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Title: Determination of ethylene regulated changes in anthocyanin composition during ripening in blueberry

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Objectives:

To characterize anthocyanin composition at different developmental stages, and to determine the effect of ethephon and 1-aminocyclopropane-1-carboxylate (ACC) on blueberry ripening

Justification and Description:

Anthocyanins are a group of phenolic compounds responsible for coloration in many flowers, fruits, and vegetables. The concentration and composition of anthocyanins can vary among different species. Blueberries contain a high concentration of different classes of anthocyanins. Previous studies have demonstrated the health benefits of these compounds as they have high antioxidant activities, preventing chronic diseases, cancer, and heart problems (Hou, 2003; Prior, 2004). Due to increasing consumer awareness of various health benefits of blueberries, their demand has been continuously increasing. From 2000 to 2017, worldwide production of blueberries increased by 100% (acreage) and 42% (yield per unit area) (FAO, 2019).

Ethephon and ACC are ethylene releasing plant growth regulators (PGRs) and have the potential to be developed as ripening aids (Ban et al., 2007, Wang et al., 2018). A survey in 2019 taken by 43 growers in the southeastern United States indicated that approximately 86% of blueberry growers placed high to moderate value on reducing harvest frequency. Our previous research has already demonstrated the efficacy of both these PGRs in concentrating ripening (Figure 1) (Wang et al., 2018). There are advantages in developing both the PGRs to concentrate ripening. Ethylene release from ACC is more controlled and less dependent on temperature and therefore ripening effects can be more consistent compared to that with ethephon. On the other hand, ethephon is relatively less expensive and is already registered for use as a ripening aid in many crops. Ethephon has been used to facilitate ripening and improve skin coloration in climacteric fruits such as kiwifruit and tomato, and non-climacteric fruits such as lemon and grapes (Ampa et al., 2017; El-Kereamy et al., 2003; Li et al., 2017; Suehiro et al., 2019; Zhang and Zhou, 2019). Similarly, ethephon and ACC applications can increase the number of blue fruit in bluberries (Figure 1). However changes in anthocyanin composition after applying of these PGRs have not yet been characterized in bluberries.

Hence, in this study, we evaluated the effects of ethephon and ACC on anthocyanin composition during ripening. To achieve this objective, we first characterized anthocyanin composition during different stages of fruit ripening. Next, changes in the anthocyanin composition after ethephon and ACC applications were determined.

Preliminary data:

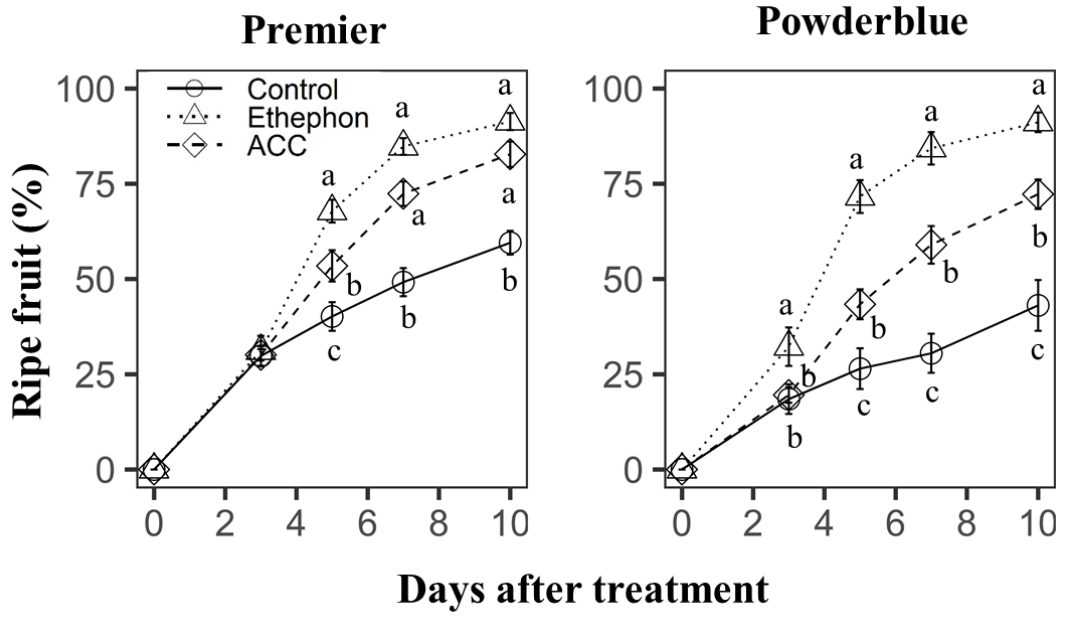

Figure 1: Effect of ethephon and ACC on percentage of ripe fruit in rabbiteye blueberry in 2020 in 'Premier' and 'Powderblue'. Means within days after treatment followed by different letters are significantly different by Least significant difference (LSD) at 0.05% level.

