Title

# Investigating the Transmission of Systemic Southern Highbush Blueberry Diseases Via Softwood Cuttings

Progress Report for SRSFC Project # 2011-14

Research Proposal


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## Objectives

1) Take symptomless summer and fall softwood cuttings from field-grown Star plants known to be infected with one of the following:

- *Xylella fastidiosa* (bacterial leaf scorch)
- *Blueberry red ringspot virus*
- Blueberry necrotic ringblotch disorder ("funky spot")
- Healthy (asymptomatic control)

2) Root cuttings, pot up to containers, and assess for survival, growth, and disease over the course of two growing seasons by measuring the following:

- Cutting mortality
- Presence or absence of rooting
- Volume of root system if present
- Vegetative growth and flower production
- Type of leaf symptoms

3) Confirmation of the respective pathogen using polymerase-chain reaction (PCR) to calculate rate of transmission from infected mother plant to cutting

## Justification

With an annual farm gate value exceeding $100 million, blueberries are the top fruit commodity in Georgia (http://www.caed.uga.edu/publications/2010/pdf/AR-10-01.pdf). Much of this value is being generated by the early-maturing, high-value southern highbush blueberry crop.

Several new or emerging diseases affect southern highbush blueberries in south Georgia, but it is largely unknown to what extent these are transmitted through vegetative propagation. Softwood cuttings currently are the most common method of blueberry propagation. It is crucial that growers, propagators, and nurseries be not only aware of the effect of these diseases but also understand whether or not their propagation method is appropriate and preventative in the transmission of disease.

The symptoms of **bacterial leaf scorch** (Fig. 1C), caused by the xylem-limited bacterium *Xylella fastidiosa*, are necrotic leaf margins (scorching), yellowing of stems, dieback, and eventual death of the plant within a few years of infection. This serious systemic disease is transmitted in the field by leafhoppers, most likely in blueberries by the glassy-winged sharpshooter (*Homalodisca vitipennis)*. In a 2008 field survey, most farms (71.1%) were positive for the disease in at least one field, and the disease was present in 41.9% of the 167 fields surveyed. Rabbiteyes and some other cultivars, such as Emerald and Millenia, appear to be resistant to the disease, but the widely planted V1 (FL 86-19) has shown high susceptibility, along with a few other southern highbush varieties. In grape, a crop susceptible to Pierce's Disease caused by a different strain of *X. fastidiosa*, hardwood cuttings from infected plants proved to be less successful in rooting than cuttings from asymptomatic plants, but there is no information on survival or the rate of pathogen transmission of *X. fastidiosa* via softwood cuttings on blueberries.

***Blueberry red ring spot virus*** (BRRV) (Fig. 1A) is a common virus disease in Georgia, affecting 19 out of 45 farms (42.2%) and 25 of 167 (14.9%) southern highbush fields according to a survey in 2008. Although pronounced in the fall, symptoms of infected plants are not easy to recognize in the spring/ early summer when spring softwood cuttings are taken. BRRV is readily transmitted via grafting, giving reason to believe that the disease is highly transmissible (perhaps close to 100%) through cuttings. Although BRRV does not appear to have a significant impact on fruit yield, the study of this disease in cuttings could indicate the need for alternative propagation methods for blueberries.

**Blueberry necrotic ring blotch disorder** (BNRBD) (Fig. 1B), commonly called funky spot, is a new disease/ disorder that is suspected to be caused by a novel virus, based on research by Dr. Robert Martin, USDA-ARS small fruits virologist in Oregon. Double-stranded RNA has been isolated from symptomatic leaves collected at several sites in Georgia, Florida, and North Carolina. So far, symptoms are only found on the leaf. It is not clear, whether the pathogen inhabits other areas of the plant. Symptoms are irregularly shaped red or brown leaf spots with some having green centers depending on the cultivar. These "funky spots" may eventually coalesce to cover the leaf surface. No information is available whether BNRBD can be transmitted through cuttings. Disease severity in the same plants is highly sporadic across years, and this – along with the fact that symptoms are shown only on the leaves – may indicate that the disease is not very systemic. This would indicate limited transmission via cuttings, a hypothesis that needs to be confirmed through controlled experiments.

