online. 16 Oct. 2012.

18. Pascon, R. Kitajima, J.P., Breton, M.C., Assumpção, Greggio, C., Zanca, A.S., Okura, V.K., Alegria, M.C., Camargo, M.E., Silva, G.C., Cardozo, J.C., Vallim, M.A., Franco, S. F., Silva, V.F., Junior, H.J., Oliveira, F., Giachetto, P.F., Ferrari, F., Aguilar-Vildoso, C.L., Franchiscini, F., Silva, J., Arruda, P., Ferro, J.A., Reinach, F., and Rasera da Silva, A.C. 2006. The complete nucleotide sequence and genomic organization of Citrus leprosis associated virus, Cytoplasmic type (CiLV). Virus Genes 32:289-298.

19. Quito-Avila, D., Brannen, P.M., Cline, W.O., Harmon, P.F., and Martin, R.R. 2013. Genetic characterization of *Blueberry necrotic ring blotch virus*, a novel RNA virus with unique genetic features. J. Gen. Virol. 94:1426-1434.

20. Rodrigues, J.C.V., Kitajima, E.W., Childers, C.C., and Chagas, C.M. 2003. *Citrus leprosis virus* vectored by *Brevipalpus phoenicis* (Acari: Tenuipalpidae) on citrus in Brazil. Experimental and Applied Acarology 30:161-179.
21. Scherm, H. and Krewer, G. 2003. Blueberry production in Georgia: Historical overview and recent trends. Small Fruits Rev. 2(4)83-91.
22. Scherm, H. and Krewer, G. 2008. Disease management in organic rabbiteye blueberries. International Journal of Fruit Science 8 (1-2):69-80.
23. Scherm, H., Savelle, A. T., Brannen, A. 2008. Occurrence and prevalence of foliar diseases on blueberry in Georgia. Online. Plant Health Progress doi:10.1094/PHP-2008-0421-01-RS.
24. Sorrow, A.R. 2010. Disease threatens Georgia blueberry crop. Georgia FACES. College of Agricultural and Environmental Sciences, University of Georgia, Athens.
25. Tzanetakis, I. 2010. The Guardian knot of small fruit virology: Emerging diseases and their control. Online. APS Feature Article.

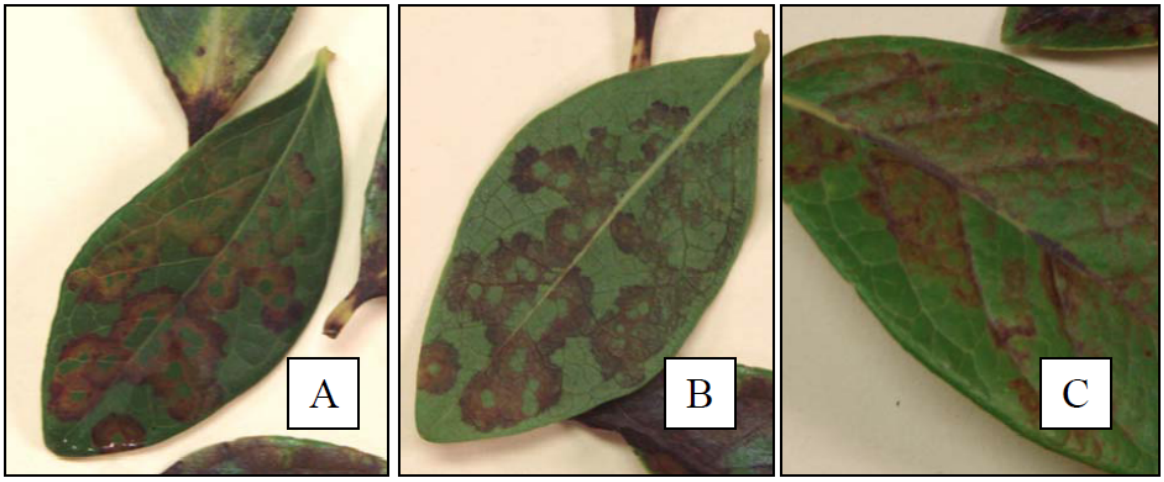


Figure 1.1. Symptoms of blueberry necrotic ring blotch are observed as circular blotches with green centers on the adaxial leaf surface (A) and abaxial leaf surface (B). A variant of this symptom, often observed on the same plant, is a greasy or oily appearance on the leaves, which resembles a chemical burn injury (C).

Figure 1.2. Ring color can vary from dark to purplish-black (left), but rings often coalesce to form solid brown to black necrotic spots and blotches (right), and severely diseased leaves can become totally covered in spots before they abscise.

CHAPTER 2

IN-FIELD AND IN-PLANTA SPATIO-TEMPORAL SPREAD OF *BLUEBERRY NECROTIC RING BLOTCH VIRUS* IN SOUTHERN HIGHBUSH BLUEBERRIES IN GEORGIA[1]

[1] Robinson, T.S., Brannen, P.M., Deom, C.M., and Scherm, H., 2013. To be submitted to *Plant Disease.*

In-field and *in-planta* spatio-temporal spread of *Blueberry necrotic ring blotch virus* in southern highbush blueberries in Georgia.

T.S. Robinson, P.M. Brannen, C.M. Deom, and H. Scherm

Department of Plant Pathology, University of Georgia, Athens 30602

ABSTRACT

Blueberry necrotic ring blotch, caused by *Blueberry necrotic ring blotch virus* (BNRBV), is a new disease in the southeastern United States whose epidemiology is largely unknown. In a 2-year field study, susceptible 'Star' blueberry plants were monitored to quantify the field-level and plant-level spatio-temporal spread of BNRBV in naturally infected commercial blueberry plantings. At the field-level, disease severity was assessed visually in a 25 X 25 block of contiguous 'Star' plants over time (4 to 6 assessments per season). A total of thirty-six field distribution maps were constructed across sites, assessment dates, and years. Edge effects (disease significantly more severe at the edge of the field) occurred in 19.4% of fields during at least one assessment date and were more common along row edges vs. column edges of the field. No significant association was found between the presence of an edge effect and specific neighboring vegetation. Within-field aggregation of symptomatic plants was detected by ordinary runs analysis, with clustering in both within and across row directions. However, the percentage of maps having within row aggregation (94.4%) was greater than the percentage of maps for across-row aggregation (83.3%). At each assessment date, 10 individual whole plants were examined in detail for within-plant distribution of disease using a grid system. Final mean disease severity was significantly lower in the top stratum of plants than in the middle or bottom

strata. Furthermore, disease severity was higher in the interior quadrats of the plants than in exterior strata. When data from individual shoots were analyzed, no consistent differences in leaf disease severity between top, middle, and bottom shoot sections were found. This suggests that leaves can become infected at any leaf stage. This is the first study to quantify the spatio-temporal spread of BNRBV in southern highbush blueberries.

INTRODUCTION

Blueberry is Georgia's most important fruit crop in both acreage and farm gate value. Georgia is currently ranked as the third-largest blueberry producer in the nation (9). According to the 2011 Georgia Farm Gate Value Report, the blueberry industry produced over $254 million in total sales (4). More than 9,300 hectares in the state are devoted to blueberry farming compared with 3,237 hectares 10 years ago (6,16). Historical estimates of relative shares of rabbiteye blueberry (*Vaccinium virgatum = V. ashei*), and southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) production were at 90% and 10%, respectively (20). Although no formal surveys have been conducted recently, southern highbush blueberries have increased in production, possibly accounting for 20-30% of current production (P.M. Brannen, *personal communication*). While rabbiteye is the more widely grown blueberry type in Georgia, the early-maturing southern highbush blueberries are economically important due to a highly favorable market window for fresh-picked fruit from late April to mid May.

Southern highbush blueberries are more susceptible to diseases than the native rabbiteye blueberries. Among the diseases occurring on southern highbush blueberry, Blueberry necrotic ring blotch is becoming a major concern in Georgia and other southeastern states. This disease, caused by *Blueberry necrotic ring blotch virus* (BNRBV) (18), was first observed in 2006 in

Bacon County, Georgia. By 2008, most southeastern states with southern highbush blueberry production reported the disease. Many southern highbush cultivars are susceptible (5), whereas the disease has not been observed on rabbiteye blueberries. Symptoms appear as irregularly shaped concentric rings or blotches with or without green centers on both leaf surfaces. Ring spots are generally more prevalent on older tissue (i.e. the lower leaves of plants), but they can affect a bush from bottom to top. In severe cases, BNRBV can lead to premature defoliation, which is presumed to result in reduced return yield the following year, as observed in previous studies in which premature defoliation was incited by manual leaf removal or through naturally occurring fungal leaf spot epidemics (17, 22). This is a major concern for producers as it could reduce profitability.

BNRBV possesses a 14 kb genome divided into four ssRNA segments (18). Phylogenetic analysis revealed that BNRBV clustered with members of the family *Bromoviridae* based on the methyltransferase domain. An analysis of the RNA-dependent RNA polymerase domain grouped BNRBV with members of the *Virgaviridae*, specifically with *Citrus leprosis virus* (CiLV, genus *Cilevirus*), and the newly characterized *Hibiscus green spot virus* (HGSV: proposed genus *Higrevirus*). BNRBV possesses two helicases, which is a unique genetic feature among known viruses. The diverse lineages of the helicases suggest that BNRBV is a result of a recombination event between members of the *Bromoviridae* and *Virgaviridae* family. Overall, the findings above suggest that BNRBV represents a new genus; the name *Blunervirus* is proposed.

Since BNRBV causes a new disease, there is no information relative to the epidemiology of the disease. Most importantly, in-field vectors of the virus have yet to be identified, although circumstantial evidence suggests that eriophyid mite(s) may be involved in disease transmission

(6, 18). In the absence of data from controlled transmission experiments, studies of spatio-temporal spread may serve as the first step toward elucidating biotic or abiotic modes of disease transmission. Based on the above considerations, the objectives of this study were to quantify the spatio-temporal spread of BNRBV across naturally infected blueberry plantings and within individual plants. This will be the first epidemiological study to better understand disease development and progression of BNRBV under field conditions.

MATERIALS AND METHODS

Field sites. The study was carried out in six commercial blueberry plantings affected by BNRBV located in Atkinson, Bacon, Berrien, Clinch, and Ware counties (key areas of blueberry production in Georgia) in the spring and summer of 2011 and 2012. Field sites were selected in cooperation with county extension agents and growers based on the history of the occurrence. Selected sites were Alma (31 32.14 N 82 25.10 W), Enigma 1 (31 21.05 N 83 24.35), Enigma 2 (31 22.23 N 83 19.11), Homerville (30 59.46 N 82 40.47 W), Waycross (31 34.52 N 82 18.62 W), and Willacoochee (31 15.57 N 83 06.17 W). Disease monitoring focused on 'Star' southern highbush cultivar, as it has previously been shown to be highly susceptible to necrotic ring blotch. Five of six fields surveyed consisted of alternating rows of 'Star' with other southern highbush cultivars (e.g. 'Rebel', 'Emerald', and 'O'Neal'). The standard spacing for southern highbush blueberries in Georgia is 1.2 m between plants in a row and 3.0 m across rows (14). One planting in Alma, GA, was a high-density pine bark bed of only 'Star' with a closer spacing of 0.9 m X 1.5 m (13). Plant age varied from field to field, but typically ranged from 5 to 8 years.

Plants were managed according to each individual grower's standard practices, generally following Cooperative Extension recommendations for blueberry production in Georgia (13). All plantings were treated with fungicides to manage fungal leaf spot diseases, and fungal leaf spots were suppressed at all locations in both years.

Field distribution of symptomatic plants. In each planting, a 25 X 25 block of contiguous 'Star' plants was monitored for necrotic ring blotch severity every 3 to 4 weeks during the spring/summer growing seasons of 2011 and 2012. Disease severity was assessed visually by inspecting each bush for the percentage of leaves (0 to 100%) affected by symptoms typical of necrotic ring blotch. All six fields were monitored in 2011, whereas three fields (Enigma 1, Homerville, and Willacoochee) were monitored in 2012. Plot maps of disease severity were developed for each site and each assessment date, resulting in a total of 36 individual plots maps across sites, assessment dates, and years (Fig. 2.13 through 2.21).

Plot maps were analyzed for the presence of edge effects separately at each of the four edges of the 25 X 25 plant block. An edge effect was considered present when the average disease severity in the outermost three plant rows (or columns) was at least three times the overall mean disease severity across all 625 plants. The presence or absence of edge effects was tabulated for each planting and each assessment date and related by Chi-Square association analysis to the neighboring vegetation at each of the four edges of the block. Neighboring vegetation (i.e., pine, oak, grassland, shrubland, etc.) was classified as to whether it was within the row edge or across column edge of the plant block, and any other vegetation that may be surrounding the plant block.

To test for aggregations of necrotic ring blotch in each plot map, an ordinary runs analysis (8) was conducted. The analysis was performed separately for each assessment date and

for within- vs. across-row patterns. Instead of analyzing 25 rows and 25 columns separately for each map, adjacent rows (or columns) were joined such that the last plant of the first row (or column) was considered the neighbor of the last plant in the second row (or column). For this analysis, disease severity values were converted to binary values (0, 1) based on whether or not disease severity on a given plant was below or above the median disease severity for the entire 625-plant block at a given assessment date. Under the null hypothesis, symptomatic plants occurred in an aggregated pattern if the *z*-statistics was less than -1.64 (P=0.05). The code for the analysis was programmed in SAS (v. 9.2; SAS Institute, Inc., Cary, NC).

Distribution of symptoms within individual plants. Ten plants (showing initial stages of necrotic ring blotch symptom development) were chosen arbitrarily from each of the blocks utilized for the in-field disease assessments described above. These plants were then monitored every 2 to 3 weeks in detail for within-bush disease spread for each of the 2 years. The disease assessments utilized a two-dimensional grid system superimposed on one side of the bush to determine disease severity separately for up to 60 quadrats. The physical infrastructure for the grid consisted of a metal frame (2.0 X 1.2 m) having metal handles attached for easy manipulation and sharp metal pegs welded to the bottom to allow the grid to stand upright (Fig 2.22). Heavy-duty plastic baler twine was strung from side-to-side and top-to-bottom to construct a grid system, consisting of 60 quadrats, each 20 cm X 20 cm. The grid was constructed at the University of Georgia Instrument Design and Fabrication Shop, Athens, GA. During disease assessment, the grid was centered on the plant and staked approximately 0.6 m away from it. Each quadrat of the plant was assessed visually for disease severity (percentage of symptomatic leaves in each quadrat within the grid). Data were averaged across the ten plants per field to obtain one grid map per field and assessment date (Fig. 2.23 through 2.31).

For data analysis, each grid map was divided into three strata, top, middle, and bottom, based on the location of the quadrats relative to overall plant height. Kruskal-Wallis tests (SAS PROC NPAR1WAY) were then applied to determine whether average disease severity differed across the three height strata. In a separate analysis, quadrats were classified as to whether they were located at the exterior or interior of the bush, whereby exterior quadrats were those that had empty quadrats (i.e. no plant growth) on at least one side. Kruskal-Wallis tests were applied to determine whether average disease severity differed between exterior and interior quadrats of the bush.

Distribution of symptoms on individual shoots. Ten actively growing shoots were selected and tagged on each of the ten plants used in the individual-plant study described above and assessed for disease progression at each assessment date. Each leaf present on each shoot was tracked individually during the sampling period, assigning a number to indicate the position of the leaf on the shoot. New leaves at the tip of the shoot were added as they emerged. Disease severity was recorded as the percentage of necrotic leaf area on each individual leaf.

For data analysis, leaves were assigned to one of three strata (bottom, middle, or top) based on their position along the individual shoot. Analysis of variance (PROC GLIMMIX in SAS), followed by Tukey's test, was applied to compare average disease severity across the three strata (fixed effect), with the ten plants serving as replicates (random effect). The analysis was conducted only for final disease severity at the end of the monitoring period.

RESULTS

Field distribution of symptomatic plants. Edge effects, defined as the average disease severity in the outermost three rows (or columns) being at least three times the average disease

severity of the entire block for at least one assessment date, were observed in three out of six plantings in 2011 and in one out of three planting in 2012 (Tables 2.1 through 2.9) i.e., in 44.4% of plantings overall. Of the 36 plot maps (site-assessment date-year combinations), 13 displayed edge effects, which were much more common in row edges (12 cases) than in column edges (1 case). Interestingly, in all fields that displayed edge effects, the effect was always present during the earliest assessment date.

In those cases where edge effects were observed, they occurred most commonly on edges that neighbored pine woodlands (42.9% of cases). However, Chi-Square analysis indicated no statistically significant association between the presence of an edge effect and the presence of pine woodlands ($P = 0.2545$).

The ordinary runs analyses showed that plants symptomatic for necrotic ring blotch were highly aggregated both within and across rows (Tables 2.10 through 2.13). For the within-row analyses, 34 of 36 plot maps (94.4%) showed significant aggregation, whereas 30 of 36 maps (83.3%) showed significant across-row aggregation. The only site where a random pattern of disease was common was Homerville in 2012, where randomness was indicated for two of six assessment dates within row and four of six assessment dates across row (Tables 2.12 and 2.13).

Distribution of symptoms within individual plants. As observed visually on the plant grid maps (Fig. 2.23 through 2.31), disease often began to develop in the center of the bush, and as the season progressed, disease spread to the outer portion of the bush. In both years, final disease severity in quadrats in the top stratum of plants was significantly lower than that in the middle and bottom strata (Tables 2.14 and 2.15). Furthermore, in 2011, disease severity in quadrats within the interior of plants (6.0%) was significantly higher than that in exterior quadrats (2.8%) (Table 2.14). Although a higher average disease severity was also observed in

the interior quadrats in 2012 (7.4% vs. 4.9%), the difference was not statistically significant (Table 2.15).

Distribution of symptoms on individual shoots. Individual shoots were monitored over time for final mean disease severity at six commercial blueberry sites in 2011 and 2012. Based on the *P*-values of the analysis of variance, only 4 out of the 28 site-assessment date-year combinations showed significant differences among shoot sections (top, middle, and bottom) at the 5% level (Table 2.16). In three of those four cases, disease severity was greatest at the bottom levels of the shoot segment.

DISCUSSION

This research provides the first epidemiological report concerning the spatio-temporal dynamics of necrotic ring blotch epidemics over the course of two growing seasons. Results demonstrated that (i) edge effects are present in commercial plantings, (ii) disease is highly aggregated (not random) within and across rows in the field, (iii) disease severity is higher in the middle and bottom strata of whole plants and in the interior of the bush, and (iv) disease severity is similar on leaves along shoots, suggesting that leaves on individual shoots can become infected at any leaf stage.

Through the within in-field disease distribution analyses, we found that edge effects were present in 42.9% of fields during at least one assessment time. This may indicate introduction of the virus from adjacent vegetation at the beginning of the season. However, we could not confirm that edge effects were associated consistently with any particular surrounding vegetation. Initial visual observation of field plot maps suggested that field sites that displayed edge effects were in close proximity to wood lines, which were primarily composed of mixed

pine/hardwoods. However, a Chi-Square analysis test indicated no significant association between the presence of a wood line and edge effects. If there is an edge effect relationship of necrotic ring blotch with surrounding vegetation, additional detailed studies would be required to discern the associations.

The rapid progression of symptom development across fields is indicative that an aerial vector may be present. In Georgia, there have been significant populations of an eriophyid mite(s) found on leaf tissue associated with necrotic ring blotch in commercial fields. Observational information supports the premise that at least one eriophyid mite is vectoring the disease. Significant populations of an eriophyid mite were found associated with southern highbush blueberries showing symptoms of necrotic ring blotch in commercial fields in Georgia and Florida. In addition, introduction of tissue-cultured, virus-free 'Star' plants into a University of Florida greenhouse containing both symptomatic plants and eriophyid mites has twice resulted in rapid transfer of both mites and disease symptoms to the disease-free plants (P.M. Brannen and P.F. Harmon, *personal communication*). An eriophyid mite vector would also be consisitent with observations of eriophyid mite-transmitted viruses in other plant systems. As with necrotic ring blotch, edge effects have been confirmed for *Wheat streak mosaic virus,* vectored by the wheat curl mite, *Aceria tosichella*, a type of eriophyid mite. Thomas and Hein (20) reported that disease development begins at the edges of the wheat fields that are surrounded by volunteer wheat and other grass vegetation, where the mites oversummer. They have also reported that as the season progresses a strong disease gradient develops (20). The wheat curl mite is solely dependent upon wind-borne movement to spread and initiate viral infection. We speculate that for necrotic ring blotch, the eriophyid mites are dispersed to and through commercial blueberry fields in a similar fashion. Bassanezi and Laranjeira (2) reported edge effects in citrus groves in

Brazil for *Citrus leprosis virus* (CiLV), which is transmitted by the false spider mite, *Brevipalpus phoenicis* (2). Edge effects in CiLV symptoms were much lower (in percentage) than in our study. However, the probability of detecting primary infestations or new infestations was low for citrus leprosis (2). If it were possible to follow the entire leprosis epidemics, then edge effects for this perennial crop would be more frequently detected (2). This would be true for our study as well. To date, alternate hosts have not been identified for BNRBV, but it is assumed that surrounding vegetation provides a reservoir of viral inoculum to bridge epidemics.