The effect of ethephon and ACC has been tested in multiple years at the rate of 250 ppm in one southern highbush cultivar 'Ms. Lilly' and two rabbiteye cultivars 'Premier' and 'Powderblue'. Consistently, these PGRs accelerated ripening. Ethephon increased the percentage of ripe fruits by 69, 72, and 53%, at 5, 7, and 10 days after treatment (DAT) in 'Premier', and by 74, 170, 176, 112% at 3, 5, 7, and 10 DAT respectively in 'Powderblue', compared to the control treatment in 2020 (Figure 1). On the other hand, ACC increased the percentage of ripe fruits by 33, 47, and 39% in 'Premier', and by 63, 93, 68% at 5,7, and 10 DAT respectively in 'Powderblue' compared to control treatment in 2020 (Figure 1). These findings suggest that the above PGRs can lead to greater percentage of harvested fruits 10 d after application. We also determined the effect of ethephon and ACC on the composition of major sugars and organic acids at harvest. Overall, these PGRs did not consistently change the concentration of sucrose, glucose, fructose, malic acid, citric acid and quinic acid (data not presented). It is also important to determine the effect of these PGRs on anthocyanin composition. There is no prior information on the effect of ethylene on anthocyanin composition and this area of investigation is important due to the health benefits associated with these compounds. For the proposed work, we used samples that were already collected for this study. Details of sample collection is described in the Experimental Plan section. Moreover, we developed a protocol for anthocyanin determination using high-performance liquid chromatography (HPLC) coupled with a photodiode array detector (PAD)

(Waters, Milford, MA). After protocol development, identification of anthocyanin compounds in blueberry fruit were developed by using liquid chromatography-mass spectrometry (LC-MS), and m/z ratio were compared with the literature (Prior et al., 2001; Wu and Prior., 2005). A list of anthocyanin compounds in 'Premier' and 'Powderblue' fruit and their mass to charge ratios are given in Table 1.

Table 1. Retention times, mass spectral data, and anthocyanin compounds from 'Premier' and 'Powderblue'

Peak	Elution time (min)	$[M]^+$ (m/z)	MS/MS (m/z)	Anthocyanin
1	8.86	465.1	303.05	delphinidin 3-galactoside
2	10.96	465.1	303.05	delphinidin 3-glucoside
3	12.35	449.1	287.06	cyanidin 3-galactoside
4	13.62	435.1	303.05	delphinidin 3-arabinoside
5	15.76	449.1	287.06	cyanidin 3-glucoside
6	18.29	479.1	317.07	petunidin 3-galactoside
7	18.29	419.1	287.06	cyanidin 3-arabinoside
8	21.99	479.1	317.07	petunidin 3-glucoside
9	23.18	463.1	301.07	peonidin 3-galactoside
10	25.2	449.1	317.07	petunidin 3-arabinoside
11	27.5	463.1	301.07	peonidin 3-glucoside
12	28.73	493.1	331.08	malvidin 3-galactoside
13	29.56	433.1	301.07	peonidin 3-arabinoside
14	30.5	493.1	331.08	malvidin 3-glucoside
15	31.45	463.1	331.08	malvidin 3-arabinoside

Experimental Plan:
The two main objectives are to characterize anthocyanin composition during blueberry ripening and after application of ethylene-releasing PGRs.

Objective 1: Characterization of anthocyanin composition during the blueberry fruit ripening. Fruit samples were collected at the immature green, mature green, pink, and ripe stages from the Durham Horticulture Farm, University of Georgia, Athens, Georgia, in 2020. Samples were defined as follows: immature green stage is fully green, having 9-12 mm in diameter; mature green stage fruit display a slight color change to pink (< 25%); pink stage fruit are completely pink, and ripe stage fruit are fully blue. Samples were collected from 'Premier' and 'Powderblue'. For each stage, multiple fruit were pooled from each replicate and immediately frozen in liquid nitrogen and stored at -80°C until analysis. Fruits were collected in four replications. These samples were used for the determination of anthocyanin changes during the fruit ripening.

Results for objective 1:

Table2: Concentrations of anthocyanins at various developmental stages in blueberry.