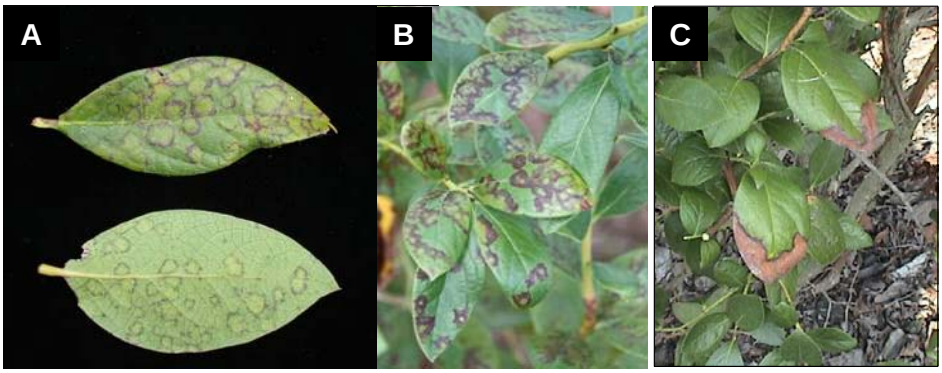


**Fig. 1.** Symptoms of *Blueberry red ringspot virus* (A), Blueberry necrotic ringblotch disorder (B), and bacterial leaf scorch (*Xylella fastidiosa*) (C) on southern highbush blueberry.

Methodology

Softwood cuttings were taken in four different Star southern highbush blueberry fields in south Georgia in June and again in September of 2011. (Similar collections had been done already in 2010, although different fields had been used.). These are referred to, respectively, as the spring and fall cuttings henceforth. The four fields were selected such that they were similar in age but differed in the types of diseases present, as determined by visual assessment and – in some cases – PCR testing: 1) asymptomatic (healthy) plants; 2) plants with BRRV symptoms; 3) plants with BNRBD symptoms; and 4) plants with bacterial leaf scorch (*Xylella*) symptoms. These plants are referred to as mother plants henceforth. Cuttings were 15-20 cm in length, and care was taken to collect only asymptomatic cuttings from the symptomatic mother plants. Ca. 300 cuttings were collected per field and stored in large plastic bags on ice overnight in a cold room.

The next day a sterile scalpel was used to make a 2-cm vertical incision at the base of each cutting from the fall collection to facilitate rooting; there was no such wounding for the spring cuttings. The bases of the cuttings were subsequently dipped into 2,500 ppm potassium-indole-acetic acid plant growth hormone before sticking in 36-well trays containing milled pine bark. Four trays (replicates) were prepared for each of the four treatments. The trays were arranged in a randomized complete block design and placed on a mist bench in a greenhouse for rooting. During the rooting of the Fall 2011 cuttings, 3 weeks after sticking, the temperature in the greenhouse rose temporarily to 99°F due to a heater malfunction, resulting in leaf scorching and defoliation.

Ca. 10 weeks after sticking in the four trials, a visual assessment of the cuttings was made, examining each for characteristic visual symptoms of the three diseases. Also, it was noted whether the cutting was alive, had rooted, had formed new vegetative growth, and whether defoliation (leaf loss) had occurred. Cuttings were uprooted and the volume of the root system determined by measuring length, width, and depth with a digital caliper. On the same day, 20 live cuttings per treatment were transplanted into 1-gal pots with 3:1 fine pine bark:sand and 14-14-14 NPK fertilizer, and grown in a greenhouse at 22.8 to 23.8°C. The remaining cuttings were frozen and stored for future PCR testing.

The transplanted cuttings were watered every other day and fertilized once a month with a 20-20-20 liquid fertilizer. A visual disease and vegetative growth assessment was done 1.5 months after transplanting, and again 3 months after transplanting. The growth assessment consisted of measuring the cumulative length of all vegetative shoots formed on each plant.

In the spring of 2012, plants will be moved to an insect-proof screenhouse and monitored for vegetative growth as well as symptom development during the season. Flower bud set will be determined at the end of the 2012 season. Any characteristic symptoms on the BNRBD and *Xylella* plants will be sampled and frozen for PCR analysis. All plants, regardless whether symptomatic or asymptomatic, will be sampled at the end of 2012, or – if very limited symptoms are apparent – carried over into the 2013 season to allow more time for symptom development. Our experience has shown that neither *Xylella* nor BNRBD are detected readily in asymptomatic plants by PCR testing, hence it is important to allow time for symptom development before molecular confirmation is attempted.