In both years of the study, spatial patterns of BNRBV were highly aggregated both within and across rows. This suggests that disease is contagious and spreads from plant to plant (i.e., diseased plants are likely neighbors of other diseased plants). Eriophyid mites are known to be nomadic foliar feeders, walking or crawling from plant to plant. This could explain the clustering effect in the field, since close proximity to a point source would increase both contact with the virus and viruliferous mite populations.

Other studies of some insect and mite-vectored viruses have also shown high aggregation of virus symptoms within and across rows in the field. For example, Byamukama and Nutter (7) conducted a quadrat-based sampling method to quantify within-field spread of *Bean pod mottle virus* (BPMV) in soybean, which is predominantly vectored by the bean leaf beetle. The pattern of BPMV-infected quadrats was highly aggregated (7). The authors concluded that the quadrat-to-quadrat spread of BPMV is more widespread within-row by point source, where quadrats are located in close proximity of BPMV-positive quadrats. This is referred to as "local transmission" where the infection of one plant leads to the infection of neighboring plants (11). Also, it is important to note that BPMV-infected quadrats were highly aggregated, strongly suggesting that the bean leaf beetle population density is an important driver of BPMV

epidemics (7). It has been reported that aggregated patterns of BPMV-positive quadrats may be due to limited movement of bean leaf beetles within soybean fields, where they crawl or fly short distances (7). Due to their short flights, the bean leaf beetles were not able to move as quickly across rows vs. within row, thus creating a clustering effect in-field. If BNRBV is vectored by an eriophyid mite, the slow movement of these vectors could likewise explain the greater within row aggregation observed in our studies.

As previously mentioned, BNRBV is closely related to CiLV which is vectored by the false spider mite, *Brevipalpus phoenicis*. Spatial studies conducted by Bassanezi and Laranjeira (2) also reported aggregation in commercial citrus groves in Brazil. Spatial patterns of citrus leprosis and its mite vector were previously unstudied, with only anecdotal evidence describing this disease as occurring in clusters (2), which is similar to our studies. Bassanezi and Laranjeira (2) studied spatial patterns between mite-infested plants and those plants displaying symptoms of CiLV (diseased plants). Results from their study were similar to those found in our study, where aggregation was higher within-row vs. across-row. In the CiLV study, aggregation of diseased plants in the within-row direction was always greater than that of the across-row direction for either mite or symptom maps. Although preliminary studies suggest that wind is an important factor of *B. phoenicis* dispersal inside a grove (1), mites can also be dispersed mechanically. The spread of the leprosis mite by humans or machines should be considered, including cultural practices (i.e., hedging/pruning) and harvest, which are usually conducted within-row (2). Mites can also be transported on machines or other harvest equipment from plant to plant, hence previously non-infested trees of the same grove, or even new areas in close proximity to infected fields, can become infested (2). This could explain the clustering effect in citrus groves seen in

CiLV, where mites can be moved down a row more readily than across rows due to mechanical movement, not only by ambulation or wind.

Bassanezi and Laranjeira (2) also report that the degree of aggregation of diseased plants found in their study, was higher than that reported in other citrus diseases such as, citrus variegated chlorosis (CVC), citrus tristeza, and citrus huanglongbing. Winged vectors cicadelids, aphids, and psyllids, transmit all citrus diseases listed above respectively. Although we cannot directly compare our study to those in citrus crops, we can certainly use this information as a stepping-stone to better understand the epidemiology of BNRBV for future studies.

In the individual plant analyses, we first found that necrotic ring blotch tends to be less severe in quadrants of the top stratum of the blueberry bush, and more prevalent at the bottom of the bush. Typically, when the disease is first observed in the field (early growing season), symptom development is seen at the lower part of the bush, and over time disease symptoms spread to leaves throughout the entire plant. The reason for earlier development of disease in lower strata of the plant is currently unknown. We also found that symptoms are more concentrated in quadrats within the interior of the bush vs. the exterior of the bush. This may indicate that potential vectors may prefer to settle and/or feed in the center of infested plants, where it is cooler and protected from direct sunlight. It has been reported that eriophyid mites, specifically the wheat curl mite, can die within a few hours if exposed to low humidity and moderate to high temperatures (15). Most eriophyid mites desiccate where there is lack of humid and moist conditions (15). Further studies on the presumed vector should be conducted to better understand vector-host interactions of BNRBV of blueberry.

Furthermore, disease may also appear first in the lower part of the plant canopy if the virus were picked up from weeds on the orchard floor. Weeds can act as reservoirs for certain viruses, especially those vectored by insects (12). Insects are often attracted to weeds and survive on them because weeds can provide food for insects when preferred food is scarce, or weeds can provide shelter from unsuitable conditions such as bad weather or pesticide applications (12). Assuming that eriophyid mites are transmitting this disease, it is known that adult females overwinter among bud scales and other protected surfaces on or near the host plant (10). Mites emerge at bud break in the spring when weather is conducive for reproduction and feeding.

Lastly, our studies on the distribution of disease severity along individual shoots showed that there were no consistent differences for leaves at the tip vs. those at the bottom or the middle of the shoot. This may imply that leaves can become infected at any stage of development, since location along the shoot can serve as a proxy for leaf age (leaves lower on the shoot are older). It is possible that there may have been a difference in disease severity among strata earlier in the season as compared to later in the season, but this was not analyzed here. For example, it is plausible that disease severity on the new flushes of growth in the spring and early summer would have been lower. This should be evaluated in detail in further studies.

Eriophyid mites are amongst the most specialized of herbivorous organisms, feeding on few closely related species or genera of plant (3). During spring growth, eriophyid mites migrate from overwintering sites (e.g., bud scales) and feed on newly emerging foliage (10). Because differences in leaf disease severity were inconsistent amongst segments (bottom, middle, and top of shoots) in this study, the mites could have been blown to any part of the plant, and commenced feeding (and transmitting the virus) wherever they landed.

ACKNOWLEDGEMENTS

Supported by the Georgia Agricultural Commodity Commission for Blueberries, the Southern Region Small Fruit Consortium, and the USDA-NIFA Specialty Crop Research Initiative (grant no. 2008-51180-19579). We thank lab technician Amy Savelle, and undergraduate summer interns John Morgan, Patrick Savelle, Andy Shirley, Ben Shirley, Mason Westmoreland, and Laura Williford for their assistance in sampling and data collection during the course of this study. We also like to thank county extension agents Elvin Andrews, Tim Flanders, James Jacobs, John Ed Smith, and Danny Stanaland for their help and cooperation in field site selections, and communication with blueberry growers.

LITERATURE CITED

1. Alves, E.B. Casarin, N.B. and Omoto, C. 2005. Dispersal mechanisms of *Brevipalpus phoenicis* (Geijskes) (Acari: Tenuipalpidae) in citrus groves. Neotrop. Entomol. 34(1):89-96.
2. Bassanezi, R. B., and Laranjeira, F.F. 2007. Spatial patterns of leprosis and its mites vector in commercial citrus groves in Brazil. Plant Pathol. 56:97-106.
3. Bettini, L. 1994. Eriophyid mites. Publication 100C-1-066. Alaska Cooperative Extension, U.S. Department of Agriculture, and University of Alaska, Fairbanks.
4. Boatright, S. and McKissick, J. 2012. Georgia Farm Gate Value Report 2011. AR12-01, University of Georgia, Center for Agribusiness and Economic Development, Athens.
5. Brannen, P.M. and Scherm, H. 2009. Blueberry Necrotic Ring Blotch. Small Fruits News. 9(2)1-18.

6. Burkle, C., Olmstead, J.W., and Harmon, P.F. 2012. A Potential vector of *Blueberry necrotic ring blotch virus* and symptoms on various host genotypes (Abstr.). Phytopathology 102:S4.17.
7. Byamukama, E., and Nutter, F.W., Jr. 2011. Quantifying the within-field temporal and spatial dynamics of *Bean pod mottle virus* in soybean. Plant Dis. 95:126-136.
8. Campbell, C.L., and Madden, L.V. 1990. Introduction to Plant Disease Epidemiology. Wiley, New York.
9. Chapman, D. 2012. Georgia's peachy image turns blue. The Atlanta Journal-Constitution. 25 June 2012 main ed.: A1. Online. 16 Oct. 2012.
10. Davis, R. and Beddes, T. 2011. Eriophyid mites (bud, blister, gall, and rust mites). Utah Fact Sheet ENT-149-11. Utah State University and Utah Plant Pest Diagnostic Laboratory, Logan, UT.
11. Gibson, G.J., and Austin, E. J. 1996. Fitting and testing spatiotemporal stochastic models with applications in plant pathology. Plant Pathol. 45:172-184.
12. Goyal, G., Gill, H.K., and McSorley, R. 2012. Common weed hosts of insect-transmitted viruses of Florida vegetable crops. ENY-863. Entomology and Nematology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL.
13. Krewer, G., and NeSmith, D.S. 2008. Blueberry fertilization in soil. Fruit Publication 01-1, College of Agricultural and Environmental Sciences, Univ. of Georgia, Athens.
14. Krewer, G., and NeSmith, D.S. 2012. Home garden blueberries. Bulletin C946, College of Agricultural and Environmental Science, Univ. of Georgia, Athens.

15. Murray, G.M., Knihinicki, D., Wratten, K., and Edwards, J. 2005. Wheat streak mosaic and the wheat curl mite, Bulletin DPI-99. New South Wales Department of Primary Industries, New South Wales, Orange.
16. Ohlemeier, D. 2012. Georgia blueberry production goes big. The Packer. 2 Apr. 2012: B1 and B3. Online. 16 Oct. 2012.
17. Ojiambo, P. and Scherm, H. 2006. Optimum sample size for determining disease severity and defoliation associated with Septoria leaf spot of blueberry. Plant Dis. 90:1209-1213.
18. Quito-Avila, D., Brannen, P.M., Cline, W.O., Harmon, P.F., and Martin, R.R. 2013. Genetic characterization of *Blueberry necrotic ring blotch virus*, a novel RNA virus with unique genetic features. J. Gen. Virol. 94:1426-1434.
19. Scherm, H. and Krewer, G. 2003. Blueberry production in Georgia: Historical overview and recent trends. Small Fruits Rev. 2(4):83-91.
20. Thomas, J.A., and Hein, G.L. 2003. Influence of volunteer wheat plant condition on movement of the wheat curl mite, *Aceria tosichella*, in winter wheat. Exp. Appl. Acarol. 31:253-268.
21. Williamson, J. G., and Miller, E. P. 2002. Early and mid-fall defoliation reduces flower bud number and yield of southern highbush blueberries. HortTechnology 12:214-216.

Table 2.1. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring blotch in a 25 row X 25 column block of plants at Alma, 2011.

Assessment date	Dis. incidence (%)	Disease severity (%)	Presence of edge effects[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
20 Jun	40.1	6.6	No	No	No	No
6 Jul	49.8	6.1	No	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: pond surrounded by mixed pine/hardwood and grass.
[c] Neighboring vegetation: grass.
[d] Neighboring vegetation: blueberries surrounded by fallow area with mixed broadleaf and grass.
[e] Neighboring vegetation: blueberries surrounded by fallow area with mixed broadleaf and grass.

Table 2.2. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring blotch in a 25 row X 25 column block of plants at Enigma site 1, 2011.

Assessment Dates	Dis. incidence (%)	Disease severity (%)	Presence of edge effects[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
28 Jun	74.0	1.3	Yes	No	No	No
12 Jul	90.6	30.8	Yes	No	No	No
26 Jul	69.0	6.9	Yes	No	No	No
13 Aug	81.4	7.9	Yes	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: pine stand.
[c] Neighboring vegetation: blueberries.
[d] Neighboring vegetation: pine/hardwood shrubs.
[e] Neighboring vegetation: blueberries of other variety.

Table 2.3. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring blotch in a 25 row X 25 column block of plants at Enigma site 2, 2011.

Assessment Dates	Dis. incidence (%)	Disease severity (%)	Presence of edge effects[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
27 Jun	96.0	7.8	No	No	No	No
12 Jul	94.4	31.0	No	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: pine/hardwood shrubs.
[c] Neighboring vegetation: pasture surrounded by pine stand.
[d] Neighboring vegetation: pine stand.
[e] Neighboring vegetation: blueberries of other variety and fallow area with pine/hardwood shrubs.

Table 2.4. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring blotch in a 25 row X 25 column block of plants at Homerville, 2011.

Assessment Dates	Dis. incidence (%)	Disease severity (%)	Presence of edge effect[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
22 Jun	91.8	8.1	Yes	No	No	No
11 Jul	97.4	17.8	No	No	No	No
19 Jul	97.1	15.0	No	No	No	No
12 Aug	94.2	12.5	Yes	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: pine/hardwood shrubs.
[c] Neighboring vegetation: blueberries of other variety.
[d] Neighboring vegetation: grass.
[e] Neighboring vegetation: blueberries of other variety bordered by pine stand.

Table 2.5. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring in a 25 row X 25 column block of plants disease at Waycross, 2011.

Assessment Dates	Dis. incidence (%)	Disease severity (%)	Presence of edge effects[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
16 Jun	42.4	0.9	Yes	No	No	No
7 Jul	66.1	2.2	No	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: grass and pine stand (dirt road separates blueberry plot and pine stand).
[c] Neighboring vegetation: grass.
[d] Neighboring vegetation: blueberry of other variety and mixed broadleaf and grass weeds.
[e] Neighboring vegetation: blueberries of other variety, grass, and housing with landscape.

Table 2.6. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring blotch in a 25 row X 25 column block of plants at Willacoochee, 2011.

Assessment Dates	Dis. incidence (%)	Disease severity (%)	Presence of edge effects[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
22 Jun	97.2	4.5	No	No	No	No
11 Jul	97.3	27.0	No	No	No	No
26 Jul	99.9	53.0	No	No	No	No
13 Aug	99.0	59.0	No	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: grass.
[c] Neighboring vegetation: pine stand.
[d] Neighboring vegetation: pine/hardwood shrubs.
[e] Neighboring vegetation: blueberry of other variety bordered by pine stand.

Table 2.7. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring blotch in a 25 row X 25 column block of plants at Enigma site 1, 2012.

Assessment Dates	Dis. incidence (%)	Disease severity (%)	Presence of edge effects[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
12 Jun	29.6	1.4	No	No	No	No
25 Jun	28.0	3.1	No	No	No	No
9 Jul	26.4	3.2	No	No	No	No
23 Jul	42.9	7.5	No	No	No	No
25 Sep	37.3	7.1	No	No	No	No
18 Oct	45.3	9.9	No	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: pine stand.
[c] Neighboring vegetation: blueberries.
[d] Neighboring vegetation: pine/hardwood shrubs.
[e] Neighboring vegetation: blueberries of other variety.

Table 2.8. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring blotch in a 25 row X 25 column block of plants at Homerville, 2012.

Assessment Dates	Dis. incidence (%)	Disease severity (%)	Presence of edge effects[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
12 Jun	1.4	0.0	No	Yes	No	No
25 Jun	3.5	0.1	Yes	Yes	No	No
9 July	1.3	0.1	No	Yes	No	No
23 July	2.6	0.2	No	No	No	Yes
25 Sept	16.3	0.2	No	Yes	No	No
18 Oct	44.2	2.8	Yes	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: pine/hardwood shrubs.
[c] Neighboring vegetation: blueberries of other variety.
[d] Neighboring vegetation: grass.
[e] Neighboring vegetation: blueberries of other variety bordered by pine stand.

Table 2.9. Incidence, severity, and presence of edge effects[a] of blueberry necrotic ring blotch in a 25 row X 25 column block of plants at Willacoochee, 2012.

Assessment Dates	Dis. incidence (%)	Disease severity (%)	Presence of edge effects[a]			
			Row edge 1[b]	Row edge 2[c]	Column edge 1[d]	Column edge 2[e]
12 Jun	50.7	1.3	No	No	No	No
25 Jun	73.0	5.3	No	No	No	No
9 July	99.8	25.0	No	No	No	No
23 July	99.7	54.0	No	No	No	No
25 Sep	99.8	63.0	No	No	No	No
18 Oct	99.8	41.0	No	No	No	No

[a] An edge effect was considered present if the average disease severity in the first or last three rows or columns of plants was at least three times the overall field mean.
[b] Neighboring vegetation: grass.
[c] Neighboring vegetation: pine stand.
[d] Neighboring vegetation: pine/hardwood shrubs.
[e] Neighboring vegetation: blueberries of other variety boarded by pine stand.

Table 2.10. Within-row ordinary runs analysis of 'Star' southern highbush blueberry plants affected by *Blueberry necrotic ring blotch virus* in six commercial fields in 2011.

	Field site					
Date	Alma	Enigma1	Enigma 2	Homerville	Waycross	Willacoochee
Late June	-4.12[a]	-12.36	-14.61	-13.81	-12.71	-17.09
Early July	-3.92	-10.16	-12.49	-15.26	-13.44	-17.37
Late July		-16.18		-14.21		-13.53
August		-11.92		-12.56		-14.11

[a] *z* values of the analysis < -1.64 indicate aggregation, whereas *z* > -1.64 denotes a random pattern of diseased plants.

Table 2.11. Across-row row ordinary runs analysis of 'Star' southern highbush blueberry plants affected by *Blueberry necrotic ring blotch virus* in six commercial fields in 2011.

	Field site					
Date	Alma	Enigma1	Enigma 2	Homerville	Waycross	Willacoochee
Late June	-4.94[a]	-9.21	-7.65	-7.70	(0.562)	-13.22
Early July	-3.76	-6.96	-7.52	-6.59	(-1.39)	-12.23
Late July		-8.75		-6.98		-6.96
August		-10.16		-6.95		-8.76

[a] *z* values of the analysis < -1.64 indicate aggregation, whereas *z* > -1.64 (values in parentheses) denotes a random pattern of diseased plants.

Table 2.12. Within-row ordinary runs analysis of 'Star' southern highbush blueberry plants affected by *Blueberry necrotic ring blotch virus* in six commercial fields in 2012.

	Field site		
Date	Enigma 1	Homerville	Willacoochee
Early June	-11.91[a]	(-0.349)	-4.48
Late June	-11.47	-3.54	-10.78
Early July	-11.84	(1.15)	-14.01
Late July	-14.76	-3.81	-16.03
September	-17.87	-2.34	-13.82
October	-17.47	-6.31	-15.53

[a] *z* values of the analysis < -1.64 indicate aggregation, whereas *z* > -1.64 (in parentheses) denotes a random pattern of diseased plants.

Table 2.13. Across-row ordinary runs analysis of 'Star' southern highbush blueberry plants affected by *Blueberry necrotic ring blotch virus* in six commercial fields in 2012.

	Field sites		
Date	Enigma 1	Homerville	Willacoochee
Early June	-6.53[a]	(-0.349)	-4.84
Late June	-6.90	(0.290)	-5.62
Early July	-6.46	(1.15)	-8.04
Late July	-11.33	(1.07)	-9.04
September	-13.93	-4.12	-10.56
October	-13.91	-4.04	-11.81

[a] z values of the analysis < -1.64 indicate aggregation, whereas z > -1.64 (in parentheses) denotes a random pattern of diseased plants.

Table 2.14. Disease severity of blueberry necrotic ring blotch on individual 'Star' southern highbush blueberries in southeast Georgia in 2011[a].

Site and date	Overall disease severity (%)	Top disease severity[a] (%)	Middle disease severity[a] (%)	Bottom disease severity[a] (%)	Bush interior dis. severity[b] (%)	Bush exterior dis. severity[b] (%)
Alma 2011						
20 June	1.0	0.1	2.2	0.4	2.2	0.1
6 July	0.6	0.0	1.2	0.4	1.1	0.1
Enigma 1 2011						
23 June	0.6	0.0	0.5	0.9	0.6	0.5
12 July	0.4	0.1	0.4	0.6	0.4	0.4
26 July	3.5	0.7	3.7	4.8	4.0	3.0
13 August	4.4	2.4	3.4	5.9	6.6	2.5
Enigma 2 2011						
27 June	3.9	0.2	3.0	6.0	5.1	2.4
12 July	5.0	3.7	4.4	5.8	7.5	2.3
Homerville 2011						
21 June	3.9	0.3	3.7	6.8	5.4	2.3
7 July	2.6	0.2	3.5	3.3	3.8	1.1
19 July	8.0	2.9	10.5	10.2	12.0	4.4
12 August	20.0	16.3	22.5	19.2	23.5	17.5
Waycross 2011						
16 June	3.4	0.2	1.3	6.6	2.7	4.0
7 July	1.2	0.0	1.5	1.4	1.4	1.1
Willacoochee 2011						
27 June	3.1	0.2	2.8	5.1	4.7	1.8
11 July	1.1	0.0	1.7	1.3	2.3	0.0
26 July	5.9	1.4	8.2	6.7	9.8	2.2
13 August	9.2	0.1	8.2	12.0	14.3	4.2
Mean	4.3	1.6	4.6	5.4	6.0	2.8
Kruskal-Wallis Test		Top vs. bottom: Chi-Square = 13.9, $P = 0.0002$ Top vs. middle: Chi-Square = 12.6, $P = 0.0004$ Middle vs. bottom: Chi-Square = 0.46, $P = 0.5120$			Chi-Square = 5.4, $P = 0.0200$	

[a] Data was collected from ten plants (replications) using a vertical point frame grid (Fig. 2.22) consisting of 60 quadrats 20 cm^2 each. Each grid map was subsequently divided into three strata, top, middle, and bottom, based on the location of the quadrats relative to overall plant height.

