Title: The Correlation of Firmness Loss with Flavonoid Gene Expression and Pigment Synthesis in Rabbiteye Blueberries (*Vaccinium ashei* Reade)

Progress Report

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Note: Dr. Anish Malladi has taken over the responsibilities of a PI on this project after the original PI's departure from UGA in October 2011.

Objective:

The objective of this study is to investigate the relationship between blueberry pigment production with the onset and loss of fruit firmness. Ultimately, this study will determine to what extent blueberries 'blue-up' after harvest, and whether harvest strategies can be altered in order to increase postharvest fruit firmness, without compromising the final color of the fruit.

Justification and Description:

Blueberries are a multi-million dollar industry that were worth over $100 million dollars in Georgia in 2009 (Boatright and McKissick, 2010). The two types of blueberries grown in Georgia are the native rabbiteye (*Vaccinium ashei* Reade) and southern highbush (*Vaccinium corymbosum* L.) (Sherm and Krewer, 2003). These berries are grown for both fresh market and processed use. While the majority of commercial blueberry farms are found in southeast Georgia, there are small pick-your-own operations all over the state.

Blueberries have recently become very popular because of their high concentrations of a class of secondary plant metabolites called flavonoids. Flavonoids, which include the pigmented anthocyanins, have become extremely popular with consumers because of their health-promoting benefits. Dietary intake of blueberries has been shown to help reduce the risk of cardiovascular disease and cardiovascular disease-related death (Basu et al., 2010). Increasing berry fruit consumption has been shown to help lower low-density lipoprotein (LDL) oxidation and lower cholesterol. Anthocyanins have also been shown to lower lipid peroxidation in the bloodstream. These secondary plant metabolites also have a high antioxidant capacity which can help prevent certain cancers (Taruscio et al., 2004).

The anthocyanin pathway is regulated by a few key enzymes (Jaakola et al., 2002). Anthocyanin biosynthesis in bilberry (*Vaccinium myrtillus* L.), a close relative of the blueberry, is regulated by the enzymatic activity of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), flavonoid 3'hydroxlyase (F3'H), dihydroflavonol 4-redyctase (DFR), and anthocyanidin synthase (ANS). The evidence suggests that flavonoid biosynthesis happens at two stages during bilberry fruit development, first at flowering and then when the developing fruit begins to darken. At flowering, expression of theses enzymes is very high, after which they gradually decrease. When the skin of the fruit begins to darken, but the inside of the berry is still light, expression peaks again and then decreases as the bilberry continues ripening. This process is supported by evidence from studies with other berry fruit (Castrejón, et al., 2008). However, to the best of the author's knowledge, there is no information correlating the production of pigment with the loss of blueberry firmness. Furthermore, there is little scientific evidence supporting the notion that blueberries retain the capacity to produce blue pigment after harvest.

In general, blueberries do not improve in quality postharvest. They tend to soften quickly, and as a consequence, become damaged during postharvest handling and transportation, and become increasingly susceptible to postharvest rots. Thus, any strategy to increase the harvest firmness of the fruit without compromising postharvest quality would be of great benefit to the industry. Currently, harvest maturity is based primarily on the amount of blue color. If a fruit does not possess adequate color, the fruit is not harvested (hand harvest), or is rejected by sorting line cameras or humans (mechanical harvest). However, based on discussions with growers and personal observation, there is a strong indication that fruit are capable of increasing in color after harvest. If this is truly the case, then it should be possible to harvest prior to full-blue, when fruit are in the midst of accelerated pigment production, but prior to the loss of firmness.

The purpose of the proposed study is to not only determine the relationship between gene expression and flavonoid concentrations, but to also determine these relationships in conjunction with fruit firmness. This study will also determine if fruit have the ability to 'blue-up'

postharvest. Together, this information will potentially allow for an altered harvesting strategy, whereby fruit are harvested more firm, with the knowledge that fruit will continue to produce pigment after harvest, or, in a similar manner, reduce the amount of fruit that are rejected during sorting due to improper color.