Results

*Performance of cuttings* – After the 10-week rooting period, mortality of cuttings taken from healthy (asymptomatic) mother plants was generally low (<5%), except for the Fall 2011 trial (15.9% mortality) in which the greenhouse had overheated temporarily. In all four trials (including the two 2010 trials), mortality was highest in cuttings taken from BRRV and *Xylella*-infected mother plants (Table 1). However, this effect was statistically significant only in two of the four trials: in the Spring 2010 and Fall 2010 trials, respectively, BRRV cuttings (22.9%) and *Xylella* cuttings (9.03%) had significantly higher mortality than all other treatments. The variability across the four trials suggests that although there is the potential for increased mortality in cuttings taken from BRRV and *Xylella*-infected plants, this effect may be dwarfed by differences associated with environmental factors or origin and vigor of the cuttings.

The rooting percentage for the healthy check 10 weeks after sticking ranged from 67.7 to 99.3% (Table 1). Although BRRV cuttings generally had lower rooting percentages, this effect was not statistically significant. Thus, whether a cutting formed roots was unaffected by the cutting source, i.e., whether or not the mother plants had been infected. Root volume was similarly unaffected by treatment (Table 1). Overall, the Fall 2011 trial had the lowest root volumes, presumably because of the damage caused when the greenhouse overheated.

New vegetative growth was generally produced by <30% of the cuttings during the rooting period and was affected more by trial than by treatment (Table 1). Interestingly, the trial with the weakest root growth (Fall 2011) had the highest frequency of cuttings producing new growth. In only one trial (Spring 2011) was there a significant effect of cutting source, viz. a significantly higher frequency of new growth in the BNRBD cuttings compared with all other treatments. Overall, however, there was no compelling evidence for an effect of cutting source on vegetative growth during the rooting phase.

Data on defoliation of cuttings was collected in only three of the four trials (the Fall 2011 trial was omitted because of the leaf damage caused by overheating of the greenhouse). In two of three trials, BRRV or *Xylella*-infected cuttings had significantly higher defoliation levels (up to 21.2%) than the remaining treatments (Table 1). Thus, increased defoliation was one variable significantly affected by disease status of the mother plant in the majority of trials.

*Disease symptoms on rooted cuttings* – As stated previously, care was taken to obtain asymptomatic cuttings from symptomatic plants when the cuttings were collected in the field. As such, when the cuttings were stuck, they were asymptomatic. After the rooting period only the BRRV cuttings consistently showed symptoms typical of the corresponding disease (chlorotic mottling and spotting), with incidence levels ranging from 16.6 to 83.2% (Table 2). In 2010, a high percentage of the *Xylella* cuttings (57.2 and 40.2% for spring and fall, respectively) also showed symptoms of BRRV. This was the case because BRRV was relatively prevalent in the planting where the 2010 *Xylella* cuttings were taken, despite our best efforts to avoid *Xylella*-affected mother plants that also had BRRV symptoms. In 2011, a different *Xylella* planting was used for propagation, and infection with BRRV was no longer a problem. Overall, our data show that BRRV transmits readily from mother plant to cutting, and that BRRV symptoms appear relatively soon during the propagation process.

Symptoms typical of BNRBD were observed very rarely in the rooting trays, with only cuttings in the Spring 2011 trial from the BNRBD-affected mother plants being symptomatic at an incidence level of 4.86% (Table 2). For *Xylella*, no characteristic symptoms were observed during the rooting period in any of the trials.