[b] Quadrats were classified as to whether they were located at the exterior or the interior of the bush, whereby exterior quadrats were those that had empty quadrats (i.e., no plant growth) on at the least one side.

Table 2.15. Disease severity of blueberry necrotic ring blotch on individual 'Star' southern highbush blueberries in southeast Georgia in 2012[a].

Site and date	Overall disease severity (%)	Top disease severity[a] (%)	Middle disease severity[a] (%)	Bottom disease severity[a] (%)	Bush interior dis. severity[b] (%)	Bust exterior dis. severity[b] (%)
Enigma 1 2012						
12 June	0.5	0.0	0.2	1.1	0.3	0.7
26 June	1.0	0.0	0.8	1.9	1.2	0.7
9 July	2.4	0.2	1.8	4.2	3.3	1.8
23 July	5.0	0.0	6.0	8.2	6.0	4.0
25 Sept	3.3	0.0	2.5	6.6	4.1	3.3
16 Oct	2.9	0.0	3.0	5.3	3.8	2.2
Homerville 2012						
12 June	0.0	0.0	0.0	0.0	0.0	0.0
25 June	0.0	0.0	0.0	0.0	0.0	0.0
9 July	0.0	0.0	0.0	0.0	0.0	0.0
23 July	0.0	0.0	0.0	0.0	0.0	0.0
27 Sept	0.2	0.0	0.5	0.1	0.5	0.0
18 Oct	0.1	0.0	0.3	0.1	0.3	0.0
Willacoochee 2012						
11 June	0.3	0.0	0.5	0.4	0.4	0.2
25 June	2.1	0.0	2.8	3.5	2.3	2.0
9 July	7.3	3.3	9.7	8.1	9.2	5.4
23 July	19.2	13.5	27.2	16.9	27.6	10.9
25 Sept	40.9	37.5	49.2	36.3	45.7	36.0
16 Oct	24.8	24.2	30.8	18.4	29.3	20.6
Mean	6.1	4.4	7.5	6.2	7.4	4.9
Kruskal-Wallis Test		Top vs. bottom: Chi-Square = 5.6, $P = 0.0176$ Top vs. middle: Chi-Square = 5.7, $P = 0.0168$ Middle vs. bottom: Chi-Square = 0.5, $P = 0.0823$			Chi-Square = 0.4, $P = 0.5120$	

[a] Data was collected from ten plants (replications) using a vertical point frame grid (Fig. 2.22) consisting of 60 quadrats 20 cm^2 each. Each grid map was subsequently divided into three strata, top, middle, and bottom, based on the location of the quadrats relative to overall plant height.

[b] Quadrats were classified as to whether they were located at the exterior or the interior of the bush, whereby exterior quadrats were those that had empty quadrats (i.e., no plant growth) on at the least one side.

Table 2.16. *P*-values of analysis of variance to compare final symptom severity of *Blueberry necrotic ring blotch virus* along individual shoots (top, middle, bottom) in commercial blueberry fields in southeast Georgia in 2011 and 2012.

Site and date	Assessment date 1[a]	Assessment date 2[b]	Assessment date 3[c]	Assessment date 4[d]
2011				
Alma	0.418	0.524	0.598	0.350
Enigma 1	0.064	0.379	0.442	**0.022**
Enigma 2	**0.008**	0.959	0.538	0.986
Homerville	0.107	0.910	0.215	0.511
Waycross	0.074	**0.044**	0.178	0.648
Willacoochee	0.157	**0.044**	0.231	0.694
2012				
Willacoochee	0.387	0.796	0.253	0.171

[a] assessment date 1: mid to late June
[b] assessment date 2: early July
[c] assessment date 3: late July
[d] assessment date 4: early August

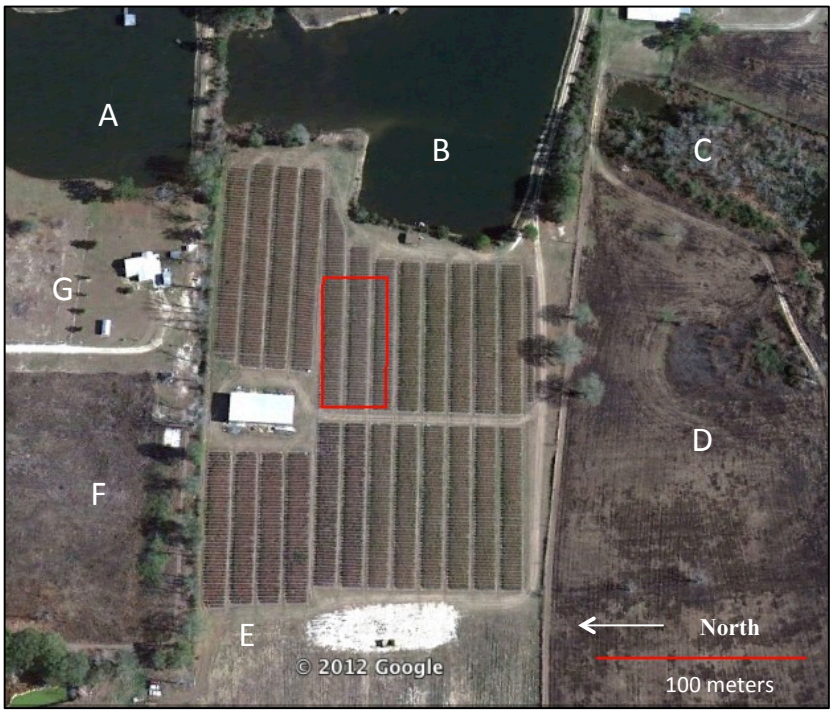


Figure 2.1. Blueberry field plot in Alma, GA (Bacon County) where Blueberry necrotic ring blotch virus (BNRBV) is endemic. The outlined area in red designates the approximate area of 'Star' plants that were surveyed in 2011. Surrounding landscape and vegetative growth of significance included: (A) pond surrounded by causeway with mixed hardwood stand and grass, (B) pond surrounded by mixed pine/hardwood and grass, (C) mixed pine and hardwood shrubs, (D) fallow area with mixed broadleaf and grass weeds, (E) grass, (F) fallow area with mixed broadleaf and grass weeds with mixed pine/hardwood surrounding, and (G) grass with mixed hardwoods and pines surrounding.

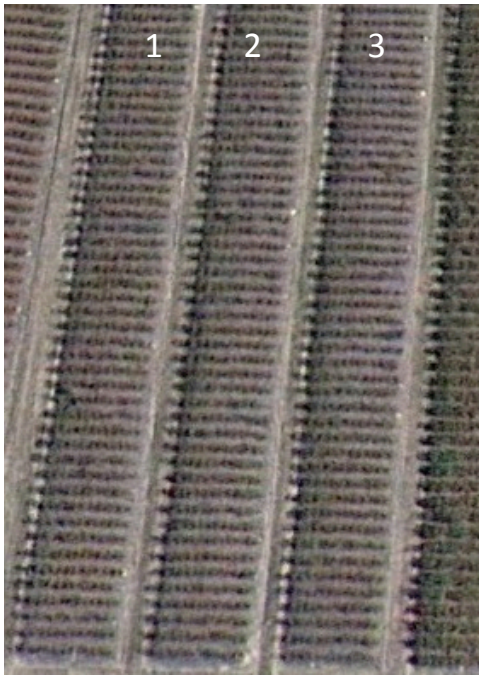


Figure 2.2. Area surveyed (enlarged). The three blocks of blueberry plants are of 'Star' variety.

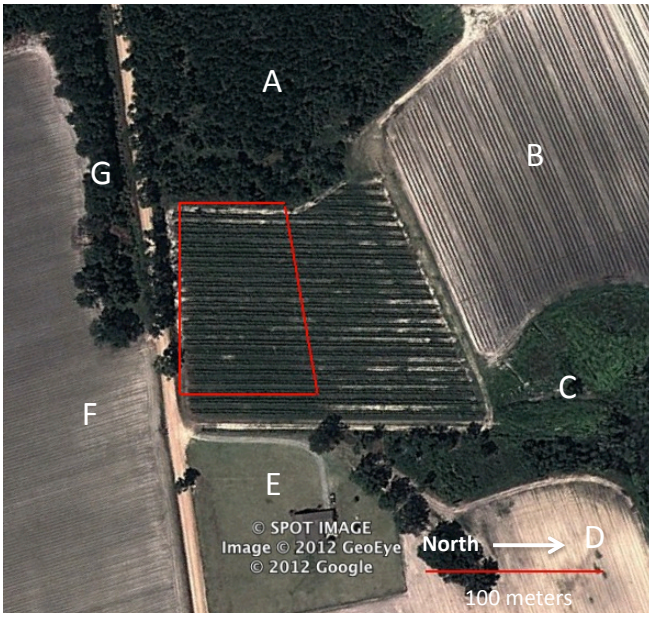


Figure 2.3. Blueberry field plot in Enigma, GA (Berrien County) where Blueberry necrotic ring blotch virus (BNRBV) is endemic. The outlined area in red designates the approximate area of 'Star' plants that were surveyed in 2011 and 2012. Surrounding landscape and vegetative growth of significance included: (A) pine stand surrounded by dirt road, (B) pumpkin field (C) pine/hardwood shrubs (D) cotton plot surrounded by pine, mixed broadleaf and grass (E) grass (F) peanut plot and (G) pine/ hardwood shrubs.

Figure 2.4. Area surveyed (enlarged). Blueberry plants are of 'Star' and 'Emerald' variety.

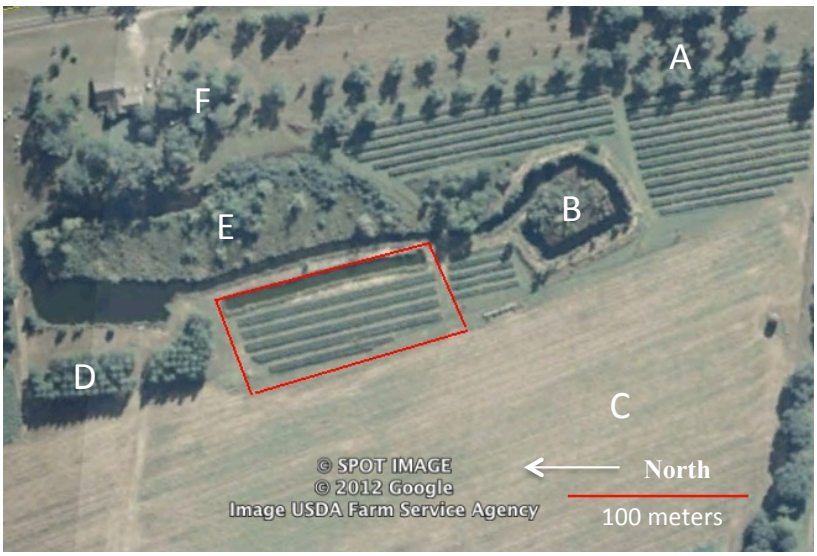


Figure 2.5. Blueberry field plot in Enigma, GA (Berrien County) Blueberry necrotic ring blotch virus (BNRBV) is endemic. The outlined area in red designates the approximate area of 'Star' plants that were surveyed in 2011. Surrounding landscape and vegetative growth of significance included: (A) pine/hardwood shrubs surrounded grass (B) fallow area with mixed pine/hardwood shrubs (C) pasture surrounded by pine stand (D) pine stand (E) pine/ hardwood shrubs with mixed broadleaf and grass weeds, and (F) pine/hardwood shrubs.

Figure 2.6. Area surveyed (enlarged). The five blocks of blueberry plants are of 'Star' variety.

Figure 2.7. Blueberry field plot in Homerville, GA (Clinch County) Blueberry necrotic ring blotch virus (BNRBV) is endemic. The outlined area in red designates the approximate area of 'Star' plants that were surveyed in 2011 and 2012. Surrounding landscape and vegetative growth of significance included: (A) fallow area with mixed broadleaf and grass weeds with mixed pine/hardwood surroundings, (B) pine stand (C) mixed pine and hardwood shrubs (D) small vineyard (Muscadines) and (E) Pine stand surrounded by dirt road.

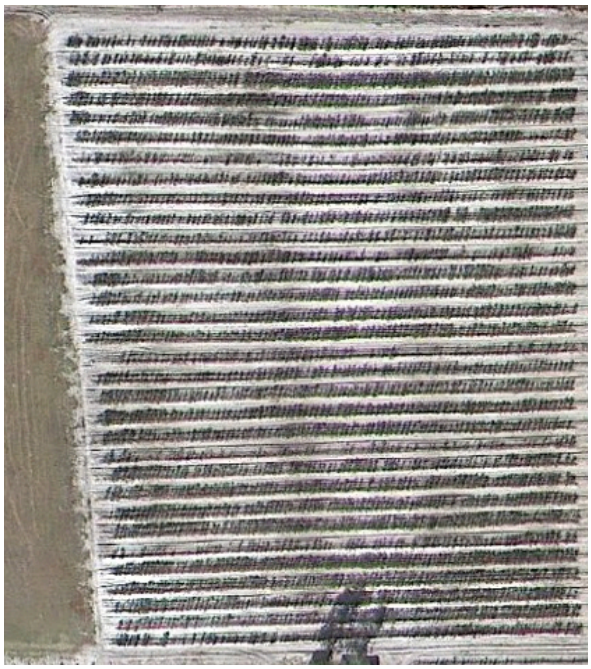


Figure 2.8. Area surveyed (enlarged). Blueberry plants are of 'Star' and 'Emerald' variety.

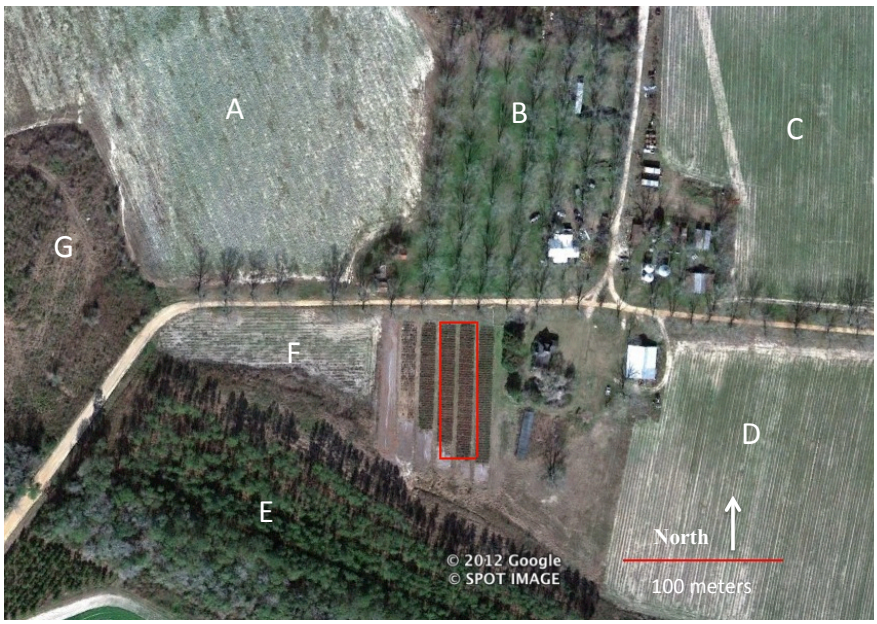


Figure 2.9. Blueberry field plot in Waycross, GA (Ware County) where Blueberry necrotic ring blotch virus (BNRBV) is endemic. The outlined area in red designates the approximate area of 'Star' plants that were surveyed in 2011. Surrounding landscape and vegetative growth of significance included: (A) grass/ grass weeds and mixed broadleaf (B) pine stand (C and D) grass (E) mixed pine and hardwood shrubs (F) mixed broadleaf and grass weeds (G) fallow area with mixed broadleaf and grass weeds.

Figure 2.10. Area surveyed (enlarged). The two blocks of blueberry plants are of 'Star' and 'Rebel' variety.

Figure 2.11. Blueberry field plot in Willacoochee, GA (Akinson County) where Blueberry necrotic ring blotch virus (BNRBV) is endemic. The outlined area in red designates the approximate area of 'Star' plants that were surveyed in 2011 and 2012. Surrounding landscape and vegetative growth of significance included: (A) pine/hardwood shrubs surrounded by dirt road (B) pine stand (C) pine stand (D) retention pond surround by clay mine, grass weeds, with pine/hardwood shrubs and (E) pine/hardwood shrubs surrounded by mixed broadleaf and grass.

Figure 2.12. Area surveyed (enlarged). Blueberry plants are of 'Star' and 'Rebel' variety.

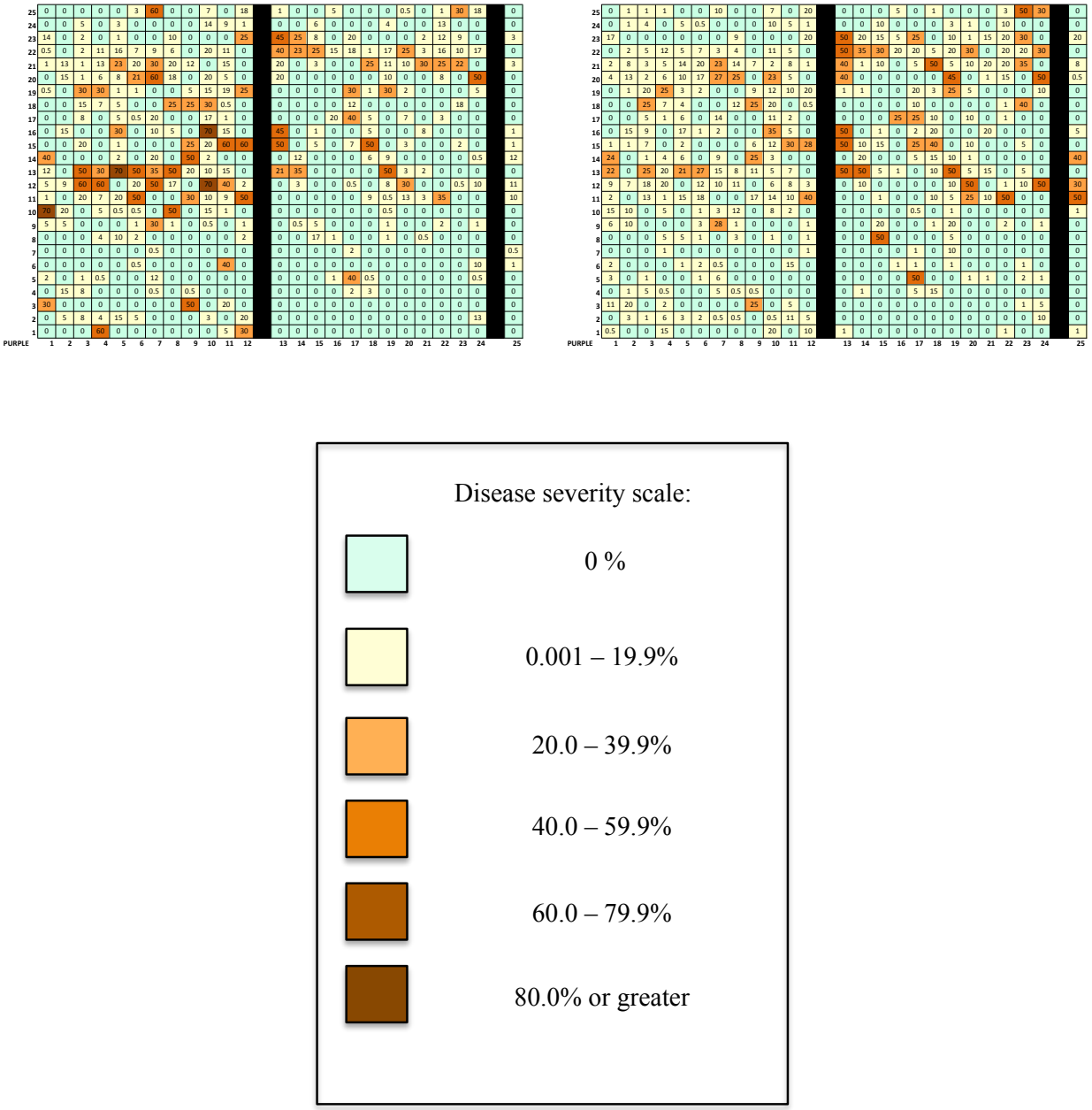


Figure 2.13. Temporal disease progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry in Alma, GA in 2011. Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 20 June and 6 July (from left to right). Blueberries at this site were planted in high-density pine park beds; the black vertical bars indicate non-planted alleys.

