Screening for Resistance to Bacterial Fruit Blotch in Watermelon

Project update to
Vegetable Seed Companies

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Summary
Bacterial fruit blotch (BFB), caused by *Acidovorax citrulli*, is a seed-borne disease. It poses a significant threat to watermelon production worldwide. Fruit blotch can lead to 100% yield loss under warm, wet conditions. Watermelon cultivars range from susceptible to slightly resistant, and sources of high resistance may be available. Thus, the disease can be controlled by breeding resistant cultivars, or seed sanitation programs. Excluding the pathogen from seeds, fruit, and transplants is difficult because the pathogen can live under the seed coat and can present inconspicuous symptoms. Resistant cultivars would provide more effective control. The objective of this project is to screen the watermelon germplasm collection for resistance to bacterial fruit blotch, and to identify accessions that can be used in breeding for resistance. Associated benefits include disease resistant germplasm for industry use in the development of cultivars, and improved understanding of the interaction between host and pathogen.

Introduction
Bacterial fruit blotch (BFB), caused by *Acidovorax citrulli*, affects the watermelon plant from cotyledon and true leaves to the fruit, causing varied symptoms in each stage. Symptoms on infected fruit start with small, dark, olive-green stains, known as blotches, that develop into necrotic spots after 7 to 10 days, followed by an increase in spot size. Secondary organisms enter the diseased areas and cause decay and collapse of fruit. On the leaves, the lesions are small, dark brown and angular. Symptoms on seedling leaves usually follow the major leaf veins. Foliar symptoms in the field are not distinctive and may be inconspicuous to growers (Hopkins et al., 1993). Under an ideal environment, BFB can cause 100% yield loss.

The first reported BFB outbreak in commercial watermelon production in the United States was in 1989 (Hopkins, 1989; Latin and Range, 1990). It was then found infecting muskmelon, honeydew, acorn squash, cucumber, pumpkin, yellow squash, zucchini squash, and wax gourd, either in production or in research studies (Isakeit et al., 1997; Isakeit et al., 1998; Langston et al., 1999, Walcott et al., 2000; Kubota and Masaharu, 2012). All cucurbit crops are potential hosts (Hopkins et al., 2003). Before 1999, BFB outbreaks were reported primarily on watermelon in the U.S. and Guam. Outbreaks were reported throughout the central, eastern and southeastern U.S. in Florida, Texas, Georgia, South Carolina, North Carolina, Illinois, Iowa, Missouri, Delaware, Oregon, and Oklahoma (Black et al., 1994; Hamm et al., 1997; Jacob et al., 1992; Latin and Rane, 1990; Somodi et al., 1991).

Because BFB is seed-borne, the primary source of inoculum in the field and in the transplant production greenhouse is through the contaminated seeds (Hopkins and Thompson, 2002). Treatments have been evaluated for decontamination of cucurbit seeds. Of those, streptomycin sulphate and NaOCl were reported to reduce transmission of BFB to seedlings (Sowell and Schaad, 1979). Also reported to be effective were 1600 ug/ml peroxyacetic acid for 30 min, dry heat seed treatment, fermentation of seeds in watermelon juice for 24-48 h followed by treatment with 1% HCl for 15 min, chlorine gas exposure for 9 h, and acidic electrolyzed water (Hopkins, 1996; Hopkins et al., 2003; Shirakawa, 2002; Kubota et al.,

2012; Stephen et al., 2008; Feng et al., 2009). However, none of the external treatments were completely effective. A possible explanation is the bacteria surviving under the seedcoat (Burdman and Walcott, 2012). In addition, contaminated volunteer watermelons, other cultivated cucurbits, and wild cucurbits were able to transmit BFB to the crop in the field (Isakeit et al., 1998; Latin and Hopkins, 1995; Hopkins and Thompson, 2002).

Since there is a zero tolerance for BFB in seedling transplant facilities, seed health testing has been developed and is considered to be critical for disease management. A PCR-based assay was developed to test seeds for *A. citrulli* (Bahar et al., 2008; Park et al., 2008; Walcott and Gitaitis, 2000). However, as cucurbit seeds contain PCR inhibitors, a range of techniques has been developed to improve test sensitivity and accuracy (Walcott et al., 2006; Walcott and Gitaitis, 2000; Zhao et al., 2009). Despite its improvement, the PCR-based technique is not routine in commercial production. The most common test is a seedling grow-out bioassay. The test requires the destruction of many seeds (n=10,000 to 50,000 seeds/lot), and is labor intensive. To date, the most effective treatment for BFB control in the field is to apply copper-based bactericide including cupric hydroxide, copper hydroxosulfate, or copper oxychloride (Hopkins, 1991; Hopkins and Thompson, 2002). Bactericide resistance might become an issue.

