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Title: Changes in flavor-related compounds, sugars and acids, after application of Ethephon and 1-aminocyclopropanae carboxylic acid.

Name, Mailing and Email Address of Principal Investigator(s):

Principal Investigator	Co- Principal Investigator	Co- Principal Investigator
Savithri Nambeesan	Anish Malladi	Renée Holland
Asst. Research Scientist/ Faculty	Associate Professor	Area Blueberry Agent
University of Georgia/ CAES	University of Georgia	Extension-Bacon County
Department of Horticulture	Department of Horticulture	University of Georgia
1111 Miller Plant Sciences	1111 Miller Plant Sciences	203 S. Dixon St., Suite 3
Athens, GA 30602	Athens, GA 30602	Alma, GA 31510
Email: sunamb@uga.edu	Email: malladi@uga.edu	Email: reneemh@uga.edu
Phone: 706-542-0777	Phone: 706-542-0783	Phone: 912-632-5601

Objectives: To determine changes in sugars and acids after application of two plant growth regulators, ethephon and 1-aminocyclopropanae carboxylic acid (ACC).

Justification and Description:

Blueberries are among the leading crop in Georgia with a farm gate value of over $300.4 million (2018; Georgia Farm Gate Value Report) and is currently cultivated in over 30,000 acres across the state. There are two main types of cultivated blueberry in GA; the southern highbush blueberry and rabbiteye blueberry. Blueberry fruits grow in a cluster and fruit maturity times vary among fruit within a cluster resulting in a non-uniform ripening, the duration of which can extend over several weeks (Suzuki et al., 1997), thus requring multiple harvests for each cultivar. In January 2019, we conducted surveys at the Annual Blueberry Meeting in Alma, GA and the Southeast Fruit and Vegetable Conference, in Savannah, GA. The survey was taken by 43 growers. Around 37% of the growers indicated that manual/machine harvesting is the most expensive among the top 5 production/management-related costs (Fig. 1A). Also, when growers were asked about value placed on reducing harvesting frequency, ~ 86% of blueberry growers placed high to moderate value on reducing harvest frequency (Fig. 1B). **Thus, synchronized ripening is a desired trait that will save production related costs in commercial blueberry production.**

Here, we propose to test the effects of two ethylene related plant growth regulators (PGRs), ethephon and ACC on fruit flavor-related compounds, mainly sugars and acids. Ethephon releases ethylene after application and absorption into plant cells. On the other hand, ACC is an ethylene precursor and gets converted to ethylene via an enzymatic reaction involving ACC oxidase. There are advantages to developing both these compounds as PGRs to aid in ripening. Ethephon has already been registered for uniform ripening, mainly in northern highbush blueberries at rates of ~500-1500 ppm (US-EPA, 2010). At these rates, ethephon has the potential to abscise fruit which is not desirable (Malladi et al., 2012). Our research over the

past few years support the effect of ethephon applications (at 250 ppm) in concentrating maturity. At this rate ethephon does not appear to induce fruit drop (Malladi et al., 2012).