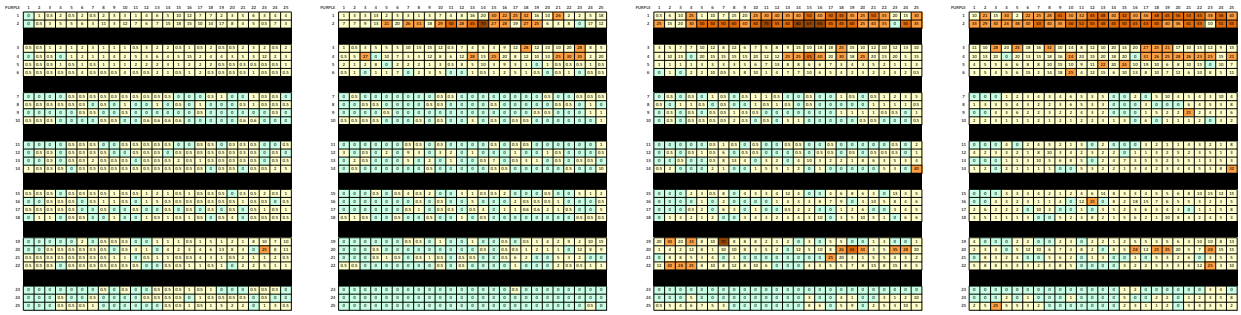


Figure 2.14. Temporal disease progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry in Enigma, GA in 2011 (Site 1). Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 28 June, 12 July, 26 July, and 13 August (from left to right). Blueberries at this site were planted in double-row beds; the black horizontal bars indicated a blueberry cultivar of different variety. A color scale for disease severity is given in Fig. 2.13.

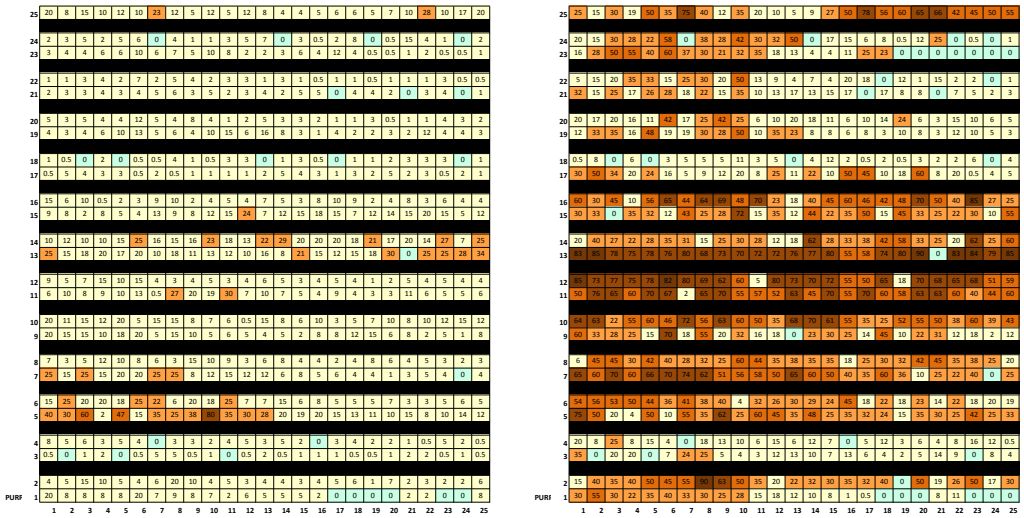


Figure 2.15. Temporal disease progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry in Enigma, GA in 2011 (Site 2). Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 27 June and 12 July (from left to right). Blueberries at this site were planted in double-row beds; the black horizontal bars indicate a blueberry cultivar of a different variety. A color scale for disease severity is given in Fig. 2.13.

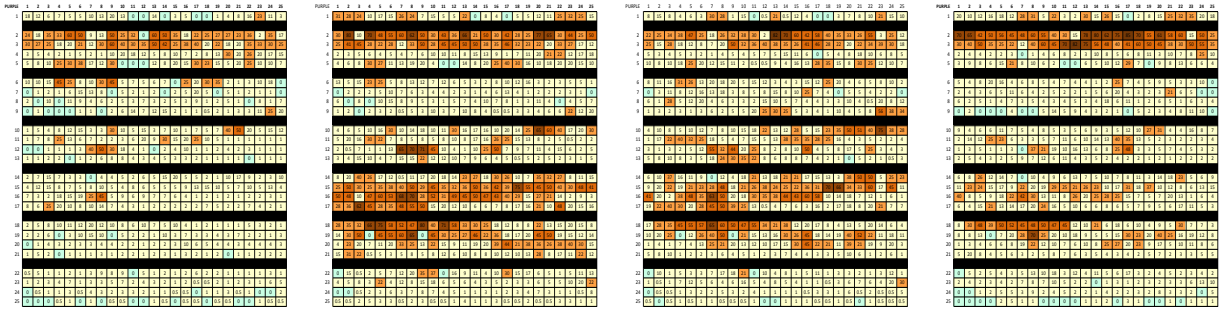


Figure 2.16. Temporal disease progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry in Homerville, GA in 2011. Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 21 June, 7 July, 19 July, and 12 August (from left to right). Blueberries at this site were planted in double-row beds; the black horizontal bars indicate blueberry cultivar of different variety. A color scale for disease severity is given in Fig. 2.13.

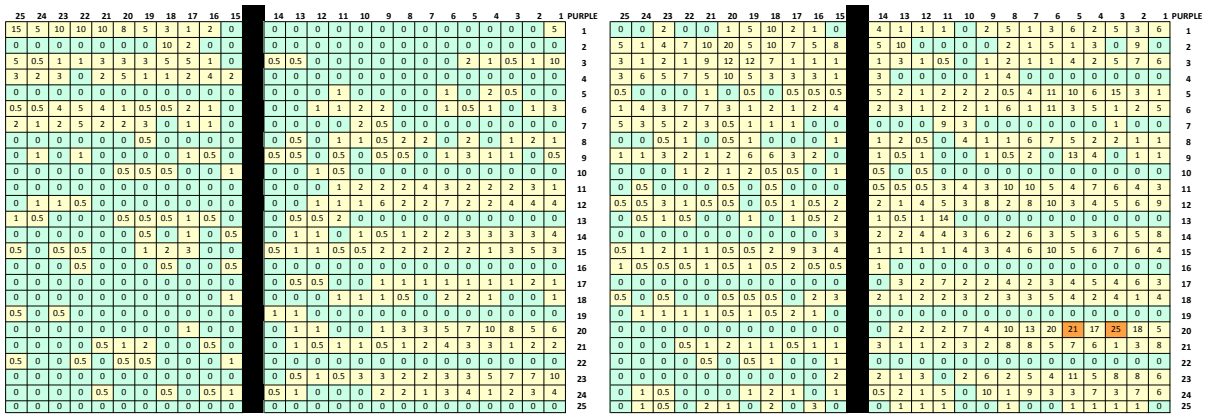


Figure. 2.17. Temporal disease progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry Waycross, GA in 2011. Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 16 June and 7 July (from left to right). Blueberries at this site were planted in double-row beds; the black horizontal bars indicate blueberry of different variety. A color scale for disease severity is given in Fig. 2.13.

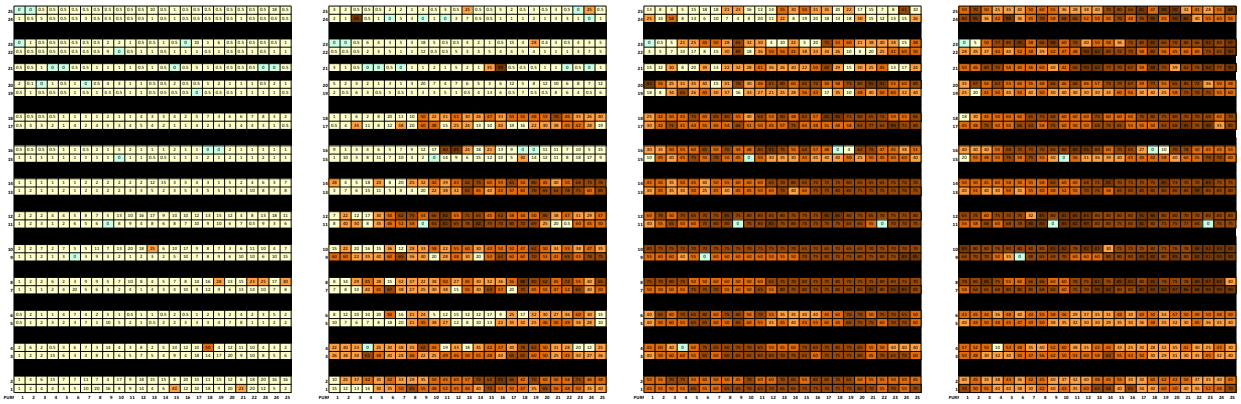


Figure 2.18. Temporal progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry in Willacoochee, GA in 2011. Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 27 June, 11 July, 26 July, and 13 August (from left to right). Blueberries at this site were planted in double-row beds; the black horizontal bars indicate blueberry of different variety. A color scale for disease severity is given in Fig. 2.13.

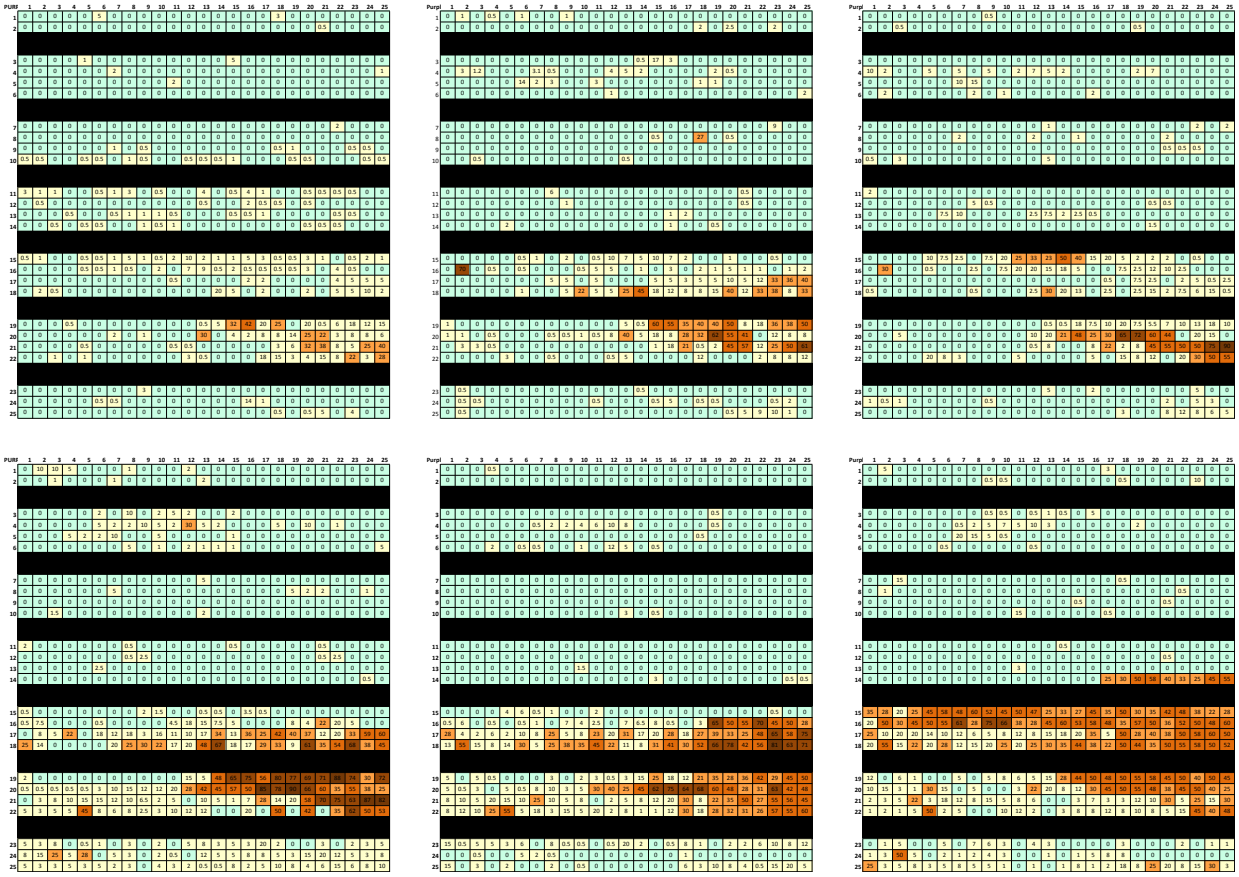


Figure 2.19. Temporal progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry in Enigma, GA in 2012 (Site 1). Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 12 June, 26 June, 10 July, 23 July, 25 September, and 16 October (from left to right, top and bottom). Blueberries at this site were planted in double-row beds; the black horizontal bars indicate blueberry cultivar of different variety. A color scale for disease severity is given in Fig. 2.13.

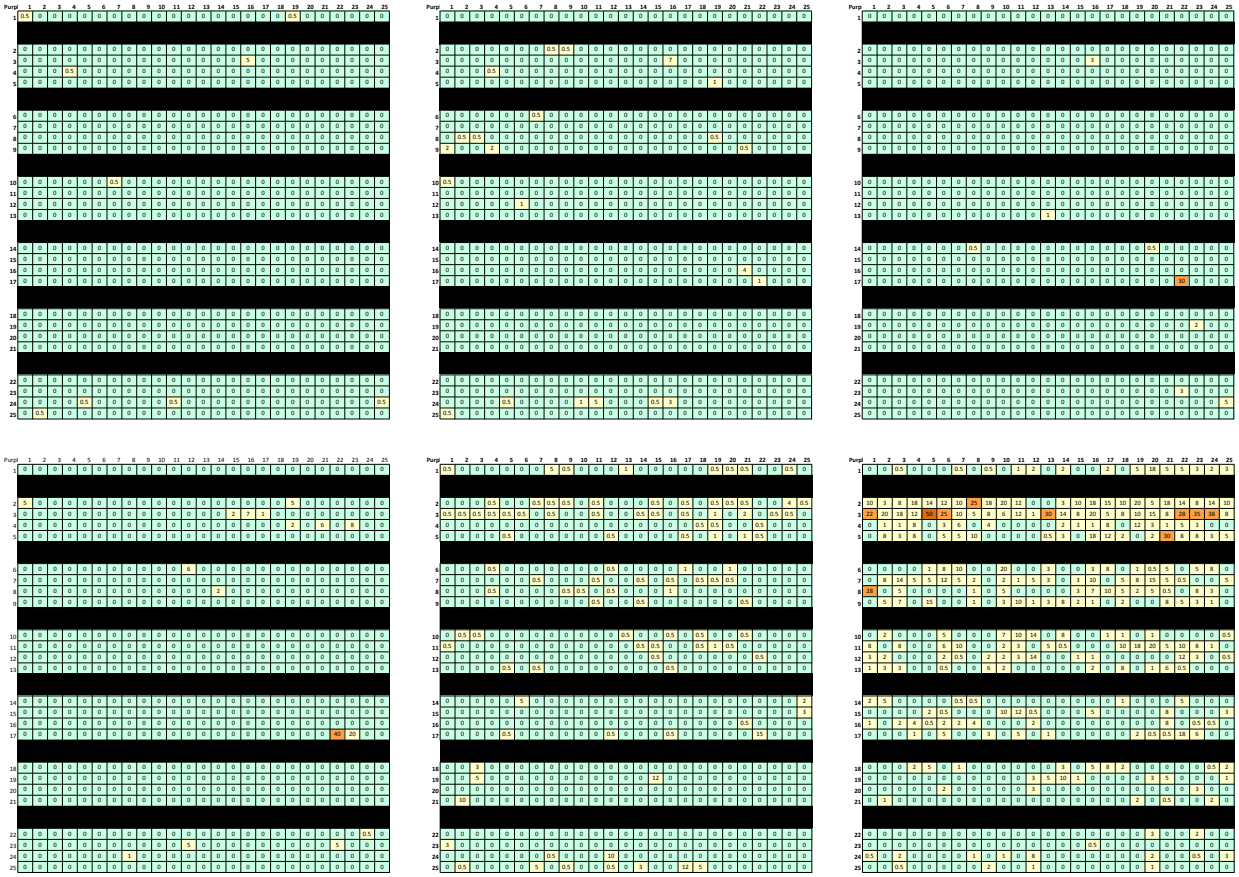


Figure 2.20. Temporal progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry in Homerville, GA in 2012. Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 6 June, 25 June, 9 July, 23 July, 27 September, and 18 October (from left to right, top to bottom). Blueberries at this site were planted in double-row beds; the black horizontal bars indicate blueberry cultivar of different variety. A color scale for disease severity is given in Fig. 2.13.

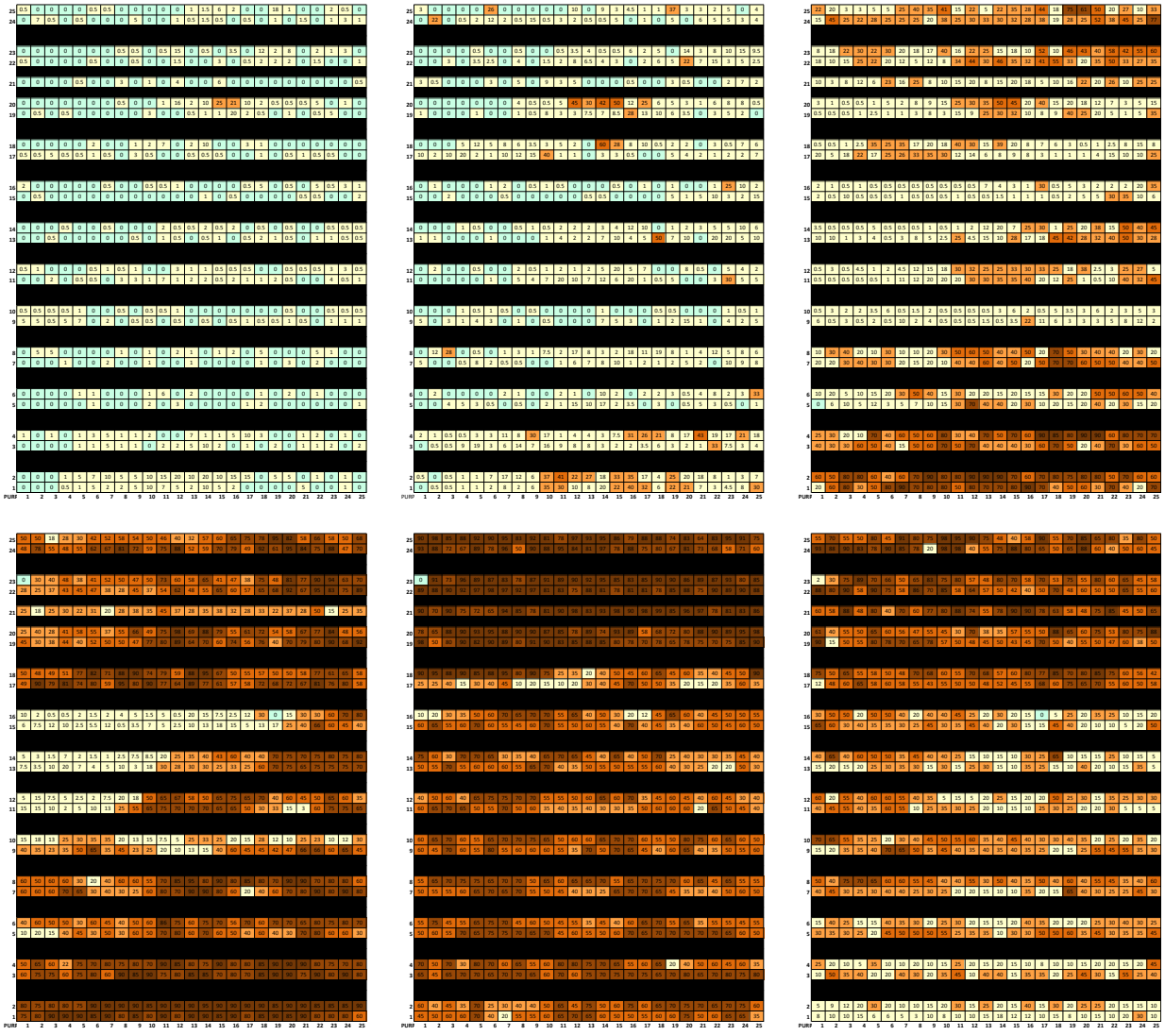


Figure 2.21. Temporal progression of *Blueberry necrotic ring blotch virus* in a 25 X 25 plant block of 'Star' southern highbush blueberry in Willacoochee, GA in 2012. Numbers in each square correspond to the disease severity per plant. Disease severity is the relative number of leaves per bush showing symptoms of necrotic ring blotch disease. Assessment dates were 6 June, 25 June, 9 July, 23 July, 25 September, and 16 October (from left to right, top to bottom). Blueberries at this site were planted in double-row beds; the black horizontal bars indicate blueberry cultivar of different variety. A color scale for disease severity is given in Fig. 2.13.

Figure 2.22. Vertical point frame grid utilized to assess disease severity of whole plants. The grid was 2.1 m high and 1.4 m wide, with 60 quadrats of 20 cm^2 each.