*Performance of plants 1.5 months after transplanting* – Data on plant performance after transplanting of the cuttings into 1-gal pots in the greenhouse is only available for three trials since the Fall 2011 cuttings are still too young for the first post-transplant assessment. In general, vegetative plant growth was more vigorous for the Spring 2010 and 2011 plants than for the Fall 2010 plants (Table 3). This was likely due to more favorable conditions for growth during summer than in the fall. Consistently in the three trials vegetative growth (cumulative length of all shoots produced per plant) was significantly lower in plants derived from *Xylella* cuttings than in those from healthy or BNRBD cuttings, even though the *Xylella* plants were still asymptomatic. This was not unexpected since the *Xylella*-affected mother plants from which these cuttings were taken were considerably less vigorous (due to the effects of the disease) than the mother plants belonging to the other three treatments. As such, the reduced growth is more likely due to reduced overall vigor of the cuttings than to a direct effect of *X. fastidiosa* on the cuttings after sticking or transplanting. What was more surprising, however, was the significant reduction in vegetative growth in the BRRV plants in two out of three trials (Table 3), given that the corresponding mother plants showed little evidence of reduced vigor in the field. Thus, the reduced plant growth in the BRRV cuttings was most likely a direct effect of the virus on growth. This effect could also have been due to the increased defoliation in BRRV cuttings mentioned above (Table 1). In contrast, the BNRBD plants grew as well as those derived from healthy mother plants.

At 1.5 months after transplanting between 33.1 and 97.5% of the plants derived from BRRV cuttings showed symptoms of the disease (Table 3). BRRV incidence was higher for the plants from the fall cuttings than from the spring cuttings. There were no symptoms of BNRBD, except for the Spring 2011 trial in which the incidence of typical BNRBD symptoms was 1.25%. (This percentage was lower than for the corresponding cuttings at the earlier assessment date because not all cuttings were transplanted.) No symptoms typical of *Xylella* were observed 1.5 months after transplanting, confirming the earlier observations made at the cutting stage.

*Observations at later plant growth stages* – Detailed growth and disease assessments were also made 3 months after transplanting and again at the end of each growing season in the fall. These data have not yet been analyzed fully, but preliminary observations indicate that the plants derived from the *Xylella* cuttings have caught up in vegetative growth relative to those from the other treatments. Close to 100% of the plants derived from BRRV cuttings show symptoms of the disease at later plant growth stages. However, most importantly, no additional *Xylella* or BNRBD symptoms have appeared on these larger plants, suggesting that the two diseases are either not transmitted readily through the propagation process or that symptoms appear not until much later. No comprehensive PCR testing of plants has been conducted to date since our experience has shown that neither *Xylella* nor BNRBD are detected readily in asymptomatic plants. As such, we will maintain these plants for at least another year to increase the potential for symptom development. At the end of the experiment in Fall 2012, all plants will be tested by PCR regardless of whether or not they are symptomatic at that time.

Conclusions

- When asymptomatic cuttings were taken from mother plants affected with BRRV, BNRBD, or *X. fastidiosa* and grown on a mist bench, survival and rooting were not consistently affected compared with cuttings from healthy control plants. However, BRRV and *Xylella* cuttings had significantly higher levels of leaf loss than cuttings from the other two sources.
- The only cuttings that consistently developed symptoms of the corresponding disease during the rooting phase were those taken from BRRV-affected mother plants, regardless of whether the cuttings were collected in the spring or fall. Only very sporadic BNRBD and no *Xylella* symptoms appeared in rooted cuttings.
- After the cuttings had rooted and were transplanted, plants from *Xylella* and BRRV cuttings grew more slowly at first than those from healthy or BNRBD cuttings. However, several months later, the *Xylella* and BRRV plants had caught up with their healthy counterparts in all aspects of vegetative growth.
- Following additional growth in the greenhouse (2011 plants) or screenhouse (2010 plants), close to 100% of the progeny from BRRV-infected plants showed symptoms of the disease. In contrast, there has been no characteristic symptom development on plants derived from *Xylella* cuttings, even those from Spring 2010 which are now 1.5 years old. Only a handful of the plants from BNRBD cuttings showed symptoms. This suggests that the latter two diseases are either not transmitted readily through the propagation process or that symptoms appear not until much later.
- No comprehensive PCR testing of plants has been conducted to date since neither *X. fastidiosa* nor BNRBD are detected readily in asymptomatic plants. As such, we will maintain these plants for at least another year to increase the potential for symptom development. At the end of the experiment in Fall 2012, all plants will be tested by PCR regardless of whether or not they are symptomatic.