Genetic resistance is the single most effective strategy for managing plant disease. In addition to cost effectiveness, resistance-based strategies are compatible with other integrated disease management approaches. Unfortunately, there are no watermelon cultivars with resistance to BFB.

Watermelon and melon are the two major crops seriously threatened by BFB, and studies have been done to identify resistant germplasm for the two crops (Sowell and Schaad, 1979; Somodi and Jones, Hopkins et al., 1993; Hopkins and Thompson, 2002; Carvalho et al., 2012; Bahar et al., 2009; Wechter et al., 2011). An attempt was made to increase watermelon cultivar resistance to BFB (Hopkins and Levi, 2008). Different levels of resistance were reported. Two South African PI accessions (PI 295843 and PI 299378) and 'Congo' were reported to be resistant (Sowell and Schaad, 1979), but were later proven susceptible. 'Garrisonian' was reported to be immune (Goth and Webb, 1981), but was later proven susceptible (Hopkins et al., 1993). The failure of resistance is likely caused by the introduction of exotic strains or mutation of the predominant strains.

Hopkins and Thompson (2002) published the first evaluation for BFB resistance of US watermelon PI accessions, the largest screening of the USDA collection before ours. Of the 1344 *Citrullus spp.* and *Praecitrullus fistulosus* accessions, they identified two accessions with high resistance: PI 482279 from Zimbabwe and PI 494817 from Zambia. Resistance was defined as less than 20-50% necrotic lesions having chlorosis on cotyledons based on a rating made 10 days after inoculation. However, the resistant PI accessions segregated for resistance. That was also observed in screening for resistance in melon PI accessions (Wechter et al., 2011). Disease ratings of F_1 and F_2 generations from susceptible cultivars crossed with resistant PI 482279 or PI 494817 found that resistance was controlled by more than one gene, with a complex mode of inheritance. In crosses of resistant accessions with 'Crimson Sweet' it was difficult to maintain resistance while also selecting for elite characteristics (Hopkins and Levi, 2008). In addition, this study assumed that the resistance to BFB at both seedling and flowering stage would be found in a single accession (Hopkins and Thompson, 2002). By eliminating the susceptible accessions at seedling stage before testing at flowering stage in the field, they may have missed the accessions having resistance at the later stages of growth. The hypothesis that resistance at different stages might be controlled by different gene(s) was supported by the observation that disease severity varied when the same accessions when tested at different stages in both watermelon and melon (Carvalho et al., 2012; Bahar et al., 2009; Wechter et al., 2011).

Recently, Shen and Wehner (2015) conducted an extensive mature foliar resistance screening of 1699 watermelon cultigens over three years in Clinton, NC. Consequently, 23 exceptionally resistant cultigens,

consisting mostly of citrons (*C. amarus*) and some watermelons (*C. lanatus* var. *lanatus*), were identified. To further validate the resistance these elite lines crosses will need to be created and extensively tested using a wider range of isolates.

From an economic point of view, fruits are the most crucial organs affected by BFB (Bahar et al., 2009). Of course, foliar screening provides a convenient way to test for resistance. Also foliar resistance is important in restricting inoculum from infecting the fruit. However, with inoculum contributed by volunteer fruits in the field, other cucurbits, and cucurbit weeds (Isakeit et al., 1998; Latin and Hopkins, 1995), together with the fact that the correlation between foliar resistance and fruit resistance is unknown, it is important to investigate watermelon resistance to BFB at discrete stage with current available USDA watermelon germplasm and combine the resistance contributed from different stage to enhance the overall performance in fighting against bacterial fruit blotch.

A recent study of watermelon resistance to BFB by a Brazilian group reported one of 74 accessions immune to BFB, BGCIA 979. Also, BGCIA 34 and 'Sugar Baby' showed high levels of resistance at most plant developmental stages. They suggested these three accessions should be used as sources of resistance in breeding programs (Francisco et al., 2012).

Methods have been developed for testing BFB resistance including a greenhouse seedling test and a field screening test. In the tests, high temperature and high relative humidity are important to promote symptom development to get proper disease ratings (Hopkins et al., 1993; Hopkins and Thompson, 2002; Francisco et al., 2012; Bahar et al., 2009; Wechter et al., 2011; Carvalho et al., 2012). Because BFB can occur at any plant growth stage, Carvalho et al. (2012) evaluated watermelon resistance at multiple stages, including seed, seedling, flowering, and fruiting. They reported differences in resistance at different stages. Similar results were reported for resistance to BFB in melon (Bahar et al., 2009). Thus, it appears that the USDA watermelon germplasm collection should be re-screened for resistance at the major growth stages to select accessions having high resistance at any of the stages.

