Title: Evaluation of Red Drupelet Reversion in Blackberries using High-Throughput Digital Image Analysis

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Introduction

The improvement of postharvest quality parameters is an important objective in US blackberry breeding programs (Finn and Clark, 2012). Red drupelet reversion (reddening) is a major postharvest disorder affecting fresh market blackberries (Clark and Finn, 2001). Blackberries often develop red discoloration after they are returned to room temperature following cold storage. This discoloration is perceived as unattractive by consumers and limits fruit marketability (Perkins-Veazie and Clark, 2005).

Genotypic differences in red drupelet reversion were first documented by Perkins-Veazie et al. (1996), who found that 'Navaho' had superior retention of fruit firmness and maintenance of fully-black drupelet color compared to other popular cultivars. The relationship between fruit texture and red drupelet reversion has been further supported by Salgado and Clark (2016), who found that genotypes with novel crispy texture retained firmness in storage and developed less red drupelet reversion than non-crispy genotypes. Only 13% of berries from crispy genotypes experienced color reversion after storage at 5°C and 80% relative humidity for seven days, compared to 41% of berries from standard textured genotypes (Salgado and Clark, 2016).

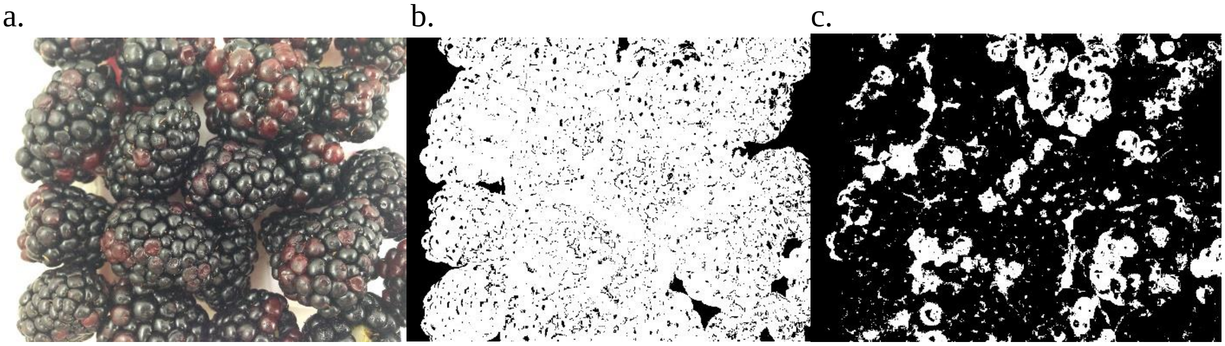
Selection for black color retention cannot be conducted in the field, but is possible through postharvest evaluations (Clark and Finn, 2011). Clark and Perkins-Veazie (2011) developed a subjective postharvest evaluation protocol that is used to characterize advanced selections and commercial check cultivars in the University of Arkansas blackberry breeding program for firmness, visible mold, leaking, and color retention. However, because of the expense and difficulty of postharvest evaluations, fewer than 50 genotypes are evaluated each year.

Given the economic importance of color retention in blackberry and obstacles to large-scale phenotypic selection for the trait, private sector breeders have expressed interest in developing a DNA marker based test for red drupelet reversion (personal communication, Ellen Thompson, Pacific Berry Breeding). However, hundreds of genotypes from large breeding population(s) would need to be evaluated in a successful mapping project. Furthermore, red drupelet reversion is a complex trait affected many environmental factors including temperature during harvest (McCoy et al., 2016) and the maturity of harvested fruit (Perkins-Veazie et al., 1996). Thus, each genotype would need to be evaluated at multiple sites, years, and harvest dates to generate reliable estimates of red drupelet reversion. The existing protocol for postharvest evaluation of red drupelet reversion cannot practically accommodate the thousands of samples that would need to be evaluated in a large mapping project. Additionally, the subjective nature of this rating scale might complicate the combined analysis of sampled handled by different personnel across sites.

Digital image analysis may be an effective option to scale-up phenotyping capacity for red drupelet reversion. The capture and analysis of digital images is rapid, non-destructive, and unbiased (Steddom et al., 2005). Researchers can develop and apply set criteria to evaluate samples in the exact same manner across time and sites, increasing accuracy and reducing sample error variation (Maloney et al., 2014). This methodology is also highly economical as modern digital imaging software such as ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD) is open source and free to use. Digital image analysis has been used for many agricultural applications including quantification of disease symptoms (Steddom et al., 2005; Maloney et al., 2014), insect damage (Mirik et al., 2006), biomass (Bumgarner et al., 2012), and abiotic stress (Berger et al., 2012).