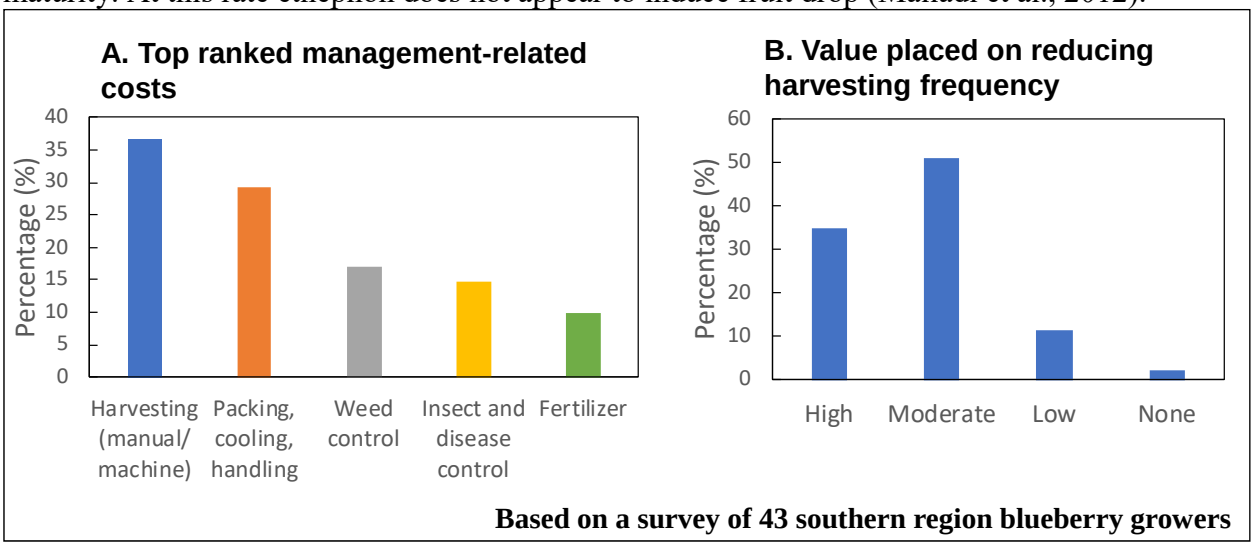

Figure 1: Top ranked management-related costs (A) and Value placed on reducing harvesting frequency (B) from a survey conducted.

However, ethylene release from ethephon is temperature dependent leading to inconsistencies in plant responses. In the past few years, our research has also demonstrated that ACC applications (at 250 ppm) can increase ripening rate. In comparison to ethylene, developing ACC to concentrate maturity may have some advantages as ACC oxidase, the enzyme that converts ACC to ethylene, is often not rate-limiting, and is likely to be abundant in ripening fruit, thereby reducing the variability associated with temperature fluctuations. This should allow for a more controlled release of ethylene using ACC. However, ACC is not yet registered for use as a PGR to concentrate maturity. Whether ethephon and ACC are viable ripening aids also depends on their effects on fruit quality characteristics such as fruit-flavor related compounds, sugars and acids. It is important that these PGRs while accelerating ripening, maintain fruit quality. Measuring total soluble solids and titratable acidity (sugar acid ratios), provides a general indication of fruit quality. However, these tests are a crude measure of fruit quality since interfering compounds such as anthocyanins may substantially influence the results. Thus, in this proposal we plan to determine the effect of PGRs on individual sugars and acids to determine the effects of these PGRs on fruit quality.

Preliminary data:

Ethephon and ACC application at 250 ppm increased the rate of ripening in two rabbiteye cultivars, 'Premier' and 'Powderblue' (Fig. 2). In 'Premier', at 7 days after treatment (DAT), approximately 50% of fruit were ripe in the control treatment, whereas 72% and 85% of fruit were ripe with ACC and ethephon treatments, respectively (Fig. 2A). This trend continued further at 10 DAT, but the rate of ripening was similar between ethephon and ACC treatments (Fig. 2A). In 'Powderblue' at 7 DAT, 31% of fruit were ripe in control treatment, whereas 59% and 84% of fruit were ripe with ACC and ethephon treatments, respectively (Fig. 2B). This trend

continued further at 10 DAT, with ethephon showing greater ripening rate compared with ACC (Fig. 2B).

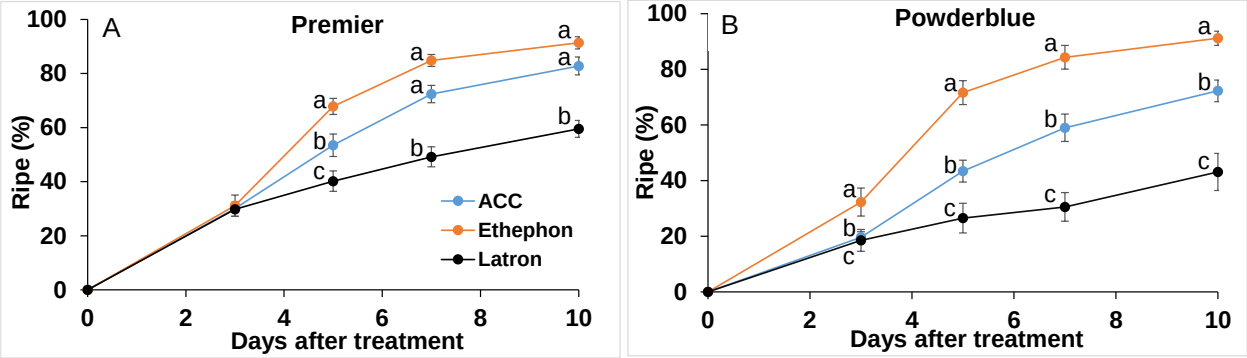

Figure 2: Effect of pre-harvest application of Ethephon, ACC, and Latron (Latron B-1956; control) on the percentage of ripe fruit in two rabbiteye blueberry cultivars, Premier (A) and Powderblue (B) in 2020. Means at days after treatment, separated by the same letters are not significantly different from each other ($\alpha = 0.05$).

