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Studies in Blackberry: Development and Implementation of a Phenotyping Protocol for Blackberry Seedling Populations and Impact of Time of Day of Harvest on Red Drupelet Reversion for University of Arkansas Blackberry Genotypes

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Studies in Blackberry: Development and Implementation of a Phenotyping Protocol for
Blackberry Seedling Populations and Impact of Time of Day of Harvest on Red Drupelet
Reversion for University of Arkansas Blackberry Genotypes

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Horticulture

by

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Abstract

There are two major public blackberry (*Rubus* L. subgenus *rubus* Watson) breeding programs in the United States: one at the University of Arkansas (UA) and another at the U.S. Department of Agriculture Agricultural Research Service Horticultural Crops Research Unit (USDA-ARS HCRU) based in Corvallis, OR. The germplasm and breeding objectives of these two breeding programs are diverse, but frequent collaboration necessitated a standardized method of characterizing plant and fruit traits. A phenotyping protocol for blackberry was developed at UA and implemented for two years on UA seedling populations. The protocol included plant traits (health, vigor, estimated crop load, peak bloom date, and number of canes) and fruit compositional traits (firmness by compression, berry weight, soluble solids content, pH, and titratable acidity). Multivariate analysis indicated that quantitative measurements, rather than qualitative, were best for phenotypic resolution, particularly for specific traits contributing to larger overall characteristics. Fruit compositional traits were evaluated on juice from one year from populations of blackberries grown at UA and USDA-ARS HCRU, and included soluble solids content, pH, titratable acidity, and organic acids and sugars. Analysis of variance of fruit compositional traits indicated that population means in UA material had more significant differences for several attributes when compared to USDA-ARS HCRU material. Generally, UA seedlings had lower soluble solids content, higher pH, and lower titratable acidity as well as acid content than USDA-ARS HCRU seedlings. Another interesting attribute of fresh-market blackberries is red drupelet reversion, a postharvest phenomenon in which drupelets on the berry turn red. Nine UA advanced selections and cultivars were harvested at different times of days and evaluated for weight lost by berry, change in firmness, and incidence of red drupelet reversion after storage. Results indicated that a novel “crispy” genotype performed better than

other genotypes in regard to red drupelet reversion. However, for both change in firmness and incidence of reversion, a significant genotype and harvest time interaction effect was observed. Overall, it is suggested that growers harvest blackberries at earlier, cooler times to avoid the postharvest disorder, regardless of genotype.

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Overall Introduction

Blackberry (*Rubus* L. subgenus *rubus* Watson) is a member of *Rosaceae*, which encompasses many horticulturally prominent crops, including apples (*Malus domestica* Borkh.), peaches (*Prunus persica* (L.) Batsch), pears (*Pyrus communis* L.), sweet (*Prunus avium* L.) and tart cherries (*P. cerasus* L.), strawberries (*Fragaria x ananassa* Duch.), and roses (*Rosa* L.).

Blackberries are cultivated globally, with North America being the largest producer by weight (Kaume et al., 2012). In 2014, U.S. blackberry production was valued at \$50.1 million, an increase of \$6.9 million from 2011 (Giesler, 2015). This figure was comprised of: \$45.2 million from processed blackberries and \$4.91 million from fresh-market blackberries (Giesler, 2015). In the United States, 75% of production occurs in the Pacific Northwest, 90% of which is for processed products. Fresh-market blackberry production has seen substantial growth, particularly in California and the South (Clark and Finn, 2011).

Interest in blackberry has increased due in part to several studies documenting high *in vitro* antioxidant activity in comparison to other fruits (Kafkas et al., 2006; Kaume et al., 2012). The potential nutraceutical benefits of blackberry consumption include reducing the risk of diseases associated with oxidative stress to human cells, such as cancer and heart disease (Kaume et al., 2012).

Cultivated blackberries are almost always derived from interspecific crosses and thus lack a common species epitaph (Clark and Finn, 2011). Several foundational crosses that preceded the current U.S. Department of Agriculture-Agricultural Research Service Horticultural Crops Research Unit (USDA-ARS HCRU) blackberry breeding effort in Corvallis, OR included material from *R. ursinus* (Focke), *R. armeniacus* (Focke), red raspberry (*R. idaeus* L.), wild plants from the *Rubus* genus, and New Zealand germplasm (Finn and Strik, 2016). Additionally,

commercially relevant blackberries vary in ploidy level: most erect and semi-erect plants are tetraploids, while trailing plants typical of western U.S. cultivars exhibit hexaploid or greater ploidy levels (Meng and Finn, 2002).

The genetic diversity found within currently available blackberry breeding germplasm illustrates not only the potential for improvement in most characteristics, but also the challenges for manipulating the inheritance of horticulturally important traits and enabling marker-assisted breeding techniques.

RosBREED is a USDA-Specialty Crop Research Initiative-funded grant in its second phase, RosBREED 2, and focuses on eight rosaceous crops: apple, peach, pear, sweet and tart cherries, rose, strawberry, and blackberry. The project aims to enable marker-assisted breeding techniques for combining horticultural quality with disease resistance in new rosaceous cultivars through high throughput genotyping and high quality, standardized phenotypic data (Iezzoni et al., 2010). Unlike row and vegetable crops, most rosaceous crops are grown as perennials and cultivar development using traditional breeding techniques is an extensive endeavor; for blackberries, the timeline from initial cross to cultivar release often takes between nine and 17 years (Clark and Finn, 2011). DNA-informed or marker-assisted breeding expedites the traditional breeding timeline, using sequence information to identify quantitative trait loci (QTL) and associated molecular markers whose nucleotide-level polymorphisms are predictably and consistently associated with changes in phenotype. Blackberry has limited molecular data and while several phenotypic data sets exist, they are not comparable due to their incongruent protocols.

In the United States, there are two major blackberry breeding programs: one at the University of Arkansas (UA) in Fayetteville, AR, and one at the USDA-ARS HCRU (USDA-

ARS) in Corvallis, OR. These two programs address different sectors of the blackberry market, with the USDA-ARS program focused on producing fruits primarily for processing and the UA program focused on the development of fresh-market blackberries (Clark and Finn, 2011; Finn and Strik, 2016). In addition to differing ploidy levels, the two programs contrast in target market, sources of genetic material, and environment, resulting in distinct fruit characteristics. The USDA-ARS processing blackberries generally have higher acid and sugar content and more powerful aromatic profiles than the UA fresh-market blackberries, which place greater emphasis on postharvest storage and transportation capacity (Clark and Finn, 2011; Finn and Strik, 2016). Despite these phenotypic differences and their contributing factors, both the USDA-ARS and UA programs are members of the RosBREED blackberry team, and their respective materials should be characterized under the same standardized protocol for RosBREED QTL and molecular marker analysis.

The implementation of marker-assisted breeding requires high quality phenotypic data to most effectively utilize the resolution offered by high throughput genotyping. Phenotype is the manifestation of genotype, environment, and time interactions; the minimization of confounding environmental and temporal factors yields the most accurate, “true” phenotype of an individual instead of measuring the individual’s ability to mitigate external stresses. Due to their perennial life cycles and generally large growth habits, rosaceous crops are primarily grown in the field environment where control over environment and yearly fluctuations is minimal. Consequently, protocols for phenotyping in *Rosaceae* should be standardized to reduce the additional variance from user error and differences in field and laboratory equipment.

Though RosBREED aims to combine horticultural quality with disease resistance, the blackberry project in RosBREED focuses on improving consistency of fruit characteristics. In

blackberry, improving fruit quality while maintaining high yield is a main breeding objective as consumers tend to prefer sweeter berries with more flavor (Clark, 2005; Finn and Strik, 2016).

Investigating the compositional elements that contribute to overall fruit quality and sensory perception is an avenue that has notably been pursued in crops such as strawberry and apple and yielded gene candidates and functional markers for individual sugars, acids, and aromatic compounds (Chambers et al., 2014; Guan, 2013; Schwieterman et al., 2014).

The breeding program at UA produces blackberry cultivars mainly intended for the fresh market, in which postharvest quality is of great importance. The capacity a cultivar holds for maintaining structural and compositional integrity is a determining factor of its marketability and consequently its potential for becoming a successful commercial product. At UA, postharvest quality has been explored in several ways including berry firmness, changes in juice chemistry over storage, red drupelet reversion (RDR) and variability amongst genotypes (Kim et al., 2015; Perkins-Veazie et al., 1999; Salgado and Clark, 2016; Segantini et al., 2017). A recently increased concern is RDR, a postharvest disorder in which black drupelets become irreversibly red or purple after harvest. This discoloration can negatively impact consumer perception and marketability. Though the causal mechanism is not known, it has been proposed that RDR is an effect of harvest time, temperature, and physical damage to fruit. Limited work has been conducted in RDR, and understanding how genotypic and environmental factors influence the disorder could give growers practical information on mitigation.

Objectives

1. To develop and implement a phenotyping protocol for blackberry plant and fruit quality characteristics on six UA biparental seedling populations.
2. To implement the phenotyping protocol for fruit composition on six UA and eight USDA-ARS biparental seedling populations.
3. To determine the effects of harvest time of day on RDR in nine UA blackberry advanced selections and cultivars.

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Literature Review

Blackberry as a cultivated crop

Blackberry (*Rubus* L. subgenus *Rubus* Watson) is a specialty fruit crop that has experienced increasing interest in the past 10 years (Clark and Finn, 2011; Strik et al., 2012). Blackberry is cultivated globally, with North America being the largest producer by weight and Europe the largest producer by area planted (Finn and Clark, 2011; Kaume et al., 2012; Strik et al. 2012). Recently, production area in the United States has expanded due to large operations in California, Georgia, North Carolina, Arkansas, and Texas (Clark et al., 2007; Clark and Finn, 2011). From the 1980s to early 1990s, Chilean blackberry production was substantial, but the formidable cost of transporting these berries by air freight contributed to the decline of South American production. Beginning in the 1990s, blackberry production saw significant and steady increase in Mexico. This transition can be attributed to two main factors: improvements in blackberry management, particularly for artificially inducing flowering and fruiting, and improved postharvest characteristics of new cultivars which could be transported more cost-effectively by truck (Clark et al., 2007). Blackberry production from Mexico is especially important for supplying the United States and other countries with more temperate climates with fruit in the winter months, as these blackberries are produced between October and June (Clark et al., 2007).

Fresh blackberries appeal to consumers due to several factors, including consistent availability and distinct flavor (Threlfall et al. 2016). In addition, interest in blackberry has increased due in part to several studies documenting high *in vitro* antioxidant activity in comparison to other fruits (Kafkas et al., 2006; Kaume et al., 2012). The potential nutraceutical benefits of blackberry consumption include reducing the risk of diseases associated with

oxidative damage to human cells, such as cancer and heart disease (Esselen et al., 2011; Kaume et al., 2012; Overall et al., 2017; Sarkar et al., 2016).

Eastern and western U.S. blackberries

In the United States, blackberries are largely grown in the Pacific Northwest, which experiences warm dry summers with cool nights. These conditions are optimal for blackberry production and are conducive to high fruit quality (Finn and Strik, 2016). About 90-95% of the region's production volume is sold for processing (Finn and Strik, 2016). Though blackberry breeding in the Pacific Northwest can be traced to the late 1850s with Seth Lewelling's 'Lawton' blackberry, the U.S Department of Agriculture (USDA) breeding effort that continues to this day began in 1927 in Oregon with Dr. George Darrow, who focused on amassing *Rubus* germplasm. Some foundational USDA crosses were made from this collection, using wild material, *R. ursinus* Focke, *R. armeniacus* Focke, and red raspberry (*R. idaeus* L.) (Finn and Strik, 2016). More recently, germplasm material from New Zealand (Plant and Food Institute of New Zealand) and the breeding program at the University of Arkansas (UA) were incorporated into the USDA-Agricultural Research Service, Corvallis (USDA-ARS) breeding program. The diverse germplasm contributing to the USDA-ARS blackberry material can be seen in that individuals are generally hexaploid or greater, with trailing or semi-erect architecture (Clark, 2016; Finn and Strik, 2016). The combination of ideal growing conditions and genetic variation from across *Rubus* lends to the distinctive and sought-after flavor profiles of western blackberries (Clark, 2016; Clark et al., 2007).

Fresh-market blackberries constitute a smaller portion of the blackberry market in the United States. In 2014, \$4.91 million of \$45.2 million garnered from blackberry sales were from

fresh-market sales (Geisler, 2015). However, promising advances in genotype-specific postharvest storage potential are likely to aid in the expansion of the fresh-market blackberry industry

The UA serves as the eastern counterpart to the USDA-ARS breeding efforts in Corvallis, OR, producing genotypes primarily intended for fresh-market fruit, with an emphasis on erect architecture, increased sweetness, reduced acidity, improved postharvest storage potential, and as of the 1990s, primocane fruiting (Clark, 2016). Blackberry cultivar development began at UA under Dr. J.N. Moore in 1964, emphasizing improvement of the native plant by targeting larger fruit size, high fruit quality, thornlessness, increased productivity, and erect cane architecture. Moore used the New York-developed ‘Darrow’, Texas-developed ‘Brazos’, and USDA-ARS germplasm as the foundational material for his breeding program (Clark, 2016). An important decision made by Moore was to pursue and maintain breeding at the tetraploid level, including the incorporation of ‘Merton Thornless’ as the UA source of thornlessness (Clark, 2016).

Blackberry plant characteristics

Cultivated blackberries are usually derived from two or more species of *Rubus* and as such demonstrate wide phenotypic variation (Clark et al., 2007; Finn and Clark, 2012). Blackberry plants are perennial with biennial canes; from emergence through the first year of growth, canes are referred to as primocanes and in the second year they are referred to as floricanes, which senesce after fruiting in the second growing season (Finn and Clark, 2012). Most blackberry cultivars are floricanes fruiting, with flowers and fruit developing only on second-season wood. However, breeding for primocane fruiting has received increased attention, beginning in the 1990s at UA. Primocane-fruiting blackberries flower on both first and second-

year canes, giving growers the opportunity for both producing a crop on first-year plants, and also the “insurance” of a crop if late spring freezes affect florican bloom.

The primocane-fruited trait was first described in ‘Hillquist’, a wild genotype from near Ashland, VA. An initial UA cross of ‘Brazos’ × ‘Hillquist’ in 1967 gave rise to the material from which the primocane-fruited trait has been incorporated in UA primocane-fruited genotypes (Clark, 2016).

Cane characteristics also vary in architecture and thorniness. Cultivated blackberries range from trailing to semi-erect to erect, with trailing genotypes growing prostrate to the ground and erect genotypes displaying strongly negative gravitropism (Clark et al., 2007; Strik et al., 2012). Thorniness, or more accurately the presence of botanical spines, is a characteristic nearly synonymous with wild blackberry “brambles” and negatively impacts labor costs for hand-harvested blackberries and marketability of machine-harvested blackberries (Clark et al., 2007). The thorniness of a blackberry can range from completely smooth to long, thickly clustered spines on both canes and petioles. There are four main single genetic sources of thornlessness that have been identified, including ‘Merton Thornless’ (recessive gene *s*), ‘Austin Thornless’ (dominant gene *S_T*), non-chimeral derivatives of ‘Thornless Evergreen’ (undetermined inheritance of gene *S_{TE}*), and ‘Lincoln Logan’ (dominant gene *S_L*) (Clark et al., 2007; Finn and Clark, 2012). As mentioned previously, ‘Merton Thornless’ was selected as the source of thornlessness for UA (Clark, 2016).

Some major pests and diseases of note for blackberry production include: anthracnose (*Elsinoe veneta* [Burkholder] Jenk.), cane botrytis and botrytis fruit rot (*Botrytis cinerea* Pers.: Fr.), cane blight (*Leptosphaeria coniothyrium* (Fuckel) Sacc.) and orange cane blotch (*Cephaluros virescens* Kunze ex E.M. Fries) while notable pests include: raspberry crown borer

(*Pennisetia marginata* [Harris]), red-necked caneborer (*Agrilus ruficollis* [Fabricius]), strawberry weevil (*Anthonomus signatus* Say), brown and green stink bugs (*Euschistus* spp. and *Acrosternum hilare* Say, respectively), Japanese beetle (*Popillia japonica*, Newman), thrips (*Frankliniella tritici* Fitch and *F. occidentalis* Pergande), and spotted wing drosophila (*Drosophila suzukii* Matsumura) (Clark, 2016).

A number of virus diseases affecting *Rubus* are documented in blackberries. Blackberries are vegetatively propagated for both home garden and commercial operations, and are susceptible to viral diseases during initial propagation, vegetative growth, and production (Martin et al., 2013). At least 30 well-documented viruses have been observed on *Rubus* throughout North and South America, Europe, Asia, Australia, and New Zealand, and can be transmitted via pollen, seed, nematodes, and a number of arthropods (Martin et al., 2013). Some of these diseases primarily affect vegetative structures, indicated by symptoms such as mottling, severe yellowing, leaf distortion, while some two-plus virus complexes result in drupelets crumbly, unharvestable fruit (Martin et al., 2013).

One blackberry-associated virus of particular significance is blackberry yellow vein associated virus (BYVaV) (Poudel et al., 2013). In the southeastern United States, BYVaV is of serious concern, as it is the most prevalent of viruses associated with blackberry yellow vein disease (BYVD). Floricanes affected by BYVD experience yellowing of veins, irregular-patterned chlorosis, oak-leaf and line patterns, and ultimately cane dieback during fruiting (Poudel et al., 2013). Blackberry plants affected with BYVD require replanting after five to seven years to remain economically viable to growers, while healthy blackberry plants unaffected by the disease can remain productive for 20 or more years (Martin et al., 2013). Most available blackberry cultivars fruit most heavily on floricanes, and dieback of floricanes during

the fruiting season is highly detrimental to yield. This virus is transmitted by whiteflies (*Trialeurodes* Cockerell), which are highly mobile; strict management of these vectors is necessary for minimizing BYVD-induced losses in blackberry (Poudel et al., 2013).

A newly discovered virus, currently named blackberry leaf mottle-associated virus (BLMaV) was found in cultivated and wild blackberries affected with BYVD in several U.S. states, including Arkansas and Oregon (Hassan et al., 2017). The vector for BLMaV appeared to be mites (*Eriophyidae* Nalepa), though the particular species or genus was determined to possibly belong to a previously unidentified species or genus (Hassan et al., 2017).

Another virus of concern for blackberry is blackberry chlorotic ringspot virus (BCRV), which is pollen and seed-transmitted, without the need of a biotic vector (Poudel et al., 2014). Though unaccompanied BCRV infections do not cause detrimental symptoms in blackberry, dual infections with raspberry mosaic virus and/or BYVD may contribute to the development of disease (Poudel et al., 2014).

As with other fruit crops, the main concerns of blackberry in relation to abiotic stresses are associated with losses in fruit set and quality. Extreme temperatures pose the most threat to successful blackberry crops. Cold winter temperatures of -15 °C and lower can result in cold damage and cane dieback, reducing fruit potential on floricanes; late spring frosts can kill flower buds or damage flowers, resulting in reduced bloom, vigor, and yield (Clark et al., 2012). Daytime temperatures meeting or exceeding 29.4 °C tend to inhibit normal drupelet development and may suppress or mask primocane-blooming habit and bearing depending on the severity of the temperature (Stanton et al., 2007).

Blackberry fruit characteristics

Blackberries are aggregate fruits composed of a fleshy central torus surrounded by juicy drupelets, each containing a single pyrene, usually referred to as a seed. The torus abscises from the receptacle, a morphological characteristic distinguishing blackberry from raspberry.

Blackberry fruits are borne panicle-like with primary fruits ripening first followed by secondary fruits and other berries on the inflorescence (Clark and Finn, 2011).