Preliminary data suggests that it is possible to use hue, saturation, and brightness thresholds in ImageJ to estimate total berry area (b) and red drupelet area (c) from overhead photos of clamshells filled with berries (a). These values can then be used to generate percent drupelet reversion estimates for each sample. The objective of this research is to **develop a digital image analysis protocol to estimate percent red drupelet reversion in blackberry and evaluate the usefulness of this protocol by comparing results with standard phenotyping protocols.** If this alternative phenotyping protocol is successful, we hope to apply it in a large breeding population segregating for red drupelet reversion as part of a molecular mapping study.

a. b. c.



Methods

Plant Materials and Harvest

Fifteen advanced selections and cultivars from the University of Arkansas breeding program representing a range of blackberry textures were selected for this study.

Floricane-fruiting: Natchez, Osage, Ouachita, A-2428T, A-2538T, A-2444T, A-2453T, A-2454T, A-2491T, A-2524T

Primocane-fruiting: Prime-Ark™ Freedom, Prime-Ark™ Traveler, APF-77 (Black Magic™), APF-205T, APF-268

Fruit from selected genotypes was harvested from the University of Arkansas Fruit Research Station (FRS) in Clarksville, AR. Floricane fruit was harvested from floricane-fruiting and primocane-fruiting selections and cultivars. Fruit were harvested at "peak" ripeness, when 50% or more of fruit present on plants were at the shiny black stage. For each genotype, two replicate clamshells were harvested from floricanes before noon or before temperatures exceeded 80°F on two separate dates, about a week a part. Only fully black, shiny fruit was harvested. Fruit was harvested into ~½ pint hinged plastic clamshells with absorb pads (Pactiv; as used for fresh market) and placed in vented cardboard flats within a portable cooler with ice packs until all genotypes were harvested for that day. Clamshells were filled so that they were marketable, but not so full that any drupelets were in contact with the lid of the clamshell when closed.

Image Capture

After harvest was complete, fruit was transported to an indoor location where clamshells were photographed in photo box (LimoStudio 16" x 16" Table Top Photo Photography Studio Lighting Light Tent Kit in a Box, AGG349). The photo box was constructed on the countertop with a tripod (Targus Grypton Pro Xl Flexible Tripod with GoPro Hero Attachment) holding an Apple iPhone situated directly above the clamshell to capture a full frame of berries. Default settings for "photo" with the "HDR" setting were used. Three photos of the same clamshell were taken, with the fruit gently tossed between each photograph to reveal different sides of the fruit. Immediately after photos were taken, clamshells were placed in Tupperware containers to maintain relative humidity >80% and refrigerated at 4-5°C for 7 d.

After 7 d of cold storage, clamshells were removed and photographed in the photo box while fruit was still cold. Three photos of the open clamshell, gently tossed between each photograph to reveal different sides of the fruit were taken as before storage. Fruit was subsequently brought to room temperature (RT). Images of RT fruit were captured as before three photos of the open clamshell, gently tossed between each photograph to reveal different sides of the fruit.

Red Drupelet Subjective Evaluation

After all images were captured, each clamshell was subjectively evaluated using two methods; percent reverted berries and percent reverted drupelets. For percent reverted berries, the number of berries in each clamshell was recorded then each berry was individually inspected for reverted drupelets, with a berry having three or more red drupelets being scored as reverted while a berry with two or fewer red drupelets was not scored as reverted following Clark and Perkins-Veazie (2011). For percent reverted drupelets, five berries from each clamshell were selected at random. Each berry was mounted on a toothpick through the abscission scar to aid in viewing berries and examined individually under bright lights. Red drupelets, including fully red and any deviated from standard black toward red, were counted and marked with a paint pen. After red drupelet count, the remaining drupelets were counted for a total drupelet count per berry. Percent reverted drupelets was calculated for each of the five berries per clamshell.