Significance: There is a lot of interest among the growers to manipulate ripening as mentioned previously based on the survey we conducted. Work proposed here will be vital to determine the effect of ethephon and ACC application on subsequent fruit quality. Results from this work will help develop informed recommendations on the use of ethephon and ACC as ripening aids, in relation to their effects on fruit quality.

Experimental Plan

Plant material: Two sets of fruit samples were collected from two rabbiteye cultivars Premier and Powderblue from the Durham Horticulture Farm in Athens, GA, in 2020. One set of fruit samples were collected from five developmental stages categorized as stages S4, S5, S6, S7, and S8 based on the Zifkin et al. (2012). Stages 4 and 5 were collected based on the size (S4: < 9 mm, and S5: > 9 and < 12 mm in diameter), and stages 6, 7, and 8 based on the color (S6: 25-50% of surface is pink; S7: predominately pink with some blue; and S8: fully blue (Fig. 3). Fruit ethylene production rate was measured during the above developmental stages (Fig. 3). The selection of 'Premier' and 'Powderblue' was based on their high and low ethylene production levels at ripening stages. 'Premier' displays higher ethylene evolution, whereas 'Powderblue' displays lower ethylene evolution during the ripening stages (Fig. 3). Each sample was also frozen in liquid nitrogen and stored at -80°C for metabolites analysis.

The second set of sample collection was performed after ACC and ethephon treatments. The application of PGRs was done when 25-40% fruit were ripe. Before the application of PGRs, all ripe fruits were removed from the plant and several branches were tagged. Ethephon and ACC (Valent Bioscience LLC, Long Grove, IL) were applied at 250 $mg \cdot L^{-1}$ with 0.15% Latron B-1956 (Southern Agricultural Insecticides, Inc. Hendersonville, NC). Fruit samples were collected randomly from the tagged branches at 3, 5, and 10 DAT. These samples were used to determine ethephon-induced early and late changes in metabolite composition.

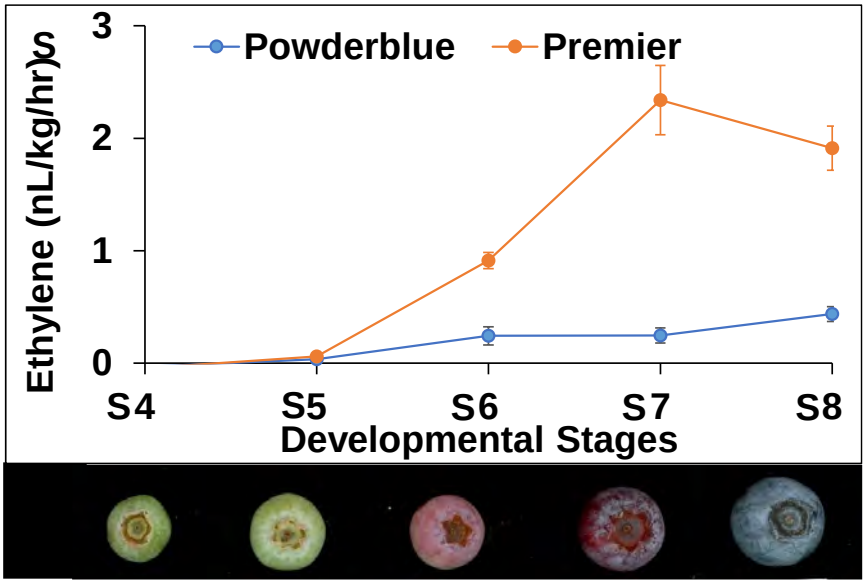

Figure 3: Ethylene production during 5 developmental stages including S4, S5, S6, S7 and S8 in blueberry fruit development in two rabbiteye cultivars, Premier (orange) and Powderblue (blue).