Although, fruit resistance represents a crucial step in disrupting the *A. citrulli* infection cycle, fruit resistance screening has been largely neglected in the literature in favor of relatively simpler foliar tests. Fruit infection occurs as *A. citrulli* bacterium, from infected leaf tissue, penetrate through stomata early in fruit development (Frankle et al., 1993) and not systemically through the vine (Rane and Latin, 1992). Based on the findings by Frankle et al. (1993), the fruit infection appears to be primarily governed by the accumulation of waxy cuticle. Over a five week period the percent decrease of diseased fruit correlated with the percent decrease of exposed stomata, with the fruit being most vulnerable (over 90% infection) during the first two weeks post anthesis (Frankle et al., 1993) This interestingly implies that effective 'resistance' or 'tolerance' could be based on avoidance, rather than pathogen triggered immunity or effector triggered immunity. It can be ventured that genes that promote the early and rapid biosynthesis of cuticle will shorten the infection window, ultimately decreasing the percentage of infected fruit and diminishing seed infestation. Selecting for barrier resistance has the potential to be effective regardless of the strain, decreasing the danger of mutant or exotic strains overcoming resistance. From a breeding standpoint, early selections for resistance may only require the selection of lines that develop waxy cuticles early in fruit development or lines that have fewer fruit stomata.

Objectives

The primary objective of this project is the identification of resistance to BFB through screening the currently available watermelon PI collection and a broad selection of commercially available lines. Additionally, identified resistance will be extensively tested through selfing resistant lines and crossing them with resistant lines in order to gain an understanding of the inheritance of BFB resistance. This project differs importantly from the only other large-scale watermelon screening conducted by Hopkins and Thompson (2002) and Shen and Wehner (2015) in that a wider diversity of *A. citrulli* isolates will be

used, resistance will be evaluated at the fruit stage, and commercially available varieties will be extensively tested. Lines demonstrating resistance during the initial screenings will be selfed and tested under high replication. Resistant lines will be increased and made available for breeders and researchers.

Secondarily, because the incidence of disease of the fruit has been correlated with cuticle formation (Frankle et al., 1993), we hypothesize that resistant fruits will acquire cuticle faster than susceptible varieties. In order to test this hypothesis, resistant and susceptible lines will be water and pathogen inoculated and formation cuticle measurements will be taken over a similar five week period to Frankle et al. 1993. If a correlation exists, it is expected that resistant lines will have infection windows that are significantly shorter than susceptible varieties, which will lead to an overall decrease in diseased fruit and infested seeds. The development of a rapid phenotyping method for cuticle formation would be of immense practical benefit for breeding resistant watermelons.

Methods

The screenings and retests will be conducted during the 2015-2017 spring seasons at the Horticultural Crops Research Station near Clinton, NC. Screening of 1600 lines (1500 PI's and 100 commercial varieties) will be planted in plots 4' long (50 per row) on 10' rows will be planted in a field having 30 rows, The inoculum will consists of a combination of 9 isolates representing a wide genetic diversity of *Acidovorax citrulli*. The experiment will be a randomized complete block replicated nine times over three years, and plants will be rated using a 0-9 scale based on percent diseased surface.

Resistance Retest

The most resistant and susceptible lines identified during the screening will be extensively retested during following seasons to verify reaction. After retesting, we will self-pollinate the most resistant plants from the most resistant accessions, select progeny on the basis of high resistance and uniformity, and release the resistant sublines for use by industry. Accessions with a resistant reaction will be verified and made available to the industry for use in development of resistant cultivars.

Genetic inheritance and allelism

A concurrent study of inheritance of foliar resistance the most resistant lines of the 23 elite lines identified by Shen and Wehner (2015) will involve the creating and testing of a segregating population (parent A, parent B, F1, F1 reciprocal, backcross to parent A, backcross to parent B, and F2). Each population will consist of a highly resistant parent previously identified in a multiyear germplasm screening study crossed with a susceptible or moderately resistant parental line. The set of populations will initially tested at the seedling stage and then the best candidates will be grown at both research stations and rated for disease traits.

Timeline

The research schedule will be as follows:
2013: Conduct inoculation methods test, screen 1600 watermelon cultigens reps 1 to 3 for leaf resistance
2014: Screen 1600 watermelon cultigens reps 4 to 6 for leaf resistance; retest of best and worst
2015: Conduct fruit inoculation methods test, screen 1600 watermelon cultigens reps 1 to 3 for fruit resistance, create F1 crosses of foliar resistant PI's and commercial lines and conduct seedling resistance studies, develop cuticle measurement methodology
2016: Screen 1600 watermelon cultigens reps 4 to 6 for fruit blotch resistance, retest resistant and susceptible lines to verify reaction (reps 1-3), measure cuticle formation of in select individuals, self and cross fruit resistant varieties; generate foliar resistance population (F2, BC1R, BC1S) and conduct a seedling resistance study
2017: Screen 1600 watermelon cultigens reps 7-9 for fruit blotch resistance, retest resistant and susceptible lines to verify reaction (reps 1-6), measure cuticle formation of select individuals, conduct inheritance study of foliar resistance