Methodologies:

Harvest: Fruit was harvested from the University of Georgia Alapaha Blueberry Research Farm from a 4 year old block of 'Vernon', 'Powderblue', and 'Ochlockonee' plants. Fruit from 'Powderblue' and 'Ochlocknee' was harvested by hand into pails at four different stages of maturity, green, pink, 50-80% blue (immature blue) and blue. Fruit from 'Vernon' was harvested at immature blue and 100% blue. Fruit was transferred into lugs, and held at 14°C in an insulated walk-in mobile cold storage unit for transport to the Vidalia Onion Research Laboratory, located at the University of Georgia, Tifton, GA. Upon arrival, fruit was sorted into 1-pint clamshells prior to the initiation of storage treatments and stored at 0°C for the first night to remove field heat and then stored at room temperature for the rest of the experiment.

Firmness and color: Flesh firmness (g/mm) and size (mm diameter) was monitored using a Bioworks FirmTech II fruit firmness tester (Bioworks Inc., Wamego, KS). A sample of 25 fruit was measured for each repetition. Color was evaluated using a handheld Konica-Minolta CR-400 colormeter, using CIE *L*, a*, b** color space. Color was evaluated on the stem-end and blossom end of the fruit (stem removed). Pictures were also taken daily.

RNA extraction and real-time PCR analysis: Blueberry RNA from different stages of ripeness will be extracted using a modified lithium precipitation technique. Following cDNA synthesis, numerous genes of the flavonoid biosynthetic pathway (PAL, CHS, F3'H, DFR and ANS), as well as genes encoding for cell wall disassembly (PME) will be monitored using an ABI two-step plus real-time PCR system. Primers for each gene will be designed based on sequence homology with flavonoid biosynthetic genes from other systems in the NCBI database.

HPLC analysis of flavonoid content: Anthocyanins were separated and identified using an Agilent 1200 series HPLC (Foster City, CA, USA) system equipped with an inline continuous vacuum solvent degasser, binary pump, temperature controlled autosampler and column compartments, and a photodiode-array detector (PDA), all controlled by Chemstation (rev. B.03.01) software package. Eluted compounds were monitored at 520 nm for the detection of anthocyanins.

Compound Identification and Quantification: Fractions for each peak were collected and analyzed using Bruker Autoflex MALDI-TOF/MS. Ions were compared against published literature (Wu and Prior, 2005) to identify peaks of interest. Anthocyanin content was expressed in cyanidin 3-*O*-galactoside (Ideain chloride, Indofine Chemical Company, Hillsborough, NJ) equivalents.

Sensory Panel: 30 participants were asked to sample 3 'Vernon' blueberry samples. The first 15 participants were given 2 samples of 100% ripe fruit and 1 sample of immature blue fruit. The second 15 participants were given 2 samples of immature blue fruit and 1 sample of 100% ripe fruit. The participants were then asked to pick which sample they thought was different from the others.

Results:

The results from the sensory panel showed that a significant number of participants could tell a difference between the 80% ripe fruit and the 100% ripe fruit ($P<0.0001$). Changes in color over time were also detected (Figure 1). Maturity and day affected firmness ($P=0.0003$ and $P=0.0089$, respectively), with the least mature berries having the highest firmness (Figures 2-3). As time progressed, the fruit stored at room temperature softened. Fruit maturity also affected the anthocyanin concentrations. The less mature fruit had lower concentrations of total anthocyanins than more mature fruit (Figures 4-5). Research on the expression of genes associated with anthocyanin pigment development is currently underway. Genes associated in the flavonoid biosynthetic pathway and in cell wall disassembly have been identified using the blueberry sequence information available with the current PI. Primers will be developed for these genes and their expression will be quantified using quantitative RT-PCR.