ImageJ

Digital images were analyzed in ImageJ with color thresholds (hue, saturation, and brightness) to measure total berry area (not counting clamshell container edges, shadow, or gaps between berries) and to distinguish between black and red drupelets. ImageJ macros were written to batch process (open, filter, analyze, record, and save data) multiple images in a single program statement. The proportion of red drupelet area to total berry area values were used to calculate percent red drupelet reversion. Two macros were written to accommodate the two different color threshold specifications, one for total berry area and the second for red drupelet area; they are virtually identical the only difference being the hue, saturation, and brightness parameters. To find total berry area HSB thresholds were 0-255, 5-255, and 5-205, respectively. Settings for HSB were 19-235, 16-255, and 0-205, respectively, to find red drupelet area.

Statistical Analysis

Data from FRS was analyzed using PROC GLM in SAS 9.4 (SAS Institute Inc., Cary, NC). Mean separation was tested with Fisher's protected LSD, with harvest date considered nested within genotype. PROC CORR was used to calculate Pearson's correlation coefficients between image-based and visual estimates of red drupelet reversion for each treatment.

Results and Discussion

Results reported below are from genotypes A-2428T, A-2444T, A-2453T, A-2454T, A-2491T, A-2538T, APF-205T, APF-268, Natchez, Osage, Ouachita, and Prime-Ark™ Traveler. Three genotypes (A-2524T, APF-77, and Prime Ark™ Freedom) were not included in analysis as partial clamshells were collected and these genotypes tended to leak during storage which confounded ImageJ results due to its inability to distinguish red juice on the absorb pad from red drupelets.

Significant differences in reversion were found between genotypes using all five methods (percent reverted berry, percent reverted drupelets, image analysis before cold storage (ImageJ original), image analysis immediately after cold storage (ImageJ cold), and image analysis of berries brought back to room temperature after cold storage (ImageJ RT)). For the percent reverted berry method (Clark and Perkins-Veazie, 2011), the percent reverted berries ranged from 1.35% (A-2454T) to 97.62% (APF-205T). For the drupelet count method, the percent of reverted drupelets ranged from 0.37% (A-2454T) to 19.73% (APF-205T). The results generated by ImageJ for each stage, room temperature before cold storage, cold after cold storage, and room temperature after cold storage, revealed the following percent area of red drupelets to total drupelet area: 4.88% (A-2454T) to 15.55% (APF-205T); 7.71% (A-2428T) to 21.04% (APF-205T); and 9.97% (A-2454T) to 25.02% (APF-205T) respectively (Table 1).

There were significant genotype, harvest date, and temperature effects on percent reversion for all three ImageJ analyses (Table 2). Although both genotype and temperature treatment main effects were highly significant ($P < 0.01$), there was no significant interaction between genotype and temperature treatment ($P > 0.05$). Because no significant interaction was found between genotype and temperature treatment, pooled mean separation of genotypes with measurements of cold and room temperature berries is reported here (Table 3).

Estimates of percent reverted berries and percent reverted drupelets for the twelve evaluated genotypes were positively correlated with each other as well as with all three image analysis methods (ImageJ original, ImageJ cold, and ImageJ RT) (Table 4).

Conclusion

In this project we showed that there are significant genotypic differences in red drupelet reversion. The 'crispy' genotypes A-2454T and A-2453T were among the best genotypes in this study, with consistently low reversion for all evaluation methods. This finding was not surprising given 'crispy' types have been reported to dually retain firmness and black color during storage (Salgado and Clark, 2016). The genotype with the highest reversion rates was APF-205T, a soft-textured berry. Interestingly, APF-205T has not expressed severe reversion in the past.

During the 2017 harvest season there were seven rain events which undoubtedly effected post-harvest quality. Seasonal variations in precipitation and temperature likely accounted for the highly significant effect of harvest date on percent reversion. Estimates of reversion could be elevated for some genotypes harvested shortly after rain events, possibly causing those genotypes to appear more disposed to reverting than they would in drier years.

Significant correlations were found for genotypic estimates of reversion using the subjective methods, percent reverted berries and percent reverted drupelets, and data generated by image analysis using ImageJ. We expected to find the highest correlation between subjective methods and analysis of images of berries at room temperature after cold storage. Interestingly, we found a stronger correlation between the subjective methods and the ImageJ original images taken before cold storage. This finding was unexpected because the subjective methods were carried out on day seven whereas the original images were captured on harvest day within one to three hours of harvest. It is possible that leakage impacted the results for day seven images or some immediate post-harvest reversion took place. Reversion has been observed to completely occur within 24-48 hours of harvest. Thus, it may be possible that reversion was already beginning during the few hours of storage on cool ice packs immediately after harvest before preliminary photographs were taken.