The physical appearance of fresh-market fruit is a major component of consumer acceptability. With strawberries (*Fragaria × ananassa* Duch.), fruit shape, symmetry, malformation from unfilled achenes, achene color and position, external color, gloss, cap size, calyx position, internal fruit color, and depth of internal fruit color are all visual characteristics of interest for breeding purposes (Mathey et al., 2013). Similarly, for apple (*Malus domestica* Borkh.), ground color, over-color type, blush color and coverage, fruit shape, calyx opening, sunburn, greasiness, and russet coverage and location are traits of interest for breeding-oriented phenotyping (Evans et al., 2011). In sweet (*Prunus avium* L.) and tart cherries (*P. cerasus* L.), flesh color, skin color, and fruit dimensions are measured quantitatively for standardized phenotyping (Stegmeir et al., 2011). Peach (*P. persica* [L.] Batsch) fruit appearance is characterized by pubescence, blush, ground color, flesh color, redness of flesh, and diameter (Frett et al., 2012). Most fruit phenotyping protocols and data sets include measurements for size, symmetry, color description, and species-specific characteristics (e.g. pubescence for peaches and russetting for apples). For fresh-market blackberry, desired visual characteristics include even drupelet set, symmetry, uniform color, size, shape, and shininess.

While large fruit size is a common goal of blackberry breeders, there is usually an upper limit of approximately 10 g for fresh-market blackberries due to ease of packing full clamshells

(Finn and Clark, 2012). However, very large berries can have postharvest limitations and reduced marketability (Clark, 2005). Consequently, breeding objectives for blackberries may not necessarily be for larger fruit so much as fruit sensory qualities important to consumers. One of the most important characteristics of improvement for blackberry is fruit flavor, which is multiplex and generally divided into acidity, aromatic content, astringency, bitterness, and sweetness (Clark et al., 2007; Clark and Finn, 2011, Finn and Clark, 2012). Clark (2005) suggested that meeting consumer preferences, especially by increasing sweetness, is important to expanding the blackberry market.

Because blackberry has not had as much genetic improvement and has a smaller market presence than strawberry, fewer data sets exist for comprehensive characterization of blackberry fruit quality. However, both are members of *Rosaceae*, and share several fruit quality similarities; blackberries and strawberries are both noted for their distinctive “berry” flavors, balance between sweetness and tartness, delicate postharvest qualities, and high phytochemical content.

Cultivated strawberries are a small fruit crop that has been extensively studied, and popular cultivars are favored for their large size, attractive red color, agreeable texture, distinct aroma, and characteristically “fruity” flavor (Schwieterman et al., 2014). As with most “berry” fruits—strawberries, red raspberries, and blueberries (*Vaccinium* L. spp.) being other examples—sugars are not necessarily the most significant contributors to flavor profile. Secondary metabolites that influence acidity, astringency, and aroma are often considered just as important as total sugars in consumers’ perception of “berry” flavor and palatability (Kafkas et al., 2006; Mahmood et al., 2012). When considering texture, sweetness, sourness, flavor, harvest time, total sugars, titratable acidity (TA), and total volatiles of 35 strawberry accessions,

Schwieterman et al. (2014) found that overall consumer liking was most highly correlated with sweetness intensity ($R^2 = 0.742$, p-value <0.001), followed by flavor intensity ($R^2 = 0.604$, p-value <0.001), texture liking ($R^2 = 0.490$, p-value <0.001), and total sugars ($R^2 = 0.489$, p-value <0.001).

Soluble solids content (SSC) includes sugars, organic and amino acids, soluble pectins, and other compounds (Garner et al., 2014). In fruit juice, sugars contribute the most to SSC and this measurement can be used to estimate overall sugar content. However, sweetness is a broad quantitative trait that can be broken down into individual sugars and when applicable, sugar alcohols, glycosides, proteins, and other compounds. Interestingly in strawberry, total sugar content only accounted for approximately two-thirds of sweetness intensity variation, and total volatile content was positively correlated with sweetness intensity, accounting for up to 13.9% of sweetness intensity variation (Schwieterman et al., 2014).

The percent TA is usually used as a standard measure for acid content of fruit juice, and is a measurement of the amount of acid that can be neutralized with the addition of a base with known concentration. However, like SSC, it is an estimation of a broad quantitative trait and both acids and other secondary metabolites such as volatiles affect the experience of acidity or tartness. Additionally, the biosynthesis of secondary metabolites from primary metabolites such as sugars, fatty acids, and amino acids is upregulated during the later stages of ripening (Fait et al., 2008). The relationships between sugars, acids, and other phytochemicals illustrates that manipulation of fruit flavor and quality is a nuanced process.

Guan et al. (2013) found that in apple, the ratio of sugar to malic acid concentration was highly correlated with sensory sweetness, which is in agreement with findings in blackberry (Mikulic-Petkovsek et al., 2012). Guan (2013) further examined these relationships through

genetic analysis, identifying quantitative trait loci (QTL) highly associated with each sugar and sugar alcohol. This identification could be important to establishing genetic markers for future use in marker-assisted breeding in for rosaceous crops.

Blackberry postharvest

Except for pick-your-own operations, fresh-market blackberries are subjected to cold storage before shipping, distribution, and consumption. Beginning in 1989 with ‘Navaho’, followed by ‘Arapaho’, and ‘Apache’, the UA blackberry breeding program released cultivars with postharvest storage potential for shipping (Clark, 2005; Clark et al., 2007). Starting in the late 1990s, blackberries have been consistently and increasingly imported in large quantities from South America and Mexico to supply the United States market with fresh blackberries during winter months (Clark, 2005; Finn and Clark, 2011). Prior to the late 1990s, more limited quantities of blackberries were often shipped by air from Chile. After cultural management practices were developed to allow blackberry production particularly in Central Mexico, the resulting trucking of the fresh fruit from Mexico supplanted air transport of berries from Chile (Finn and Clark, 2012). An additional consequence of increased production in Mexico was the greater presence of blackberries as a common grocery store fruit in the “off season,” which contributed to U.S. expansion of fruit for retail markets, particularly in the southern states and California (Finn and Clark, 2011; Finn and Clark, 2012; Clark et al., 2007).

A substantial contributing factor to blackberry postharvest storage potential is the firmness of the berry, which relates directly to the fruit’s ability to retain structural integrity and resist pathogenic growth. Blackberry fruit firmness has been measured by: penetration of individual drupelets and the receptacle using a 0.3 mm diameter pin, or compression of the

whole fruit using a cylindrical plane probe, or on a subjective scale (Clark and Perkins-Veazie, 2011; Perkins-Veazie et al., 2000). Previous studies indicated that evaluating firmness by penetration was not as effective, but the firmness by compression had more effective distinguishing power (McCoy et al., 2016; Salgado, 2015).

As previously discussed, fruit appearance is critical to consumer acceptance. Red drupelet reversion (RDR) is a postharvest disorder that affects fresh-market blackberries after harvest, and is characterized by the irreversible change in pigmentation of individual or groups of drupelets from black to red or purple (Clark and Finn, 2011). While the mechanism of this color change and its physiological causes are not known, Salgado and Clark (2016) found that “crispy” blackberry genotypes had significantly lower RDR incidence and better shelf life when compared to conventional genotypes like ‘Natchez’. When examined under a confocal microscope, cross sections of ripe “crispy” genotype A-2453 appeared to maintain structural integrity while cross sections of ripe ‘Natchez’ appeared to fall apart and cell walls and organelles could not be distinguished (Salgado, 2015; Salgado and Clark, 2016). Segantini et al. (2017) also found that the “crispy” A-2453 experienced the least RDR when compared to 10 other advanced selections and released cultivars from the UA blackberry-breeding program, with 0% RDR after a cold storage for 7 d.

Color in plants is attributed to the presence of particular pigments. Anthocyanins are responsible for the distinctive red, blue, and purple hues associated with berries and are important for antioxidant activity (Filip et al., 2012). In blackberries, the primary anthocyanins are mostly cyanidin derivatives (Kaume et al., 2012). Blackberry anthocyanins are accumulated in the relatively acidic central vacuole, preventing oxidation and interactions with plastid and cell wall-localized polyphenol oxidase (Murata et al., 1997).

Commercially, blackberries are harvested at the “shiny black” stage, during which drupelets are fully black, turgid, and shiny, characteristics that are preferred by consumers (Clark et al., 2007). Because blackberries mature from green to red to black, RDR negatively impacts marketability and consumer acceptance due to the appearance of red drupelets. Some blackberry growers in the eastern United States have suggested that warmer temperatures at time of harvest contribute to higher RDR. McCoy (2016) found significantly less RDR across eight UA blackberry genotypes harvested at 7 AM when compared to other harvest times (10 AM, 1 PM, and 4 PM).

Blackberry traditional breeding

Plant breeding is dependent on the base genetic variability available to breeders. When controlled crossing and selection are successful, phenotypic stabilization of desired genotypes result in advanced selections and potential cultivars for release. However, traditional breeding, particularly in fruit crops, relies heavily on phenotyping and morphological markers, which are not indicative of the genetic stability of a trait and often require mature plants in the case of fruit phenotyping (St. Clair, 2010). Traditional breeding is most successful when segregating populations are large and representative of the genetic variability shared by each cross in question. However, it is often not economically feasible to have excessive seedlings per cross, and this contributes to difficulty in identifying individuals that possess the most desirable trait combinations possible for each cross, especially if they demonstrate medium or low heritability (Collard et al., 2005; St. Clair, 2010).

After emasculating flowers, pollination, seed harvest, and stratification, it takes 18 to 28 weeks to produce a field-transplantable seedling from a seed. Under ideal cultural conditions,

these seedlings grow vegetatively for a year before the first-year primocanes mature into floricanes and bear fruit, which may then be evaluated for selection (Clark and Finn, 2011; Finn and Clark, 2012). Usually, 100-200 seedlings per cross are planted, with most selections made two years after planting, and evaluation continues after the selections are field-transplanted. More seedlings represent and allow for greater genetic variability to be expressed, which leads to a higher chance of rapid improvement. In a realistic setting however, it is often not practical to maintain a large number of seedlings due to the land, labor, and resources required to sustain them for evaluation of fruiting and growth quality. Of seedlings from each year's crossing plan, less than 1% are selected for potential as advanced selections (Clark and Finn, 2011; Finn and Clark, 2012).

RosBREED

RosBREED is a USDA-Specialty Crop Research Initiative (SCRI)-funded project involving both United States and international institutions. The initial RosBREED project, RosBREED 1, spanned September 2009 to August 2014, focusing on enabling marker-assisted breeding (MAB) of fruit quality traits in five main rosaceous crops: apple, peach, strawberry, and sweet and tart cherries (Iezzoni et al., 2014). The current RosBREED project, RosBREED 2, extends to 2019 and aims to continue enabling MAB, combining disease resistance with horticultural quality. Research on the initial five crops was expanded to include blackberry, pear (*Pyrus communis* L.), and rose (*Rosa* L.) for a total of eight focus crops (Iezzoni et al., 2014). The UA is a member of both the RosBREED blackberry and peach breeding teams, acting as a demonstration breeding site, contributor to phenotypic data sets, and collaborator for genetic work.

Molecular markers

DNA or molecular markers are genotypic differences amongst individuals that are linked to loci controlling or contributing to a trait. Usually, these markers act as “flags” and do not affect the phenotype of the individual, and the strength of a molecular marker depends on its proximity to the gene of interest (i.e. tightly linked) (St. Clair, 2010). Because DNA markers can be represented by point mutations, insertions or deletions, or errors in replication of tandemly repeated DNA, they are extremely numerous and unaffected by plant growth stage, age, or environmental factors (Collard et al., 2005; Winter and Kahl, 1995). In addition to “flagging” the locations of important genetic loci and constructing linkage maps, these markers can be used to identify cultivars and ascertain parentage (Winter and Kahl, 1995).

The development of molecular markers begins with genetically segregating populations that show phenotypic differences. The parents of these populations are screened with markers for polymorphisms that span the genome (St. Clair, 2010). The number of polymorphic markers determines the ability to identify recombination break points. Using polymorphic markers to genotype each individual of the populations considered, a marker genotype data set is constructed. This data set is then used to generate a linkage map of the population with specialized software (e.g. JoinMap) (St. Clair, 2010).

In addition to genotyping, complementary phenotyping must be conducted on the same individuals in the segregating populations. Trait mapping software (e.g. mapQTL) is used to analyze the phenotypic and genotypic data sets in tandem to determine statistically significant marker-trait associations, inferring probable locations of genes controlling or contributing to a trait and identifying the marker polymorphisms that co-segregate or are associated with significant phenotypic variation (Collard et al., 2005; Winter and Khal, 1995).

When consistently predictive molecular markers are confirmed, marker-assisted selection (MAS) or marker-assisted breeding (MAB) can be conducted. Employing MAS as a selection tool can reduce the number of seedlings planted in the field, because seedlings may be scored for desirable and undesirable traits as soon as there is enough tissue for DNA extraction. The ability to visualize and confirm certain traits can also help breeders avoid crosses with high linkage drag, pyramid desirable allelic combinations of QTLs (MAS backcrossing), analyze pedigrees, and transfer traits with low or moderate heritability (Lecomte et al., 2004; Quilot et al., 2004).

Molecular research in blackberry genetics

Blackberry molecular genetics is a relatively unexplored field. So far, work has largely focused on pedigree analysis and identification of polymorphic regions. A genetic map was constructed by Castro et al. (2013), focusing on the primocane-fruiting and thornless traits in a population of tetraploid blackberries. More recently, the blackberry transcriptome was analyzed, producing a cDNA library from which over 8,000 simple sequence repeats (SSRs) and 67,000 single-nucleotide polymorphisms (SNPs) were detected (Garcia-Seco et al., 2015).

Using random amplified polymorphic DNA (RAPD) markers, Stafne et al. (2003) was able to calculate mean genetic contribution for 16 cultivars of blackberry and red raspberry. Pedigree analysis and 157 RAPD loci were used to distinguish between blackberry and red raspberry cultivars. By comparing these molecular results with pedigree records, Stafne et al. (2003) determined that the use of RAPD markers was able to determine percentage similarity between 1% and 5%, though ultimately RAPD and pedigree data were not completely correlated, with RAPD markers overestimating relatedness of individuals.

An F1 mapping population was derived from a cross between APF-12 (a primocane fruiting blackberry) × ‘Arapaho’. To account for the hybridization within *Rubus*, SSR primer pairs were selected from: the spineless red raspberry ‘Glen Moy’ whose genetic background includes several species of *Rubus*, a European weed *R. alceifolius* Poir., strawberry, and diploid strawberry (*F. vesca* L.). The SSR screening of the APF-12 × ‘Arapaho’ population indicated that 19 of 45 primer pairs were able to detect base-pair differences between the two parents, and thus could be used for future mapping of blackberry (Stafne et al., 2005).

Bassil et al. (2010) evaluated 29 dinucleotide SSR primer pairs and identified 10 of these pairs to distinguish between 16 North American blackberry cultivars. Nine of these 10 SSRs produced six to 12 alleles under multiplex PCR, with the remaining one producing three alleles. The alleles generated were comparable, and there was no indication of interaction amongst the primer pairs amplified in the same multiplex reaction.

A six SSR set was developed by Bassil et al. (2012) at the USDA-ARS National Clonal Germplasm Repository in Corvallis, OR. Initially, 24 SSRs were tested and evaluated for their ease of scoring and polymorphism for 35 red raspberry accessions from the United States and United Kingdom. The final six SSR set was easy to score in two separate multiplexes, polymorphic, able to map to five of the seven red raspberry linkage groups known, and able to differentiate between unique accessions.

Castro et al. (2013) utilized a population of 188 individuals, derived from a cross of APF-12 × ‘Arapaho,’ segregating for thorniness and primocane fruiting. After conducting phenotyping of these two traits and screening for SSRs, they found that 93 of 167 amplifying primer pairs showed polymorphism distinguishing between APF-12 and ‘Arapaho.’ Phenotypic data from thorniness and primocane fruiting evaluation was paired with 119 molecular markers

to construct a linkage map of this population using TetraploidMap. The linkage map indicated nine linkage groups for APF-12 and eight linkage groups for ‘Arapaho,’ and also identified 83 out of 85 bi-parental markers appearing in the same linkage group for both parents.

Lewter et al. (2015) found that DNA could be extracted from the torus of blackberry fruits and screened for polymorphisms using SSRs. As few as three markers were required to correctly identify 30 UA-developed genotypes.

Garcia-Seco et al. (2015) utilized RNA-seq analysis of the blackberry transcriptome, generating short cDNA reads from ‘Lochness’ fruit. These cDNA reads were assembled de novo and also aligned to wild strawberry *F. vesca* spp. *vesca* as a reference genome due to 73.6% non-redundant similarity. Using parameters of five or fewer mismatches in the alignment, the gene map rate of wild strawberry and blackberry was lower than 7%, and 12,077 genes were found to have high similarity to strawberry genes. Further, Garcia-Seco et al. (2015) found 34,552 annotated genes out of 42,062 assembled for ‘Lochness’. Relying on sequence homology, 40 functional groups were identified, mainly belonging to the categories of molecular function, cellular components, and biological processes.

Garcia-Seco et al. (2015) also reported that over 8,500 SSRs were detected in ‘Lochness’ cDNA using MISA software, including over 4,000 dinucleotides, 3,000 trinucleotides, and 365 hexanucleotides with similar numbers for tetra and pentanucleotides. SOAPaligner/SOAP2 analysis detected approximately 67,000 SNPs. The sequence information is available in EBI databases (PRJEB6680), and can be used in marker identification for traits of interest in blackberry (Garcia-Seco et al., 2015).

Bassil et al. (2016) developed a set of 13 tri-nucleotide SSRs to screen 13 genetically diverse blackberry cultivars from the USDA-ARS National Clonal Germplasm Repository

(NCGR). From this set of 13 SSRs, 10 were selected for ease of scoring, polymorphism, and ability to amplify consistently. The 10 SSR set was able to distinguish amongst the 13 blackberry accessions as well as the 13 SSR set, and was equally effective in simplex and multiplex amplification.

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Chapter 1

Development and Implementation of a Phenotyping Protocol for Blackberry Seedling Populations

Abstract

A foundational step of implementing molecular markers for DNA-informed breeding is establishing reliable protocols for creating phenotypic data sets to complement sequence information. Eight U.S. Department of Agriculture Agricultural Research Service (USDA-ARS) and six University of Arkansas (UA) populations were designated as phenotyping populations for blackberry, with each set representing the variation within these two major public blackberry breeding programs. The blackberry populations for UA were evaluated for plant and fruit traits in 2015 and 2016, while both UA and USDA-ARS populations were evaluated for fruit composition traits in 2015. Individual seedlings were treated as replications for their respective populations. The 2015 and 2016 data for UA were analyzed using multivariate methods to identify important distinguishing traits and to explore relationships between variables. For UA characteristics evaluated both years, the variation amongst individuals and populations in titratable acidity, pH, and soluble solids content had the greatest segregating power. Single-year fruit composition data indicated that UA populations generally had lower soluble solids content, titratable acidity, and organic acid content and higher pH than USDA-ARS populations. While the phenotyping protocol was able to distinguish between populations for some characteristics, it was not able to distinguish amongst individuals within populations, and should be further refined to increase this distinguishing ability. For future pairing of phenotypic data with genetic information and QTL analysis, quantitatively measured traits, such as fruit composition, are recommended for the higher resolution they offer in comparison to subjectively scored traits.

Introduction

The RosBREED program is a U.S Department of Agriculture (USDA) Specialty Crop Research Initiative project with an objective of using DNA-informed breeding techniques to combine disease resistance and horticultural quality in new rosaceous cultivars (Peace, 2017). For blackberries (*Rubus* L. subgenus *rubus* Watson) the primary focus is the improvement of fruit quality, and in the case of fresh-market blackberries, post-harvest storage capacity.

Fruit quality can be described qualitatively and quantitatively, but is largely represented by flavor. Blackberry flavor is complex and multifaceted, and includes sweetness, acidity, aromatic content, astringency, and bitterness (Clark et al., 2007; Clark and Finn, 2011). The RosBREED blackberry program aims to identify quantitative trait loci (QTL) that significantly impact fruit flavor. In order to achieve this, sequence information and phenotypic data must be paired for quantitative trait locus (QTL) analysis, association mapping, or other methods for enabling DNA-informed breeding in blackberry.