Conclusions:

While harvesting fruit at immature blue stage may help improve firmness, consumers were able to tell a difference between the 100% ripe fruit and immature blue. The participants in the sensory panel commented that the immature blue fruit was very "acidic" and "sour" while the 100% ripe fruit was "sweet." The immature blue fruit and pink fruit appeared to turn blue during the time it was stored at room temperature. Most of our findings agree with conventional thought that the more mature a blueberry is, the softer it will be and the more mature fruit will have more anthocyanins than less mature fruit.

Impact Statement:

Since consumers can tell a difference between fruit harvested at immature blue and 100% ripe, it may not be useful for growers to pick the fruit early in hopes of keeping the fruit firmer for a longer period of time. The gene expression work will help advance our understanding of how blueberries develop their characteristic blue color and how the concentrations of anthocyanins affect color development.

Citations: No publications have yet been developed from this study.

Literature Cited

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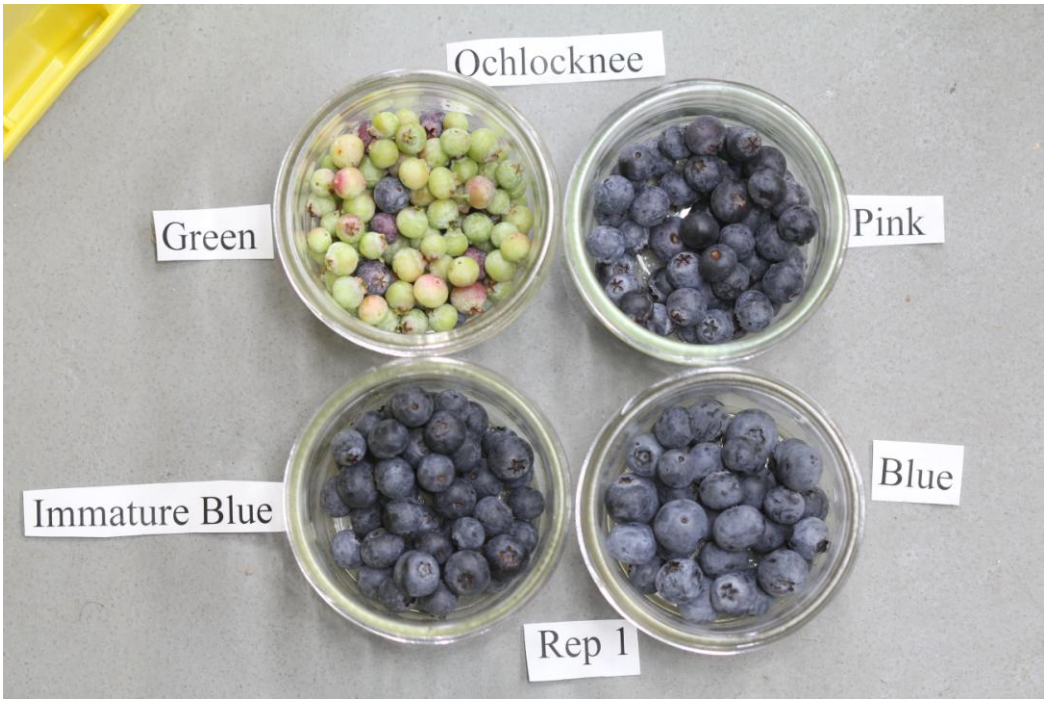
B. 

Figure 1. Blueberry fruit color changes in 'Ochlocknee' over time. A. was taken at harvest and B. was taken 4 days after harvest.