Metabolite analyses: For each replicate, six fruit were ground to a fine powder in liquid nitrogen using a mortar and pestle. The concentration of key sugars and organic acids were determined using Gas Chromatography-Flame Ionization Detector (GC-FID) based on (Chapman Jr and Horvat 1989). In brief, 125-150 mg powder was extracted in 1.5 mL methanol. Samples were centrifuged for 30 min at 22000 x *g*. 100 µL supernatant was taken out and dried under the N_2 gas. After addition of 50 µL of methoxyamine HCl, samples were heated for 30 minutes. Derivatization of samples were performed using the *N*-Trimethylsilyl-*N*-methyl trifluoroacetamide **(**MSTFA) with 1% tri-chloromethylsilane (TMCS). An internal standard (phenyl β-D-glucoside) was added to each sample during extraction to determine extraction efficiency. 1 µL of extracted sample was injected into the GC-FID having HP-5 capillary column (Agilent Technologies Inc., USA). Helium was used as a carrier gas. The oven temperature was set at 150 °C for 1 min, ramped to 190 °C at 3 °C/min, set at 190 °C for 0.5 min, ramped at 1 °C/min to 210 °C, set at 0.5 min at 210 °C, ramped to 260 °C at 10 °C/min and set at 260 °C for 15 minutes. The total run time was around 53 minutes. Sugar and acid standards were prepared for all identified metabolites and were analyzed similar to that of the samples. Standard curves were generated and used for metabolite quantification.

Results

Accumulation of major sugars and organic acids during fruit development

Sugar metabolism: The two cultivars, Premier and Powderblue exhibited similar patterns in sugar concentration changes at different fruit developmental stages including ripening (Fig. 4). Fructose, glucose, and sucrose were the three major sugars that accumulated during blueberry fruit development. Fructose, glucose, and sucrose concentrations increased steadily during early and mid-fruit development stages (S4-S7), and continued to increase between S7 to S8 stages in both cultivars (Fig. 4). Fructose and glucose were the major sugars that accumulated during fruit development and were similar in concentration, whereas sucrose concentration was relatively low (Fig. 4).

In 'Premier', fructose increased by 3.7, 1.8, 0.5, and 0.9-fold between S4 to S5, S5 to S6, S6 to S7, and S7 to S8 stages, respectively (Fig. 4). Similarly, glucose increased by 4.0, 1.9, 0.5, and 0.8-fold between S4 to S5, S5 to S6, S6 to S7, and S7 to S8 stages, respectively. Sucrose

increased by 0.7, 0.4, and 0.8-fold between S5 to S6, S6 to S7, and S7 to S8 stages, respectively (Fig. 4). In the case of 'Powderblue', fructose increased by 1.7 and 2.0-fold between S5 to S6, and S7 to S8 stages, respectively. Similarly, glucose increased by 1.9-fold between S5 to S6, and S7 to S8 stages. Sucrose increased by 1.3-fold between S5 to S6, and S7 to S8 stages. However, fructose, glucose, and sucrose between S4 to S5 and S6 to S7 were not significantly different in 'Powderblue' (Fig. 4). Myo-inositol, another sugar alcohol was overall less abundant during fruit development in both the cultivars (Fig. 4)

Organic acid metabolism: The two cultivars exhibited similar patterns in organic acid concentration at different stages of ripening (Figs. 5-6). Malic acid, citric acid, quinic acid, and shikimic acid were the major acids in blueberries (Figs. 5-6). Of these four acids, quinic acid was the predominant acid during early fruit development (Fig. 6). Compared to citric acid, malic acid was greater at earlier stages during development, whereas both acids were similar at later stages.

Malic acid increased from S4 to S5 stages by 0.3 and 0.4-fold in 'Premier' and 'Powderblue', respectively, and then decreased continuously in both cultivars. In 'Premier', malic acid decreased by 0.21, 0.19, and 2.1-fold between S5 to S6, S6 to S7, and S7 to S8 stages, respectively. Similarly, in 'Powderblue', malic acid reduced by 0.33 and 3.3-fold between S6 to S7, and S7 to S8 stages, respectively. However, the citric acid concentration at all developmental stages was similar in 'Premier'. In 'Powderblue', citric acid was maximum at the S6 stage (increased by 0.63-fold from S5 to S6), then slightly declined by 0.30-fold between the S7 and S8 stages, respectively (Fig. 5).

The concentration of quinic acid continuously decreased, whereas shikimic acid increased during fruit development (Fig. 6). Quinic acid decreased by 0.24, 0.30, 0.59-fold between S4 to S5, S5 to S6, and S7 to S8 stages, respectively in 'Premier', and by 0.17 and 1.61-fold between S6 to S7, and S7 to S8 stages, respectively in 'Powderblue'. On the other hand, shikimic acid increased by 0.71 and 0.35-fold between S7 to S8 stages in 'Premier' and 'Powderblue', respectively.