	Premier		Powderblue	
Anthocyanin	**S7**	**S8**	**S7**	**S8**
Del 3-gal	0.00 b	16.60 a	0.00 b	18.58 a
Del 3-glu	0.00 b	0.28 a	0.00 b	9.75 a
Cya 3-gal	1.93 b	14.85 a	3.10 b	25.95 a
Del 3-ara	0.00 b	6.48 a	0.00 b	11.35 a
Cya 3-glu	0.00 b	0.55 a	1.03 b	14.15 a
Pet 3-gal + Cya 3-ara	1.00 b	21.03 a	1.53 b	28.15 a
Pet 3-glu	0.00 b	0.38 a	0.00 b	13.05 a
Peo 3-gal	0.50 b	6.63 a	0.38 b	8.05 a
Pet 3-ara	0.00 b	5.5 a	0.00 b	7.60 a
Peo 3-glu	0.00 b	1.10 a	0.65 b	13.28 a
Mal 3-gal	1.15 b	34.15 a	0.83 b	30.95 a
Peo 3-ara	0.18 b	1.68 a	0.20 b	2.80 a
Mal 3-glu	0.00 b	1.60 a	0.58 b	28.63 a
Mal 3-ara	0.40 b	11.85 a	0.45 b	16.13 a
Total anthocyanin	5.08 b	122.65 a	8.83 b	228.40 a

Different letters in each row for each cultivar are significantly different according to Fischer's LSD (α=0.05). Del:delphinidin, Cya: cyanidin, Pet: petunidin, Peo: peonidin, gal: galactoside, glu: glucoside, ara: arabinoside.

We did not detect anthocyanin during the early fruit developmental stages, in the S4 and S5 developmental stages. In the S6 developmental stage, only one anthocyanin (Cya 3-gal) in Premier, and three anthocyanins (Cya 3-gal, Pet 3-gal/Cya 3-ara) were detected in Powderblue (data not presented). In S7 developmental stage where ripening initiates, seven different anthocyanin compounds (Cya 3-gal, Pet 3-gal/Cya 3-ara, Peo 3-gal, Mal 3-gal, Peo 3-ara, Mal 3-ara) were detected in Premier, whereas in Powderblue 10 different anthocyanin compounds (the additional three included: Cya 3-glu, Peo-3-glu, Mal 3-glu) were detected (Table 2). All anthocyanins in the S8 stages were significantly higher than in the S6 and S7 developmental stages. Finally, the total anthocyanin compounds were calculated by the sum of individual anthocyanins. The total anthocyanin concentration in the S8 stages increased by 23-fold in Premier and 25-fold in Powderblue, compared to the S7 stage (Table 2).

Objective 2: To determine ethylene-regulated changes in anthocyanin composition during ripening. Before the application of PGRs, three 50-100 cm long shoots, each containing approximately 50-150 fruits, were tagged for all experimental units. Next, small immature developing fruit and ripe (blue) fruit were removed from the tagged branches. Subsequently, ethephon and ACC (Valent Bioscience LLC, Long Grove, IL) were applied @ 250 mg per liter of water along with 0.15% Latron-1956 (control). Additionally, Latron-1956 application (0.15%) was used as the control treatment. All applications were performed in the morning before 9:00 am. After treatment, fruit were collected randomly at 0, 3, and 5 days after PGR application from tagged branches. We also harvested fully ripe fruit at 10 days after PGR application. After harvest, fruit were immediately frozen in liquid nitrogen and stored at -80°C until analysis. For each treatment four replications were harvested. These samples were used for the evaluation of ethylene-regulated anthocyanin changes during blueberry ripening.

For extraction of anthocyanins, a previous protocol with slight modifications was used (Downey, Mazza, & Krstic, 2007; Downey & Rochfort, 2008). About 100-125 mg samples were extracted in 1 mL of 50% (v/v) methanol. The samples were left undisturbed in the dark for one hour, followed by sonication (Bransonic 220, Parrot Drive, CT) for 20 minutes. Finally, samples were centrifuged at 12,000 rpm for 10 minutes. The supernatant was purified by passing through a 0.45 µm filter and transferred to the HPLC autosampler vial. The injection volume for each analysis was 30 µL. Anthocyanin quantification was performed using a Waters 2690 separation module HPLC connected with autosampler/injector and a PAD detector. A discovery C-18 column (15cm x 4.6 mm, 5µm, Sigma-Aldrich Inc, ST. Louis, MO) was used for this study. The two mobile phases include, 10% formic acid in water (Solvent A), and 10% formic acid in methanol (solvent B), with a 1 ml/min flow rate. The gradient flow rate was maintained as: 0 min of 10% B, 14 min of 12% B, 25 min of 16% B, 28 min of 25% B, 32 min of 50% B and 35 min of 10% B. Chromatograms were recorded at 520 nm. Statistical data analysis for all parameters were performed through software R, version 4.0.5 (R core 2021, Vienna, Austria).