For future years, we intend to make adjustments to improve the rapid image analysis protocol and produce stronger results. This experience has revealed that ImageJ is sensitive to shadows and background colors, and when presented with non-uniform images does not operate ideally. With the base macro, ImageJ had difficulty in accurately calculating areas for partially filled clamshells and clamshells where the fruit leaked red juice on the white absorb pad. To minimize these issues next season, some options include: using only full clamshells or writing a separate macro for partially filled clamshells and using green paper under fruit to provide a stark contrast between fruit and background. Using a different background will likely mitigate falsely perceived color areas, such as red juice or dark shadows in ImageJ. Another way to potentially reduce shadow would be to photograph a single layer of fruit.

This protocol has the potential to be expanded or modified for other applications, including white drupelet analysis in blackberry, or characterizing color differences or postharvest disorders in other fruit crops. The ability to utilize ImageJ for quantifying red drupelet reversion would allow more genotypes to be analyzed without bias in a reasonable timeframe compared to subjective methods.

Table 1. Mean reversion (%) of subjective methods (reverted berries and reverted drupelets) and ImageJ analysis at different temperature treatments (original, cold, room temperature (RT)).

Genotype	N	Reverted berries	Reverted drupelets	Original	Cold	RT
APF-205T	4	97.62 a[z]	19.73 a	15.55 a	21.04 a	25.02 a
A-2444T	4	90.79 a	14.88 ab	13.09 ab	16.39 bc	18.64 bc
A-2538T	4	62.51 b	14.24 ab	9.61 bc	15.46 bcd	21.66 ab
APF-268	4	55.94 b	11.88 b	14.23 a	11.22 def	10.22 d
Ouachita	4	52.18 b	13.86 ab	8.60 cd	19.49 ab	23.69 ab
Natchez	4	35.18 c	3.49 c	5.30 d	11.51 def	13.88 cd
Osage	4	23.33 d	4.76 c	7.71 cd	14.58 cde	15.14 cd
A-2428T	4	17.23 de	4.48 c	7.18 cd	7.71 f	12.18 d
PA Traveler	4	14.52 def	1.44 c	8.08 cd	10.14 ef	13.95 cd
A-2491T	4	8.72 efg	0.89 c	5.19 d	9.46 f	11.90 d
A-2453T	4	4.58 fg	1.62 c	5.56 cd	12.13 cdef	11.20 d
A-2454T	4	1.35 g	0.37 c	4.88 d	7.71 f	9.97 d

[z] Means followed by the same letter are not significantly different (α=0.05).

Table 2. Mean reversion (%) between ImageJ temperature treatments original, cold, and room temperature (rt).

ImageJ temperature treatment	N	Reversion (%)
Rt	48	15.62 a[z]
Cold	48	13.18 b
Original	48	8.75 c

[z] Means followed by the same letter are not significantly different (α=0.05).

Table 3. Mean reversion (%) of genotypes from ImageJ analysis of images captured after cold storage with both cold and room temperature (RT) berries.

Genotype	N	Reversion (%)
APF-205T	8	23.03 a[z]
Ouachita	8	21.59 ab
A-2538T	8	18.56 bc
A-2444T	8	17.51 cd
Osage	8	14.86 de
Natchez	8	12.70 ef
PA Traveler	8	12.05 ef
A-2453T	8	11.66 ef
APF-268	8	10.72 f
A-2491T	8	10.68f
A-2428T	8	9.94 f
A-2454T	8	9.50 f

[z] Means followed by the same letter are not significantly different (α=0.05).

Table 4. Pearson correlation of means for reverted berries (%), reverted drupelets (%), and percent reversion (%) for image analysis before cold storage (ImageJ original), immediately after cold storage (ImageJ cold), and room temperature berries after cold storage (ImageJ RT).

	Reverted berries	Reverted drupelets	ImageJ original	ImageJ cold	ImageJ RT
Reverted berries		0.96**	0.88*	0.79*	0.76*
Reverted drupelets			0.89*	0.84*	0.82*
ImageJ original				0.61*	0.53NS
ImageJ cold					0.92**
ImageJ RT					

**Significant at p<0.001
*Significant at p<0.05
NS Not significant at p>0.05

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