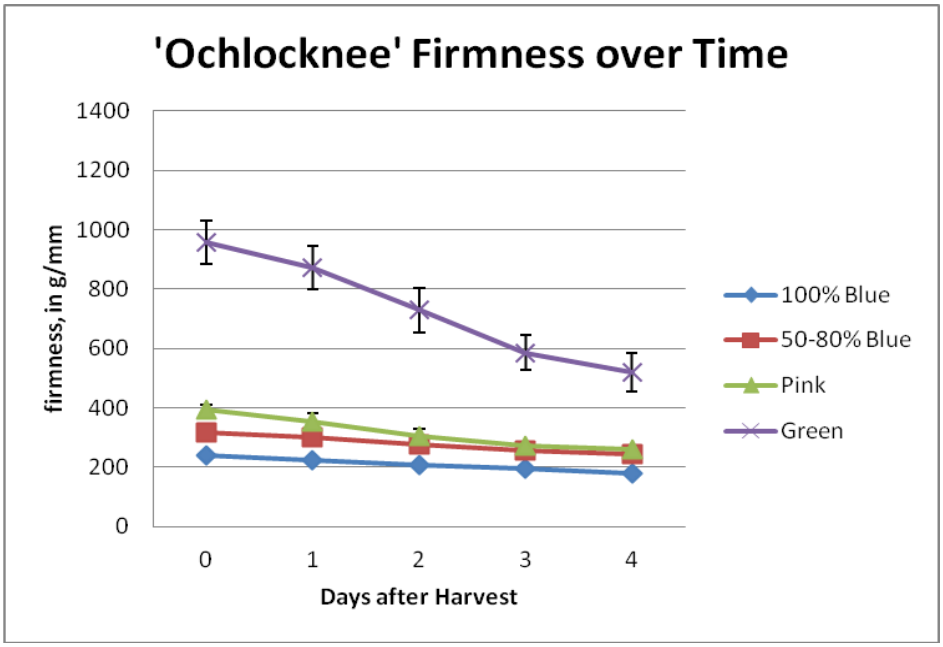


Figure 2. Changes in firmness over time in 'Ochlocknee' fruit. Error bars represent 1 standard deviation.

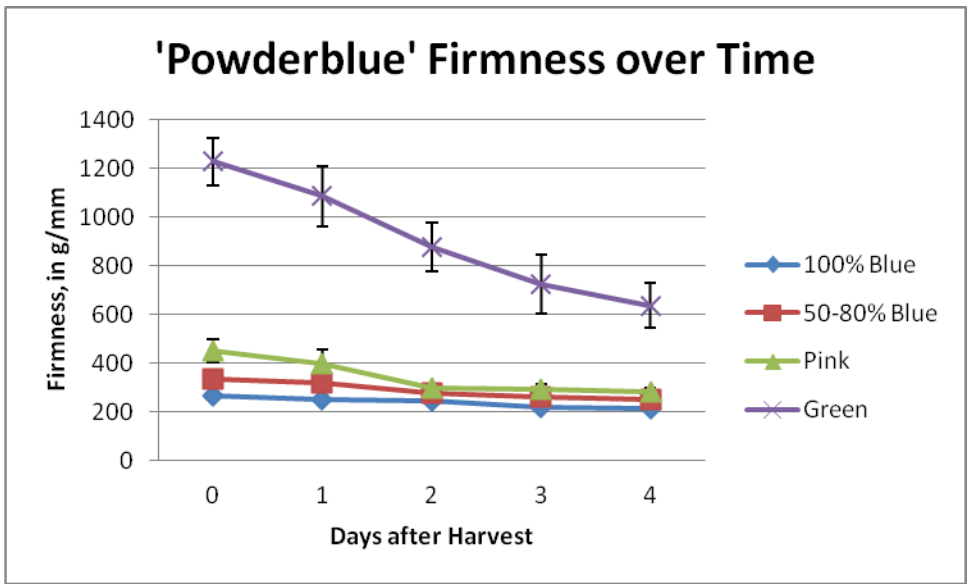


Figure 3. Changes in firmness in 'Powderblue' fruit over time. Error bars reperesent 1 standard deviation.