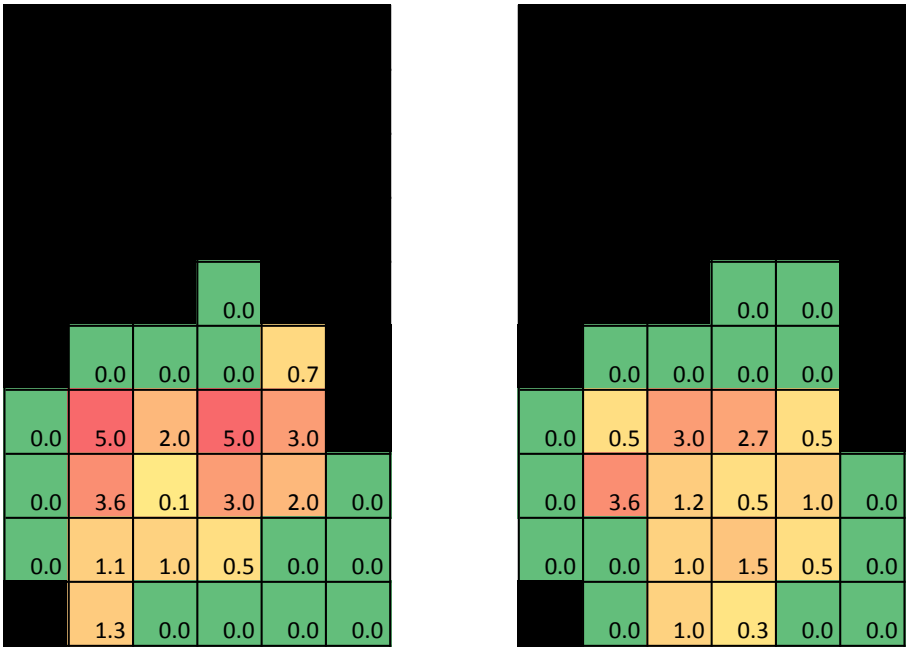


Figure 2.23. Temporal progress of blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Alma, GA in 2011. Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean disease severity levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 20 June and 6 July (from left to right). Shaded black areas designate where there is no plant growth.

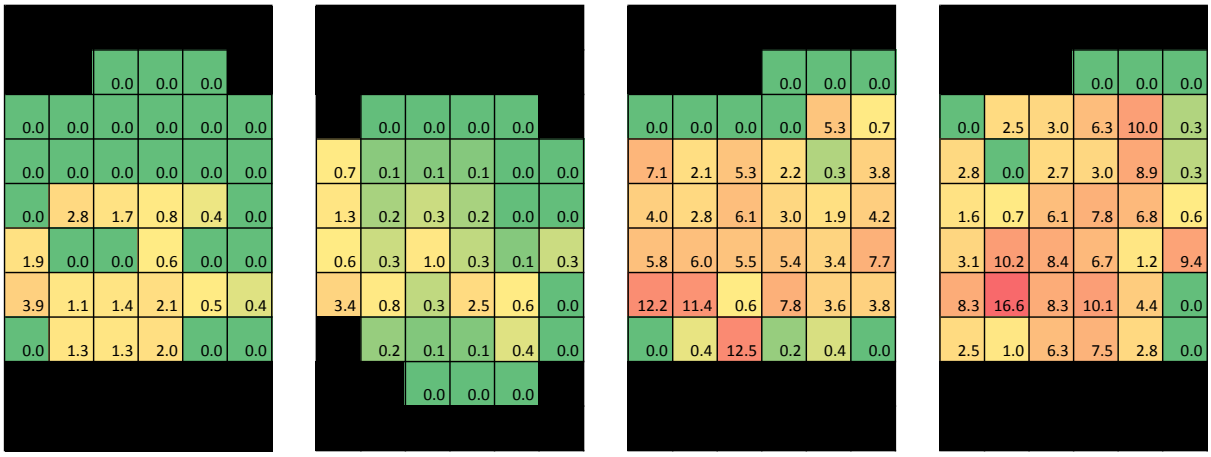


Figure 2.24. Temporal progress of blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Enigma, GA in 2011 (Site 1). Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean severity disease levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 23 June, 12 July, 26 July, and 13 August (from left to right). Shaded black areas designate where there is no plant growth.

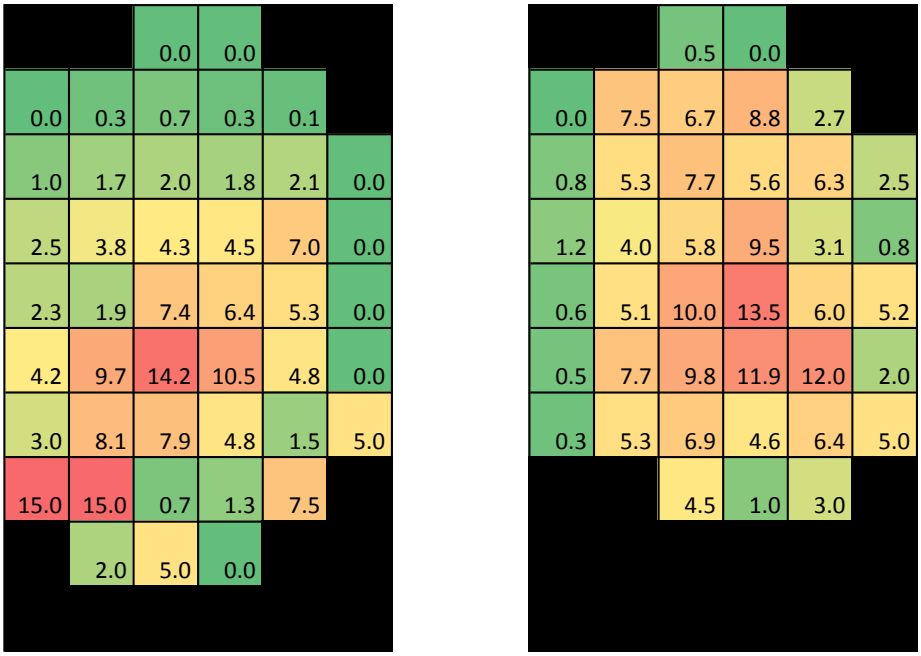


Figure 2.25. Temporal progress of blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Enigma, GA in 2011 (Site 2). Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean severity disease levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 27 June and 12 July (from left to right). Shaded black areas designate where there is no plant growth.

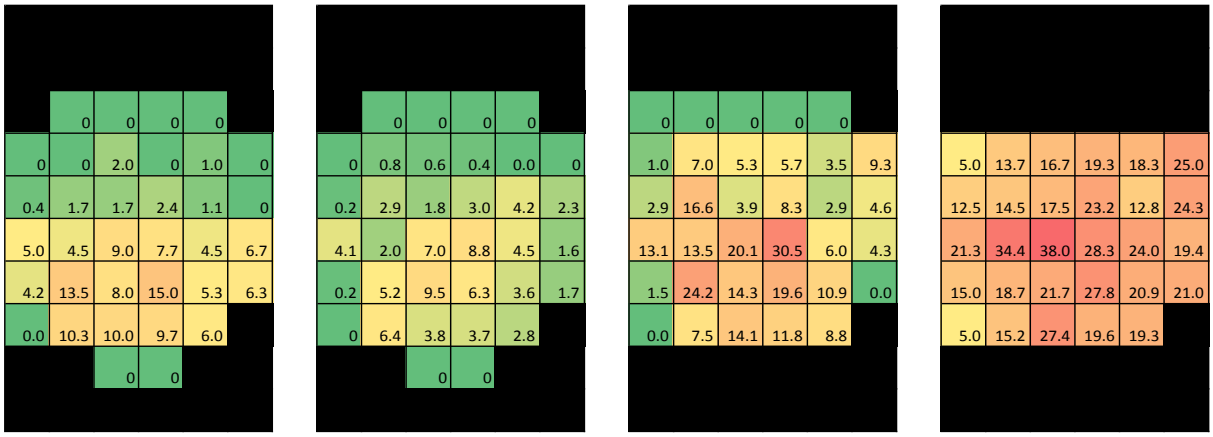


Figure 2.26. Temporal progress of blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Homerville, GA in 2011. Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean severity disease levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 21 June, 7 July, 19 July, and 12 August (from left to right). Shaded black areas designate where there is no plant growth.

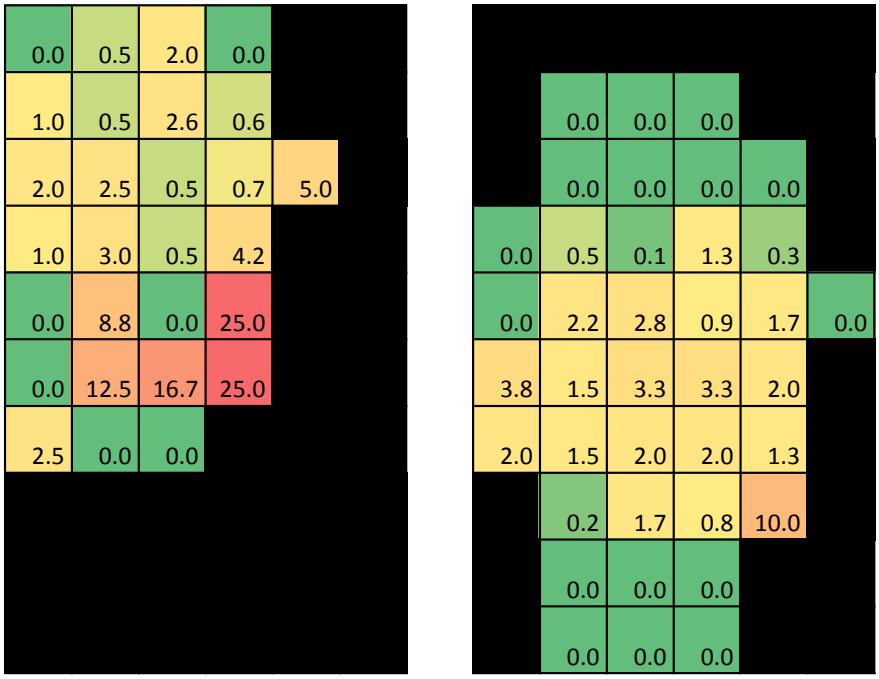


Figure 2.27. Temporal progress of blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Waycross, GA in 2011. Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean severity disease levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 16 June and 11 July (from left to right). Shaded black areas designate where there is no plant growth.

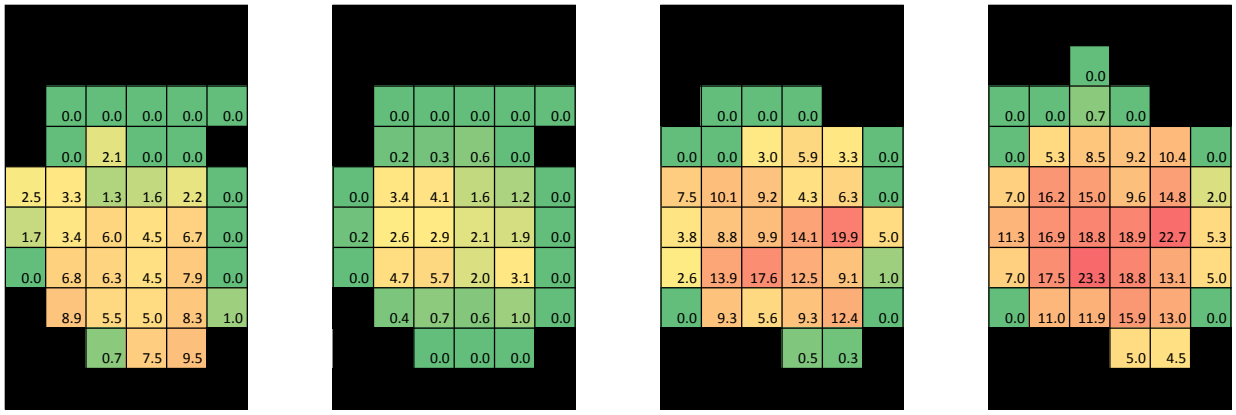


Figure 2.28. Temporal progress of Blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Willacoochee, GA in 2011. Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean severity disease levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 27 June, 11 July, 26 July, and 13 August (from left to right). Shaded black areas designate where there is no plant growth.

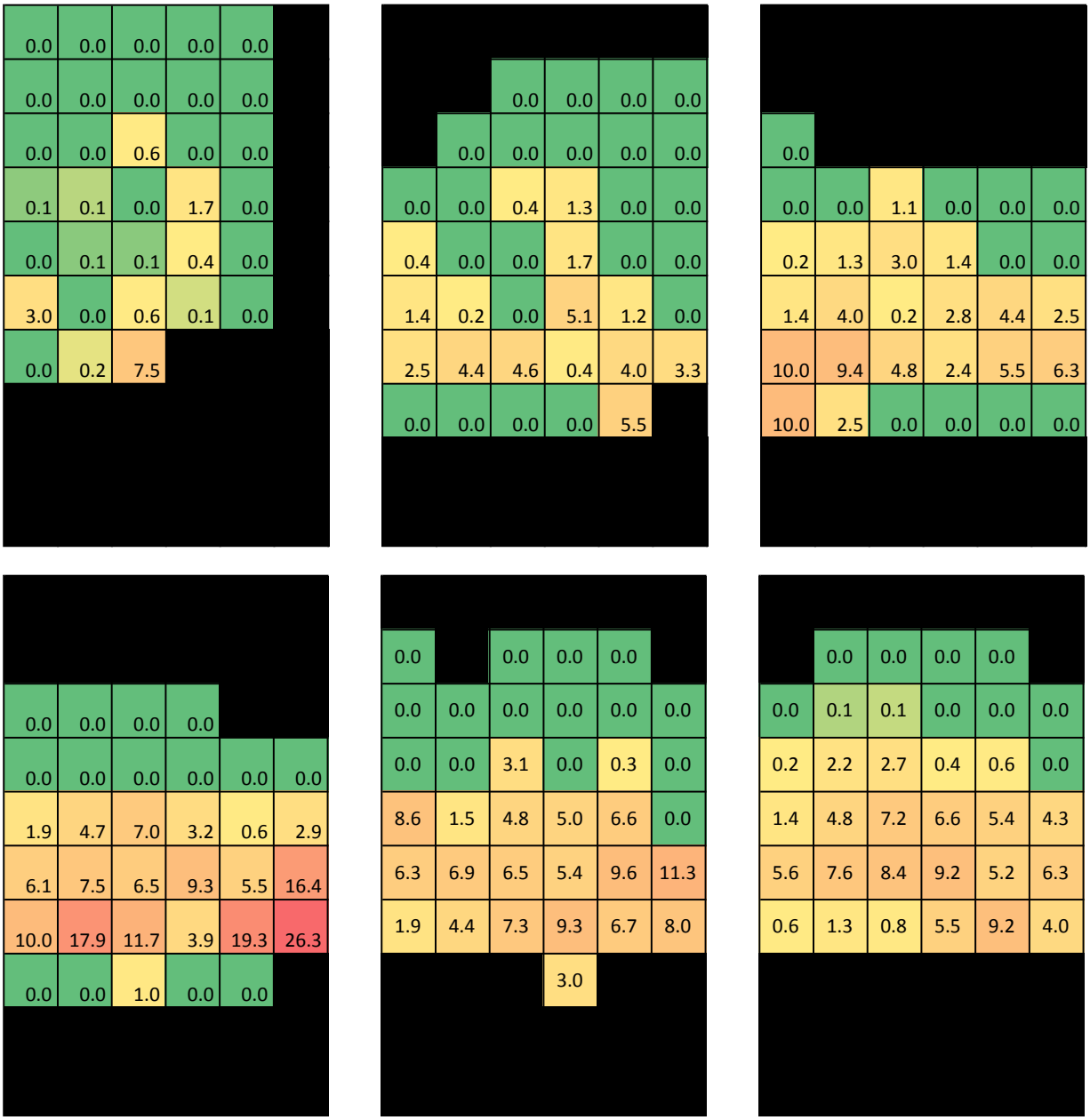


Figure 2.29. Temporal progress of blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Enigma, GA in 2012 (Site 1). Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean severity disease levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 12 June, 26 June, 9 July, 23 July, 25 September, and 16 October (from left to right). Shaded black areas designate where there is no plant growth.

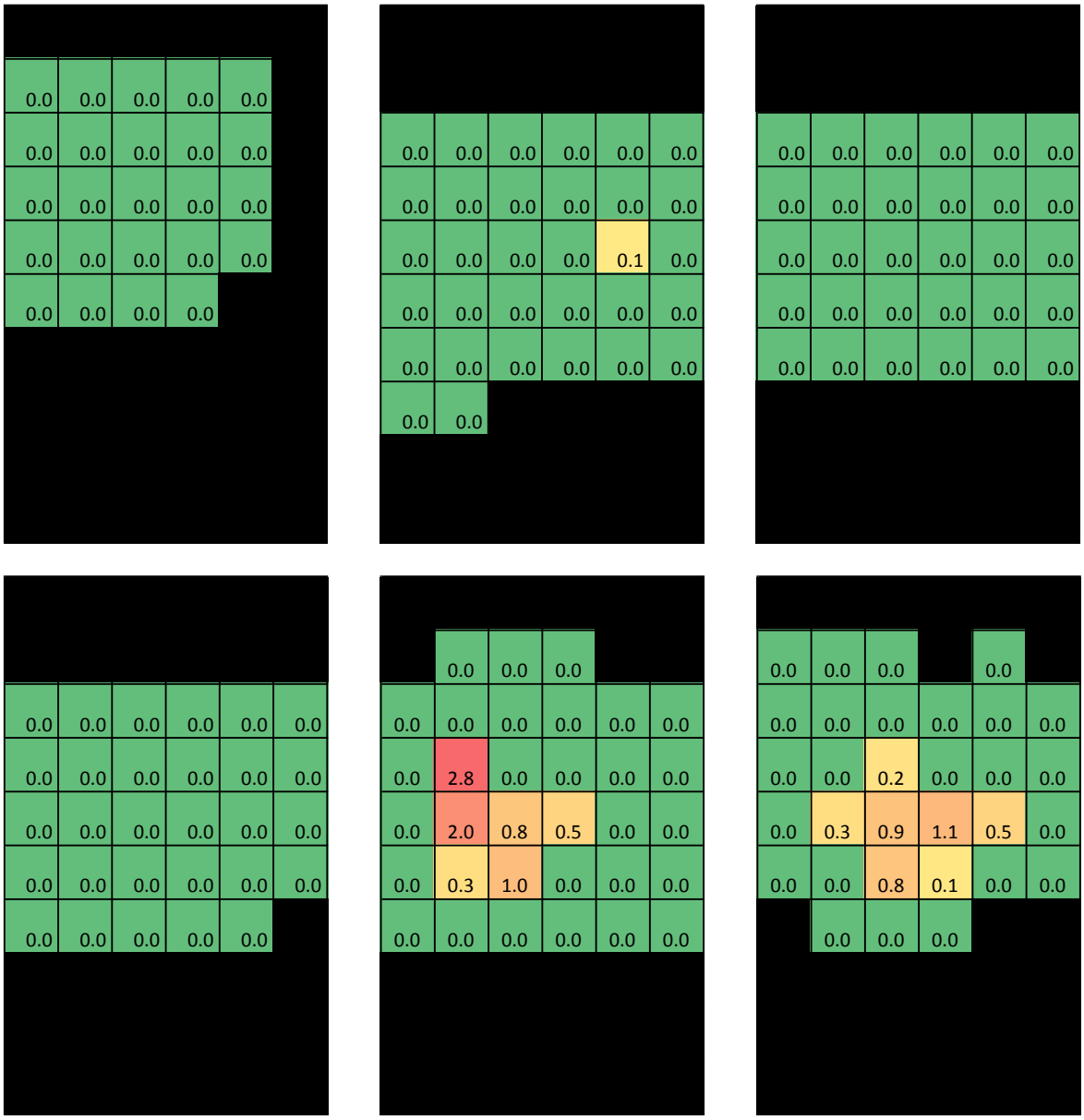


Figure 2.30. Temporal progress of blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Homerville, GA in 2012. Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean severity disease levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 12 June, 25 June, 9 July, 23 July, 27 September, and 18 October (from left to right). Shaded black areas designate where there is no plant growth.