The bottleneck for DNA-informed breeding in many crops is limited phenotypic data. For blackberries, little molecular work has been done, and no phenotyping protocol exists for DNA-informed breeding. In strawberry (*Fragaria* × *ananassa* Duch.), peach (*Prunus persica* (L.) Batsch), apple (*Malus domestica* Borkh.), and sweet (*Prunus avium* L.) and tart cherries (*P. cerasus* L.), standardized phenotyping protocols were developed as previous RosBREED projects, and have helped advance their respective crops towards DNA-informed breeding. Some successful RosBREED QTL discovery and validation studies based on reliable phenotypic data include: QTL for sugars and soluble solids content in apple (Guan et al., 2015), remontancy in strawberry (Sooriyapathirana et al., 2015), and fruit size and weight in peach (Fresnedo-Ramirez et al., 2016).

The objectives of this study were:

1. To develop and implement a phenotyping protocol for blackberry plant and fruit quality characteristics on six University of Arkansas (UA) biparental seedling populations.
2. To implement the phenotyping protocol for fruit composition on six UA and eight USDA-Agricultural Research Service Horticultural Crops Research Unit in Corvallis, OR (USDA-ARS) biparental seedling populations.

Materials and Methods

This study was divided in two parts to address the two objectives. In Part 1, which focused on phenotyping the UA seedling populations, characteristics were evaluated for both 2015 and 2016 seasons. These characteristics were: plant architecture, plant health, plant vigor, crop load potential, berry weight, berry firmness, soluble solids content (SSC), pH, and titratable acidity (TA). For Part 2, which evaluated both UA and USDA-ARS seedling populations, characteristics were evaluated for the 2015 season only. These characteristics were: SSC, pH, TA, and individual sugars and acids.

Plant material, management, and selection

This study was conducted on six UA and eight USDA-ARS biparental seedling blackberry populations. The blackberry seedlings used in this study were products of the UA and USDA-ARS blackberry breeding programs and were not replicated at either location. The seedlings in the UA program were grown at the UA Fruit Research Station (FRS) in Clarksville, AR (lat. 35°31'58"N and long. 93°24'12"W). The seedlings in the USDA-ARS program were grown at Oregon State University's North Willamette Research and Extension Center in Aurora, OR (lat. 45°16'52.7"N and long. 122°45'00.1"W). The populations at both locations were selected by optimizing balanced unselected average allelic representation focusing on highest-priority important breeding parents as determined by Dr. C. Peace at Washington State University. With this approach, six biparental seedling populations were selected in Arkansas: 1222, 1229, 1236, 1250, 1253, and 1261. For USDA-ARS, eight biparental seedling populations were selected: 4534, 4540, 4647, 4650, 4651, 4660, 4665, and 4674.

The crosses for all UA seedling populations were made in 2012, with seedlings transplanted in the field in April 2013 at a spacing of 0.5 m in rows spaced 3.7 m. The seedlings were two years old when data collection began in year 2015. Parents were maintained in a nearby selection planting, and consisted of a single 6 m plot of each genotype. Parent plot ages varied from six to 15 years according to when they were selected in the breeding program.

In the UA seedling planting as well as selection planting, standard cultural practices for erect blackberry production were used including annual pre-emergent and post-emergent herbicide applications, and annual spring nitrogen fertilization (56 kg/ha N) using ammonium nitrate (Fernandez et al., 2016). Plants were not trellised. All seedlings were mechanically hedged at approximately 1.1 m height once in late summer, 2014. In December 2015, all seedlings were hand-pruned to remove dead material and then tipped to improve harvest efficiency and yield per plant. Canes were pruned to a maximum height of 1.5 m according to the vigor of the plant, and canes that were not within 8 cm of labeled crowns were removed. Parents were managed similarly, but were hedged in late summer both years, and pruned in October each year to remove old floricanes and shorten laterals to 0.5 m in length.

All plants received a single application of liquid lime sulfur (94 L/ha) at budbreak for control of anthracnose [*Elsinoë veneta* (Burkh.) Jenkins]. This was the only fungicide applied to any plants. Raspberry crown borer (*Pennisetia marginata* Harris) was controlled by a single application of a labeled insecticide with bifenthrin as the active ingredient in October of each year. All plots were irrigated as needed using overhead sprinkler irrigation (Fernandez et al., 2016).

Although the selected UA populations had up to 200 plants, only 50 individuals for each population were selected in May 2015. Seedlings were labeled linearly from the beginning of

each seedling plot, and were selected based on plant health and vigor indicating likely survival for two seasons, and also potential for fruiting. Each selected seedling was labeled using an engraved aluminum tag with population and individual number indicated. Individuals were relabeled in the same manner during the spring of 2016 to ensure consistent seedling identity.

The crosses for USDA-ARS Corvallis seedling populations were made in 2011 (populations 4534, 4540) and 2012 (populations 4647, 4650, 4651, 4660, 4665, 4674). Standard cultural practices for trailing blackberry production were used to manage these seedling populations as well as parents, including: annual pre- and post-emergent herbicides, spring nitrogen fertilization (78 kg N/ha), postharvest removal of floricanes, a two-wire trellising system for canes, and overhead sprinkler irrigation during the growing season as needed (C.E. Finn, personal communication). For preemptive pest and disease control, dormant applications of liquid lime sulfur and copper hydroxide were used to manage leaf and cane spot (*Septoria rubi* Westend), purple blotch [*Septocya ruborum* (Lib) Petr.], rust [*Kuehneola uredinis* (Link) Arth.], and anthracnose.

A multiplex simple sequence repeat blackberry seedling fingerprinting set was used to screen all UA and USDA-ARS seedlings and parents in a method similar to that outlined by Bassil et al. (2016). This method determined that all individuals evaluated were both true-to-type and genetically distinguishable from one another for the six and eight populations used for UA and USDA-ARS, respectively. True-to-typeness was determined by screening seedling DNA for alleles found in one or both parents; the presence of alleles found in neither parent indicated that the seedling was not true-to-type and should be discarded. Seedlings were screened for genetic distinctness by evaluating individuals' electropherograms representing the allele products of the eight SSR multiplex. Seedlings were determined to be genetically distinct if differences in allele

products could be visualized consistently over three identical multiplex amplifications of the SSR set. If a set of seedlings were found to be genetically indistinguishable, only one of these seedlings was selected for further phenotyping.

Evaluation of plant characteristics

Plant characteristics were evaluated for seedling plants. In May 2015, each labeled seedling in Arkansas was evaluated for plant architecture, presence or absence of thorns, health, and vigor. Plant architecture described the growth habit of the plant and was categorized into trailing, semi-erect, and erect, denoted “1,” “2,” and “3,” respectively. Plant health was a visual estimation rated on a 1-9 scale, with 1 representing a plant that was nearly dead and 9 representing a plant that appeared green and without visible vegetative discoloration or physical damage. Plant vigor was also evaluated on a subjective 1-9 scale, describing the amount of growth for the current season: a rating of 1 was given to very small, stunted plants with no new growth and a rating of 9 was given to plants with excessively vigorous growth, with many primocanes developing and new leaves growing.

Crop load potential was evaluated visually and determined by the intensity of floricanes bloom. This characteristic was also rated on a 1-9 subjective scale, with 1 indicating no flowers and 9 indicating a potentially excessive crop.

In May 2016, UA seedling characteristics were expanded to include those evaluated in 2015 as well as peak bloom date and primocane and floricanes count. Peak bloom was the date during which approximately 50% or more of flowers had opened fully and was denoted as the days from 1 Jan. of the year. Primocane count included new canes emerging from the crown exceeding 15 cm in length, not including suckers or canes located more than 8 cm from the main

crown. Floricane count included actively growing canes from the previous year no more than 8 cm away from the main crown.

Harvest

Blackberries from seedlings that produced fruit were harvested at the shiny-black stage, without red drupelets, blemishes, or compromised structure from physical damage. Fruit was harvested only in the morning, before the temperature exceeded 27 °C. In the event of precipitation, fruit was harvested after a 24 h window of dry weather.

At the UA in 2015 and 2016, 15 berries from each seedling were harvested and placed into three labeled zip-top bags to represent three repeated measurements. In the field, berries were kept in an insulated cooler containing ice packs, then transferred to a ~4 °C refrigerator as soon as possible.

At the USDA-ARS in 2015, blackberries were harvested, then juice was extracted from the berries, frozen (-20 °C) and shipped overnight on dry ice to UA for compositional analysis.

Evaluation of fruit characteristics

In 2015 and 2016, fruit physical and compositional characteristics were evaluated at the UA.

Physical characteristics: The physical characteristics evaluated included berry weight and firmness. Average berry weight was evaluated using five berries per plant. Firmness was measured on two berries per replication using an Icon Texture Analyzer (Texture Technologies Corp., Hamilton, MA) equipped with a cylindrical plane probe measuring 7.6 cm in diameter was used to determine the force in Newtons (N) required to compress each individual berry 5

mm from the point of contact, with a trigger force of 0.02 N. For berry firmness by compression, berries were placed such that the longest side of the fruit was parallel to the platform of the texture analyzer, or as the berry would naturally sit. Firmness of berries was evaluated on fruit at room temperature, approximately 25 °C.

After fruit attributes were evaluated, berries were returned to their respective bags, and transferred to a -20 °C freezer for later compositional analysis.

Compositional characteristics: The compositional characteristics included basic compositional analysis and analysis of individual sugars and acids. For UA, each five-berry sample per replication was removed from the -20 °C freezer and thawed at 4 °C overnight. Fruit were then brought to room temperature and processed for analysis. The berries for each replication were transferred to a borosilicate beaker lined with a two-ply square of grade-60 cheesecloth and then squeezed. The juice was squeezed to exclude seeds or other visible debris. Before proceeding to each step of analysis, juice was mixed thoroughly by stirring. Juice samples were at room temperature for analysis. All samples from USDA-ARS and UA were analyzed for fruit composition (basic composition and acid and sugar composition) at UA.

Basic composition: Basic composition of juice from UA and USDA-ARS blackberries was evaluated. Juice was evaluated for SSC using a refractometer (Abbe Mark II refractometer, Bausch and Lomb Inc., Rochester, NY). The pH and TA were measured using an 862 Compact Titrosampler (Metrohm AG, Herisau, Switzerland). The pH value was recorded after 3 min. The TA of each sample was determined by measuring 3 mL juice diluted with 50 mL of deionized and degassed water, and titrating with 0.1N sodium hydroxide (NaOH) to an endpoint of pH 8.2. Titratable acidity was expressed as percent citric acid. The remaining juice from each sample

was placed in 15 mL screw-top plastic falcon tubes and stored at -20 °C for sugar and organic acid analysis.

Acid and sugar composition: Individual sugar and acid composition of juice from UA and USDA-ARS blackberries were evaluated. Individual components (isocitric/citric acid, isocitric lactone, malic acid, glucose, and fructose) were analyzed using high performance liquid chromatography (HPLC) with a method slightly modified from Walker et al. (2003). Juice samples were thawed at 4 °C overnight. The day of analysis, samples were brought to room temperature and homogenized by gently inverting individual tubes. Blackberry juice was diluted with deionized, degassed water for a 1:5 juice: water dilution. The dilutions were mixed and then filtered with 0.45 µm nylon Acrodisc® syringe filters, 13mm (Pall Corporation, Port Washington, NY). Samples were filtered through the syringe-filter system into a 1 mL glass shell vial (VWR, Radnor, PA) and then capped for HPLC analysis.

Samples were placed in a 96-vial capacity carousel and analyzed using a Waters HPLC system (Waters, Milford, MA), which was equipped with a Bio-Rad HPLC Organic Acid Analysis Aminex HPX-87H ion exclusion column (300 × 7.8 mm) (Bio-Rad Laboratories, Hercules, CA). This column was maintained at 65 °C. The mobile phase consisted of a sulfuric acid and water mixture maintained at pH 2.28, with a flow rate of 0.65 mL/min. The solvent delivery system was a Waters 515 HPLC pump equipped with a Waters 717 plus autosampler. Injection volumes were 10 µL, and the run time for completion was set to 25 min to ensure that all probable organic acids and sugars could be detected. A Waters 2414 differential refractometer was used to measure refractive index connected in series with a Waters 996 photodiode array detector monitored the eluting compounds. Organic acids were detected by photodiode array at 210 nm and glucose and fructose were detected by a differential refractometer. The peaks were

quantified with Waters Empower software using external standard calibration based on peak height estimation with baseline integration.

Statistical analysis

Population means for each trait, one-way analysis of variance (ANOVA) means separation via Tukey's Honest Significant Difference (HSD) test, multivariate analysis of variance (MANOVA), recursive partitioning, and iterative clustering were conducted in JMP® Pro Version 13.0.0 (SAS Institute Inc., Cary, NC). All significance levels were set to $\alpha = 0.05$.

The MANOVA procedure was used to compare populations for overall differences for UA. The MANOVA variates used to compare populations were the across-year average values by individual for berry weight, fruit firmness, SSC, pH, TA, and plant health, vigor, and crop load potential. The identity function was used to generate a canonical centroid plot with biplot rays to visualize significant differences amongst populations at a 95% confidence level.

Multivariate discriminant analysis via recursive partitioning was used to construct a decision tree with the validation portion set to 20% for UA data. This validation portion required that at least 20% of individuals to be partitioned by a specific trait value for a branch to be constructed in the decision tree. The variables used for recursive partitioning were the same as for the MANOVA procedure. The decision tree separated groups of individuals by creating binary rules for predicting groups with similar phenotypic combinations. The number of splits was set to six, the number of families for UA. The decision tree identified the most important predictors for grouping all individuals considered into families based on phenotypic similarity.

Iterative clustering using the k-means option was used for UA's seedling material, grouping individuals into clusters according to the six seedling populations. The variables used

for k-means clustering were the same as for the MANOVA procedure. Parallel coordinate plots were constructed for each cluster, and 2-D biplots were generated to visualize the interactions of the parallel coordinate plots in a single space.

Principal components analysis (PCA) for fruit composition data (SSC, pH, TA, sugars, and organic acids) was conducted in Genstat (VSN International, United Kingdom) and used to estimate correlations and relationships between traits in a multidimensional space accounting for all fruit composition characteristics. GenStat was also used for canonical variate analysis (CVA) to analyze populations and individuals using a different correlations matrix and set of assumptions than PCA. With CVA, the 95% confidence intervals for population means according to the two dimensions accounting for the most variation amongst individuals were determined, and this information indicated which populations were the most similar or dissimilar depending on these dimensions. Fruit compositional data for 2015 season was used for both PCA and CVA analysis of USDA-ARS and UA material included SSC, pH, TA, and individual sugars and acids.

Results

Part 1:

For UA, out of 305 seedlings that were phenotyped, only 209 had one or more repeated measurements for both years, while 96 seedlings had one or more measurements for just one year. This lack of data was influenced by plant characteristics and environmental conditions that affected the traits of interest. With approximately 1/3 of the data missing, an ANOVA procedure was determined to be less effective in describing populations and determining important distinguishing characteristics than multivariate analysis because of the unbalanced data. Means, minimums, maximums, and standard errors were calculated by population across years for UA berry weight, firmness, SSC, pH, TA, and plant health, vigor, and crop load potential.

Berry size, plant architecture, number of primocanes and floricanes, fruit symmetry, and peak bloom were measured on UA populations in 2016, but these one-year data will not be discussed. The population means for these traits are shown in Appendix B.

Descriptive statistics—UA

For UA, the population means for single berry weight ranged from 5.21 g to 3.48 g for populations 1229 and 1236, respectively (Table 1). Berry firmness population means ranged from 12.31 N (population 1236) to 7.99 N (population 1253) (Table 1). The population means for SSC ranged from 10.5% (population 1229) to 9.0% (population 1236) (Table 2). Population means for pH ranged from 4.04 (population 1236) to 3.40 (population 1250) (Table 2). The population means for TA ranged from 1.21% (population 1250) to 0.71% (population 1229) (Table 2). Plant health, vigor, and crop load potential were evaluated as continuous variables due to the 1-9 scale being evenly spaced between each ranking. The population means for health

ranged from 7.7 for population 1250 to 5.9 for population 1261 (Table 3). Plant vigor ranged from 7.6 for population 1250 and 5.4 for populations 1222 and 1261 (Table 3). Crop load potential ranged from 7.0 for population 1229 to 4.2 for population 1261 (Table 3).

Table 1. Blackberry fruit weight and firmness for six University of Arkansas blackberry seedling populations averaged for 2015 and 2016 at the Fruit Research Station, Clarksville, AR.

Population	Individual berry weight (g)			Berry firmness (N)		
	Mean	Min.	Max.	Mean	Min.	Max.
1222	4.39	1.97	7.39	12.29	7.92	21.50
1229	5.21	2.18	8.19	11.15	6.53	16.17
1236	3.48	1.28	5.53	12.31	6.50	21.64
1250	4.45	1.31	7.68	8.00	3.20	15.37
1253	4.06	1.37	6.39	7.99	2.83	13.30
1261	4.10	1.61	6.46	10.90	3.97	18.45

Table 2. Blackberry soluble solids content, pH, and titratable acidity for six University of Arkansas blackberry seedling populations averaged for 2015 and 2016 at the Fruit Research Station, Clarksville, AR.

Population	Soluble solids content (%)			pH			Titratable acidity (% citric acid equivalent)		
	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.
1222	10.4	7.7	13.8	3.49	2.92	4.12	0.90	0.40	1.46
1229	10.5	6.1	13.8	3.73	3.40	4.06	0.71	0.49	1.11
1236	9.0	5.3	12.4	4.04	3.68	4.53	0.59	0.30	0.90
1250	9.4	7.1	12.6	3.40	2.86	3.80	1.21	0.72	1.67
1253	9.6	6.5	12.6	3.80	3.39	4.25	0.90	0.61	1.50
1261	9.4	7.0	13.3	3.55	2.94	4.35	0.92	0.30	1.49

Table 3. Blackberry plant health, vigor, and crop load potential values for six University of Arkansas blackberry seedling populations averaged for 2015 and 2016 at the Fruit Research Station, Clarksville, AR.

Population	Plant health ^z			Plant vigor ^y			Crop load potential ^x		
	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.
1222	7.3 ^z	5.0	9.0	5.4 ^z	2.0	9.0	6.2 ^z	2.0	9.0
1229	6.5	3.0	9.0	6.4	3.0	9.0	7.0	3.0	9.0
1236	7.5	4.5	9.0	6.2	2.0	9.0	6.6	2.0	9.0
1250	7.7	6.0	9.0	7.6	4.0	9.0	6.3	2.5	9.0
1253	7.4	4.0	9.0	6.8	2.0	9.0	6.9	2.0	9.0
1261	5.9	3.0	8.0	5.4	2.0	8.0	4.2	1.0	8.0

^z Plant health evaluated on a subjective scale of 1-9, with 1 = excessive visible discoloration or physical damage to vegetative growth and 9 = no visible discoloration or physical damage to vegetative growth.

^y Plant vigor evaluated on a subjective scale of 1-9, with 1 = least and 9 = most vigorous vegetative growth.

^x Crop load potential evaluated on a subjective scale of 1-9, with 1 = absence of and 9 = excessive number of flowers observed at peak bloom.

MANOVA

The canonical centroid plot indicated that all UA seedling populations were significantly different when considering the across-year values for berry weight, fruit firmness, SSC, pH, TA, health, vigor, and crop load potential (Fig. 1). The only exception was population 1222 and 1261, which had similar berry weight and SSC values, indicated by their close positions on the plot (Fig. 1).