Results for objective 2: We have presented the data for only the major anthocyanin after applying ethylene-releasing PGRs in 'Premier' (Figure 1). Delphinidin 3-O-galactoside (Del-3-gal) was significantly higher in ethephon-treated fruits compared to the control at 3 DAT. For Malvidin-3-O galactoside (Mal 3-gal) and Mal-3-O-glucoside (Mal 3-glu), both ACC and ethephon applications increased their concentration compared to control at 3 DAT. Malvidin 3-O-arabinoside (Mal 3-ara) was significantly higher in ethephon, and ACC-treated fruits at both 3 and 5 DAT, respectively (Figure 1). In addition, we found similar results for all the remaining anthocyanins. A similar effect with ethylene releasing PGRs was also detected in the 'Powderblue' cultivar. We also determined anthocyanin concentration in blue fruits (fully ripe) at 10 DAT application of ethylene-releasing PGRs. However, 10 DAT anthocyanin concentrations were not different in blue fruit in PGR treatments compared to the control.

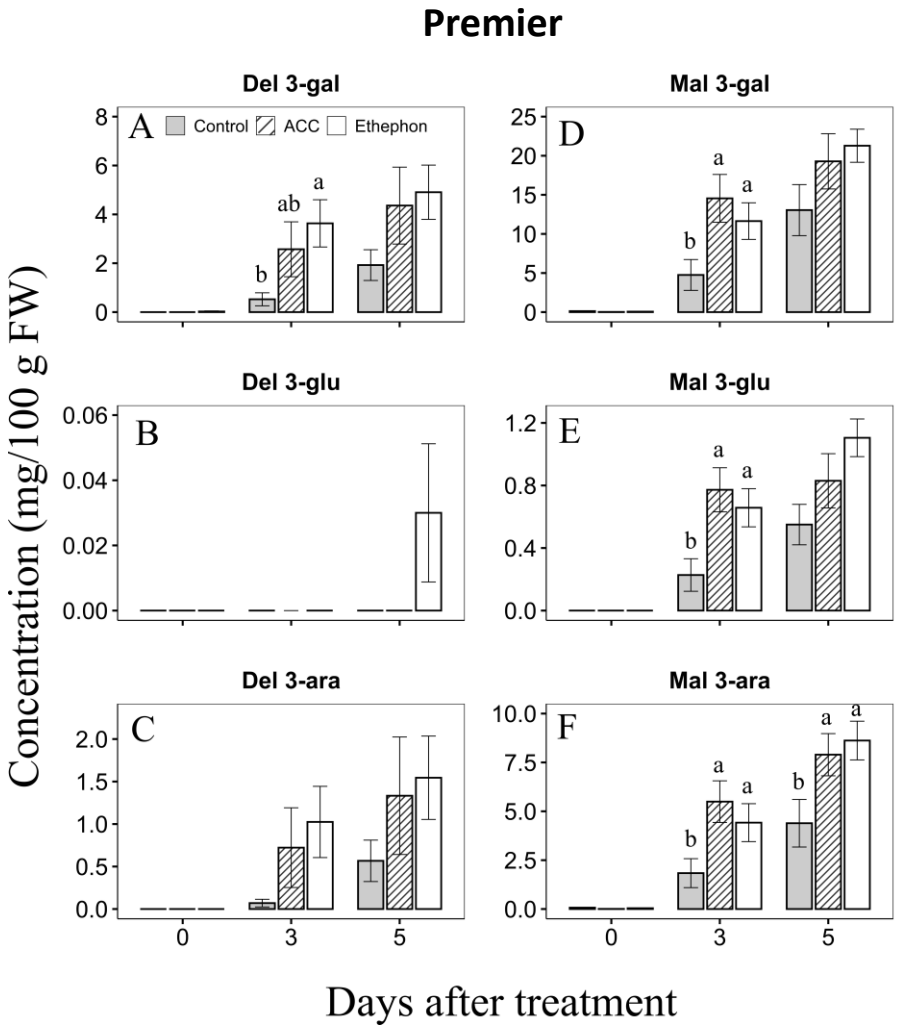

Figure 1: Concentration of major two group of anthocyanin in Premier after the application of control, ethephon and ACC**.** Different letters above symbols indicate that the means are significantly different between the treatment (within a day after treatment) according to ANOVA and Fischer's LSD (α=0.05).

Conclusion: In this study, we characterized 15 major anthocyanins in the rabbiteye blueberry. Overall anthocyanins increased during fruit ripening with highest concenterations in the ripe fruit. Application of ethephon and ACC increased rate of fruit ripening and the rate of anthocyanin accumulation. However 10 days after application ripe fruit had similar anthocyanin concentration compared to the control fruit. These data suggests that ethylene triggers anthocyanin production during fruit ripening. However there may be a threshold of anthocyanin concenteration in fruits and therfore at 10 days after applications similar concenterations were noted for treatments and control.

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