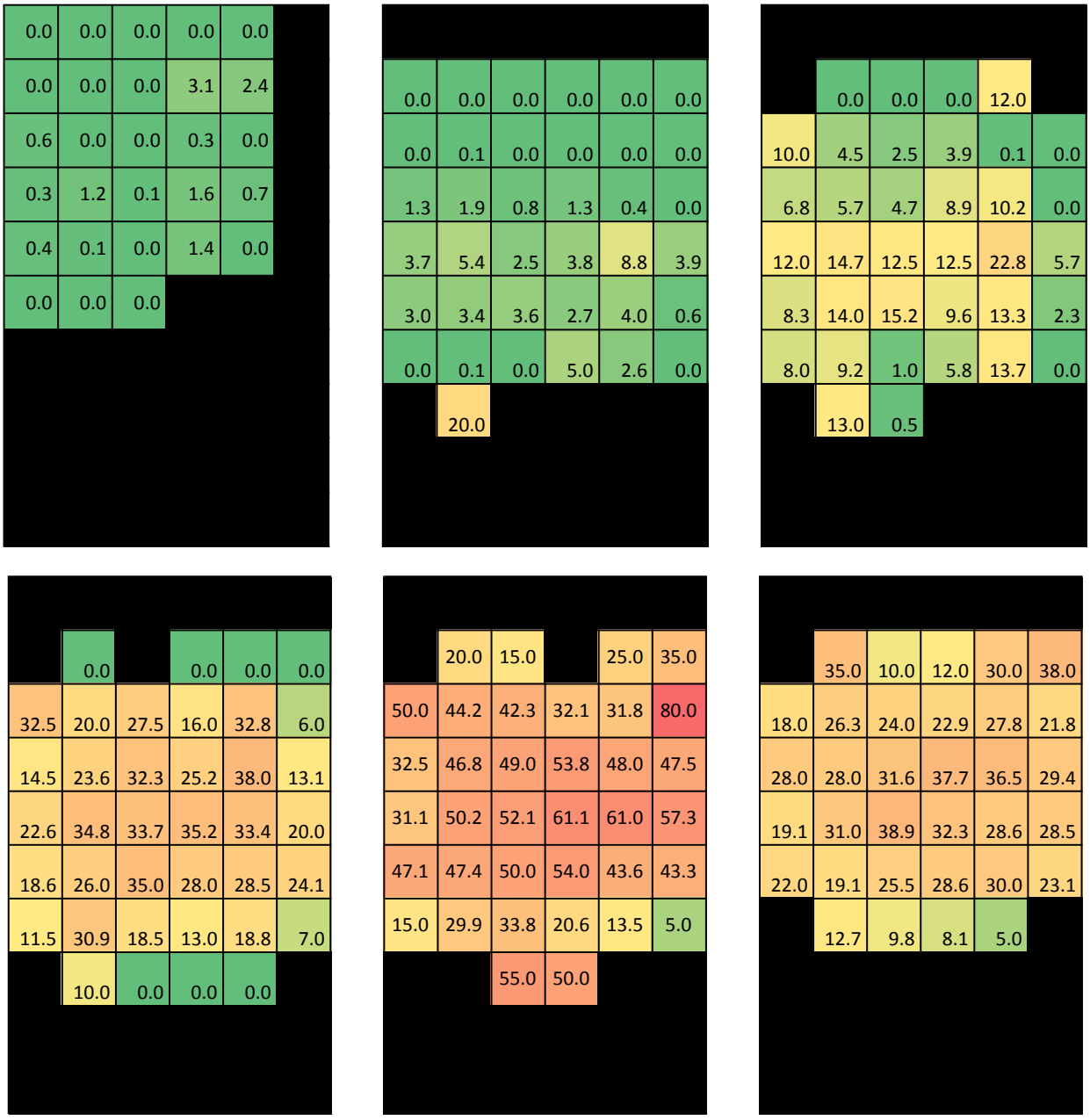


Figure 2.31. Temporal progress of blueberry necrotic ring blotch severity of whole plants of 'Star' southern highbush blueberry in Willacoochee, GA in 2012. Horizontal and vertical directions correspond to width and height of the plant, respectively. Values are mean severity disease levels per 20 cm^2 quadrat averaged across ten plants. Assessment dates were 6 June, 25 June, 9 July, 23 July, 25 September, and 16 October (from left to right). Shaded black areas designate where there is no plant growth.

CHAPTER 3

DETECTION OF BLUEBERRY NECROTIC RING BLOTCH VIRUS FROM COMMERCIAL FIELDS OF SOUTHERN HIGHBUSH BLUEBERRY IN GEORGIA[1]

[1] Robinson, T.S., Scherm, H., Brannen, P.M., and Deom, C.M. 2013. To be submitted to *Plant Disease.*

Detection of Blueberry necrotic ring blotch virus from commercial fields of southern highbush blueberry in Georgia.

T.S. Robinson, H. Scherm, P.M. Brannen, and C.M. Deom

Department of Plant Pathology, University of Georgia, Athens 30602

ABSTRACT

A novel blueberry virus, *Blueberry necrotic ring blotch virus* (BNRBV), causes a new emerging disease on southern highbush blueberries in Georgia and neighboring states in the southeastern U.S. Symptoms associated with the disease include irregularly shaped reddish brown to black concentric rings or blotches with or without green centers on both leaf surfaces. In this study, the correlation between symptoms and the presence of the virus was determined in plants between growing seasons as well as within plants in a single growing season. Previous observations indicated that there was considerable variability in disease incidence in blueberry fields from year-to-year. SHB bushes showing symptoms of necrotic ring blotch one year might be asymptomatic the following year. Leaf tissue from symptomatic 'Star' plants collected in 2011 tested positive for the presence of the virus. The following year, tissue from the same 'Star' plants, which were asymptomatic, tested negative for the virus. The absence of BNRBV in these asymptomatic plants suggests that the virus does not persist in SHB plants after defoliation in the fall, which would be indicative that BNRBV does not systemically infect SHB plants. It is not known to the degree of which the virus moves systemically throughout the SHB plant. Individual shoots that displayed mixed symptoms of asymptomatic leaves and symptomatic leaves were collected in the fall of 2012. In addition, leaves displaying symptoms

on one leaf halve, but not the other half were tested for the presence of the virus. Results from these studies showed symptoms generally correlate with the presence of virus in infected plant tissues, and the virus likely causes a local infection. Lastly, to better understand how BNRBV progresses in the field, tissue culture-derived 'Star' trap plants were placed between well-established 'Star' plants that did not show symptoms. As the season progressed, disease onset of the field plants were observed 2-3 weeks prior to symptoms on trap plants. Data obtained from the disease onset study suggest the virus is likely transmitted within the field by a slow-moving vector overwintering within the field.

INTRODUCTION

Blueberry is Georgia's most important fruit crop in both acreage and farm gate value. Georgia is currently ranked as the third largest blueberry producer in the nation (9). According to the 2011 Georgia Farm Gate Value Report, the blueberry industry produced over $254 million in total sales (2). More than 9,300 hectares in the state are devoted to blueberry production compared with 3,237 hectares 10 years ago (5,9). In 2003, rabbiteye blueberry (*Vaccinium virgatum* = *V. ashei*) accounted for approximately 90% of production, while southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) represented approximately 10% (12). Although no formal acreage surveys have been conducted since then, southern highbush blueberry (SHB) has increased considerately during the past decade, possibly accounting for 20-30% of current production (P.M. Brannen, *personal communication*). While rabbiteye is the more widely grown blueberry type in Georgia, the early-maturing SHBs are economically important due to a highly favorable market window for fresh-picked fruit early in the season from late April to mid May.

In 2006, a new disease, blueberry necrotic ring blotch disease, was first observed in Georgia on SHB, and was subsequently found in several southeastern states including North and South Carolina, Florida, and Mississippi (10). Many SHB cultivars are susceptible to the disease. Symptoms appear as irregularly shaped reddish brown to black concentric rings or blotches with or without green centers on both leaf surfaces. Eventually, the spots may coalesce to cover the entire leaf. Ring spots are generally more prevalent on older leaves in the lower portion of the canopy. In severe cases, necrotic ring blotch may lead to premature defoliation of infected bushes, resulting in crop losses (P.M. Brannen, *personal communication*). To date, disease symptoms have not been observed in the native rabbiteye cultivars.

Initial analysis failed to identify a fungal or bacterial pathogen as the causative agent of necrotic ring blotch, nor was a known blueberry virus confirmed in symptomatic tissue. However, the presence of double-stranded RNA (dsRNA) in the symptomatic leaf tissue suggested that a virus was the etiological agent. Isolation and sequencing of the dsRNA identified a novel RNA virus subsequently designated *Blueberry necrotic ring blotch virus* (BNRBV) (10). BNRBV possesses a 14 kb nucleotide genome divided into four ssRNA segments (10). RNA 1 (5.9 kb) has a single open reading frame (ORF), which encodes for a putative 215 kDa protein having a N-terminal methyltransferase domain (MT), a central cysteine protease domain (C-prot), and a C-terminal helicase domain (HEL-1). RNA 2 (3.9 kb) encodes for a putative 130 kDa protein having an N-terminal helicase domain (HEL-2) and a C-terminal RNA-dependent RNA-polymerase domain (RdRp). RNA 3 (2.6 kb) has 5 ORFs encoding putative proteins of 7, 9, 22, 28, and 31, kDa of unknown function. RNA 4 (1.7 kb) encodes for a putative movement protein.

Sequence analysis reveals that BNRBV likely resulted from recombination events between Ilaviruses (family *Bromoviridae*) and Tobamoviruses (family *Virgaviridae*). Phylogenetic analysis indicated that the closest relatives of BNRBV are *Citrus leprosis virus* (CiLV) and *Hibiscus green spot virus* (HGSV) (10), both members of *Virgaviridae*. CiLV and HGSV are transmitted by *Brevipalpus phoenicis* (false spider mite), which move over short distances on infected plants in the field (11). Furthermore, CiLV was shown to move locally from cell to cell, but failed to move systemically in infected plants. It is not known whether BNRBV moves locally or systemically in infected plants and research is needed to address this question. There is currently no information available on the epidemiology of BNRBV, including its means of spread or transmission.

Based on the above considerations, the objectives of this study were (1) to determine whether the virus overwinters in SHB plants, (2) to determine if the virus is a localized lesion virus, and (3) to determine if the disease onset of disease symptoms on field-exposed trap plants correlates with the onset of symptoms in field plants.

MATERIALS AND METHODS

RNA extraction and Reverse-Transcriptase Polymerase Chain Reaction. Leaves were washed with absolute ethanol for 1.5 minutes and dried prior to RNA extraction. Total RNA was extracted from 100 mg of fresh leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RNA from healthy leaf tissue maintained in the greenhouse was used a negative control.

RNA was subjected to reverse-transcription polymerase chain reaction (RT-PCR) (10) with virus-specific primers 5’- CCA- GTT-TGG-AGG-AAT-TGC-AT 3’(forward) and 5’-

GCG-TTT-CAG-CAC-CAC-TAA-C - 3' (reverse) using the OneStep RT-PCR kit (Qiagen, Valencia, CA). The PCR reaction volume was 25 µl, which included 13 µl of RNase free water, 5 µl of OneStep RT-PCR buffer 5X, 1 µl dNTP mix, 1.5 µl 0.6 µM forward primer 31, 1.5 µl 0.6 µM reverse primer 31, 2 µl of template RNA, and 1 µl OneStep RT-PCR enzyme mix (protocol modified, Philip F. Harmon, University of Florida, Gainesville). The following thermal profile was used: 40 cycles of 30 s at 94°C, 20 s at 55°C, 45 s at 72°C, preceded by an initial denaturation for 1 min at 95°C and followed by a 10-min extension at 72°C. The expected amplicon was 432 bp and cDNA was visualized on a 1% agarose gel.

Testing of previously symptomatic SHB plants for BNRBV in a subsequent season. Commercial blueberry fields in Enigma and Homerville, GA have a long history of BNRBV epidemics. When these field sites were first surveyed in the spring of 2011, the disease incidence was high with most plants showing severe disease symptoms. Symptomatic plants were selected in 2011 for virus confirmation; 15 individual shoots were collected (per site) from a total of 30 plants, 6 leaves per shoot were obtained to conduct RT-PCR, and 180 individual leaves were analyzed by RT-PCR as described above, to confirm the presence or the absence of the virus. To determine if plants that were asymptomatic in 2012, but were infected by BNRBV in 2011, harbored the virus, leaves from the same 30 'Star' plants now asymptomatic, were re-analyzed in 2012 for the presence of the virus.

Testing asymptomatic and symptomatic foliage of SHB for BNRBV. Individual shoots and leaves from naturally infected SHB plants ('Star') showing symptoms of necrotic ring blotch were sampled from fields in the fall of 2012. A total of 11 shoots were collected from 11 different plants from Enigma, GA. The shoots displayed mixed symptoms throughout the shoots (i.e., asymptomatic leaves and leaves with moderate to severe symptoms) (Figure 3.1). Three to

four leaves were arbitrarily taken from the top (apical), middle, and bottom of each shoot for a total of 39 samples. Leaves were analyzed by RT-PCR for the presence or absence of BNRBV. Leaves were washed with absolute ethanol for 1.5 minutes and dried prior to RNA extraction. Total RNA was extracted from 100 mg of fresh leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RNA from healthy leaf tissue maintained in the greenhouse was used a negative control.

For half-leaf analysis, 13 leaves were collected from individual plants showing symptoms of necrotic ring blotch at test sites in Homerville and Enigma, GA. Leaves chosen displayed symptoms of moderate to severe disease symptoms on one half of the leaf, whereas the other half was symptomless (Fig. 3.2). The presence or absence of the virus was determined by RT-PCR as described above, separately for each leaf.

Disease onset of potted trap plants. Thirty-nine, 2.5-month-old tissue cultured-derived SHB plants of cultivar 'Star' were used to determine how rapidly symptoms appear on newly introduced plants that previously had not been exposed to BNRBV. Tissue cultured plugs were placed in 15-cm standard containers in a potting mix containing pine bark and sand in a 3:1 vol/vol ratio. Plants were fertilized with Miller Nutri-leaf Soluble liquid fertilizer 20/20/20 for 30 days, and Scott's Osmocote was given to plants at 3-4 month intervals. Tissue-cultured plants were maintained for 2.5 months between 22°C to 24°C, under an automatic light system set for 13 hour days; plants were watered as needed. Thirteen 2.5 month old transplants (per field site) were arbitrarily placed and exposed to natural field conditions in commercial blueberry fields in Enigma, Homerville, and Willacoochee on 16 March 2012 (Fig. 3.3). Plant height varied from plant to plant, but most plants were approximately 42 cm when exposed. At each field site trap plants were placed in between well-established 'Star' plants. Symptom development was

monitored on the foliage bi-weekly until the end of the growing season. Field conditions as well as plant conditions were recorded at each rating date. Trap plants placed in the field were assessed for disease severity based on the percentage of leaves affected on the entire plant (0-100%). Control plants that were not exposed to natural field conditions were maintained in the campus greenhouses at the University of Georgia Athens, GA, and were also monitored for symptom development. All test plants were tested via RT-PCR for BNRBV and *Blueberry red ringspot virus* (BBRV) before exposure to natural field conditions.

RESULTS

Testing of previously symptomatic SHB plants for BNRBV in a subsequent season. Previous observations indicated that there was considerable variability in disease incidence in blueberry fields from year-to-year. SHB bushes showing symptoms of necrotic ring blotch one year might be asymptomatic the following year. In the 2011 growing season, symptoms were prevalent at all field sites. By June 22 and 28, disease incidence at Homerville and Enigma was at 92% and 72%, respectively (Tables 2.2 and 2.4; Figs. 2.14 and 2.16). However, during the 2012 growing season much less disease incidence was observed, with 1% and 30% of plants showing symptoms by June 12 at Homerville and Enigma respectively (Tables 2.7 and 2.8; Figs. 2.19 and 2.20). To determine if symptoms correlate with the presence of the virus, asymptomatic plants in 2012 that were symptomatic and had tested positive for BNRBV in 2011 (Table 3.1), were assayed for the presence of BNRBV. Leaf extracts from the 30 asymptomatic test plants from Enigma and Homerville tested negative for BNRBV. The results suggest that the virus does not overwinter in SHB plants and that the virus does not systemically infect SHB.

Testing asymptomatic and symptomatic foliage of SHB for BNRBV. Typically, shoots of infected plants show a mixture of symptomatic and asymptomatic leaves (Fig. 3.1). To determine if symptoms correlate with the presence of the virus in individual leaves, 11 'Star' blueberry shoots were collected from fields in southeast Georgia (Enigma and Willacoochee) where BNRBV was prevalent. For this study, there were 39 leaves total, in which 26 leaves were asymptomatic, and the remaining 13 were symptomatic. Results revealed that 9 asymptomatic leaves (35%) tested positive for the virus. The 13 leaves that displayed viral symptoms tested positive as expected (Table 3.2).

To further study if symptoms correlate with the presence of the virus, 13 leaves collected from Homerville and Enigma displayed necrotic ring blotch symptoms on one half of the leaf and were asymptomatic on the opposite half leaf (Fig. 3.2). RT-PCR analysis revealed that 3 out of the 13 symptomless leaf halves tested positive for BNRBV. As expected, the leaf halves that displayed disease symptoms tested positive for BNRBV (Table 3.3).

Disease onset of potted trap plants. To if quantify necrotic ring blotch progresses in newly exposed plants, thirteen 2.5-month-old healthy tissue-culture-derived transplants (per field site) were placed in commercial blueberry fields in Enigma, Homerville, and Willacoochee on 16 March 2012 (Fig. 3.3). Symptoms first appeared on June 25 at Enigma and July 9 at Willacoochee, which were 14 and 16 weeks after exposure, respectively, and 2 and 4 weeks after the end of the fruiting season (Table 3.4). Disease severity was moderate to severe, with individual disease severity ratings ranging from 45% to 70% (determined by visual observation – *data not shown*). Disease symptoms were observed on most leaves of the trap plants, where symptoms on older leaves were more severe than on younger leaves. By comparison, disease was observed 2-3 weeks earlier in field plants with an incidence of 28% at Enigma on June 25

and 100% at Willacoochee on July 9. Trap plants at Enigma and Willacoochee showed 100% disease incidence by July 30. RT-PCR analysis confirmed the presence of BNRBV on all symptomatic trap plants (Table 3.4). At Enigma, 6 out of 13 trap plants survived, while at the Willacoochee site, 8 out of 13 trap plants survived. Trap plants placed at the Homerville site were affected by severe herbicide damage and died early before the season survey. No symptoms were observed on control plants that were maintained in the greenhouse. All control plants tested negative for BNRBV and BRRV before and after field trials.

DISCUSSION

A novel blueberry virus, *Blueberry necrotic ring blotch virus* (BNRBV), associated with necrotic ring blotch symptoms in SHB, has recently been identified. The virus has been detected by RT-PCR in symptomatic SHB plants from several southeastern states (10). In this study, the correlation between symptoms and the presence of the virus was determined in plants between growing seasons as well as within plants in a single season. Results from this study suggest that (i) BNRBV does not persist in SHB plants, (ii) virus likely causes a local infection, and (iii) the disease is likely spread to new transplants from established field plants, and (iv) the virus is not likely systemic.

The incidence of disease can be variable from year-to-year in SHB fields. We assessed and assayed plants in 2012 that were asymptomatic, but had been symptomatic in 2011. The absence of BNRBV in these asymptomatic plants suggests that the virus does not persist in SHB plants, which would be indicative that BNRBV does not infect SHB plants systemically. It was shown previously that CiLV, which is closely related to BNRBV, does not move systemically in citrus, but rather remains localized or has limited movement for short distances in the host plant

(11). CiLV, which is vectored by the false spider mite, *Brevipalpus phoenicis*, has been very difficult to transmit to citrus by grafting, which is only successful when symptomatic tissue areas are in direct contact with receptor tissue on healthy plants (6). Symptoms develop only in adjacent tissues in close proximity of the receptor plant, supporting the suggestion that CiLV is a localized disease. With BNRBV, future studies to identify the vector and subsequent vector transmission studies in parallel with grafting studies are needed to provide more definitive evidence that BNRBV only moves locally in SHB.

In 2011, major Georgia blueberry producing counties experienced dryer weather conditions with minimal rainfall, especially early in the growing season. If the vector, presumably an eriophyid mite(s), is transmitting the disease, then these conditions may be conducive for their reproduction and/or movement. In contrast, the early part of the 2012 growing season was much wetter, with a larger number of rainfall events in the first half of the year (Appendix B). The increased rainfall and larger number of rainfall events early in 2012 relative to 2011 could have suppressed vector movement and/or decreased vector numbers by washing the vectors from the plants. This would have decreased disease incidence and/or disease severity, resulting in less virus-infected tissue being available for vector acquisition and subsequent transmission.

While there was a correlation between the presence of the virus and disease symptoms within symptomatic and asymptomatic plants in different years, a less definitive correlation existed within individual plants. When asymptomatic and symptomatic leaves were taken from the same shoot, 66% of the asymptomatic leaves tested negative for BNRBV, while 35% of the asymptomatic leaves, and all of the symptomatic leaves tested positive for BNRBV. Similarity, 77% of asymptomatic leaf halves tested negative for BNRBV, while 23% of the asymptomatic

leaf halves, and 100% of the symptomatic leaf halves tested positive for the virus. A likely explanation for both findings is that BNRBV does not move systemically, resulting in entire leaves on shoots or leaf halves remaining virus -free. Such a scenario would suggest that asymptomatic tissue testing positive for BNRBV results from virus being identified by RT-PCR early after infection before disease symptoms start developing.

The introduction of virus-free tissue culture-derived trap plants into SHB fields resulted in infection with BNRBV with a delay of approximately 2 to 3 weeks relative to the detection of symptoms on field plants. The delay suggests that the virus is being transmitted from field plants to transplants. Adult female eriophyid mites overwinter in bark and under bud scales and start feeding on new growth when buds break in the spring (7). The mites are primarily dispersed by wind (1). Therefore, if eriophyid mites vector BNRBV, then a 2-to-3 week lag between the appearance of symptoms on field plants versus trap plants, which did not contain overwintering mites, could be explained by the slow movement of mites from field plants to trap plants with a subsequent delay in symptom development. Greenhouse studies at the University of Florida support our findings. Harmon et al. (3, 8) placed virus-free, tissue-cultured 'Star' plants in greenhouses adjacent to infected plants, and within 3 weeks, BNRBV had infected most of the virus-free tissue-cultured plants. Interestingly, BNRBV spread within the greenhouses occurred in the presence of large populations of eriophyid mites of the genus *Calacarus* (4).