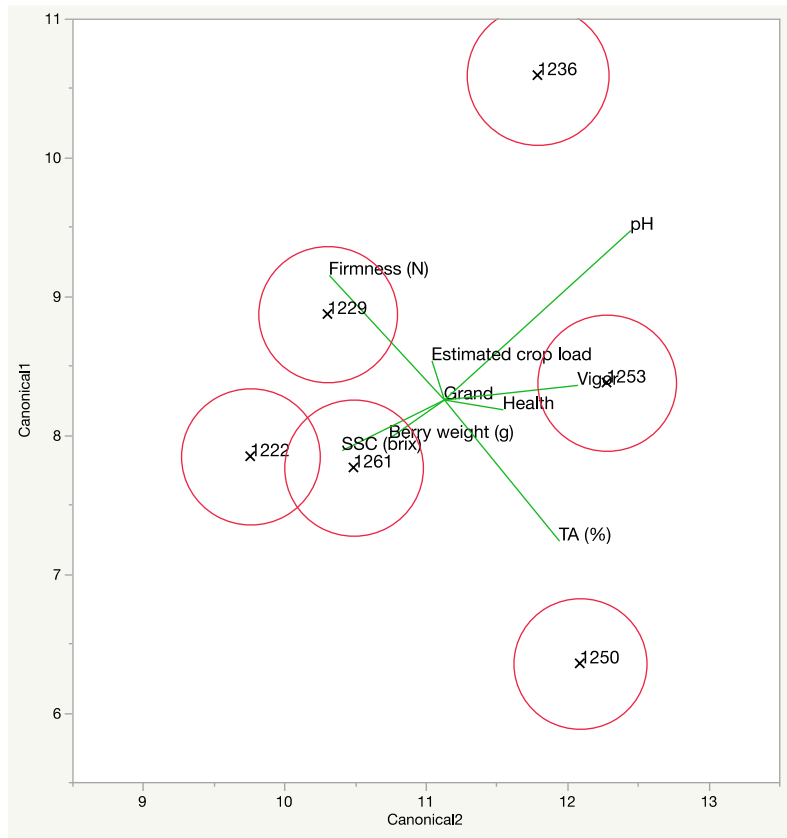


Fig. 1. Multivariate analysis of variance fit canonical centroid plot for six University of Arkansas blackberry populations for fruit firmness, crop load potential, plant vigor, plant health, berry weight, soluble solids content (SSC), pH, and titratable acidity (TA) for years 2015 and 2016 at the Fruit Research Station, Clarksville, AR.

Multivariate discrimination: recursive partitioning

As the canonical centroid analysis indicated, most populations were significantly different when considering the eight characteristics evaluated and averaged for both years. The term that had the greatest partitioning power was pH, followed by firmness, TA, crop load potential, and then berry weight. The decision tree was constructed by dividing the entire collection of individuals by term, beginning with the term with the greatest partitioning power, subsequent branches divided by the next most powerful term, and so forth, resulting in “branches” of individuals grouped according to their similarities in phenotypic values.

A recursive partitioning was able to identify that a pH of 3.605 could divide individuals into two groups. The group with a pH greater than 3.605 could be divided further into groups with firmness values of either less than or greater than 9.37 N; the group with firmness values of less than 9.37 N could be divided one more time by crop load potential, with values greater than or less than 4.5. The group with firmness values greater than 9.37 N could then be divided by berry weight, and then TA (Fig. 2).

The group with a pH values of less than 3.605 could be divided further into two groups that had firmness values either greater or less than 9.37 N. The individuals with firmness values greater than 9.37 N could be further distinguished using TA values, and then crop load potential (Fig. 2).

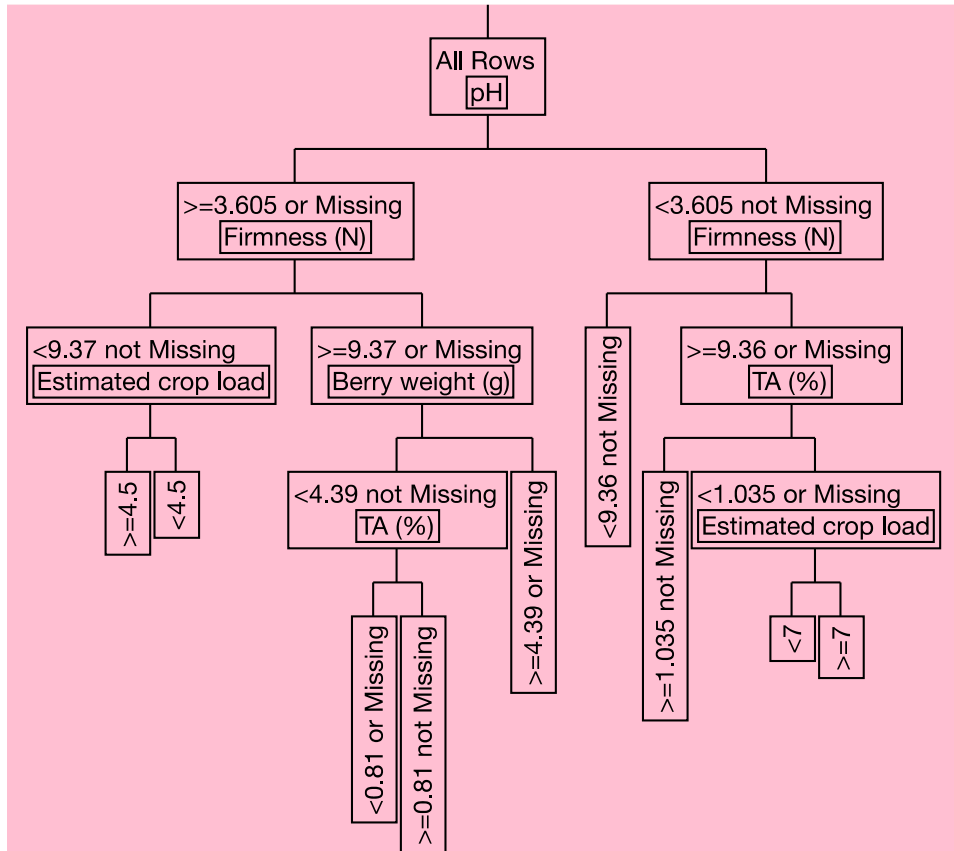


Fig. 2. Decision tree plot constructed using recursive partitioning for seedlings from six University of Arkansas blackberry seedling populations across years 2015 and 2016 at the Fruit Research Station, Clarksville, AR using fruit firmness, crop load potential, plant vigor, plant health, berry weight, soluble solids content (SSC), pH, and titratable acidity (TA) values as factors for partitioning individuals.

K-means clustering

The K-means clustering analysis identified individuals that had similar values and grouped them into a predetermined number of clusters; in this case the number was six to reflect the number of seedling populations evaluated.

The UA individuals used in the analysis were divided into clusters 1-6, ranging from 20 to 78 individuals per cluster. Cluster three had the fewest individuals, while cluster five had the most; cluster three generally had highest TA and vigor, while cluster five was characterized by

the greatest health and lowest mean berry weight (Table 4). These clusters and their relative positions in a two-dimensional space are provided in a biplot (Fig. 3).

Table 4. Mean values for blackberry weight, firmness, soluble solids content (SSC), pH, titratable acidity (TA), health, vigor, and crop load potential for six K-means clusters and respective individuals per cluster for University of Arkansas seedlings based on values averaged across years 2015 and 2016 at the Fruit Research Station, Clarksville, AR.

Cluster	Count	Berry weight (g)	Firmness (N)	SSC (%)	pH	TA (%)	Health	Vigor	Crop load potential
1	35	4.13	11.65	9.2	3.91	0.69	6.0	4.5	7.3
2	21	4.37	14.44	10.0	3.33	0.91	6.1	5.2	4.3
3	62	4.74	8.60	9.3	3.39	1.19	7.9	7.7	6.9
4	40	4.20	9.76	9.0	3.52	1.07	6.1	5.2	4.6
5	56	3.60	11.32	9.1	3.82	0.77	8.1	7.1	7.5
6	64	4.79	9.73	11.2	3.83	0.67	7.0	6.9	6.0

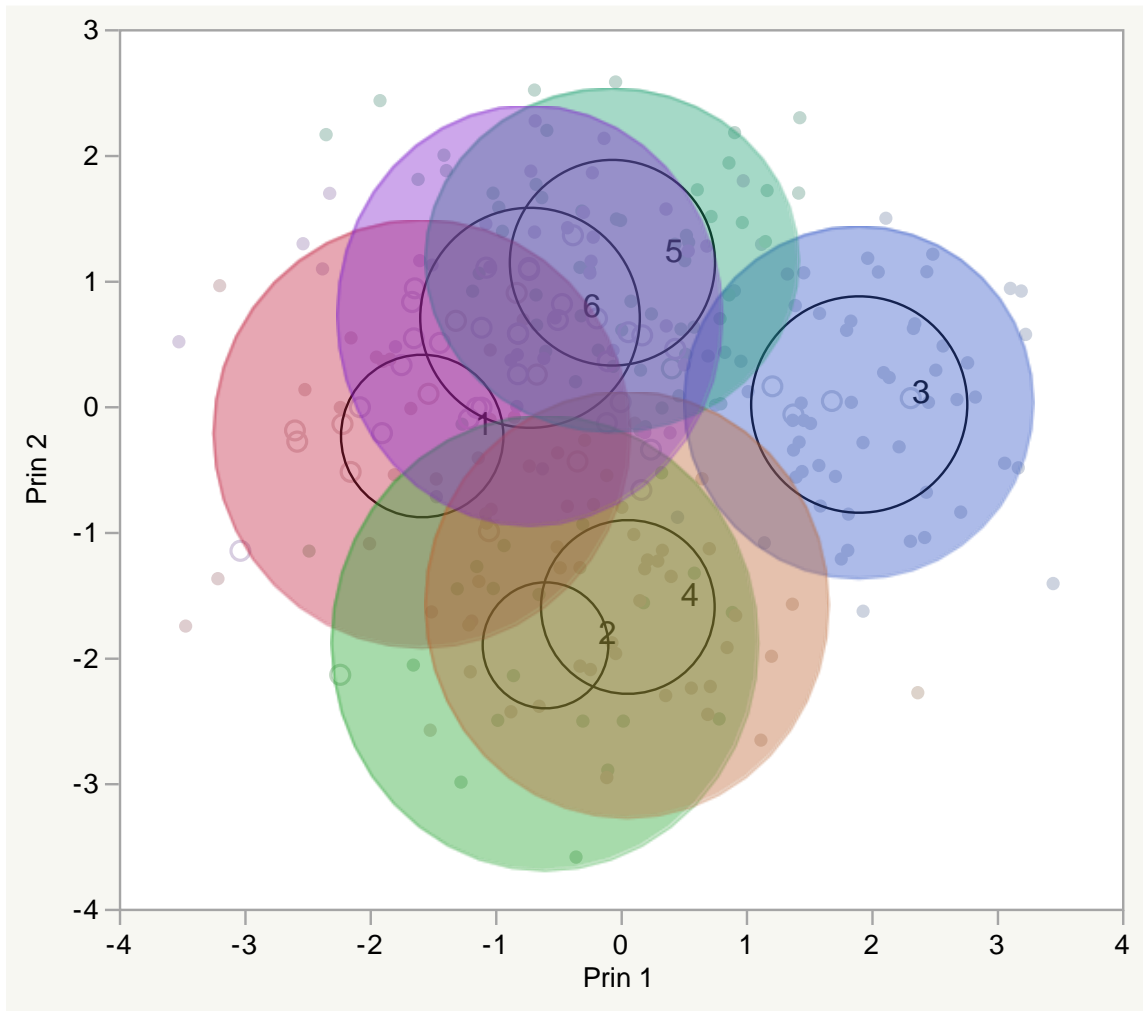


Fig. 3. Biplot of six clusters created from k-means clustering analysis, with black circles as 95% confidence intervals of weighted mean values of characteristics^z of individuals per cluster for University of Arkansas blackberry seedlings, based on values averaged across years 2015 and 2016 at the Fruit Research Station, Clarksville, AR.

^z Characteristics analyzed in k-means clustering were berry weight, fruit firmness, soluble solids content, pH, titratable acidity, health, vigor, and crop load potential.

Part 2:

Basic composition

One-way ANOVA indicated that for both UA and USDA-ARS seedlings, population means were significantly different for SSC, pH, and TA.

For UA seedlings, population 1229 had the numerically highest SSC, but was not significantly different from population 1222 (Table 5). For USDA-ARS seedlings, population 4660 had the numerically highest SSC, but was only significantly different from two other populations, 4647 and 4650 (Table 6). Population 1261 had the numerically lowest SSC for UA, but was not significantly different from populations 1250 and 1253 (Table 5). For USDA-ARS, population 4647 had the numerically lowest SSC, but was not significantly different from any other population except for 4660 (Table 6). Population 1222 had the lowest pH, while population 1236 had the highest pH for UA seedlings, and both population mean values were significantly different from that of other populations (Table 5). For USDA-ARS, population 4650 had the numerically lowest pH while population 4540 had the numerically highest pH, though the only significant difference in population means was seen between populations 4540, 4647, and 4651 and population 4650 for this characteristic (Table 6). Population 1250 had the highest TA, and population 1229 had the lowest TA for UA seedlings, and these two population means were significantly different from all other population means for UA (Table 5). Population 4650 had the numerically highest TA for USDA-ARS, but was not significantly different from populations 4534 and 4665 (Table 6). Population 4540 had the numerically lowest TA for USDA-ARS seedlings, but was not significantly different from populations 4647, 4651, 4660, or 4674 (Table 6).

Table 5. Soluble solids content (SSC), pH, and titratable acidity (TA) of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2015.

Population	SSC (%)	pH	TA (%)
1222	10.6 a ^z	3.85 c	0.91 b
1229	11.1 a	4.00 b	0.74 c
1236	9.6 b	4.17 a	0.57 d
1250	9.3 bc	3.70 d	1.21 a
1253	9.5 bc	3.98 b	0.89 b
1261	8.9 c	3.96 b	0.95 b

^z Means with different letter(s) are significantly different ($\alpha=0.05$) using Tukey's honest significant difference test ($\alpha = 0.05$).

Table 6. Soluble solids content (SSC), pH, and titratable acidity (TA) of eight U.S. Department of Agriculture Horticultural Crops Research Service blackberry seedling populations from the North Willamette Research and Extension Center, Aurora, OR 2015.

Population	SSC (%)	pH	TA (%)
4534	12.9 ab ^z	3.83 ab	1.64 ab
4540	11.9 ab	3.94 a	0.86 d
4647	10.8 b	3.86 a	1.22 cd
4650	11.4 b	3.59 b	1.99 a
4651	12.0 ab	3.90 a	1.25 bcd
4660	13.8 a	3.84 ab	1.12 cd
4665	12.7 ab	3.76 ab	1.53 abc
4674	13.0 ab	3.87 ab	0.96 d

^z Means with different letter(s) are significantly different ($\alpha=0.05$) using Tukey's honest significant difference test ($\alpha = 0.05$).

Acid and sugar composition

One-way ANOVA indicated that for both UA and USDA-ARS seedlings, population means were significantly different for isocitric/citric acid content, isocitric lactone content, and malic acid content. For UA, both glucose and fructose content population means were significantly different, but for USDA-ARS, ANOVA indicated that neither fructose nor glucose content were significantly different amongst populations.

For isocitric/citric acids, UA populations 1222 and 1261 had the highest concentration, but were not significantly different from population 1250, while population 1253 had a significantly lower concentration of isocitric/citric acid than other populations (Table 7). USDA-ARS population 4650 had significantly more isocitric/citric acid content compared to all other USDA-ARS populations, while population 4540 had the numerically lowest isocitric/citric acid content, though the population mean was not significantly different from that of populations 4647, 4660, and 4674 (Table 8). For isocitric lactone, UA population 1261 had the highest content but was not significantly different from population 1250 (Table 7). In USDA-ARS populations, 4540 had significantly higher isocitric lactone concentration than the other seedling populations (Table 8). Population 4660 had the numerically lowest isocitric lactone content, but was not significantly different from populations 4647, 4650, or 4665 (Table 8). For malic acid, UA population 1250 had the highest mean concentration, while four of the remaining five populations were not significantly different from each other (Table 7). In USDA-ARS seedlings, malic acid was numerically highest in population 4534, though not significantly different from populations 4540, 4647, or 4665 (Table 8). Population 4674 had the numerically lowest malic acid content, but was not significantly different from USDA-ARS populations 4650, 4651, or 4660 (Table 8).

In UA seedlings, population 1229 had the highest glucose and fructose content; for glucose, population 1229 had significantly higher content than all other populations, while for fructose, it was not significantly different from population 1222 (Table 7). Populations 1250, 1253, and 1261 all had the lowest glucose and fructose content, and their means were significantly different from the other three populations for both sugars (Table 7).

Table 7. Isocitric/citric acid, isocitric lactone, and malic acid content of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2015.

Population	Isocitric/citric acid (mg/100 g juice)	Isocitric lactone (mg/100 g juice)	Malic acid (mg/100 g juice)	Glucose (g/100 g juice)	Fructose (g/100 g juice)
1222	23.5 a ^z	3.75 bc	57.48 c	0.69 b	0.60 ab
1229	20.35 b	3.5 c	59.65 c	0.77 a	0.66 a
1236	16.36 d	3.4 c	58.17 c	0.65 b	0.56 b
1250	21.88 ab	5.46 a	97.9 a	0.55 c	0.47 c
1253	18.46 c	4.4 b	60.77 c	0.54 c	0.48 c
1261	22.73 a	6.03 a	73.93 b	0.54 c	0.47 c

^z Means with different letter(s) are significantly different ($\alpha=0.05$) using Tukey's honest significant difference test ($\alpha = 0.05$).

Table 8. Isocitric/citric acid, isocitric lactone, and malic acid content of eight U.S. Department of Agriculture Horticultural Crops Research Service blackberry seedling populations from the North Willamette Research and Extension Center, Aurora, OR 2015.

Population	Isocitric/citric acid (mg/100 g juice)	Isocitric lactone (mg/100 g juice)	Malic acid (mg/100 g juice)	Glucose (g/100 g juice)	Fructose (g/100 g juice)
4534	50.20 b ^z	7.24bc	186.19 a	0.74	0.73
4540	19.19 e	9.92a	170.1 ab	0.72	0.70
4647	30.19 de	4.9de	157.39 abc	0.67	0.63
4650	67.11 a	4.45de	135.94 b-e	0.62	0.61
4651	37.28 cd	5.6cd	121.18 cde	0.68	0.65
4660	30.45 de	3.8e	116.48 de	0.75	0.76
4665	45.13 bc	4.88de	149.96 a-d	0.81	0.76
4674	20.75 e	8.98ab	94.33 e	0.78	0.73

^z Means with different letter(s) are significantly different ($\alpha=0.05$) using Tukey's honest significant difference test ($\alpha = 0.05$).

Principal components analysis

The PCA for fruit composition had six dimensions, with the first three explaining 87.2% of total variation amongst individuals. Fructose, glucose, TA, and SSC contributed the most to the first dimension as indicated by their relative weights for PC 1. Dimensions 1 and 2 explained 70.3% of the first three dimensions, and were used to construct a PC biplot (Fig. 4). The biplot's accompanying table of correlations indicated that SSC, fructose, and glucose were positively and closely correlated, particularly fructose and glucose content ($r = 0.988$). The acid that was most closely correlated with TA was isocitric/citric acids ($r = 0.6298$). Isocitric/citric acid also had the closest correlation with pH ($r = -0.529$). Isocitric lactone and malic acid were positively

correlated ($r = 0.668$) and had the strongest relationship when compared to the other combinations of acids.