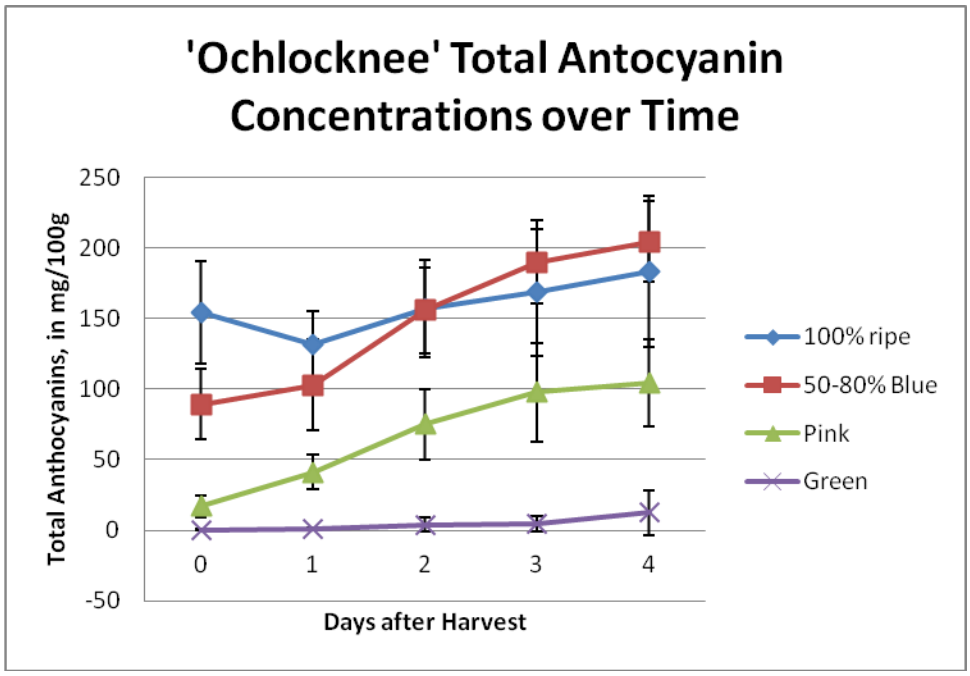


Figure 4. Changes in total anthocyanin concentrations in 'Ochlocknee' fruit over time. Error bars represent 1 standard deviation.

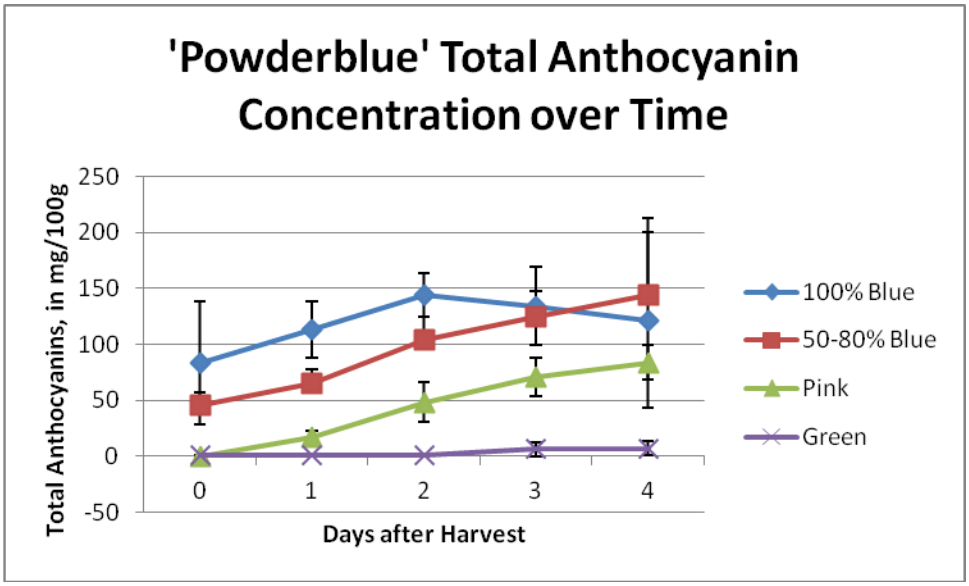


Figure 5. Changes in total anthocyain concentrations in 'Powderblue' fruit over time. Error bars represent 1 standard deviation.