The results from these studies provide preliminary insights into aspects of BNRBV epidemiology. This work lays the foundation for future research to better understand how BNRBV moves within infected plants and how the virus is transmitted in the field.

ACKNOWLEDGEMENTS

Supported by the Georgia Agricultural Commodity Commission for Blueberries, the Southern Region Small Fruit Consortium, and the USDA-NIFA Specialty Crop Research Initiative (grant no. 2008-51180-19579). We thank Benjamin Shirley for his help with the trap plant study, and Charles Laster, and Matt Seadar for providing greenhouse support. We thank county extension agents Elvin Andrews, Tim Flanders, James Jacobs, John Ed Smith, and Danny Stanaland for their help and cooperation during field site selections, and communication with producers.

LITERATURE CITED

1. Anonymous. 2012. Eriophyid mites. Missouri Botanical Garden, St. Louis, MO. Online.
2. Boatright, S. and McKissick, J. 2012. Georgia Farm Gate Value report 2011. AR12-01, University of Georgia, Center for Agribusiness and Economic Development, Athens.
3. Burkle, C. Olmstead, J.W., and Harmon, P.F. 2012. A Potential vector of *Blueberry necrotic ring blotch virus* and symptoms on various host genotypes. (Abstr.). Phytopathology 102, S4.17.
4. Cantu-Iris, M., Harmon, P.F., Londoño, A., and Polston, J.E. 2013. A variant of *Blueberry necrotic ring blotch virus* associated with red lesions in blueberry. Arch Virol. 158(10):2197-2200.
5. Chapman, D. 2012. Georgia's peachy image turns blue. The Atlanta-Journal Constitution. 25 June main ed.: A1. Online. 16 Oct. 2012.
6. Chung, K.R. and Brlanksy, R. H. 2006. Citrus diseases exotic to Florida: Citrus leprosis. Fact Sheet PP-226. Department of Plant Pathology, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, Gainesville, FL.

7. Davis, R. and Beddes, T. 2011. Eriophyid Mites (bud, blister, gall, and rust mites). Utah Pests Fact Sheet ENT-149-11. Utah State University Cooperative Extension and Utah Plant Pest Diagnostic Laboratory, Utah State University, Logan, UT.
8. Martin, R.R., Polaschock, J. and Tzanetakis, I.E. 2012. New and emerging viruses of blueberry and cranberry. Viruses 4:2831-2852.
9. Ohlemeier, D. 2012. Georgia blueberry production goes big. The Packer. 2 Apr. 2012: B1 and B3. Online. 16 Oct. 2012.
10. Quito-Avila, D., Brannen, P.M., Cline, W.O., Harmon, P.F., and Martin, R.R. 2013. Genetic characterization of *Blueberry necrotic ring blotch virus*, a novel RNA virus with unique genetic features. J. Gen. Virol. 94:1426-1434.
11. Rodrigues, J.C.V., Kitajima, E.W., Childers, C.C., and Chagas, C.M. 2003. *Citrus leprosis virus* vectored by *Brevipalpus phoenicis* (Acari: Tenuipalpidae) on citrus in Brazil. Experimental and Applied Acarology 30:161-179.
12. Scherm, H. and Krewer, G. 2003. Blueberry production in Georgia: Historical overview and recent trends. Small Fruits Rev. 2(4):83-91.

Table 3.1. Detection of *Blueberry necrotic ring blotch virus* via RT-PCR on asymptomatic foliage at commercial field sites in 2011 and 2012.

	2011				2012			
	Homerville		Enigma		Homerville		Enigma	
Plant no.[a]	Symptoms[b]	RT-PCR[c]	Symptoms	RT-PCR	Symptoms	RT-PCR	Symptoms	RT-PCR
1	+	+	+	+	-	-	-	-
2	+	+	+	+	-	-	-	-
3	+	+	+	+	-	-	-	-
4	+	+	+	+	-	-	-	-
5	+	+	+	+	-	-	-	-
6	+	+	+	+	-	-	-	-
7	+	+	+	+	-	-	-	-
8	+	+	+	+	-	-	-	-
9	+	+	+	+	-	-	-	-
10	+	+	+	+	-	-	-	-
11	+	+	+	+	-	-	-	-
12	+	+	+	+	-	-	-	-
13	+	+	+	+	-	-	-	-
14	+	+	+	+	-	-	-	-
15	+	+	+	+	-	-	-	-

[a] One shoot obtained from each plant, with six leaves tested per shoot (90 leaves total per site and year).
[b] Displays whether individual shoots express symptoms of BNRBV.
[c] RT-PCR was performed on leaf extracts from individual shoots taken from Homerville on June 25 and Enigma on July 9, 2011. In 2012, samples for RT-PCR taken from Homerville and Enigma on June 2.

Table 3.2. Detection of *Blueberry necrotic ring blotch virus* in individual leaves of 'Star' southern highbush blueberry displaying mixed symptoms of the virus from Enigma, GA in 2012.

Leaf no.		Shoot 1		Shoot 2		Shoot 3		Shoot 4		Shoot 5		Shoot 6	
		Visual[a]	PCR[b]	Visual	PCR	Visual	PCR	Visual	PCR	Visual	PCR	Visual	PCR
Top	1	-	-	-	+	-	+	-	NT	-	-	-	+
	2	-	NT[c]	-	NT	-	NT	-	-	-	NT	-	NT
	3	-	NT	-	NT	-	NT	-	NT	-	NT	-	NT
	4	-	NT	-	+	-	NT	-	NT	-	-	-	-
	5	-	NT	-	NT	-	NT	-	+	-	NT	-	NT
	6	-	NT	+	+	-	-	-	NT	-	NT	-	NT
	7	-	NT	-	NT	+	+	-	NT	-	NT	-	NT
	8	-	NT	-	+	-	NT	-	NT	-	NT	+	+
	9	-	+	-	NT	-	NT	-	NT	+	NT	-	NT
	10	+	+	-	NT	-	NT	+	+	+	+	-	NT
	11	-	NT	-	NT	-	NT	-	-	-	NT	+	NT
	12	-	NT	---	---	-	NT	---	---	-	NT	+	NT
	13	-	NT	---	---	---	---	---	---	-	NT	+	+
	14	---	---	---	---	---	---	---	---	+	NT	+	NT
	15	---	---	---	---	---	---	---	---	+	NT	+	NT
	16	---	---	---	---	---	---	---	---	+	NT	+	NT
	17	---	---	---	---	---	---	---	---	+	NT	+	NT
	18	---	---	---	---	---	---	---	---	+	NT	---	---
	19	---	---	---	---	---	---	---	---	+	NT	---	---
Bottom	20	---	---	---	---	---	---	---	---	+	NT	---	---

[a] Visual observation of whether leaf was asymptomatic or symptomatic.
[b] Leaves tested via RT-PCR to determine absence or presence of BNRBV.
[c] NT = leaves not tested via RT-PCR.
--- = End of leaf on shoot.

Table 3.2. (continued). Detection of *Blueberry necrotic ring blotch virus* in individual leaves of 'Star' southern highbush blueberry displaying mixed symptoms of the virus from Enigma, GA in 2012.

Leaf no.		Shoot 7		Shoot 8		Shoot 9		Shoot 10		Shoot 11	
		Visual[a]	PCR[b]	Visual	PCR	Visual	PCR	Visual	PCR	Visual	PCR
Top	1	-	-	-	-	-	-	-	+	+	NT
	2	-	NT[c]	-	NT	-	NT	-	NT	-	NT
	3	-	NT	-	NT	-	NT	-	NT	-	+
	4	-	NT	-	NT	-	NT	-	NT	+	NT
	5	-	NT	+	+	-	NT	-	NT	+	NT
	6	-	NT	-	NT	-	NT	+	+	+	NT
	7	-	NT	-	-	-	NT	-	NT	+	+
	8	-	NT	-	NT	+	NT	+	NT	+	NT
	9	-	-	-	NT	-	-	-	NT	+	NT
	10	+	+	-	NT	-	-	-	NT	+	+
	11	-	-	-	-	-	NT	-	-	+	NT
	12	-	NT	-	NT	+	+	-	NT	+	NT
	13	---	---	-	NT	-	NT	-	NT	+	NT
	14	---	---	-	NT	-	NT	-	NT	+	NT
	15	---	---	-	NT	-	NT	---	---	---	---
Bottom	16	---	---	-	NT	-	NT	---	---	---	---

[a] Visual observation of whether leaf was asymptomatic or symptomatic.
[b] Leaves tested via RT-PCR to determine absence or presence of BNRBV.
[c] NT = leaves not tested via RT-PCR.
--- = End of leaf on shoot.

Table 3.3. Detection of *Blueberry necrotic ring blotch virus* of individual leaf halves via RT-PCR in Enigma and Willacoochee in 2012.

	Leaf half 1		Leaf half 2	
Leaf no.	Visual[a]	PCR[b]	Visual	PCR
1	+	+	-	-
2	-	-	+	+
3	-	-	+	+
4	+	+	-	+
5	+	+	-	-
6	+	+	-	-
7	+	+	-	-
8	-	-	+	+
9	-	+	+	+
10	+	+	-	-
11	+	+	-	-
12	-	+	+	+
13	+	+	-	-

[a] Visual observation of whether leaf half was asymptomatic or symptomatic.
[b] Leaves tested via RT-PCR to confirm presence or absence of BNRBV.

Table 3.4. Symptom onset, visual disease incidence, and RT-PCR confirmation of *Blueberry necrotic ring blotch virus* of potted 'Star' trap plants in commercial blueberry fields in Georgia, 2012.

Commercial field site	Date exposed[a]	Date of first symptoms[b]	Survived transplants[c]	Visual disease incidence (%)	PCR positives for BNRBV (%)
Enigma	16 Mar	25 Jun	6/13	100.0	100.0
Willacoochee	16 Mar	9 Jul	8/13	100.0	100.0

[a] Date when transplants were exposed to natural field conditions for the first time.
[b] First date symptoms were observed on transplants; Homerville suffered from severe herbicide damage before assessment on 30 March 2012.

Figure 3.1. 'Star' southern highbush blueberry shoot displaying mixed symptoms of *Blueberry necrotic ring blotch virus* infection.

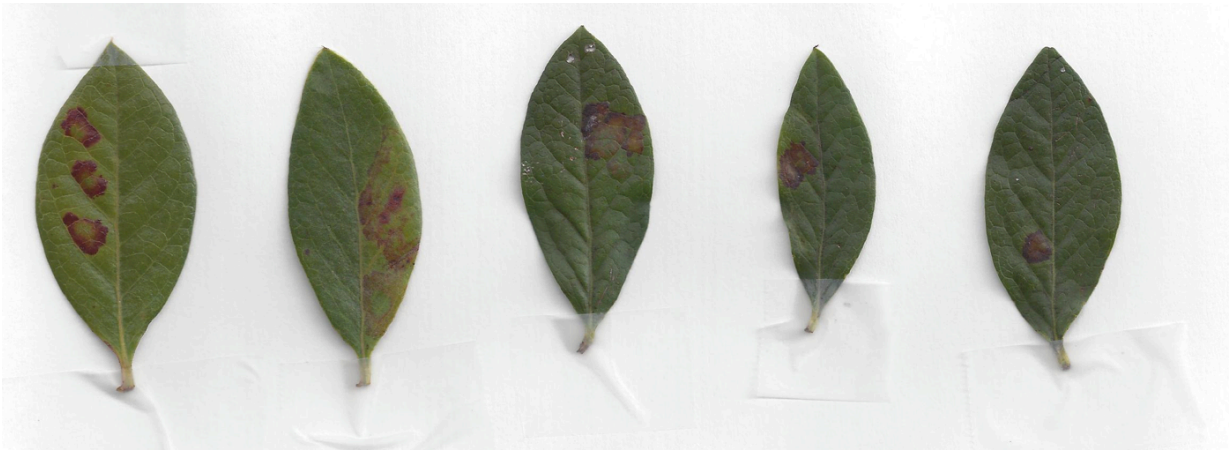


Figure 3.2. Individual leaf halves tested for presence or absence of *Blueberry necrotic ring blotch virus*.

Figure 3.3. Healthy tissue-culture-derived trap plant, planted in a commercial blueberry planting to determine time of natural infection in the field.

CHAPTER 4

CONCLUSIONS

Research in this thesis addressed various aspects of the necrotic ring blotch-blueberry pathosystem to fill critical knowledge gaps on disease epidemiology. Other than resistance, it has been very difficult to control necrotic ring blotch due to the inability to identify a viral vector. The studies presented in this thesis did not develop or test specific management guidelines, but they could provide basic information needed to understand developing epidemics they can be identified early and managed effectively.

Field distribution maps were constructed to quantify spatio-temporal progression and spread of the disease. First, we determined whether edge effects existed in the field. Edge effects in the field tend to be associated with specific vegetation types (i.e., pine, oak, grassland, shrubland, etc.). An association analysis test was conducted and did not support the idea of specific vegetation (e.g., pine/hardwood) being associated with edge effects in the field. Also, field distribution maps showed that edge effects were more common row edges than in column edges of the field. This could be due to the slower movement across than within-rows of the presumed vector, an eriophyid mite that is dispersed via wind and/or through mechanical movement (e.g. harvest equipment, field workers, etc.) Results also suggest that aggregation among plants symptomatic for necrotic ring blotch was detected by ordinary runs analysis, with clustering in both within and across row directions. This suggests that disease spreads from plant to plant (i.e. diseased plants are likely neighbors of other diseased plants). Eriophyid mites are known to be nomadic foliar feeders, walking or crawling from plant to plant; this could explain the clustering effect or aggregation in the field, since close proximity to a point source would

increase both contact with the virus and viruliferous mite populations. Data from 10 individual plants and shoots were analyzed separately to examine the final mean disease severity amongst different strata (top, middle, and bottom of plants). Final mean disease severity was significantly lower in the top stratum of plants as compared to those with the middle or bottom. Also, although disease severity was significantly higher in the interior quadrats of the plants than in the exterior of plants in one year, there was no statistical difference of disease severity in the second year the experiment was conducted. This may indicate that potential vectors may prefer to feed in the center of infected plants, where it is cooler and protected from direct sunlight. For data summarized at the shoot level, results showed there was a lack of a strong and significant association between final mean disease severity and stratum (leaf borne on the top, middle, or bottom of the shoot). This suggests that leaves could become infected at any leaf stage, given that leaf position along the shoot can serve as a proxy leaf age.

In chapter 3, the association between symptoms and the presence of the virus was determined in plants across growing seasons, as well as within plants in a single growing season. In the first study, results showed that incidence of disease can be variable from year to year. This suggests that BNRBV does not overwinter in SHB plants. Indeed, results from RT-PCR tests showed that 30 'Star' SHB plants symptomatic the previous year tested negative for the virus the next year (after winter defoliation occurred).

In the second study, individual shoots and leaves showing mixed symptoms of the virus were sampled from fields in the fall of 2012. While there was a direct correlation between the presence of the virus and disease symptoms with symptomatic and asymptomatic plants in different years, a less definitive correlation existed within individual plants. When asymptomatic and symptomatic leaves were taken from the same shoot, 66% of the asymptomatic leaves tested

negative for BNRBV, while 35% of the asymptomatic leaves, and all of the symptomatic leaves tested positive for BNRBV. Similarity, 77% of asymptomatic half leaves tested negative for BNRBV, while 23% of the asymptomatic leaf halves, and 100% of the symptomatic leaf halves tested positive for the virus. This is preliminary evidence that the virus is not able to move systemically throughout the plant, and is likely causes a local infection.

In the third study, healthy tissue culture-derived 'Star' SHB trap plants were placed into three commercial fields affected by BNRBV alongside well-established 'Star' plants to determine the onset of disease development. Results from this study revealed that disease is likely to spread to new transplants from established field plants.

In summary, results from these studies provide preliminary insights into aspects of BNRBV epidemiology. This work lays the foundation for future research for better understanding how BNRBV moves within the field, *in-planta*, and how the virus is transmitted in commercial fields.

APPENDIX A

GENETIC DIVERSITY OF *BLUEBERRY NECROTIC RING BLOTCH VIRUS* FROM VARIOUS COMMERCIAL FIELDS IN GEORGIA

Objective: To determine the genetic variability within the BNRBV genome in Georgia southern highbush blueberries.

Methodologies and Results: Leaves from 'Star' plants showing symptoms of necrotic ring blotch were collected from seven commercial blueberry sites in southern Georgia; Alma, Enigma (two sites), Homerville (two sites), Waycross, and Willacoochee. Total RNA was extracted from 100 mg of fresh leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RNA from healthy leaf tissue maintained in the greenhouse was used as negative controls. RT-PCR was performed as described in Chapter 3, *Materials and methods*. PCR products were visualized on a 1% agarose gel to confirm correct amplicon size (amplicon size = 432 bp). PCR products were ligated into plasmid vector pCR-2.1 TOPO *amp/kan* (Invitrogen, Carlsbad, CA) and transformed into *Escherichia coli* OneShot Top10 competent cells (Invitrogen, Carlsbad, CA). Plasmids were purified with PureLink Quick Miniprep Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and sequenced at Georgia Genomic Facility (University of Georgia, Athens).

Nucleotide sequences were analyzed using EditSeq (DNASTAR) and protein translations were analyzed using the ExPASy Bioinformatics Resource Portal (SIB; Swiss Institute of Bioinformatics). Sequences were compared to the GenBank database using the standard

nucleotide BLAST (BLASTn) program available at the National Center for Biotechnology Information (NCBI; http://ncbi.nlm.nih.gov/blast).

BLAST searches and nucleotide sequence alignments showed that amplicons from independent field sites were derived from BNRBV segment RNA4, and the nucleotide identity ranged from 97% at Enigma to 100% at Waycross. The nucleotide changes resulted in 18 silent amino acid changes, 5 amino acid changes showing similarity (Alma: Ile121→Val, Val130→Ile; Engima 2: Lys75→Arg, Ile121→Val, and Val 130→Ile; Homerville 2: Trp54→Arg and Phe139→Val) and 3 amino acid changes that were distinct (Homerville 1: Gln83→Lys; Homerville 2: Trp54→Arg and Phe92→Ser). The results suggest that a low level of variability exists in the virus population, which is expected with viruses having RNA genomes. No phenotype differences were detected in the symptoms virus isolates induce.

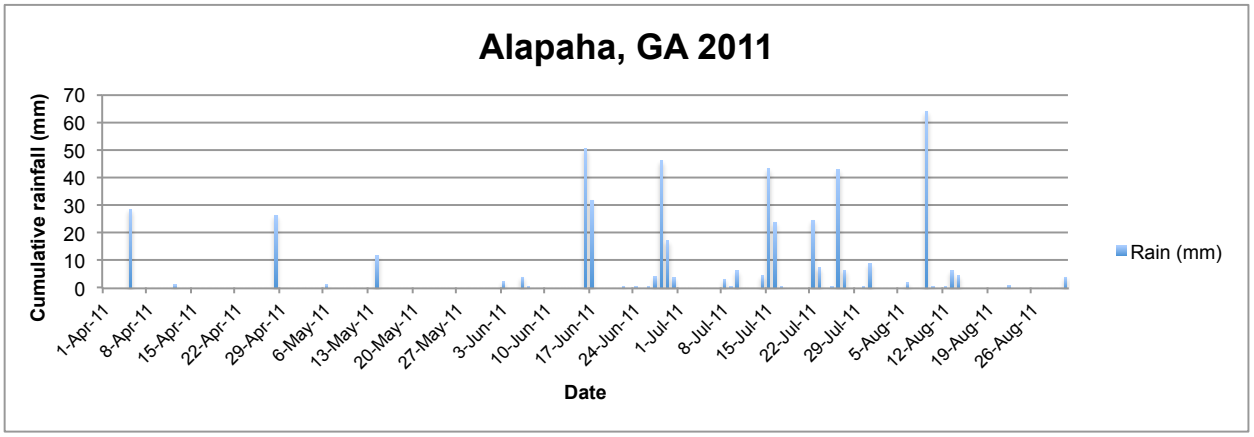
Field Site	Nucleotide changes[a]	Amino acid changes[b]
Alma	C139→T T159→A G183→T A361→G T375→C G388→A T396→C	Cys46→Cys Ala53→Ala Leu61→Leu Ile121→Val Asp125→Asp Val130 →Ile Ser132→Ser
Enigma 1	T417→C	Val139→Val
Enigma 2	T81→C C84→T C138→T T159→A G183→T A224→G A361→G T375→C A378→G G388→A T396→C	Asn27→Asn Asp28→Asp Cyst46→Cys Ala53→Ala Leu61→Leu Lys75→Arg Ile121→Val Asp125→Asp Gln126→Gln Val130→Ile Ser132→Ser
Homerville 1	C247→A	Gln83→Lys
Homerville 2	T160→C T275→C T417→C	Trp54→Arg Phe92→Ser Val139→Val
Waycross	--[c]	--
Willacoochee	T135→C A186→G T417→C	Asn45→Asn Gln62→Gln Val139→Val

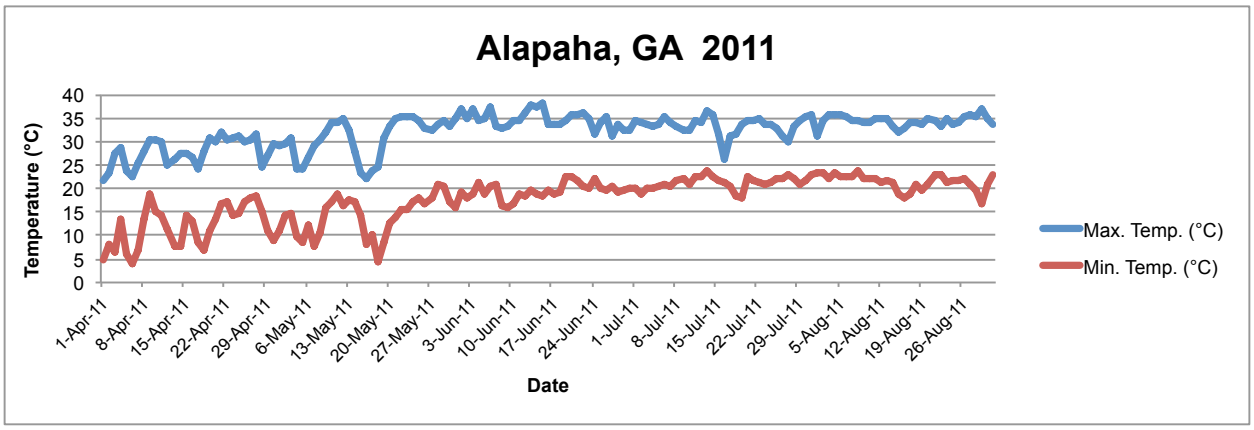
[a] First nucleotide is the published nucleotide at the position indicated (Quito-Avila et al. 2013) and the second nucleotide is the change that occurred at the nucleotide position.
[b] First amino acid is the published amino acid at the position indicated, and the second amino acid is the change that occurred at the amino acid position.
[c] Indicates that no changes occurred.