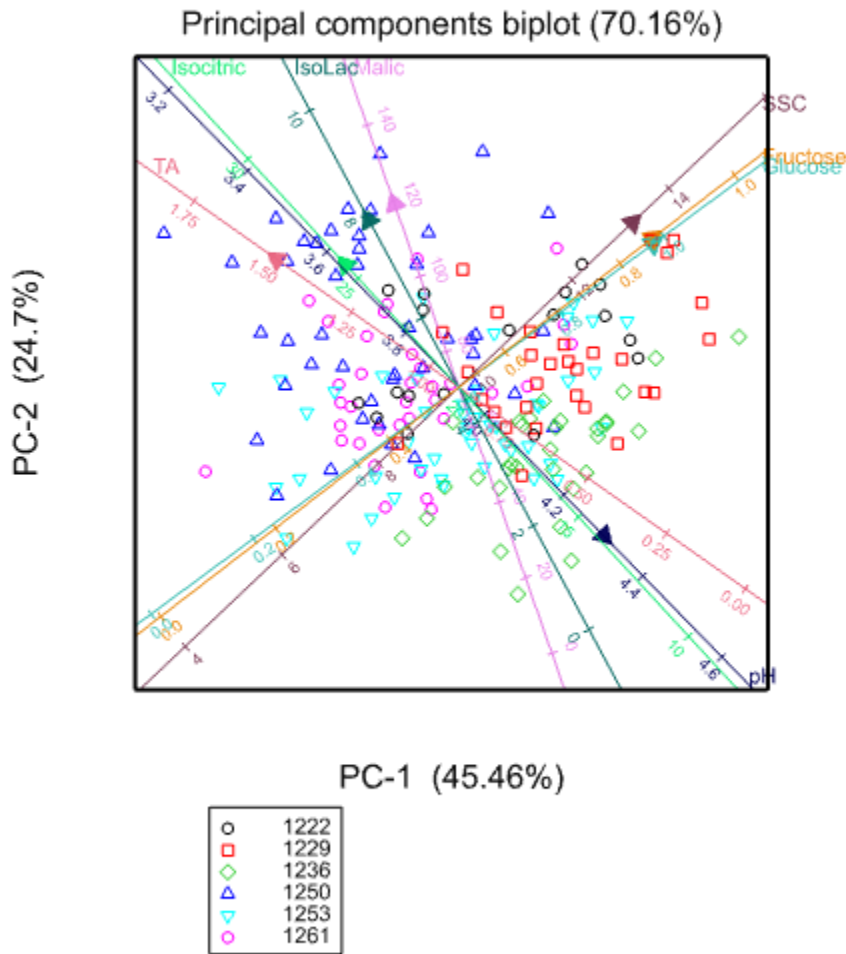


Fig. 4. Principal components biplot showing 70.2% of variation amongst blackberry seedlings in six University of Arkansas blackberry populations in 2015 using fructose, glucose, isocitric lactone, isocitric/citric acid, malic acid, titratable acidity (TA), pH, and soluble solids content (SSC) as components at the Fruit Research Station, Clarksville, AR.

The PCA for USDA-ARS juice compositional characteristics determined six dimensions, with the first three explaining 82.0% of total variation amongst individuals. The first two

dimensions contributed to 68.7% of total variation: the variation from dimension one (41.4%) was most significantly influenced by fructose, glucose, and SSC while the variation from dimension two (27.3%) was primarily influenced by isocitric acid and TA. The PC biplot constructed from dimensions one and two (Fig. 5) and the biplot's accompanying table of correlations indicated that, like for UA material, that SSC, glucose, and fructose were positively and closely correlated, especially fructose and glucose content ($r = 0.9636$). Isocitric/citric acid content was highly correlated with TA ($r = 0.8106$).

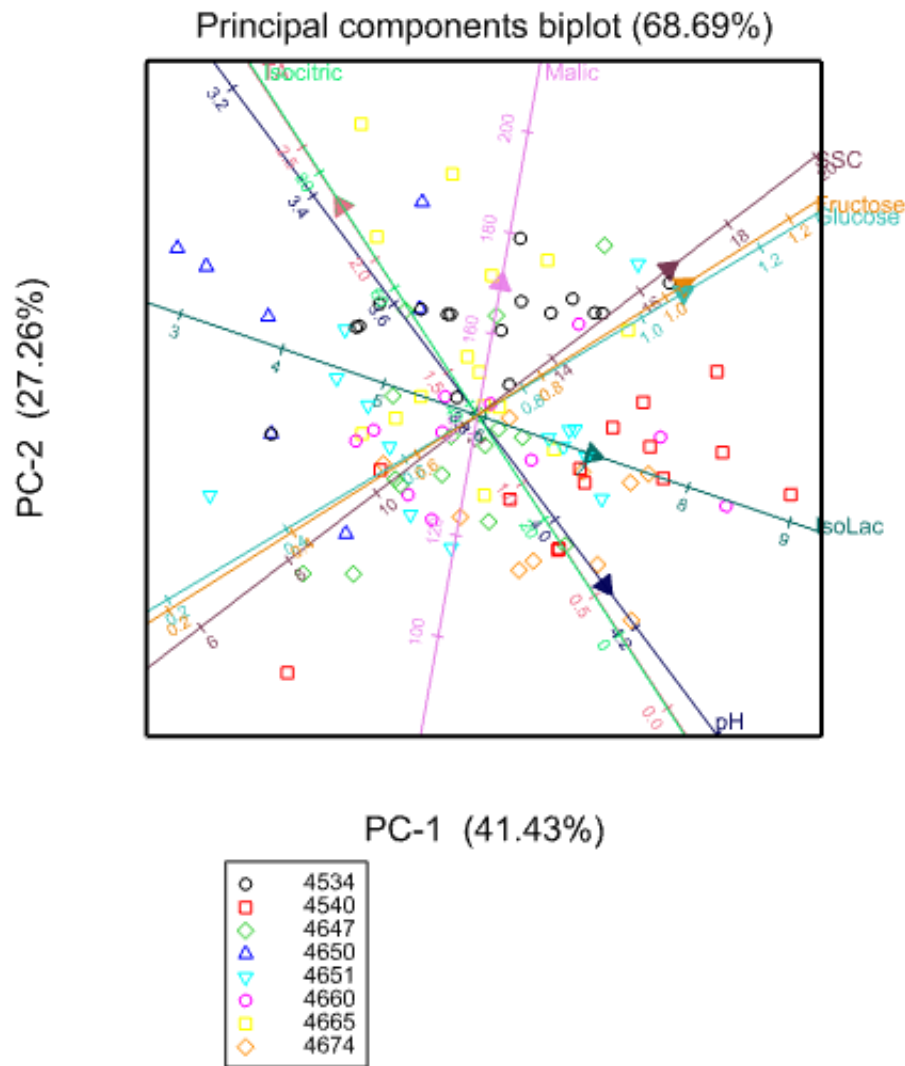


Fig. 5. Principal components biplot showing 68.7% of variation amongst seedlings in eight U.S. Department of Agriculture Agricultural Research Service Horticultural Crops Research Unit blackberry populations from the North Willamette Research and Extension Center, Aurora, OR in 2015 using fructose, glucose, isocitric lactone, isocitric/citric acid, malic acid, titratable acidity (TA), pH, and soluble solids content (SSC) as components.

Canonical variate analysis

For CVA, only fruit compositional characteristics were used. These characteristics were standardized to optimize variables in linear combinations to separate individuals into groups. The CVA determined five dimensions using a slightly different correlation matrix than PCA, and vectors 1 and 2 explained 77.4% of variation amongst individuals when considering fruit compositional traits. The CVA biplot gave 95% confidence intervals for the mean values for each population when weighing the standardized variables (Fig. 6). The confidence intervals calculated for the standardized fruit compositional variables indicated that populations 1236 and 1250 were the most different, populations 1222 and 1229 were not significantly different, and populations 1253 and 1261 were not significantly different but had many individuals with overlapping means.

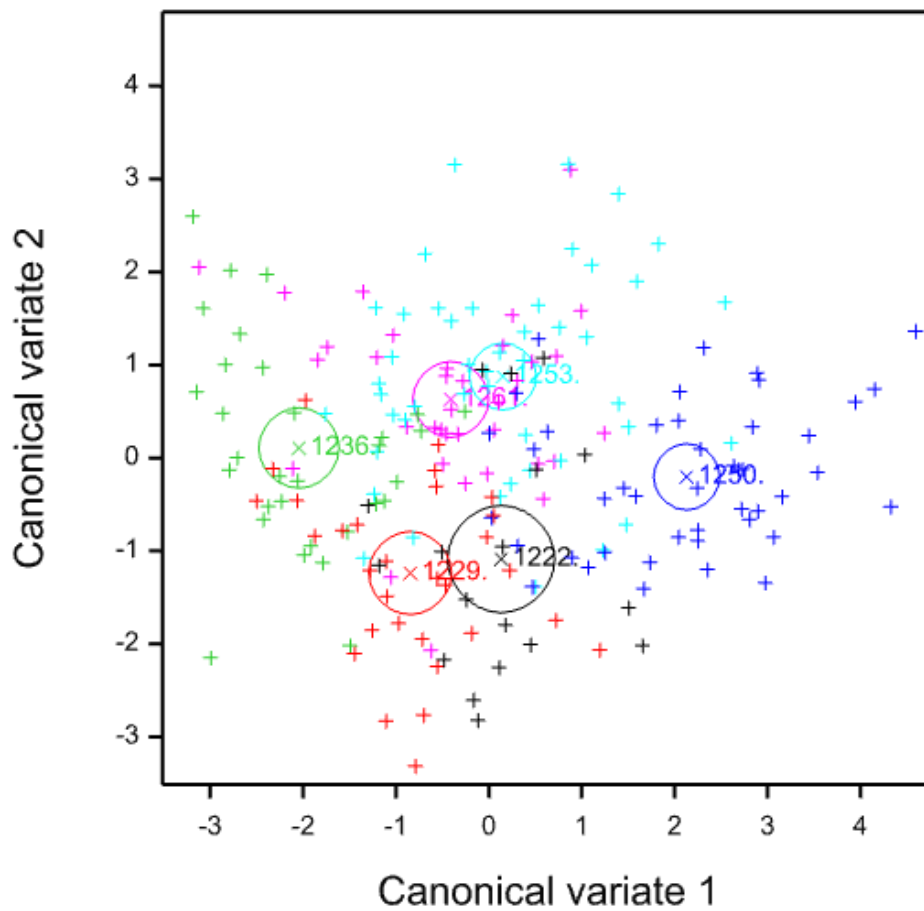


Fig. 6. Canonical variate analysis biplot showing 95% confidence intervals for population means for seedlings in six University of Arkansas blackberry populations in 2015 using standardized values for fructose, glucose, isocitric lactone, isocitric/citric acid, malic acid, titratable acidity, pH, and soluble solids content as components at the Fruit Research Station, Clarksville, AR.

The CVA for USDA-ARS material identified six dimensions, with the first two explaining 74.7% of variation amongst individuals. The CVA biplot gave 95% confidence intervals for the mean values for each population when weighing the standardized variables. These confidence intervals indicated that populations 4540, 4651, 4660, and 4674 had significantly different means from one another and the remaining four populations (Fig. 7). Population 4534 was not significantly different from population 4647, and population 4647 was not significantly different from 4665. Population 4665 was not significantly different from 4650, and the latter two populations had the greatest overlap at the 95% confidence interval.

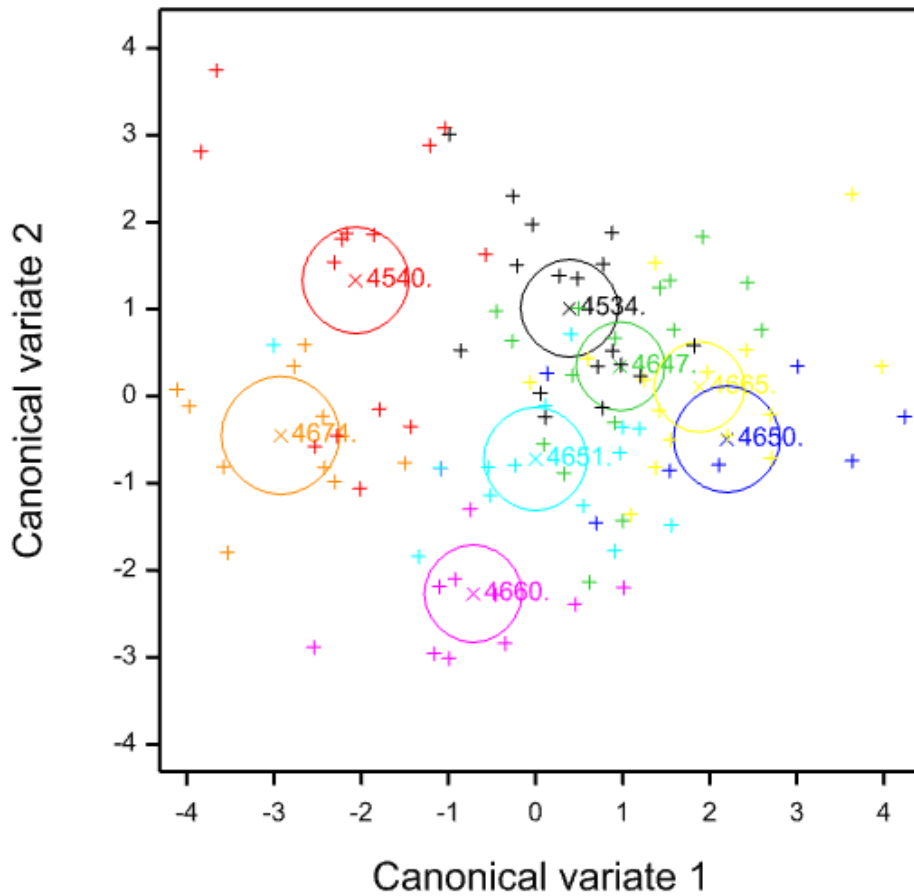


Fig. 7. Canonical variate analysis biplot showing 95% confidence intervals for population means for seedlings in eight U.S. Department of Agriculture Agricultural Research Service Horticultural Crops Research Unit blackberry populations from the North Willamette Research and Extension Center, Aurora, OR in 2015 using standardized values for fructose, glucose, isocitric lactone, isocitric/citric acid, malic acid, titratable acidity, pH, and soluble solids content as components.

Discussion

Part 1:

The phenotyping protocol was created to characterize seedlings within populations with the objective of gaining phenotypic data resolution high enough to perform fruit quality-related QTL analysis with corresponding genetic information. Horticulturally important traits for blackberry were identified and evaluated for future projects involving the original RosBREED blackberry seedling set.

The design of this experiment was a limiting factor in the analysis of the distinguishing ability of the protocol, which could have been improved with replicated plants, multiple years of usable data, and control of missing values. This should be considered at all points of interpreting any trends in data.

The effect of genotype and environment on fruit quality is a phenomenon that is easily observed when comparing the growing operations in the Pacific Northwest and those in Arkansas, and these differences could be seen in the numerical differences amongst population and location means—for example, the larger TA and SSC values in USDA-ARS material when compared to UA.

In Arkansas, more intense spring temperatures cause ripening times to occur in a tighter range than in Oregon (Finn and Clark, 2012). At the same time, the earliest trailing blackberry genotypes in Oregon begin to ripen approximately two weeks before the earliest erect blackberry genotypes in Arkansas, indicating the genotype and location effects on ripening (Finn and Clark, 2012). Differences amongst populations could be observed qualitatively in the field. For example, population 1261, a UA × USDA-ARS cross, appeared less vigorous than population

1229, a UA × UA cross that had many individuals with excessive primocane growth that consistently reached 1.8 m in height.

Similar to plant vigor, crop load potential was significantly affected by population, which could be observed qualitatively. Crop load potential ratings of 9 were not observed in population 1261, but could be observed in several UA populations. The lack of prolific bloom in population 1261 and its less vigorous plants were predicted prior to this evaluation and it was hypothesized that some level of genetic incompatibility between parents contributed to these characteristics (J.R. Clark, personal communication).

Acidity and sweetness are commonly used as basic descriptors of fruit quality (Evans et al., 2011; Mathey et al., 2013; Stegmeir et al., 2011). The partitioning power of pH in UA material and SSC in USDA-ARS material suggested that these two traits are major blackberry fruit characteristics, and that a great deal of variation can be attributed to both, across breeding programs. If seedlings from a similar genetic background are evaluated in future studies, these findings suggest that pH and SSC may be reasonable candidates for genetic analysis.

MANOVA

While the one- and two-way ANOVAs gave insight to individual traits according to population, year, and the interaction effect of the two, biological systems are multidimensional and overall quality is usually measured with this in mind. The MANOVA canonical centroid plot plotted multivariate least squares means on the first two canonical variables to indicate 95% confidence intervals for population mean values. In UA material, pH and TA contributed the most to separating the confidence intervals for populations, and only populations 1222 and 1261 had similar means due to their berry weight and SSC values. The canonical centroid plot was

used primarily to understand the general relationships amongst populations, and indicated if multivariate partitioning and clustering would be appropriate for understanding which characteristics contributed most to distinguishing power.

Multivariate discrimination: recursive partitioning

For UA populations, 262 individuals were partitioned into groups according to the variation in characteristics considered. In this model, pH contributed the most to partitioning individuals, followed by firmness, TA, crop load potential, berry weight, and SSC. This order of characteristics showed that three quantitatively measured traits, pH, firmness, and TA, accounted for 77.8% of partitioning of the 262 UA seedlings, and that these three attributes could be used to characterize genetically related populations for future studies. The range of values for SSC was the least distinguishing of the quantitatively measured traits, less so than berry weight or the qualitatively measured crop load potential. This suggests that sugar content (whether SSC or glucose or fructose content) must be evaluated with high precision due to its smaller range of values in order to distinguish UA seedlings.

Clustering

Ward's method of hierarchical clustering with a preset of six clusters indicated that TA absorbed the largest portion of total variation, followed by SSC, vigor, and pH. As before, TA appears to be important in distinguishing amongst populations and seedlings for UA material. The hierarchical clustering method emphasized the importance of SSC as well, with two of six clusters (1 and 2) distinguished from the other four by their high mean SSC values. Though SSC

had a smaller numerical range for variation than TA, firmness, crop load potential, and berry weight, it is an important characteristic in hierarchical clustering.

The K-means cluster method was used to separate individuals into a three-dimensional plot and was selected due to the number of characteristics evaluated distorting the clarity of the 2-D biplot. The fairly large cluster sizes (greater than 20) indicated that N=6 was an appropriate number of clusters for UA material.

Part 2:

Individual sugars and organic acids

Previous literature reported ranges for citric acid in cultivated blackberries to be between 0.56% and 1.20%, malic acid to be between 0.14% and 0.63%, isocitric lactone to be approximately 0.29%, and isocitric acid to be approximately 0.60% (Kaume et al., 2012; Mikulic-Petkovsek et al., 2012; Sensoy et al., 2013). Additionally, previous studies indicated that glucose content in cultivated blackberries ranged from 0.88 to 3.24% and fructose content to range from 0.81% to 2.69% (Kaume et al., 2012; Mikulic-Petkovsek et al., 2012; Sensoy et al., 2013).

While both UA and USDA-ARS glucose and fructose content were within expected values according to previous literature, the organic acids analyzed for both programs were lower than expected, with only malic acid content falling within previously suggested ranges. This deviation from previously reported values could be due to several factors. Seedling fruit were collected from both UA and USDA-ARS, and were managed differently from normal commercial production practices, possibly resulting in higher disease and abiotic stress pressure, which could have impacted fruit quality. In the case of UA fruit, which had qualitatively lower

values than USDA-ARS, the late spring and early summer months of 2015 experienced significant rainfall and associated disease pressure. The excessive rain may have had a “watering down” effect on secondary metabolite content in fruit, and resulted in a lower concentration of organic acids. However, because sugar content was within expected ranges, though on the lower end, this seems unlikely. Another aspect to consider is that the reported values from previous studies were from evaluation of different genotypes. Although some of the published data were from the same programs considered in this study, it is possible that the individuals used varied to some extent from selections or cultivars measured in previous publications as opposed to evaluation of seedlings in this study. This hypothesis could be verified with additional years of fruit quality data paired with weather data. A more likely possibility is that errors were made in identifying and quantifying sample chromatograms. Because samples were only physically filtered and no chemical purification process was implemented, sample chromatograms had a great deal of noise, with many unidentified peaks. Quantification of sample chromatograms was based on standard retention times, but contamination could have distorted organic acid peaks and led to erroneous calculations.

Overall, UA populations tended to have more significant differences when comparing population means, which is likely a function of larger population sizes when compared to USDA-ARS.

