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Title: Color Reversion in Fresh Market Blackberries, SFRC 2012

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Objectives:

1. Determine a systematic way to induce color reversion in fresh blackberry
2. Develop a key that separates color reversion from leak, freeze damage, heat buildup
3. Determine what cellular level changes are occurring in affected drupelets

Justification and Description:
Color reversion (red drupelet) in blackberry happens after storage. Blackberries with full black color are put into low temperature storage (1-2C) for one to 5 days, but when removed have developed clusters of bright red drupelets. Occurrence of this was tracked in University of Arkansas selections as long ago as 1992. At this time, the fresh market blackberry industry was less than 200 ha in the U.S. and Mexico. Red drupelet was added as a rating in selections, but not included in overall ratings (decay and softness were considered much more serious quality attributes for fresh market). However, as acreage of blackberries has dramatically increased, and winter production in Mexico has become a major addition to the US industry, color reversion now represents a large expense for repacking of berries between transit and distribution. Blackberry shippers are not sure if the red color is appearing from leak caused by fruit pack shaking in the truck,

from uneven temperature distribution in refrigerated trucks, or from rapid cooling of susceptible cultivars .
Color reversion appears to be highly related to genetics. Thornless and thorny selections ranked over 3 years showed no significant link to either thorny or thornless background , and no relation in color reversion with overall rating. Named selections do vary widely in reversion, and sometimes the level of the reversion is found only in certain production environments. For instance, Navaho rarely has a problem with color reversion, Tupy often has this problem, and Chester Thornless seems to be yes for some locations, no for others. Additionally, berries harvested at shiny black (more firm than dull black) are more likely to develop color reversion, especially in susceptible cultivars.

Methods:

Plant Material. Blackberries were harvested from research stations in Salisbury and Laurel Springs, North Carolina between May and September of 2012. Ouachita, Von and Navaho were the varieties most often used. Fruit were harvested into clamshells then placed on ice during transport to Kannapolis. Attempts were made to induce color reversion by a) holding clamshells not on ice in a hot vehicle (35-40C) for 1 hour or b) placing individual berries in a -5 C freezer for 10 minutes.

Storage studies. Blackberries were placed at 1 C for 7 to 10 days then rated for decay, leak, softness, and color reversion. Those free of decay were used for compositional analysis.

Compositional analysis. Two types of analysis were done. The first was looking at whole berries from storage studies to look at relationships among compositional variables and ratings. In this portion, samples of 10 berries free of decay were frozen, thawed, and pureed using a Polytron PT1000 equipped with a PT 1500 generator. A 0.5 g sample of puree was extracted with 10 ml solvent and total anthocyanin and phenolic content determined using microplate assays at 510 and 726 nm wavelengths. A 2 g aliquot of puree was mixed with 60 ml distilled water and pH and titratable acidity determined by automated titration (Metrohm). Soluble solids content was determined by placing a 0.5 ml of puree on a digital refractometer (Atago, P100).

The second analysis was done on a per drupelet basis. About 10 berries exhibiting red drupelet were used for Ouachita and Von, the cultivars showing most red drupelet. Red or black drupelets were individually excised using a scalpel and placed into 5 ml tubes. Three to 5 drupelets per color were placed in each tube, frozen at -20 C overnight, thawed, then homogenized using a PT 1200 Polytron equipped with a PT1272 generator. The pH of the puree was determined by inserting a stainless pH probe into puree. A small sample of puree was placed onto a digital refractometer (Atago, P100) using a transfer pipet, then removed from the refractometer back into the 5 ml tube. A 0.1 ml aliquot was weighed into a 2.0 ml centrifuge tube and 0.1 to 0.15 universal solvent added (methanol:water:formic acid at 1:2:0.1) then spun in a microcentrifuge held at 5C and 5,000 rpm for 10 minutes. Supernatants were used to determine total anthocyanins by pH differential (Giusti and Wrolstad, 2001) and total phenolics (Singleton and Rossi, 1965) using a microplate reader.