Impact Statement

**Situation**

Systemic plant diseases (those that move within the plant's vascular system and generally stay associated with an infected plant life-long) are becoming more common in southern highbush blueberries, threatening this high-value plant industry. Three potentially systemic diseases of particular concern in the south Georgia blueberry belt are *Blueberry red ringspot virus* (BRRV); bacterial leaf scorch (*Xylella fastidiosa*); and Blueberry necrotic ringblotch disorder (BNRBD, "funky spot), a disease of uncertain etiology most likely caused by one or more viruses. If these three diseases are indeed systemic or partially systemic, there is a risk of their spread and dissemination through vegetative propagation. Currently there is no information on the rates of transmission of these diseases from infected mother plants to cuttings, or on their impact on cutting survival, rooting, and subsequent plant growth.

**Response**

Asymptomatic softwood cuttings were taken from 'Star' southern highbush blueberry plants affected by one of the three diseases or from healthy control plants in the spring and fall of 2 years (2010 and 2011). Cuttings were rooted in pine bark under mist and assessed for growth and disease at the end of a 10-week rooting period. Rooted cuttings were then transplanted into larger containers n the greenhouse, and disease and growth assessments continued. Following

overwintering in the greenhouse, the 2010 plants were moved into an insect-proof screenhouse for further monitoring. Tissues with characteristic symptoms were frozen for future pathogen confirmation by PCR.

**Impact**

Disease status of the mother plant did not consistently affect survival and rooting of the cuttings, except that cuttings from *Xylella* and BRRV-affected mother plants showed increased levels of defoliation. BRRV symptoms were readily apparent on rooted cuttings, whereas BNRBD symptoms were very sporadic and *Xylella* symptoms were absent. Following transplanting, cuttings from *Xylella* and BRRV grew more slowly initially, but then caught up. In older plants that developed from these cuttings, BRRV symptoms were almost universally present whereas BNRBD and *Xylella* symptoms were absent. Pending confirmation of these results by PCR analysis, our data suggest that BRRV is transmitted from mother plants to cuttings very efficiently, whereas BNRBD and *Xylella* are either not transmitted through the propagation process or that symptoms appear not until much later.

**Table 1.** Growth of Star softwood cuttings taken from mother plants that were either asymptomatic (healthy) or symptomatic for *Blueberry red ringspot virus* (BRRV), blueberry necrotic ringblotch disorder (BNRBD), or *Xylella fastidiosa* during incubation on a mist bench for 10 weeks in four separate trials[a]

| Trial and treatment[b] | Mortality (%) | Rooting (%) | Root volume ($mm^3$) | New vegetative growth (%) | Defoliation (%) |
|---|---|---|---|---|---|
| **Spring 2010** | | | | | |
| Healthy | 3.47 b | 80.6 | 127.4 | 10.6 | 0.76 b |
| BRRV | 22.9 a | 65.4 | 109.8 | 8.32 | 8.53 a |
| BNRBD | 2.78 b | 93.7 | 158.8 | 8.56 | 0.71 b |
| *Xylella* | 7.64 b | 76.4 | 128.0 | 21.9 | 8.6 a |
| *P*-value | 0.0002 | 0.2365 | 0.3204 | 0.2418 | 0.0635 |
| **Fall 2010** | | | | | |
| Healthy | 0.69 b | 99.3 | 123.4 | 5.00 | 1.41 b |
| BRRV | 3.47 b | 100 | 135.1 | 2.78 | 0.78 b |
| BNRBD | 0 b | 99.3 | 129.1 | 0 | 0.69 b |
| *Xylella* | 9.03 a | 97.7 | 139.1 | 2.35 | 9.9 a |
| *P*-value | 0.0017 | 0.5920 | 0.2724 | 0.6432 | 0.0431 |
| **Spring 2011** | | | | | |
| Healthy | 4.86 | 67.7 b | 77.6 b | 16.1 b | 1.82 b |
| BRRV | 13.2 | 71.5 b | 75.8 b | 18.4 b | 21.2 a |
| BNRBD | 0 | 87.5 a | 91.1 a | 55.6 a | 0 b |
| *Xylella* | 5.91 | 87.8 a | 88.2 a | 13.6 b | 7.12 b |
| *P*-value | 0.1619 | 0.0099 | 0.0186 | <0.0001 | 0.0024 |
| **Fall 2011** | | | | | |
| Healthy | 15.9 | 72.9 | 43.6 | 24.8 | --[c] |
| BRRV | 29.9 | 58.3 | 31.6 | 29.9 | -- |
| BNRBD | 5.56 | 78.5 | 43.7 | 24.8 | -- |
| *Xylella* | 26.4 | 66.7 | 29.4 | 35.9 | -- |
| *P*-value | 0.2990 | 0.4182 | 0.4729 | 0.6830 | -- |

[a] All cuttings were asymptomatic when collected in the field. Means followed by the same letter for each column and trial are not significantly different according to Fisher's protected LSD test.
[b] In most cases, different sets of mother plants were used in different trials.
[c] Missing data (greenhouse overheated temporarily and caused defoliation of cuttings).