In UA populations, population 1261 stood out as having the numerically lowest SSC, though this was not directly reflected in the glucose and fructose content. Because SSC is not a direct measure of sugar content, this could indicate that this UA × USDA-ARS biparental population had fewer detectable organic and amino acids, soluble pectins, and other compounds. However, population 1261 had significantly higher organic acid content for all three organic

acids than 1229, which had the numerically highest SSC of all UA populations, suggesting that 1261 simply had fewer detectable compounds in the juice according to the instrumentation available in this study. Population 1229 had the numerically highest SSC and numerically second highest pH of all UA populations. The high SSC value was reflected primarily by the glucose and fructose content, which were also the numerically highest of all UA populations, suggesting that for 1229, SSC is considerably affected by sugar content.

In USDA-ARS populations, population 4660 was notable in that it had the numerically highest SSC, and some of the lower values for all three organic acids. Overall, the most notable factors of the USDA-ARS acids and sugars composition were that though populations ranged much more widely in organic acids than for UA populations, the glucose and fructose contents were not significantly different. While there were significant differences in population means for SSC for both UA and USDA-ARS, only UA had significantly different glucose and fructose content means. Two possible explanations for this distinct difference are: glucose and fructose content are highly environmentally dependent, and because of the more consistent temperatures during the summer at the USDA-ARS location, little difference in sugar content was observed for the USDA-ARS populations. Alternatively, it may be that sugar content is mostly genotype dependent, and the western germplasm has less variation in regard to glucose and fructose content than the eastern germplasm. Most likely, sugar content is a function highly dependent on both genotype and environment. Because the weather data collected for both experiment stations did not include light intensity but only temperatures and precipitation, it is not possible to extrapolate how much photosynthetically active radiation was available at either location and if any variation in this between locations affected the glucose and fructose content of each population.

PCA-UA

The HPLC data allowed for greater resolution when considering the quantitatively evaluated characteristics, and expanded on the SSC, pH, and TA values. The importance of quantifying glucose and fructose was especially evident in the complementary table of correlations, as SSC was highly correlated with both sugars. Though this was an expected outcome, it suggests that future molecular breeding approaches could focus on identifying the smaller number of loci contributing to individual sugars as opposed to overall SSC. Identification of loci imparting smaller genetic gains is more likely than identification of major effect QTLs, especially when considering a complex quantitative trait such as SSC.

Malic acid was the most prevalent organic acid detected in UA seedling fruit, which is in agreement with previous findings, though there are wide ranges of acceptable values for organic acids due to the variability of blackberries derived from different germplasm (Kaume et al., 2012; Sensoy et al., 2013; Vergara et al., 2016). Though isocitric/citric acid levels were lower than expected according to previous studies, these two acids were most closely related to TA and pH. Because the peaks of these two acids eluted at very similar times and were quantified together, these correlations relate the effect of the two acids on pH and TA rather than as separate acids, inaccurately magnifying the apparent effect of one peak. This preliminary PCA strengthens the argument for looking for molecular markers or advancing molecular strategies in augmenting overall SSC by looking for loci associated with individual sugars.

PCA—USDA-ARS

The PCA for USDA-ARS material showed very similar trends as for UA, with high correlations between individual sugars and SSC, though isocitric/citric acid was only highly

correlated with TA rather than TA and pH. As with UA, the correlations between individual sugars and SSC indicated promise for pursuing molecular work on advancing SSC by focusing on glucose or fructose content. A characteristic of note for the USDA-ARS material's PCA was the relationships between the organic acids, which had very weak positive correlations with one another as opposed to the moderate correlation between isocitric lactone and malic acid in UA seedlings. This can be seen in the two biplots: for UA material, most variables could be assigned to one of two qualitatively perpendicular vectors, while in USDA-ARS, two sets of perpendicular lines were visible: malic acid apparently bisecting isocitric lactone at a near 90 ° angle, and the remaining characteristics aligned as in the UA PCA biplot. These quantitative differences in correlations amongst acids, as qualitatively depicted in the biplots, indicated that the USDA-ARS and UA material had differing acid profiles, though the relationship between sugars and SSC, and the relationship between pH, TA, and isocitric/citric acids were similarly and closely correlated. Pacific Northwest blackberries have consistently been described as having different fruit flavor profiles than eastern U.S. blackberries (Clark, 2016; Clark and Finn, 2011). Two major differences that have been described both qualitatively and quantitatively are in sweetness and acidity, with western-type blackberries usually having higher perceptible sweetness and acidity than their eastern counterparts (Clark and Finn, 2011). This distinction was further demonstrated in the generally higher mean SSC and TA values for USDA-ARS material when compared to UA for most populations. A similar trend was observed for isocitric/citric acid, isocitric lactone, and malic acid contents, with UA populations generally having lower acid content, while USDA-ARS generally had higher acid content as well as a wider range than UA. However, the genotypic, environmental, and objective differences between eastern and western blackberries should be considered in this distinction: the Pacific Northwest region experiences

less intense summer temperatures than the southeastern United States, and processing blackberries can be harvested in a wider range of maturities than fresh-market blackberries.

For both UA and USDA-ARS, the one-year PCA indicated that having highly quantitative continuous variables like individual sugar and organic acid content adds resolution, even to one-year data. Future analysis of blackberry seedling populations should include HPLC or a similar methodology to optimize phenotypic resolution with one analysis.

CVA

The CVA for both UA and USDA-ARS material gave a means of evaluating variation amongst populations according to fruit composition characteristics including HPLC data. Classification of the different populations by standardizing variables allowed for a qualitative and quick way to understand how populations were organized in a multi-dimensional space. For UA, populations 1236 and 1250 were the most different, while 1229 and 1222 were similar, and 1261 and 1253 had some overlap ($\alpha = 0.05$). For USDA-ARS material, population 4651 was significantly different from other populations, though close to 4647. The CVA output underscores the importance of improving the resolution of broad characteristics like SSC, pH, and TA. For example, the two-year data for USDA-ARS indicated that population 4651 had the lowest pH and highest TA, and for the means separations it was the only population that was significantly different from others in these two categories. However, with the addition of glucose, fructose, isocitric/citric acids, malic acid, and isocitric lactone, 4651 appears to be much closer to the grand mean for USDA-ARS material when accounting for 74.7% of total variation amongst individuals.

Conclusions

The primary objective of this study was to develop and implement a phenotyping protocol for blackberry seedlings and populations which resulted in a protocol with a focus on fruit composition characteristics. An ideal phenotyping protocol would be able to distinguish amongst seedlings within populations to complement genetic sequence data.

However, this protocol was not able to identify individual seedlings by phenotype, and the MANOVA was sometimes unable to distinguish even amongst populations. While recursive partitioning using a decision tree was able to split seedlings into predetermined groups based on phenotypic data, this did not result in the populations as the study was designed—recursive partitioning did not divide the pool of seedlings into the original six populations for UA, nor the eight populations for USDA-ARS. Some of this is attributable to the fact that blackberry seedling populations, by design, segregate for all qualitative and quantitatively inherited traits, so populations would not be uniform, and would not necessarily have distinguishing characteristics except in the case of Mendelian traits. This issue illustrates the importance of a phenotyping protocol that is designed to evaluate individuals rather than populations.

Juice compositional characteristics from 2015 reflected differences in UA and USDA-ARS material, particularly with SSC, acidity, and individual sugars and acids. Generally, USDA-ARS material had higher SSC and TA than UA. For individual sugars, an interesting finding was that USDA-ARS material showed no significant differences while the UA means were significantly different amongst populations.

In similar future studies, seedlings should be replicated and locations should be added to improve the reliability and quality of data and allow for more complex multivariate models as

well as multi-way ANOVA. Phenotypic traits should include as many quantitative rather than qualitative methods as possible so that continuous distribution models can be used.

Though this phenotyping protocol was not fully successful in identifying significant differences amongst blackberry seedlings or seedling populations, it was able to test and identify traits of importance for improving distinguishing power. The quantification of individual sugars and organic acids added useful insight to the variation amongst populations and individuals, and should continue to be part of the phenotyping protocol for blackberry fruit quality. The phenotyping protocol for blackberries should be improved and the methodology should be refined for precision and accuracy, and future work should emphasize experimental design to improve statistical power.

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Chapter 2

Impact of Time of Day of Harvest on Red Drupelet Reversion for University of Arkansas Blackberry Genotypes

Abstract

Blackberry (*Rubus* L. subgenus *rubus* Watson) genotypes (cultivars and advanced selections) from the University of Arkansas (UA) are bred primarily for their value in the fresh market. In addition to resistance to decay and change in texture or flavor, the overall appearance is important for fresh-market blackberries. Fully mature blackberries are typically completely black and shiny. However, some blackberries undergo red drupelet reversion (RDR), a phenomenon in which black drupelets turn irreversibly red after berries are harvested. Commercial blackberry growers have indicated that the occurrence of RDR is related to the time of day of harvest. Performance of nine UA blackberry genotypes harvested at different times of day (7 AM, 12 PM, and 4 PM) were evaluated by measuring air and fruit surface temperature at harvest, fruit firmness before and after storage, weight loss after storage, and percent RDR after storage. Blackberries were harvested from two plants per genotype and used as replication. Air and fruit surface temperatures increased as harvest time increased during the day, with the largest increase in fruit temperature occurring between 7 AM and 12 PM and a smaller increase between the 12 PM and 4 PM harvest times. Two-way analysis of variance indicated weight loss per berry was not impacted by harvest time, but was impacted by genotype with no interaction. For berry firmness, there was a significant genotype \times harvest-time effect on change in berry firmness, with no significant genotype or harvest-time impact. For RDR, the genotype \times harvest-time interaction was significant using a generalized binomial regression model. The firmest genotype, A-2453, had the least RDR of all genotypes across all harvest times, while berries harvested for all genotypes at 7 AM generally had less RDR than those harvested at 12 PM or 4 PM. For the

nine blackberry genotypes evaluated in this study, the significant interaction effects indicated that mitigation of blackberry change in firmness and incidence of RDR are affected by both genetic and environmental factors and cannot be reliably estimated.

Introduction

Begun by Dr. J. N. Moore in 1964, the University of Arkansas (UA) blackberry-breeding program has focused on releasing blackberry (*Rubus* L. subgenus *rubus* Watson) cultivars mainly intended for the fresh market, for which postharvest quality is of great importance (Clark and Finn, 2011). Breeding objectives for UA blackberries include improving postharvest storage potential and maintaining an attractive appearance of berries for consumers (Clark, 2016).

The physical appearance of fresh-market fruit is a major component of consumer acceptability. Sensory evaluation of berries can include subjective evaluation of appearance liking, which is negatively correlated with blemishes, discoloration, lack of symmetry, or signs of disease. For example, with strawberries (*Fragaria* × *ananassa* Duch.), fruit shape, symmetry, malformation from unfilled achenes, external color, and gloss are all visual characteristics of interest for strawberry breeders (Mathey et al., 2013). For fresh-market blackberries, descriptive sensory panels can evaluate attributes such as basic tastes, feeling factors, overall aromatic impact, texture, and appearance, which can be impacted by color, uniformity of color, and glossiness (Segantini et al., 2017).

Postharvest blackberry quality has been evaluated by measuring blackberry firmness, changes in fruit composition during storage, and variability amongst genotypes (Perkins-Veazie et al., 2000; Salgado and Clark, 2016; Segantini et al., 2017). More recently, red drupelet reversion (RDR), a phenomenon in which black drupelets become irreversibly red after harvest, particularly during storage, has become an area of interest. This color change can negatively impact consumer perception and marketability of fresh-market blackberries. Though the underlying physiological mechanism is not known, some southern U.S. blackberry growers suggested that the occurrence of RDR increases at later harvest times during the day and is

associated with higher temperatures during harvest. Little is known about RDR, and understanding how genotypic and environmental factors influence occurrence could give growers techniques for mitigation.

Salgado and Clark (2016) investigated UA blackberry genotypes and found that “crispy”-textured blackberries had lower RDR incidence and better shelf life when compared to conventional softer genotypes like ‘Natchez’. When examined under a confocal microscope, cross sections of ripe UA “crispy” genotype, A-2453, appeared to maintain cellular integrity while cross sections of ripe conventional ‘Natchez’ showed that individual cells lost their structural integrity and could not be distinguished (Salgado and Clark, 2016).

Anthocyanins are phenolic compounds responsible for the distinctive red, blue, and purple pigments in berries and are important for nutraceutical properties (Filip et al., 2012). Blackberry anthocyanins are accumulated in the relatively acidic vacuole and maintenance of structural integrity of pH-specific organelles prevents the mixing of cell contents. For example, in apples (*Malus domestica* Borkh.) this sequestration helps prevent oxidation and interactions with plastid and cell wall-localized polyphenol oxidase enzymes (Murata et al., 1997).

Commercially, blackberries are harvested at the “shiny black” stage, during which drupelets are fully black, turgid, and shiny (Clark et al., 2007). Because blackberries mature from green to red to black, RDR may negatively impact marketability and consumer acceptance due to the appearance of red drupelets. McCoy et al. (2016) found a significant difference in RDR across eight UA blackberry genotypes when harvested at 7 AM compared to all other harvest times (10 AM, 1 PM, and 4 PM), with the blackberries harvested at 7 AM having the least RDR. Consequently, it was hypothesized that there was a time of harvest effect and transitively, an air temperature effect on RDR though the phenomenon appeared to be impacted by the genotype.

The purpose of this study was to further the work of McCoy et al. (2016) on red drupelets in blackberries and also evaluate the blackberry firmness and weight loss after storage at 4-6 °C for 7 d.

Materials and Methods

Plant material and culture

Plants from nine genotypes were evaluated in this study: A-2450, A-2453, APF-77, APF-268, 'Natchez', 'Osage', 'Ouachita', 'Prime-Ark® 45', and 'Prime-Ark® Traveler'. The plants were propagated using adventitious shoots from field-grown root cuttings in late winter to early spring of planting at the UA Fruit Research Station (FRS), Clarksville (west-central Arkansas, lat. 35°31'58''N and long. 93°24'12''W).

Raised beds approximately 20 cm high and 1.0 m wide were prepared with black plastic mulch laid over the beds. Two 3.1 m plots containing five plants spaced 0.6 m apart were established. All plants were planted in the prepared beds at FRS in May 2012 with the exception of A-2450 that was planted in May 2013. Plants were trained to a T-trellis with two lower wires approximately 0.5 m from the soil surface spaced 0.5 m apart and two upper wires approximately 1.0 m high spaced 0.8 m apart (Fernandez et al., 2016).

Standard cultural practices for erect blackberry production were used including annual spring nitrogen fertilization ($56 \text{ kg}\cdot\text{ha}^{-1} \text{ N}$) using ammonium nitrate (Fernandez et al., 2016). Genotypes APF-77, APF-268, 'Prime-Ark® 45', and 'Prime-Ark® Traveler' received an additional application of ($23 \text{ kg}\cdot\text{ha}^{-1} \text{ N}$) after the floricane crop was completed, which was in late June most years. Primocanes were tipped at 1.1 m height in mid-June and again in late July to early August. Dormant pruning consisted of removing dead floricanes and also removing primocane tissue to a point below the flowering area on the primocanes. The plants received a single application of liquid lime sulfur ($94 \text{ L}\cdot\text{ha}^{-1}$) at bud break for control of anthracnose (*Elsinoe veneta* [Burkholder] Jenk.). Raspberry crown borer (*Pennisetia marginata* [Harris]) was controlled by a single application of a labeled insecticide with bifenthrin as the active ingredient

in October of each year. Insecticides labeled for commercial use in Arkansas were used for spotted-wing drosophila (*Drosophila suzukii* Matsumura) control. The plants were irrigated as needed using trickle irrigation. There were two five-plant plots for each blackberry genotype in this study.

Harvest

Fruit for this study was harvested from two blackberry plants per genotype. One blackberry plant was randomly selected per plot, providing two replications of one plant per replication for each genotype. Blackberries were harvested from June and July of 2016 with all harvests for one genotype completed on the same day. The date of harvest for each genotype was determined to be when both replications for one genotype had approximately 35-50% of total floricanes fruit at the shiny-black stage. The plants were harvested at 7 AM, 12 PM, and 4 PM. Air temperature at the beginning of each harvest time for the location of the plants at FRS was recorded as a single value from the National Weather Service station located approximately 500 m from the harvested plants.

For each genotype and harvest time, two 260 g clamshells (FormTex Plastics Corp., Houston, TX) of blackberries were harvested for each replication (plant). Nine genotypes \times three harvest times \times four clamshells were harvested for a total of 108 clamshells. Clamshells were filled to a commercially acceptable volume without contact with the lid. Only shiny, completely black berries with no physical damage, discolored drupelets, or other visible imperfections were harvested for this study. In the event of precipitation, no berries were harvested and a 24 h window of dry weather was required before harvest. Fruit surface temperature was taken on five

berries on the plants immediately preceding harvest using a point-and-shoot instant-read thermometer (Raytek ST20 Pro Infrared Thermometer, Raytek Corp., Santa Cruz, CA).

Of the two clamshells harvested per plant, genotype, and harvest time, one clamshell was used for determining fresh fruit firmness and initial RDR. The second clamshell was used for evaluation of weight loss, firmness, and RDR after storage at 4-6 °C for 7 d.

Fruit evaluation

Immediately after harvest, clamshells were transported to the laboratory and their fresh weights were recorded as well as number of blackberries per clamshell. Ten berries were removed from the clamshells designated for fresh measurements only, equilibrated to room temperature (RT) and were evaluated for firmness. For firmness, blackberries were placed horizontally on an Icon texture analyzer (Texture Technologies Corp., Hamilton, MA) for compression measurements. The texture meter was equipped with a cylindrical plane probe measuring 7.6 cm in diameter to determine the force (N) required to compress each individual berry 5 mm from the point of contact, with a trigger force of 0.02 N.

The clamshells designated for cold storage were transferred to vented cardboard packing flats in a single, randomized layer of six clamshells per flat and stored at 4-6 °C for 7 d. After 7 d, the clamshells were brought to RT and weighed to determine the weight lost during storage, and this value was divided by the number of berries to determine the weight lost per berry. Ten berries were randomly selected from the clamshells and analyzed for firmness by compression. Five additional berries were randomly selected and evaluated for RDR using a paint pen to mark and count the number of red drupelets and total drupelets per berry (Fig. 1).



Fig. 1. A blackberry mounted on a toothpick for counting red and total drupelets with a paint pen.

Statistical design and analysis

The statistical design included two plant replications for each of the nine blackberry genotypes and the three harvest times. Statistical analyses were performed using JMP® Pro Version 13.0.0 (SAS Institute Inc., Cary, NC). The air temperature at each harvest time was recorded as a single value. Average fruit temperature was calculated for each harvest time for the four harvest dates, and standard errors were calculated for these average values. The difference between average fruit temperature and air temperature at each harvest date and harvest-time combination was calculated (average fruit temperature °C – air temperature °C), and standard errors were calculated for the difference between average fruit temperature and air temperature.

A two-way analysis of variance (ANOVA) was used to evaluate the main effects of genotype and harvest time and interactions on blackberry firmness and weight lost by berry. Mean separations were conducted using a Tukey's honest significant difference (HSD) test ($\alpha = 0.05$).

Red drupelet incidence was considered categorical in this study, with the two possibilities of either “red” or “not red” for each drupelet. Consequently, a generalized binomial regression model using the χ^2 test for significance was used to evaluate the degree of independence of RDR for genotype, harvest time, and genotype \times harvest-time interaction. Results for RDR were reported as percent red drupes per berry.

Results

Air and berry surface temperature

For all four harvest dates, both air and fruit surface temperatures increased with later harvest times. For fruit surface temperature the largest increase occurred between 7 AM and 12 PM, and a consistently smaller increase occurred between 12 PM and 4 PM (Table 1). Fruit surface temperature was usually higher than air temperature, except in three of four 7 AM harvest times, and one 4 PM harvest time in which air temperature was higher than fruit surface temperature (Table 1). For every harvest date, the difference between fruit surface and air temperature was greatest at 12 PM, with the fruit surface temperature higher than air temperature (Table 1).

Table 1. Air temperatures and average blackberry fruit surface temperatures on dates of harvest at 7 AM, 12 PM, and 4 PM at the Fruit Research Station in Clarksville, AR 2016. Standard error values provided for mean fruit surface temperatures and temperature difference.