HPLC analysis. A 0.1 ml extract from individual drupelets (red or black) was filtered with 20 um and injected onto a HPLC (Hitachi Elite LaChrom) equipped with photodiode array detector and Synergi 4u Hydro-RP 80A 250 x4.6 mm column (Phenomenex). Column temperature was 30 C, flow rate of 1 ml/min and a gradient system of methanol and formic acid was used. Anthocyanins were identified using external standards and comparing retention time and spectrum.

Objective 1: We had very little success in inducing color reversion with field fruit. This summer was unusually cool so it was difficult to induce high heat accumulation after harvest; even holding fruit in a vehicle for one hour after harvesting at 2 pm did not induce reddening. Holding berries in the freezer at -5 C for periods of 10 minutes did not change color. We did find more red drupelet in Von (NC430) and in Ouachita after a seven days of storage at 4 C (Table 1). Another unexpected development was the presence of spotted wing drosophila (SWD). Eggs and first instar larva are not visible but can cause damage to drupelets. Ouachita, which ripens before Von, had infestations before sprays were started. Generally Ouachita is not a blackberry that gets much color reversion; it is possible that the increased amounts we saw this year might have been from SWD.

Objective 2: key for color changes
Types of blackberry color relative to color reversion or immature drupelets are shown in Figure 1. Immature drupelets tend to be clustered near the base of blackberry fruit, are very hard, and often have an orange look. Leaky drupelets can look similar to those with color reversion but are more purplish, often look sunken, and appear right after harvest, before cold storage.

Objective 3: We tried to use a microelectrode to get individual drupelet measurements of pH. However, the tips clogged up rapidly and gave erroneous results. Instead, we excised drupelets of Ouachita and Von berries by cutting through the cortex with a scalpel then carefully disengaged drupelets from the cortex under magnification. Three to 5 drupelets per color stage per berry were placed in 5 ml tubes, held at -20 C overnight, then homogenized using a polytron with a dia blade to get maximum amounts of juice. A stainless steel pH probe was used to measure pH and about 0.5 ml was placed on the digital refractometer, then pipetted back into test tubes. A 0.1 ml sample was placed in microcentrifuge tubes, diluted to a total of 1.5 ml using extraction solvent

Stereo microscopy was used to look at the surface and structure of blackberries to look for possible physical rupture. The surface of the red drupelets was more flaccid and in some cases it appeared that there might be damage on the drupelet near the point of attachment to the cortex (Figure 2). There was a strong cuticle around each drupelet that seemed to be acting as a blanket of protection over and around the drupelets, regardless of color. Our next step will be to look at the blackberry surface using confocal microscopy.

Compositional changes in drupelets were distinct for the two cultivars used. Total anthocyanin was 30 to 50% in drupelets classified as having color reversion, compared to fully black drupelets (Table 2). The pH of red drupelets was lower than black drupelets. We noticed that black drupelets had more juice than red drupelets. Soluble solids content, composed of sugars, organic acids, and soluble polysaccharides such as pectins, was not distinctly different among color stages. Total phenolics content was generally lower in black drupelets than in red. Immature drupelets (red and hard) were distinctly different in composition from red-soft drupelets. Immature drupelets had lower SSC, high total phenolics, and half of the anthocyanin of the red-soft drupelets. Drupelets intermediate in color (red-black) generally were more similar to black drupelets, except that anthocyanin content was about 70% that of fully black drupelets. The blackberry cortex tissue was high in pH (3.33 vs 3.10), lower in SSC, and very low in total phenolic or anthocyanin content compared to black drupelets. This data indicates that cortex tissue is in the overripe stage.

Using HPLC, the primary anthocyanin in blackberries was identified as cyanidin-3-glucoside (Table 3), as reported by others. Cyanidin-3-rutionoside and peonidin-3-glucoside were the most prevalent minor anthocyanins found. Red drupelets contained about half of the cyanidin-3-glucoside and peionidin-3-glucoside found in black drupelets. Surprisingly, cyanidin-3-rutionoside values were not different between red and black drupelets. Anthocyanin pigment profiles were similar between black and red drupelets, although relative quantities were lower in red drupelets for several of the pigments.