**Table 2.** Symptom development on Star softwood cuttings taken from mother plants that were either asymptomatic (healthy) or symptomatic for *Blueberry red ringspot virus* (BRRV), blueberry necrotic ringblotch disorder (BNRBD), or *Xylella fastidiosa* during incubation on a mist bench for 10 weeks in four separate trials[a]

| Trial and treatment[b] | Visual disease incidence (%) | | |
|---|---|---|---|
| | BRRV | BNRBD | *Xylella* |
| **Spring 2010** | | | |
| Healthy | 5.30 | 0 | 0 |
| BRRV | 45.5 | 0 | 0 |
| BNRBD | 4.42 | 0 | 0 |
| *Xylella* | 57.2 | 0 | 0 |
| **Fall 2010** | | | |
| Healthy | 19.7 | 0 | 0 |
| BRRV | 83.2 | 0 | 0 |
| BNRBD | 5.56 | 0 | 0 |
| *Xylella* | 40.2 | 0 | 0 |
| **Spring 2011** | | | |
| Healthy | 0.35 | 0 | 0 |
| BRRV | 16.6 | 0 | 0 |
| BNRBD | 0 | 4.86 | 0 |
| *Xylella* | 0 | 0 | 0 |
| **Fall 2011** | | | |
| Healthy | 0 | 0 | 0 |
| BRRV | 18.8 | 0 | 0 |
| BNRBD | 0 | 0 | 0 |
| *Xylella* | 0 | 0 | 0 |

[a] All cuttings were asymptomatic when collected in the field.
[b] In most cases, different sets of mother plants were used in different trials. In the 2010 trials, some of the healthy and especially *Xylella* mother plants were also infected with BRRV, leading to symptom development in the respective cuttings.

**Table 3.** Performance of and symptom development on Star softwood cuttings taken from mother plants that were either asymptomatic (healthy) or symptomatic for *Blueberry red ringspot virus* (BRRV), blueberry necrotic ringblotch disorder (BNRBD), or *Xylella fastidiosa* 1.5 months after transplanting in the greenhouse in four separate trials[a]

| Trial and treatment[b] | Total shoot growth (cm) | Visual disease incidence (%) | | |
|---|---|---|---|---|
| | | BRRV | BNRBD | *Xylella* |
| **Spring 2010** | | | 0 | 0 |
| Healthy | 70.6 a | 0 | 0 | 0 |
| BRRV | 43.2 b | 50.6 | 0 | 0 |
| BNRBD | 66.0 a | 8.8 | 0 | 0 |
| *Xylella* | 46.8 b | 60.7 | 0 | 0 |
| *P*-value | | -- | -- | -- |
| **Fall 2010** | | | 0 | 0 |
| Healthy | 24.9 a | 0 | 0 | 0 |
| BRRV | 18.8 b | 97.5 | 0 | 0 |
| BNRBD | 25.7 a | 3.8 | 0 | 0 |
| *Xylella* | 17.4 b | 0 | 0 | 0 |
| *P*-value | | -- | -- | -- |
| **Spring 2011** | | | 0 | 0 |
| Healthy | 56.4 a | 0 | 0 | 0 |
| BRRV | 42.6 ab | 33.1 | 0 | 0 |
| BNRBD | 56.7 a | 0 | 1.25 | 0 |
| *Xylella* | 21.2 b | 0 | 0 | 0 |
| *P*-value | 0.0159 | -- | -- | -- |

[a] All cuttings were asymptomatic when collected in the field.
[b] In most cases, different sets of mother plants were used in different trials. In the 2010 trials, some of the healthy and especially *Xylella* mother plants were also infected with BRRV, leading to symptom development in the respective progeny. BRRV incidence in these plants was lower than that in the corresponding cuttings in Table 2 since not all cuttings were transplanted.