Harvest date ^z	Harvest time	Air temperature ^y (°C)	Fruit surface temperature ^x (°C)	Temperature difference (fruit surface °C – air °C)
161 (9 June)	7 AM	22.2	22.9 ± 0.4	0.7 ± 0.4
161 (9 June)	12 PM	28.8	31.5 ± 0.4	2.7 ± 0.4
161 (9 June)	4 PM	30.8	32.4 ± 0.7	1.6 ± 0.7
168 (16 June)	7 AM	25.6	25.4 ± 0.4	-0.2 ± 0.4
168 (16 June)	12 PM	31.4	35.3 ± 1.6	3.9 ± 1.6
168 (16 June)	4 PM	34.1	36.3 ± 0.8	2.2 ± 0.8
173 (21 June)	7 AM	24.4	22.7 ± 1.1	-1.7 ± 1.1
173 (21 June)	12 PM	29.7	32.8 ± 0.9	3.1 ± 0.9
173 (21 June)	4 PM	33.1	35.7 ± 0.2	2.6 ± 0.2
193 (11 July)	7 AM	21.2	20.3 ± 0.7	-0.9 ± 0.7
193 (11 July)	12 PM	26.7	28.7 ± 1.9	2.0 ± 1.9
193 (11 July)	4 PM	30.2	29.0 ± 1.0	-1.2 ± 1.0

^z Day of harvest recorded as days from 1 Jan. 2016.

^y Air temperature measured at the harvest time indicated, with a single air temperature value per harvest time and harvest date combination, using weather station located at the University of Arkansas Fruit Research Station, Clarksville, AR.

^x Average value of fruit surface temperature of blackberries harvested from the nine genotypes evaluated, with each genotype represented by two replicated plants and five berries measured for fruit surface temperature for each plant, with temperature readings taken immediately before removing berry from the plant.

Change in blackberry weight lost and firmness

Two-way ANOVA indicated weight lost per berry was not impacted by harvest time, but was impacted by genotype with the no significant interaction between the main effects (Table 2).

For berry firmness, there was a significant genotype \times harvest time effect on change in berry firmness, with no significant genotype or harvest-time impact (Table 2).

Table 2. Main and interactive effects of harvest time and genotype on blackberry fruit weight lost by berry and change in firmness for nine genotypes evaluated after storage at 4 – 6 °C for 7 d at the Fruit Research Station, Clarksville, AR 2016.

Effect	Weight lost by berry <i>P</i>	Change in berry firmness <i>P</i>
Genotype	<0.0001*	0.4543
Harvest time	0.1922	0.3906
Genotype \times harvest time	0.8006	0.0071*

^z*P* = p-value ($\alpha=0.05$) for effect on dependent variable indicated. *Significant for the F-test.

Mean separation using Tukey's HSD showed that for weight lost by berry, APF-268, APF-77, and 'Natchez' lost the most weight and were not significantly different from one another, though 'Natchez' lost the most weight, numerically (Table 3). Genotype A-2453 lost the least weight numerically, but was not significantly different from A-2450, 'Osage', or 'Prime-Ark® 45' (Table 3).

Table 3. Mean values for the nine blackberry genotypes, averaged across harvest times of 7 AM, 12 PM, and 4 PM for weight lost by berry. Genotypes were replicated using two plants (n = 2), with one full 260 g clamshell used for each replication and harvest-time combination, and evaluated in 2016 at the Fruit Research Station, Clarksville, AR.

Genotype	Weight lost per berry ^w (g)
A-2450	0.112 cd ^y
A-2453	0.088 d
APF-268	0.165 a
APF-77	0.164 a
Natchez	0.179 a
Osage	0.110 cd
Ouachita	0.123 bc
Prime-Ark® 45	0.119 bcd
Prime-Ark® Traveler	0.147 ab
<i>P-value</i>	<0.0001* ^z

^z * = significant value for F-test

^y Means with different letter(s) are significantly different ($\alpha=0.05$) using Tukey's honest significant difference test ($\alpha = 0.05$).

^x Weight lost by berry was calculated by dividing the amount of weight lost per clamshell after the 7 d at 4 – 6 °C by the number of berries per clamshell.

Change in firmness interaction means indicated different responses amongst genotypes for the different harvest times (Fig. 2). For instance, 'Osage' had rather consistent and large changes in firmness across all harvest times, while 'Ouachita' and 'Prime-Ark® 45' had small changes at each harvest time. Further, 'Natchez' had substantial change at 7 AM but much less at 12 and 4 PM (Fig. 2).

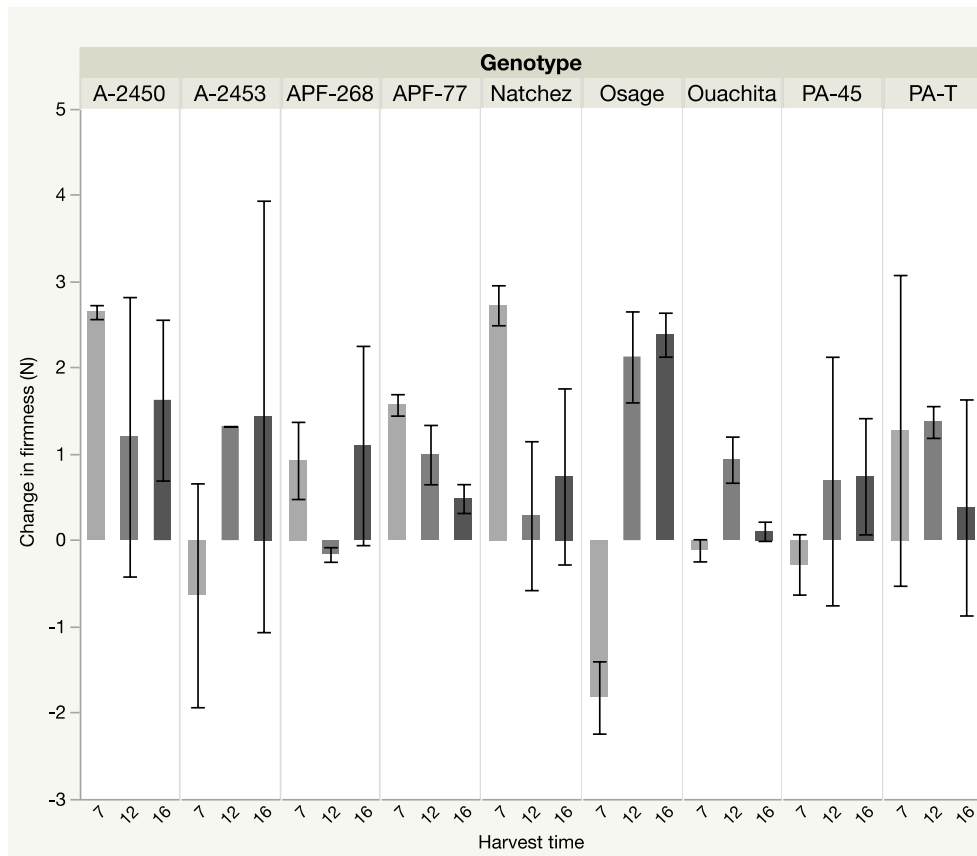


Fig. 2. Average change in berry firmness by compression values and corresponding standard error bars at three harvest times for nine University of Arkansas blackberry genotypes after 7 d of cold storage at 4 – 6 °C in 2016 at the Fruit Research Station, Clarksville, AR. (PA-45 = Prime-Ark® 45; PA-T = Prime-Ark® Traveler; harvest-time 16 = 4 PM).

Blackberry RDR

The binomial regression model showed that the genotype × harvest-time interaction effect was significant, as well as the harvest-time and genotype main effects (all p-values <0.001). Some genotypes followed a somewhat linear increase of RDR over harvest time, while others experienced an increase between 7 AM and 12 PM, and then decreased between 12 PM and 4 PM (Fig. 3).

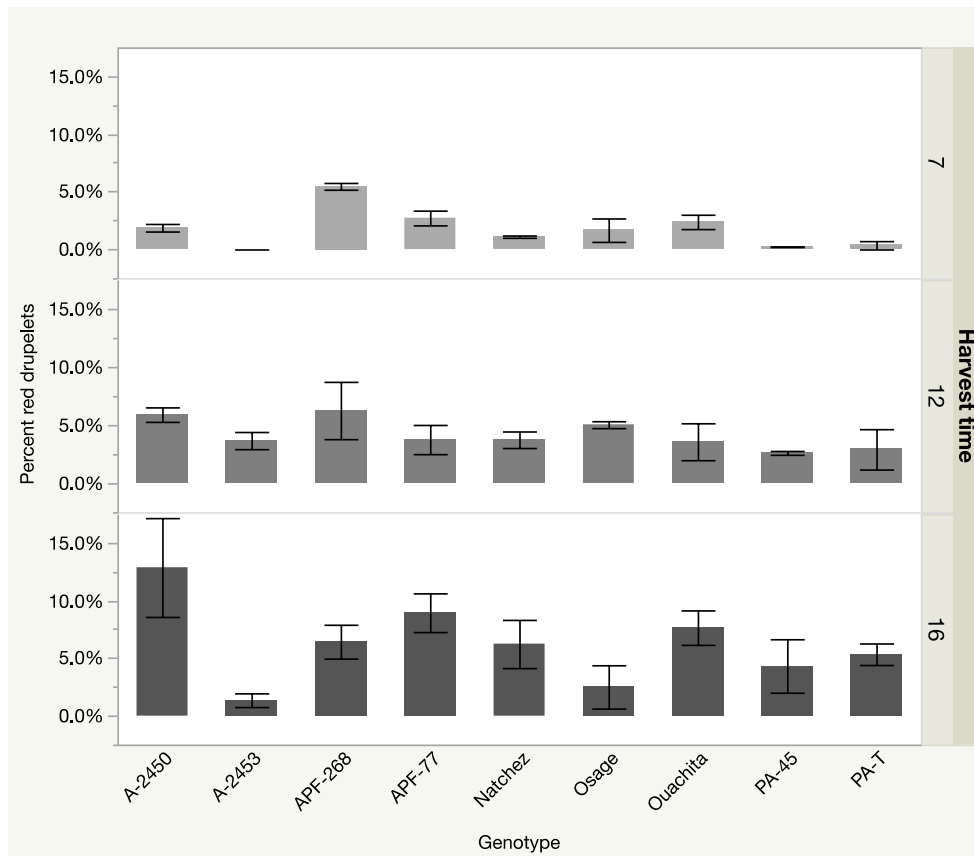


Fig. 3. Average percent of red drupelets for blackberries and corresponding standard error bars at three harvest times for nine University of Arkansas blackberry genotypes after 7 d of cold storage at 4 – 6 °C in 2016 at the Fruit Research Station, Clarksville, AR. (PA-45 = Prime-Ark® 45; PA-T = Prime-Ark® Traveler; harvest time 16 = 4 PM)

Discussion

The differences observed between fruit surface temperature and air temperature indicated that the blackberries in this study had the greatest change in temperature between 7 AM and 12 PM. Within those five hours, the fruit surface temperature changed from cooler than the air temperature (except in the case of harvest date 161) to exceeding the air temperature by 2 °C or more. This is likely explained by the fact that at 7 AM the berries had cooled overnight, with no additional solar radiation to increase fruit temperature. At this first harvest time, fruit had been exposed to sunlight for a maximum of one to two hours, while at 12 PM, fruit had been exposed to sunlight for approximately six hours. Differences amongst harvest dates for air temperatures at specific harvest times could be attributed to cloud coverage, and/or recent precipitation events. Differences in fruit temperature could also be attributed to the variability of each genotype's canopy cover of fruit.

The increase of fruit surface temperature between 12 PM and 4 PM suggested that there is an upper limit on fruit temperature. This limit is likely a combination of the plant's physiological response to heat stress, and that air temperature did not increase at the same rate throughout the day, but plateaued in the afternoon. Overall, the large increase in temperature between 7 AM and 12 PM for fruit temperature when compared to the 12 PM to 4 PM increase suggested that there might be value in investigating smaller increments of time between 7 AM and 12 PM and the associated changes in fruit surface temperature.

Weight lost per berry during storage was only significantly affected by genotype. Because weight loss in fresh horticultural products is mostly attributed to loss of water content, this genotype effect might be reflective of the fruit cuticle properties of the different genotypes, or possibly the differing rates of respiration for the fruit from each genotype. The lack of harvest-

time effect was surprising; as leaf transpiration and whole-plant water loss generally increases as temperature increases, it seemed likely that later harvest time would similarly have a significant [increasing] effect on percent weight lost per berry. However, this was not the case, and weight loss was significantly affected by genotype only.

The percent change in berry firmness had no harvest time or genotype main effects but the interaction effect was significant, indicating that different genotypes should be harvested at particular times to minimize change in firmness during cold storage. Another aspect to consider in this study is the variability in initial firmness values for each genotype. For example, APF-77 has soft fruit and is recommended as a home-garden cultivar, while A-2453 is distinguished by its crispy and accordingly high firmness value. In the case of APF-77 with a low base value for firmness, additional loss in firmness could be more detrimental than a similar percentage loss of firmness in a crispy genotype, in which the perceptible turgidity of drupelets might not change. This should be evaluated with a sensory panel, as the objective firmness-by-compression measurement in this study did not take into consideration overall mouthfeel or consumer liking, which is important in the marketability of blackberries.

For RDR, there were significant genotype and harvest-time main effects, the interaction effect was highly significant (Fig. 3). For genotypes A-2450, APF-77, ‘Natchez’, ‘Ouachita’, ‘Prime-Ark® 45’, and ‘Prime-Ark® Traveler’, the percent of red drupelets to total drupelets increased as harvest time increased. However, for A-2453 and ‘Osage’, the increase from 7 AM to 12 PM was followed by an unexpected decrease between 12 PM and 4 PM (Fig. 3). For APF-268, there was an overall increase in RDR over harvest times, there was numerically very little difference between harvest times, particularly between 12 PM and 4 PM (Fig. 3). For the six genotypes that had increased RDR over time, harvesting earlier in the morning minimized RDR,

with a harvest at 7 AM likely being the most effective for mitigating RDR. However, the lack of data between 7 AM and 12 PM indicates that in future studies, more time points between these harvest times could provide a more precise window of time for harvesting blackberries and minimizing RDR.

The best harvest time for A-2453 and ‘Osage’ was 7 AM, followed by 4 PM then 12 PM, with approximately 50% of the RDR observed at noon. This finding should be verified with additional years of data on these two genotypes.

For APF-268, harvest time did not have a major impact on RDR, with the average percent of red drupelets increasing from 5.5% to 6.4% between 7 AM and 4 PM (Fig. 3). However, it is unclear whether this is an actual property of APF-268, as the plants had some visibly stunted vegetative growth and discoloration of leaves. This could have contributed to poor fruit quality, and so the performance of APF-268 should be reevaluated with plants with normal growth and color.

One clear result for RDR was for A-2453, which experienced both the least weight loss and RDR of all genotypes. Previous work by Segantini et al. (2017) demonstrated a similar result for this “crispy” genotype, and found that A-2453 had the least percent RDR (0%) amongst 10 other advanced selections and cultivars from the UA blackberry-breeding program. The low incidence of RDR found in A-2453 in this study is also in agreement with Salgado and Clark (2016), who compared the novel texture type to ‘Natchez’ and McCoy et al. (2016) who found that A-2453 had the least RDR with earlier harvest times.

The grower in the southern U.S. states who experienced unacceptable levels of RDR in ‘Natchez’ likely had very different environmental conditions than this study at FRS, particularly with summer temperatures at lower latitudes. The results for ‘Natchez’ indicated a

numerical increase with later harvest times for RDR, and as the day progressed the RDR continued to increase. This suggests a similar finding as found in the commercial production field, although RDR was likely not as high in this study as observed in the grower's conditions.

In future studies on RDR, factors to investigate include expanding locations with more extreme temperatures and evaluating longer storage times. Hotter summer temperatures that were not reached in this study might be required to induce post-harvest physiological changes that cause change in drupelet color. Longer storage times might more accurately represent level of postharvest deterioration in commercial production, and expand results in regards to RDR. Contrastingly, reversion in some blackberries can be seen almost immediately after harvest and without cold storage, and future studies should evaluate blackberries for RDR at "Day 0" (R.T. Threlfall, personal communication).

In this study the overall amount of RDR, even in APF-268 which was ranked highest in RDR, had a maximum value of 6.4% of total drupelets or approximately six red drupelets per 100 at the 4 PM harvest time. Though a consumer panel was not consulted in this study, it seems likely that a significantly higher percentage of RDR would be required to prompt commercial rejection. However, the harvest method in this study was different from commercial practice; in this study, berries were picked individually with extreme care to prevent visible damage, and each berry was examined for physical imperfections. In a commercial setting, blackberries can be subjected to more handling at harvest and during shipment, and this could impact the berries' postharvest performance.

Conclusions

Overall, genotype A-2453, a novel “crispy” genotype had the least RDR and weight loss per berry. Change in berry firmness was significantly affected by the interaction of genotype and harvest time, indicating that genotypes should be evaluated individually for their fruit firmness in response to harvest time. The findings from this study indicate that though RDR generally does increase with later harvest times, the severity cannot be reliably estimated using a single model when considering different genotypes.

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Overall Conclusions

These studies focused on strengthening and expanding the available information on blackberries, particularly within the University of Arkansas breeding program. The phenotyping protocol was developed over the course of two years, and identified that fruit compositional characteristics are important sources of variability amongst individuals and populations. Additionally, soluble solids content, pH, and titratable acidity showed high degree of phenotypic resolution. The results of this study suggested that future RosBREED blackberry work would benefit from focusing on populations initially designed for genetic analysis. Knowledge of the challenges with data collection, statistical analysis, and experimental design in this study will be used in the future to streamline the process of blackberry phenotyping.

In the postharvest evaluation of University of Arkansas blackberry genotypes, the main objective was to identify genotypic and time of harvest effects on berry weight loss, change in fruit firmness, and incidence of red drupelet reversion after cold storage. Overall, harvesting blackberries earlier in the day, particularly at 7 AM, resulted in lower reversion. Weight lost per berry due to cold storage was significantly affected by genotype. However, significant interaction effects indicated that both change in firmness and incidence of red drupelets should be evaluated using a model considering both genotype and harvest time effects.

The incidence of RDR was significantly lower in A-2453, a finding that was supported by previous studies. This reinforces evidence that the “crispy” genotype has potential either as a cultivar with great storage capacity on top of novel texture, or as a breeding parent to impart firmness and exceptionally and consistently low reversion in the University of Arkansas blackberry breeding program. Overall, it is recommended that growers harvest blackberries earlier in the morning, or select a cultivar that is resistant to RDR. With additional years of data,

more genotypes, and diverse growing environments, the postharvest phenomenon could be better understood, and its genetic and environmental contributors could be identified more clearly.

Appendix A. Temperature and Precipitation Data for UA FRS and USDA-ARS Corvallis for 2015 and 2016

Table. A.1. Average minimum and maximum temperatures (°C) and average precipitation (mm) by month for 2015 and 2016 at University of Arkansas Fruit Research Station, Clarksville, AR.

Year	Month	Average maximum temperature (°C)	Average minimum temperature (°C)	Average precipitation (mm)
2016	January	7.9	-2.1	41.1
2016	February	12.5	1.5	30.7
2016	March	17.4	6.3	229.9
2016	April	21.1	11.0	101.9
2016	May	23.8	14.2	122.2
2016	June	31.1	20.9	42.9
2016	July	30.3	21.1	173.5
2016	August	30.5	21.8	123.7
2016	September	29.8	18.4	39.6
2016	October	25.6	13.9	18.8
2016	November	18.6	7.9	99.1
2016	December	10.2	-0.8	23.6
2015	January	7.5	-2.6	82.3
2015	February	7.0	-4.5	53.6
2015	March	14.1	5.4	195.8
2015	April	21.1	10.8	94.7
2015	May	23.2	13.8	455.9
2015	June	29.2	20.4	189.5
2015	July	31.7	22.7	124.2
2015	August	30.2	20.0	127.3
2015	September	28.9	18.6	47.2
2015	October	23.1	11.8	59.4
2015	November	15.7	7.7	306.3
2015	December	13.4	3.7	236.0

Table. A.2. Average minimum and maximum temperatures (°C) and average precipitation (mm) by month for 2015 and 2016 at USDA-Agricultural Research Service Horticultural Crops Research Unit North Willamette Research and Extension Center, Aurora, OR.