Conclusions:
Blackberry drupelets exhibiting color reversion were slightly lower in pH and had 50% less total anthocyanin. However, the similar pigment profiles in red or black drupelets, indicates that quantity of pigment rather than a change in type of pigment, is the primary cause of red drupelets. The lower pH of red drupelets may be enhancing the visible red color as well. Soluble solids content and total phenolics content were not well correlated with red drupelets. Immature red drupelets were easy to separate from drupelets with color reversion, having high total phenolic content, low SSC and pH, and very low total anthocyanin content. Inducing color reversion turned out to be much more difficult than we expected and will require new strategies.

References
Giusti, M.M. and R.E. Wrolstad. 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. Current Protocols in Food Analytical Chemistry F1.2.1 – F1.2.13.

Singleton, V.L. and J.A. Rossi. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic reagents. Amer. J. Enol. Vitic. 16*:*144-158.

Impact statement: Color reversion in blackberry drupelets appears when total anthocyanin content of black drupelets is reduced by 50%.

Table 1. Composition and subjective ratings of Von and Navaho after storage.

days storage	**storage temp**	**cultivar**	**% ssc**	**ph**	**% citric acid**	**% mold**	**%leak**	**%soft**	**%red**	**overall**
7	1	Von	12.2	3.79	1.09	3.8	7.0	20.5	25.7	68.7
10	1	Von	11.0	3.82	0.86	2.4	9.4	33.0	39.7	55.1
15	1	Von	11.5	3.91	0.70	6.0	6.9	13.4	28.5	73.7
7	1	Ouachita	10.3	3.49	1.28	2.0	9.0	24.0	32.9	64.9
10	1	Ouachita	10.1	3.67	1.04	5.8	13.4	30.5	45.3	50.3
15	1	Ouachita	9.9	3.65	1.11	6.4	13.0	23.0	26.6	57.6
10	1	Navaho	8.4	3.34	1.28	0.0	15.5	30.4	17.2	54.1
15	1	Navaho	8.7	3.42	1.16	3.0	16.0	32.4	8.9	48.6

Table 2. Drupelet composition of 'Ouachita' and 'Von' blackberries.

Drupelet Color	no. samples	% soluble solids content (SSC)	pH	Total phenolic content (mg/kg gallic acid equiv fwt)	Total anthocyanin content (mg/kg cyanidin-3-glucoside equiv fwt)
			Ouachita		
Hard red (immature)	4	6.0c	2.62b	2289a	87d
Red (color reversion)	15	7.9b	2.68b	1153c	160c
Red-black	9	8.5a	2.84a	1642b	244b
Black	21	8.5a	2.86a	1033d	363a
			Von		
Red (color reversion)	5	8.5a	2.96b	2032b	113c
Red-black	2	8.5a	3.10a	1990b	187b
Black	8	9.0a	3.10a	2272a	247a

Means separated within column and cultivar by Tukey's, P<0.05.

Table 3. Types of anthocyanin pigments in drupelets of Ouachita and Von.

Drupelet color	cyanidin-3-glucoside	cyanidin- 3-rutinoside	cyanidin- 3-glucoside malonyl	peonidin-3-glucoside	total anthocyanin (mg/kg fwt)
			Ouachita		
Red (color reversion)	54.2	0.50	0.01	3.9	65.5
Black	99.3*	0.58	0.09*	5.6*	113.3*
			Von		
Red (color reversion)	33.6	0.43	0	1.5	45.9
Black	89.0*	0.49	0.02	4.5*	105.9*

Means separated within cultivar and column by t-test, P<0.05.