Current Progress and Observations

Methods testing: We conducted a large inoculation methods test to determine the best method for inoculating fruit in preparation for screening the watermelon germplasm. All combinations of the following factor were evaluated: plastic bagging after inoculation, damaging fruit, high inoculum concentration, low inoculum concentration, flowering stage inoculation (yellow flower), and post flowering inoculation (brown flower). We found that in all but a few cases, damaging and/or bagging fruit lead to early death of the developing fruit. In addition, both inoculum concentrations produced symptoms, but many of the fruits aborted post inoculation. Using the spray method on the 1600 lines of Rep 1 we observed that slightly older fruit (~1-2 weeks old) developed infection and rarely aborted; this is accordance with by Frankle et al. (1993). Interestingly, at this stage of development the fruit have a shiny appearance and soft wax texture that retains sprayed inoculum. Later, maturing fruit eventually adopt a dull sheen and a smooth texture that beads off inoculum when sprayed, vastly decreasing inoculum retention on the fruit surface. The transition between surface wax characteristics may represent a critical marker for resistance, and that resistant varieties would have a shortened shiny-fruit period. We concluded that a spray inoculation of ~1-2 week old fruit was the most effective and practical inoculation method. Consequently, we selected the method used by Frankle and Hopkins (1993) with an additional inoculation to decrease the possibility of inoculation misses.

Resistance Screening: Through reps 1-3 we collected resistance data on 1048 lines; and we have identified lines that have exhibited various resistance levels. Resistance was quantified by the percent upper surface showing symptoms (0-9), the type of symptom, and for the presence of internal necrosis. (Please see the attached disease rating PowerPoint for more details.) Although we expected many lines showing no infection, lines exhibiting a 'blister' type reaction were unexpected. A 'blister' appears to be a hypersensitive response by the plant to counter pathogen infection and was frequently associated with many lines showing very little surface infection and no internal necrosis. Resistant lines that also had relatively commercial acceptable traits were recorded, which consisted mostly of *Citrullus lanatus* subsp. *lanatus* and *Citrullus lanatus*.

The following lines had high resistance, low variation within variety, some adaptation, and at least two replications of data for 2015:

PI 172786	*C. lanatus* subsp. *lanatus*	PI 176909	*C. lanatus* subsp. *lanatus*
PI 325248	*C. lanatus* subsp. *lanatus*	PI 277982	*C. lanatus* subsp. *lanatus*
PI 357739	*C. lanatus* subsp. *lanatus*	PI 293766	*C. lanatus* subsp. *lanatus*
PI 482247	*C. lanatus* subsp. *lanatus*	PI 500313	*C. lanatus* subsp. *lanatus*
PI 500312	*C. lanatus* subsp. *lanatus*	PI 518607	*C. lanatus* subsp. *lanatus*
PI 532723	*C. lanatus* subsp. *lanatus*	PI 534533	*C. lanatus* subsp. *lanatus*
PI 612462	*C. lanatus* subsp. *lanatus*	PI 660975	*C. lanatus*
PI 500349	*C. lanatus* subsp. *lanatus*	PI 176923	*C. lanatus* subsp. *lanatus*
PI 635637	*C. lanatus*	PI 482250	*C. lanatus* subsp. *lanatus*
PI 307750	*C. lanatus* subsp. *lanatus*	PI 357656	*C. lanatus* subsp. *lanatus*

Future Research Plans

In order to validate our current observations and identify other promising lines, we must continue to add replications over the next two years. In addition, a set of highly resistant lines will be retested under high replication in 2016 and 2017 seasons. Due to the challenging nature of the BFB fruit assay under field conditions, we missed a significant number of lines; however, we are exploring methods to increase the efficiency of the screening through greater spacing and by adding a fourth replication.

Overall, we are pleased with our decision to spray inoculate developing fruit, as per prior reporting by Frankle and Hopkins (1993) and Hopkins, Thompson, and Elmstrom (1993) and our methods test observations. While we will use the same method for 2016, we will conduct a more extensive methods test, focused on optimizing inoculum concentration and fruit age, the primary factors involved in field inoculations.

We are in the process of creating crosses based from lines showing high foliar resistance identified by Shen and Wehner (2015) in the greenhouse and thus far we have a few F1 hybrids with limited seed. The primary challenge is that most of the foliar resistant lines are *Citrullus amarus* and crossing with commercial lines (Charleston Gray and Crimson Sweet) resulted in few seeds or aborted crosses. We require more seed to evaluate these hybrids. We have recently added LED light systems to our greenhouse that may permit us to generate more seed during the winter months, possibly allowing F1 seedling evaluation and F2 generation during summer 2016.

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