Year	Month	Average maximum temperature (°C)	Average minimum temperature (°C)	Average precipitation (mm)
2016	January	9.1	2.6	221.7
2016	February	13.0	3.3	91.9
2016	March	13.8	4.2	206.0
2016	April	19.9	5.2	81.0
2016	May	21.9	7.6	22.1
2016	June	25.2	9.6	12.7
2016	July	27.0	11.4	12.7
2016	August	30.3	10.3	2.8
2016	September	24.6	7.8	18.0
2016	October	17.9	8.2	308.6
2016	November	14.4	6.1	197.6
2016	December	6.4	-1.1	131.1
2015	January	10.0	2.2	91.2
2015	February	13.8	4.6	132.3
2015	March	16.8	3.1	108.5
2015	April	16.4	3.2	45.5
2015	May	21.2	7.5	20.6
2015	June	26.8	9.4	19.3
2015	July	30.6	11.5	0.0
2015	August	30.2	11.8	10.7
2015	September	24.6	8.4	48.8
2015	October	21.1	7.8	61.5
2015	November	12.0	1.4	147.6
2015	December	9.1	3.4	355.1

Appendix B. Population Means and Bar Graphs for Plant and Fruit Characteristics Evaluated for University of Arkansas Material in 2016.

Table B.1. Blackberry fruit symmetry scores of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2016.

Population	Symmetry ^z			
	Mean	Minimum	Maximum	Standard error
1222	4.9	1	8	0.2
1229	4.7	1	8	0.1
1236	4.7	1	8	0.2
1250	4.8	1	8	0.1
1253	4.1	1	8	0.1
1261	3.9	1	8	0.1

^z Symmetry evaluated on a subjective scale of 1-9, with 1 = berry being completely misshapen and asymmetrical and 9 = berry completely symmetrical.

Table B.2. Blackberry fruit length of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2016.

Population	Berry length (mm)			
	Mean	Minimum	Maximum	Standard error
1222	23.83	13.77	32.88	0.34
1229	25.04	16.83	35.35	0.30
1236	20.24	13.57	28.67	0.28
1250	24.16	13.37	34.52	0.32
1253	19.88	11.55	27.30	0.27
1261	21.58	15.60	29.44	0.24

Table B.3. Blackberry fruit width of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2016.

Population	Berry width (mm)			
	Mean	Minimum	Maximum	Standard error
1222	19.93	15.76	24.54	0.17
1229	20.76	16.16	26.52	0.18
1236	18.25	12.44	22.60	0.17
1250	19.37	13.74	23.79	0.16
1253	19.30	15.11	24.15	0.16
1261	20.34	15.64	25.08	0.17

Table B.4. Blackberry fruit drupelet diameter near the abscission zone of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2016.

Population	Drupelet diameter abscission zone (mm)			
	Mean	Minimum	Maximum	Standard error
1222	5.54	4.31	7.07	0.05
1229	5.39	3.84	6.63	0.06
1236	4.85	2.91	6.95	0.06
1250	5.48	4.28	7.13	0.05
1253	4.89	3.62	6.19	0.05
1261	5.39	3.88	7.14	0.06

Table B.5. Blackberry fruit drupelet diameter near the fruit midpoint of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2016.

Population	Drupelet diameter midpoint (mm)			
	Mean	Minimum	Maximum	Standard error
1222	5.01	4.05	6.22	0.05
1229	4.90	3.25	5.97	0.05
1236	4.44	3.15	6.43	0.06
1250	4.83	3.72	6.33	0.04
1253	4.51	3.23	5.89	0.05
1261	4.98	3.64	6.41	0.05

Table B.6. Blackberry fruit drupelet diameter near the distal end of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2016.

Population	Drupelet diameter distal end (mm)			
	Mean	Minimum	Maximum	Standard error
1222	4.58	3.37	6.33	0.05
1229	4.49	3.43	5.52	0.05
1236	4.03	3.08	5.59	0.05
1250	4.26	2.80	6.42	0.05
1253	4.16	2.89	6.13	0.06
1261	4.59	3.52	6.61	0.06

Table B.7. Blackberry fruit abscission zone diameter of six University of Arkansas blackberry seedling populations from the University of Arkansas Fruit Research Station, Clarksville, AR 2016.

Population	Abscission scar diameter (mm)			
	Mean	Minimum	Maximum	Standard error
1222	3.61	2.46	5.89	0.06
1229	3.95	2.49	10.26	0.11
1236	3.44	1.73	5.37	0.06
1250	3.68	2.31	6.01	0.07
1253	3.28	2.18	4.73	0.05
1261	3.67	1.99	5.37	0.06

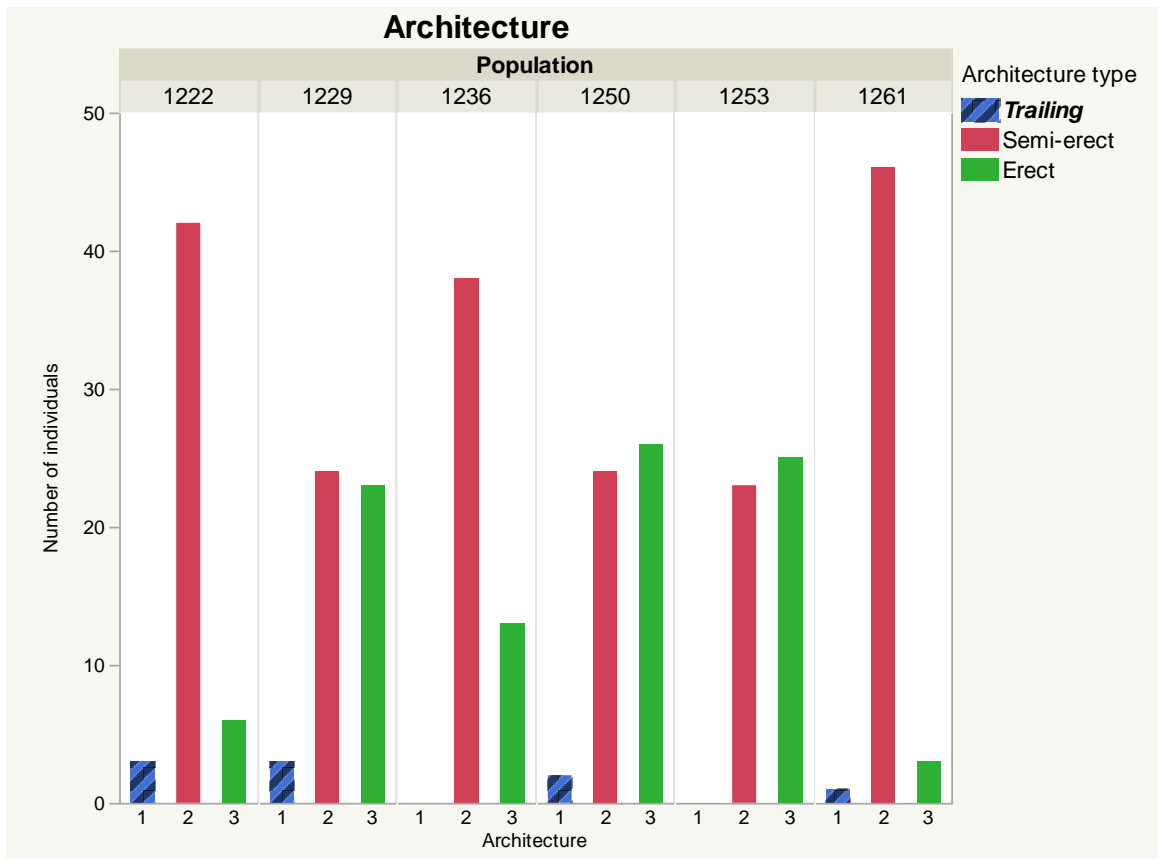


Fig. B.1. Number of blackberry seedlings categorized as “erect” (3), “semi-erect” (2) or “trailing” (1) architecture by seedling population for all University of Arkansas seedlings evaluated at the Fruit Research Station, Clarksville, AR 2016.

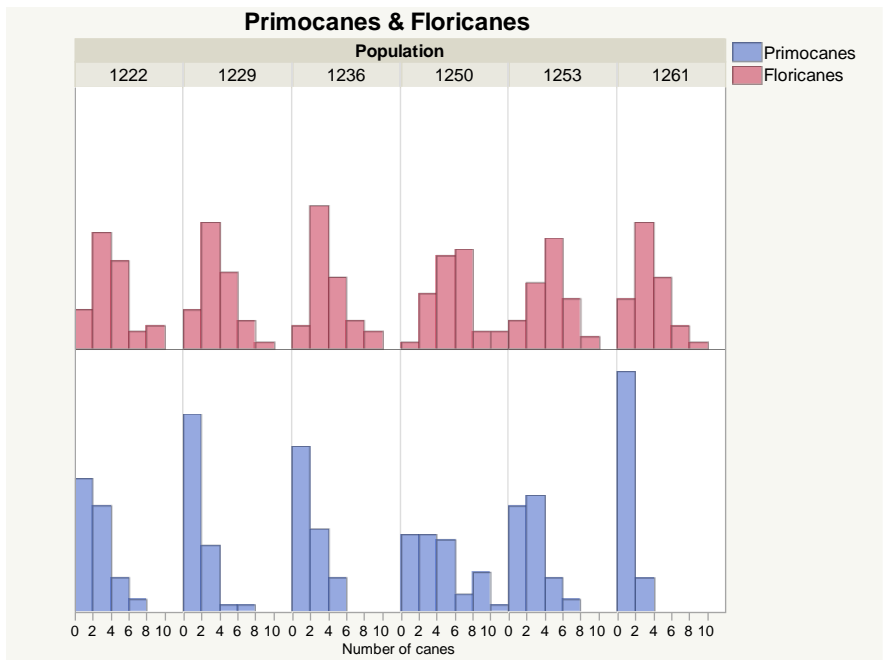


Fig. B.2. Average proportion of number of primocanes and floricanes counted for seedlings by population for all University of Arkansas blackberry seedlings evaluated at the Fruit Research Station, Clarksville, AR 2016.

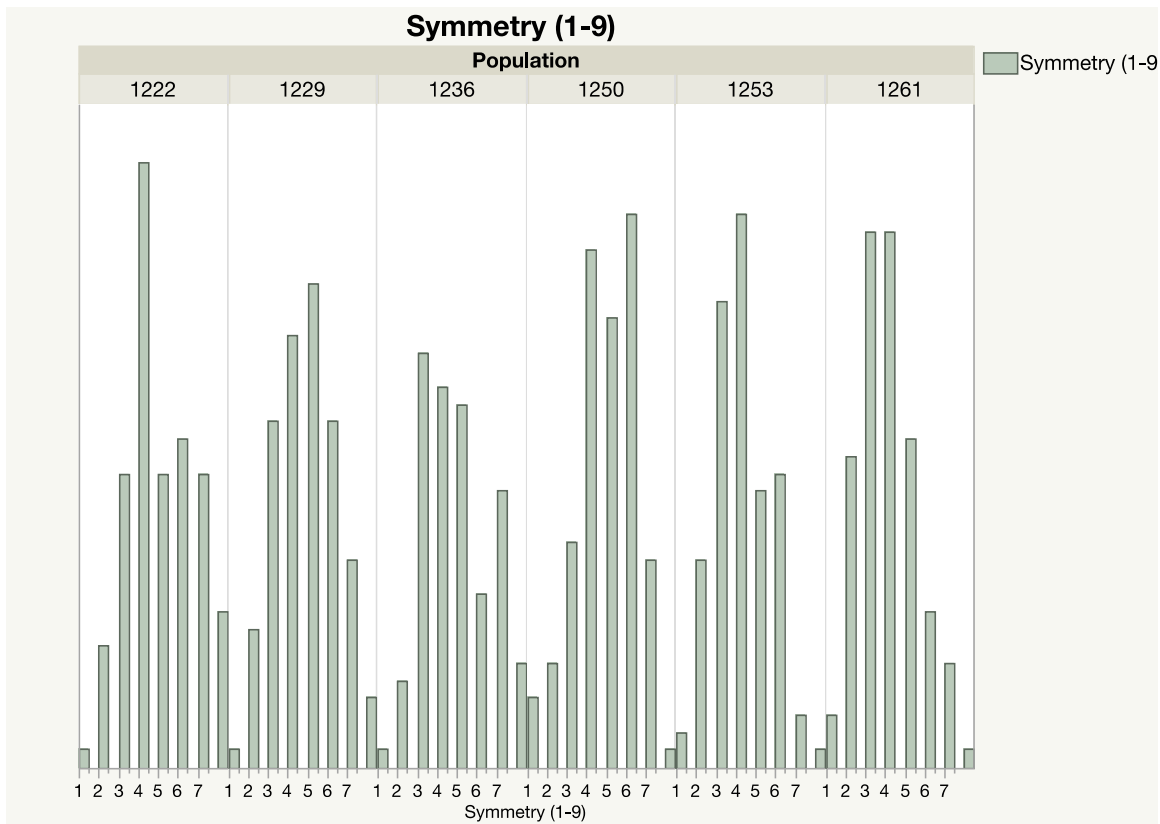


Fig. B.3. Distribution of blackberry fruit symmetry scores by seedling population for all University of Arkansas seedlings, at the Fruit Research Station, Clarksville, AR 2016. A score of “1” was completely asymmetrical and a score of “9” was perfectly symmetrical.

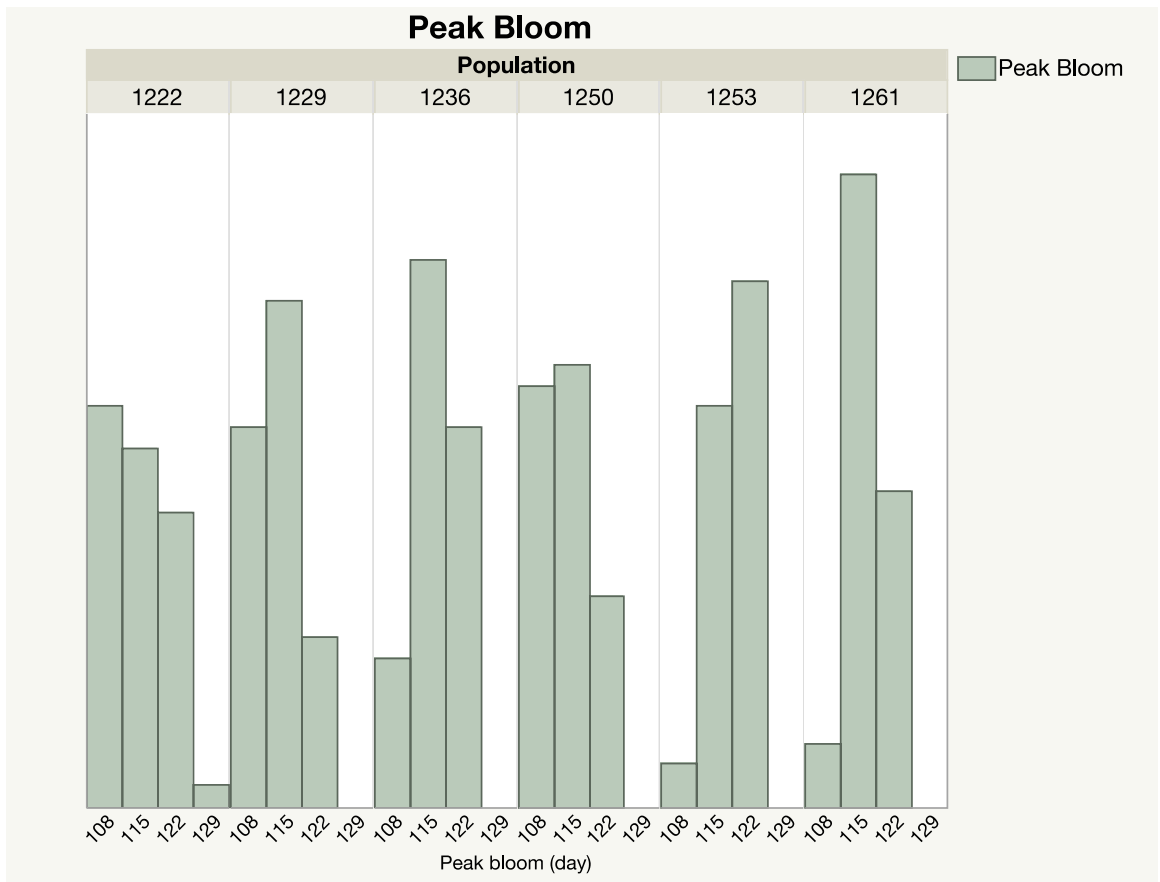


Fig. B.4. Distribution of peak bloom date for blackberry seedlings by seedling population, denoted as days from 1 Jan. 2016 for each population for all University of Arkansas seedlings evaluated at the Fruit Research Station, Clarksville, AR 2016.

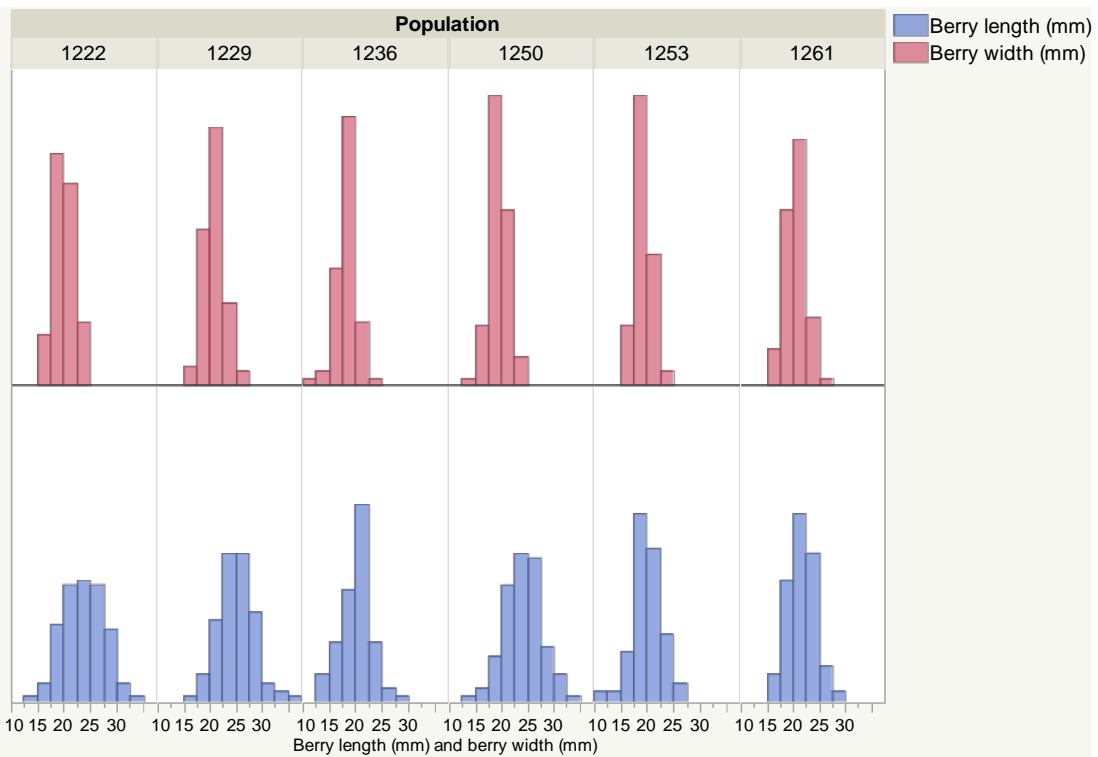


Fig. B.5. Distribution of berry length (mm) and berry width (mm) by seedling population for all University of Arkansas blackberry seedlings evaluated at the Fruit Research Station, Clarksville, AR 2016.