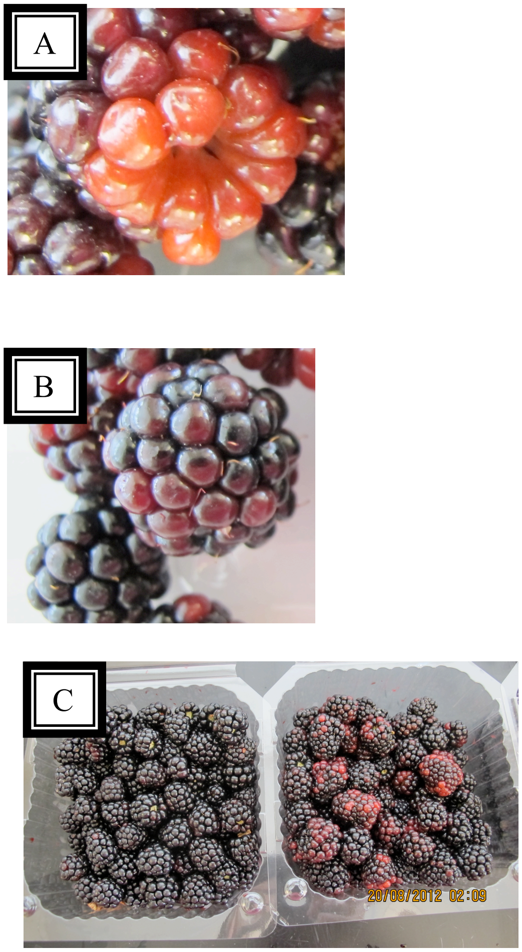

Figure 1. Types of redness in blackberry: immature drupelets (A), leaky drupelets (B) and comparison of Navaho (left) to Von (right) showing blackberries without color reversion next to those with color reversion (C).

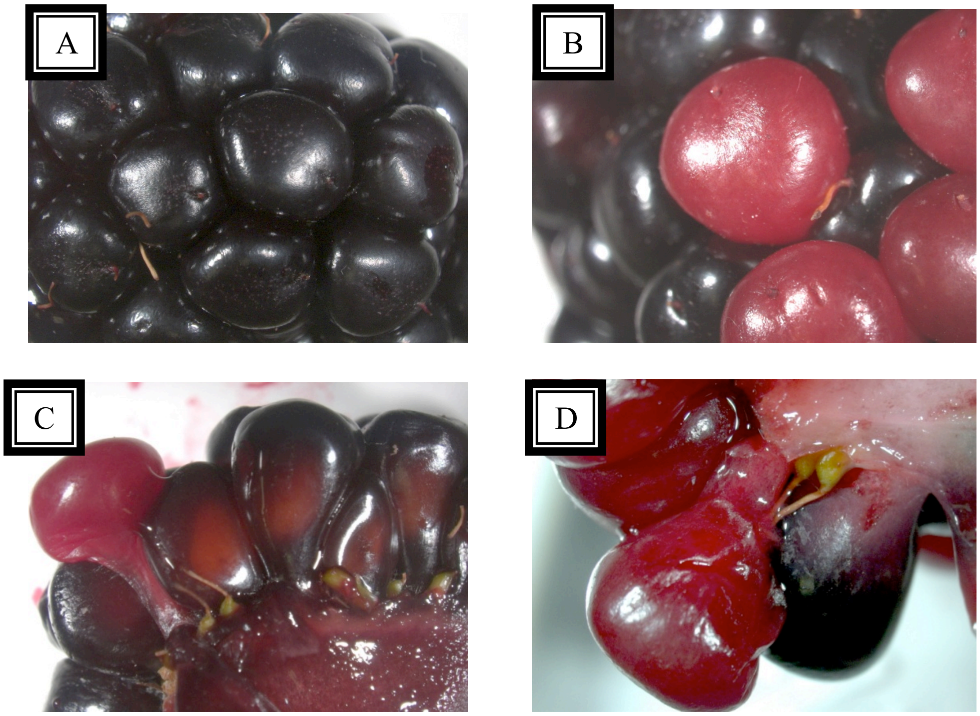

Figure 2. Micrograph of black drupelets (A), red drupelets (B), attachment of black and red drupelets to cortex (C,D).