APPENDIX B

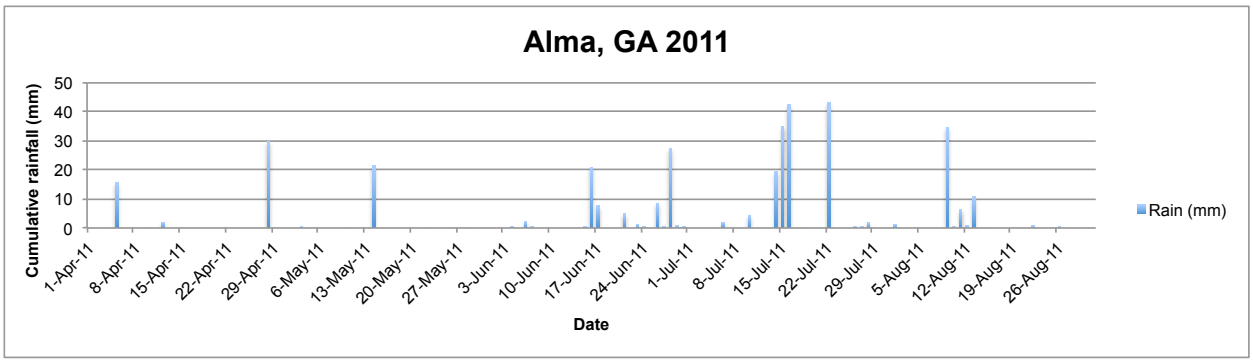
CUMULATIVE RAINFALL WITH MINIMUM AND MAXIMUM TEMPERATURES FOR COMMERCIAL BLUEBERRY FIELDS SURVEYED IN GEORGIA

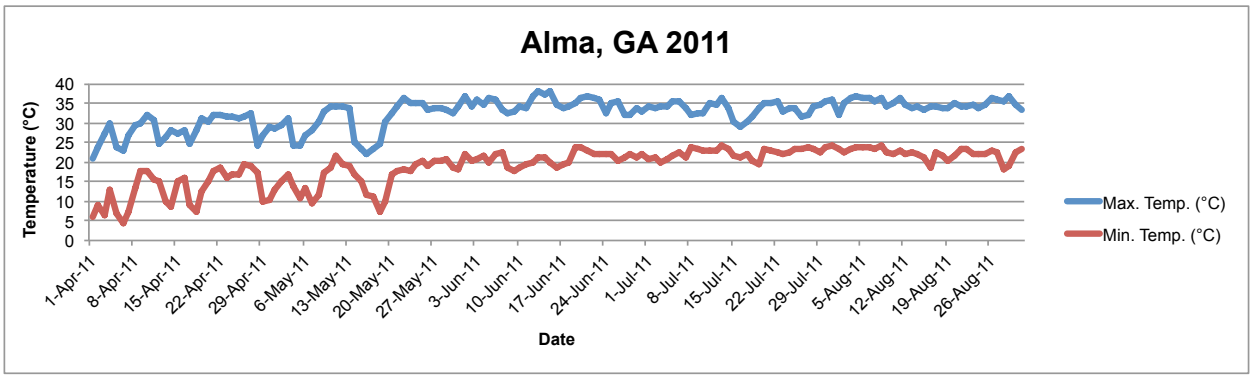
Below are cumulative rainfall graphs and temperatures from various weather stations closest to the six commercial field sites that were surveyed in the spring and summer of 2011, and three commercial field sites in the summer and fall of 2012. Historical weather data was obtained from the Georgia Automated Environmental Monitoring Network (http://www.GeorgiaWeather.net).



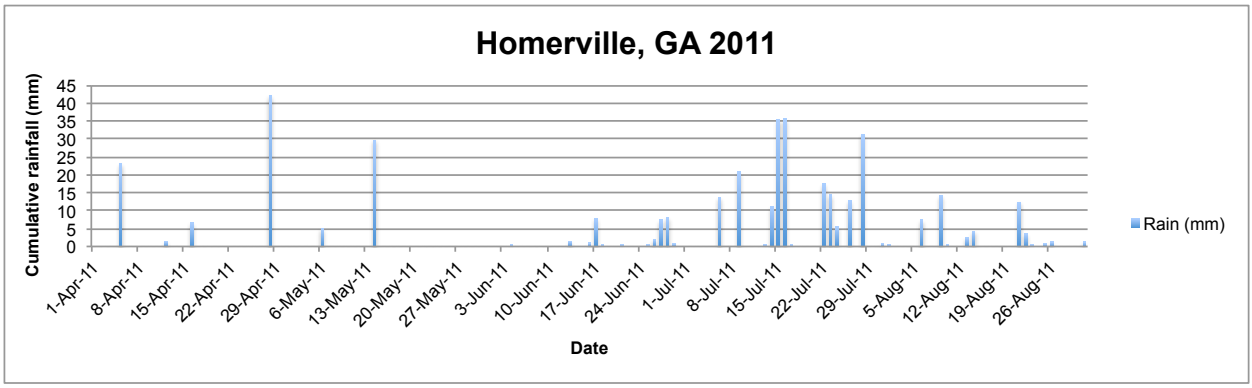


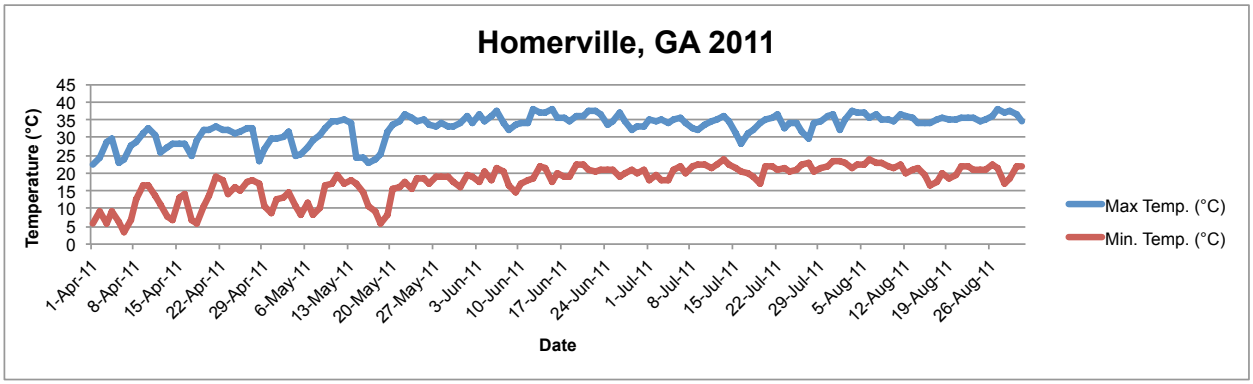
Weather station: Alapaha Range Grazing Unit – Blueberry farm



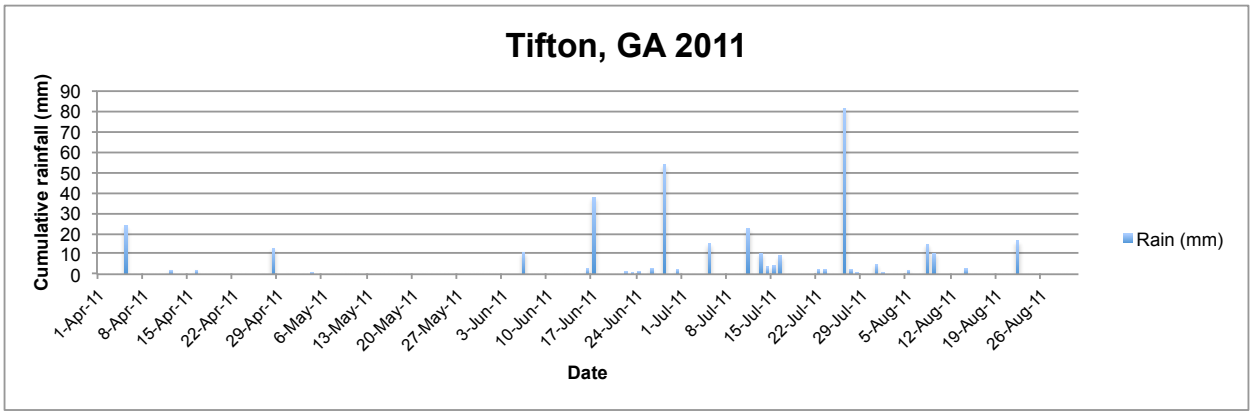


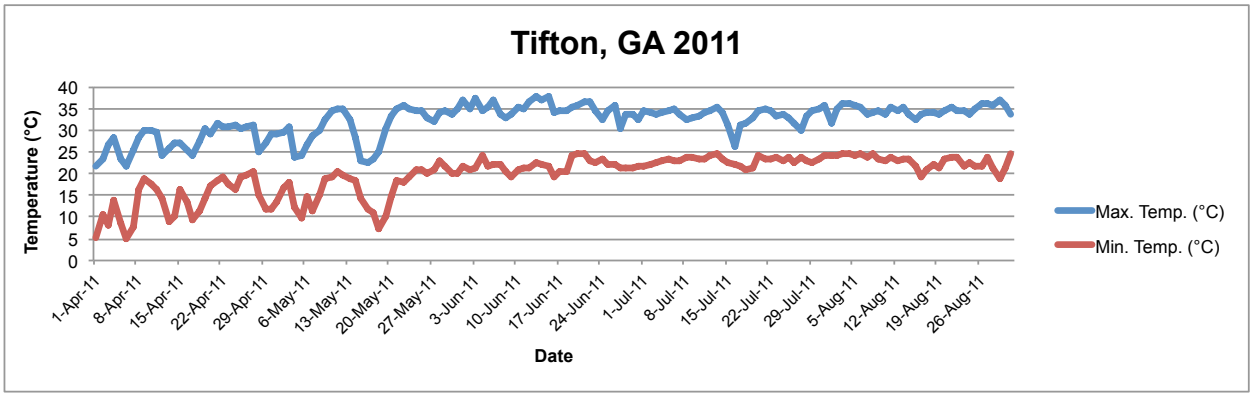
Weather Station: Bacon County Airport





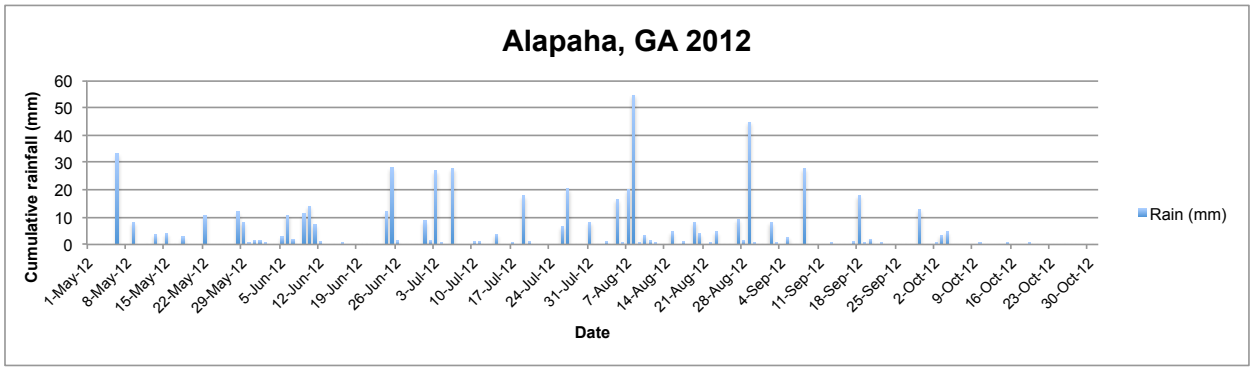
Weather Station: Booth Berry Farms

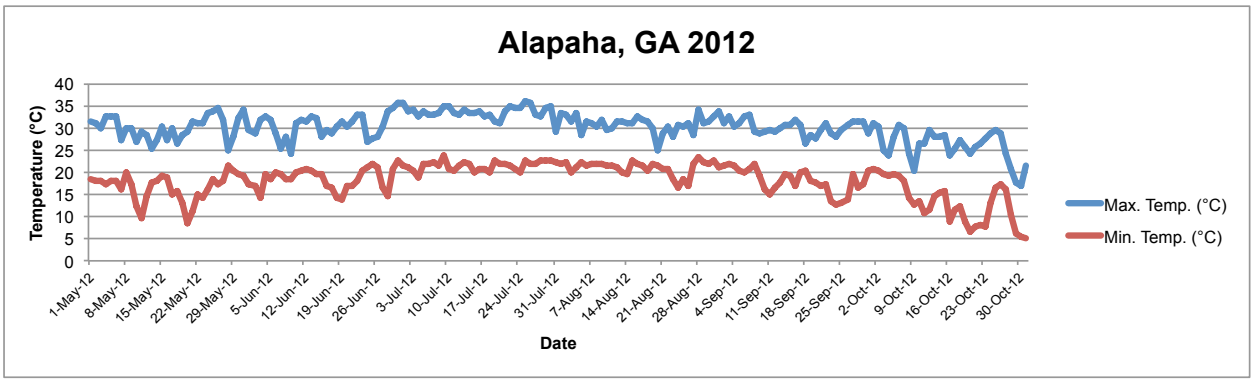




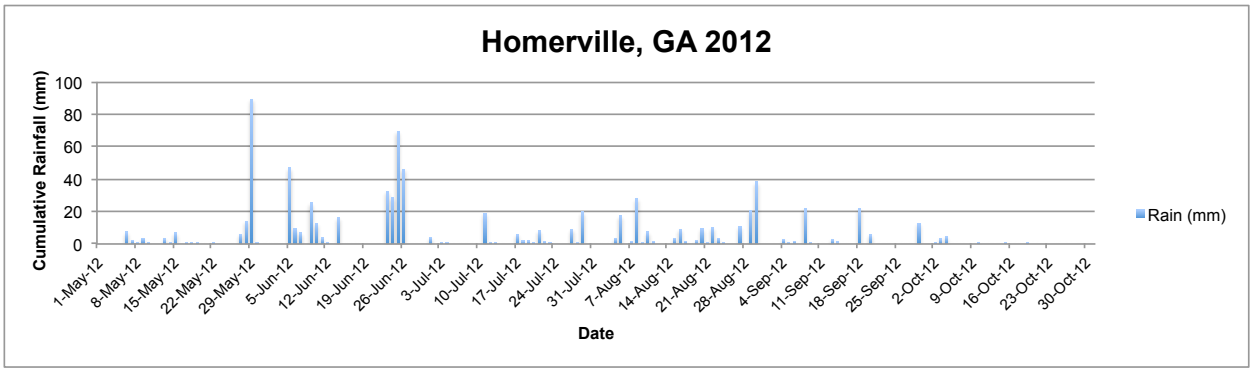
Weather station: Coastal Plain Experiment Station – The University of Georgia

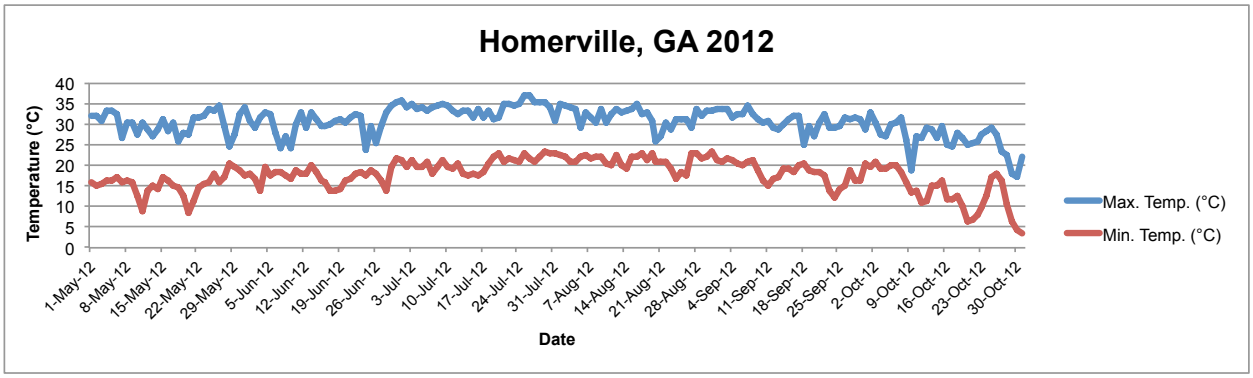
Cumulative rainfall was much heavier in 2012 as compared with 2011.



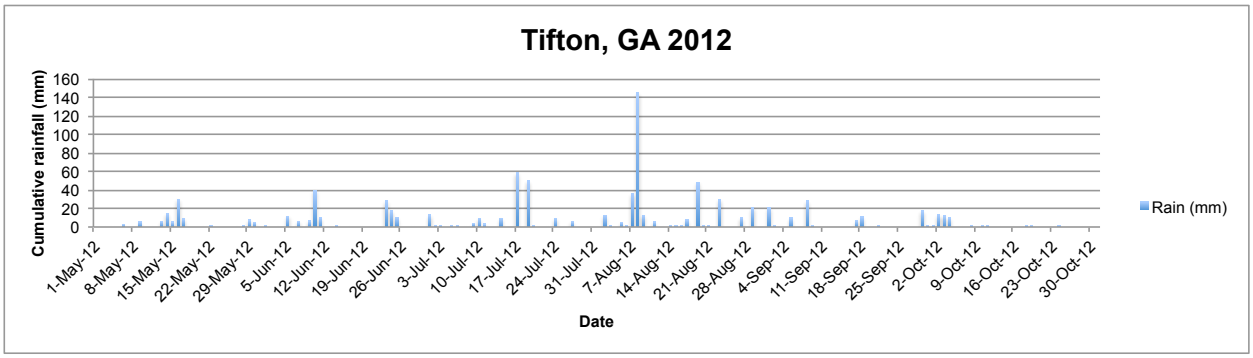


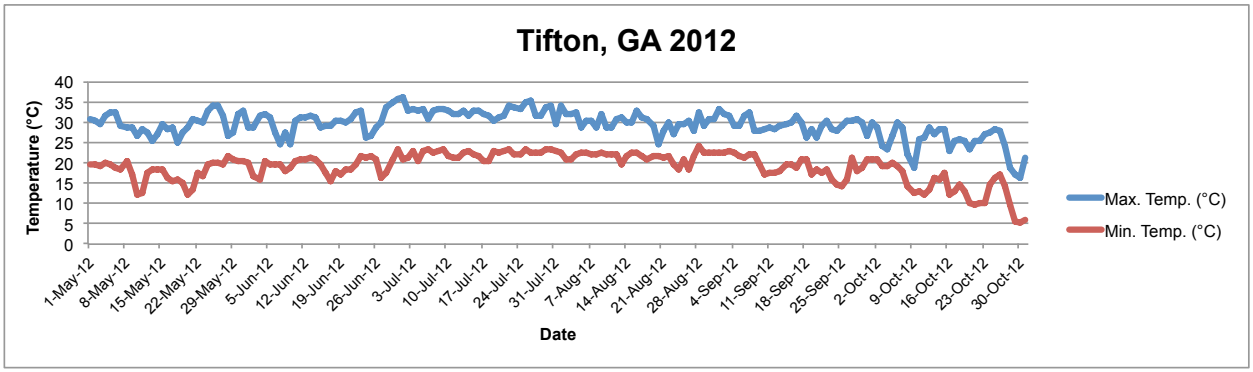
Weather station: Alapaha Range Grazing Unit – Blueberry farm





Weather station: Booth Berry Farms





Weather station: Coastal Plain Experiment Station – The University of Georgia