Sugar and acid metabolites after application of Ethephon and ACC

Overall, ethephon and ACC did not consistently influence sugar and acid concentrations in both cultivars (Figs. 7-10). At 3 DAT, ethephon and ACC treated fruits exhibited higher concentration of fructose and glucose than control in Premier; however, this was not observed in 'Powderblue'. At 10 DAT the control had more sucrose concentration in 'Powderblue' but not in 'Premier' (Figs. 7-8). Malic acid, citric acid, quinic acid, and shikimic acid were also not consistently different by applying ethephon and ACC.

Conclusions: Quinic acid is the major carbon storage form during earlier stages, whereas glucose and fructose are the major carbon storage forms during later developmental stages. Overall, application of ethylene releasing PGRs did not influence sugar and acid composition in the fruit. These results suggest that the PGRs may be effective as ripening aids to concentrate fruit ripening with minimal effects on fruit flavor related sugar and acid metabolites. These data also suggest that the PGRs can enhance ripening possibly by influencing color change. Hence, future studies will focus on the effects of ethephon and ACC applications on anthocyanin production.

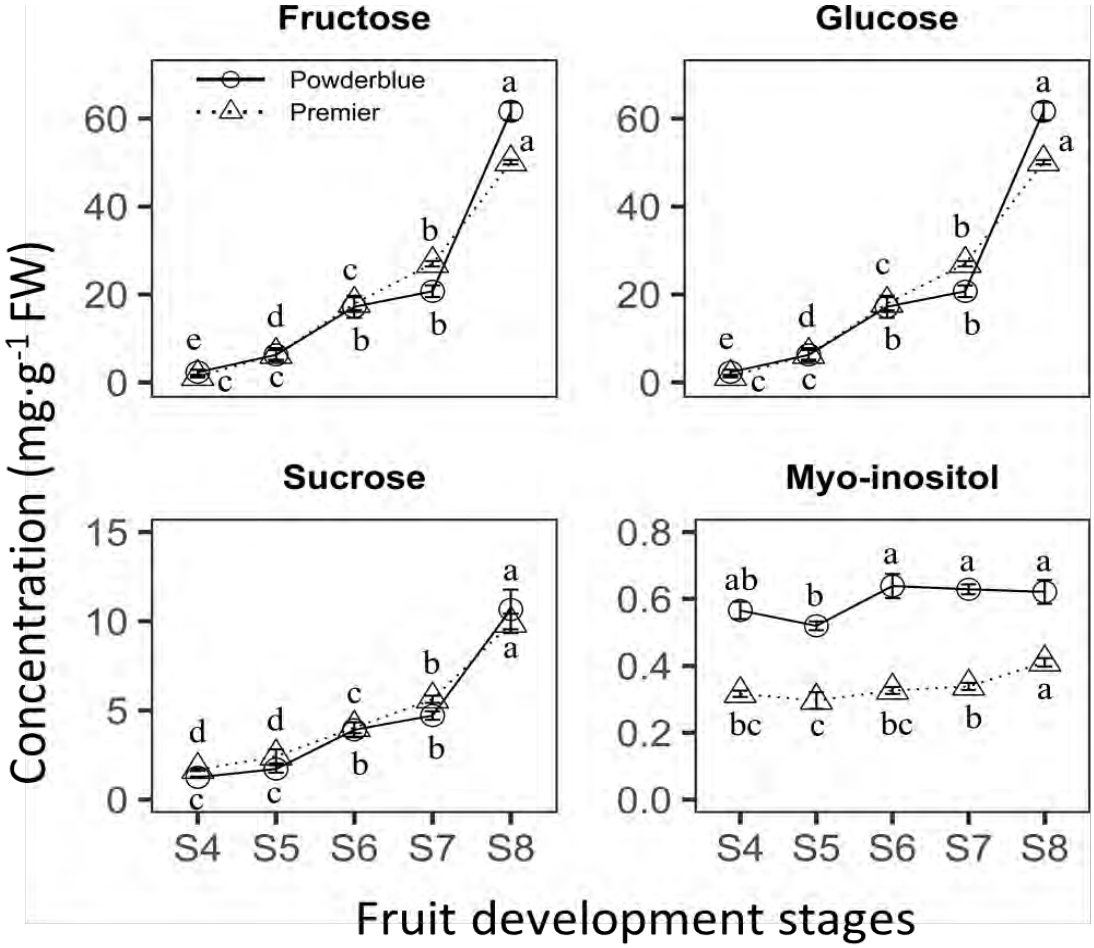

Figure 4: Major sugar metabolism at different stages of fruit ripening. Means at each cultivar fruit developmental stages followed by different letters are significantly different according to ANOVA and Fischer's LSD (α=0.05).

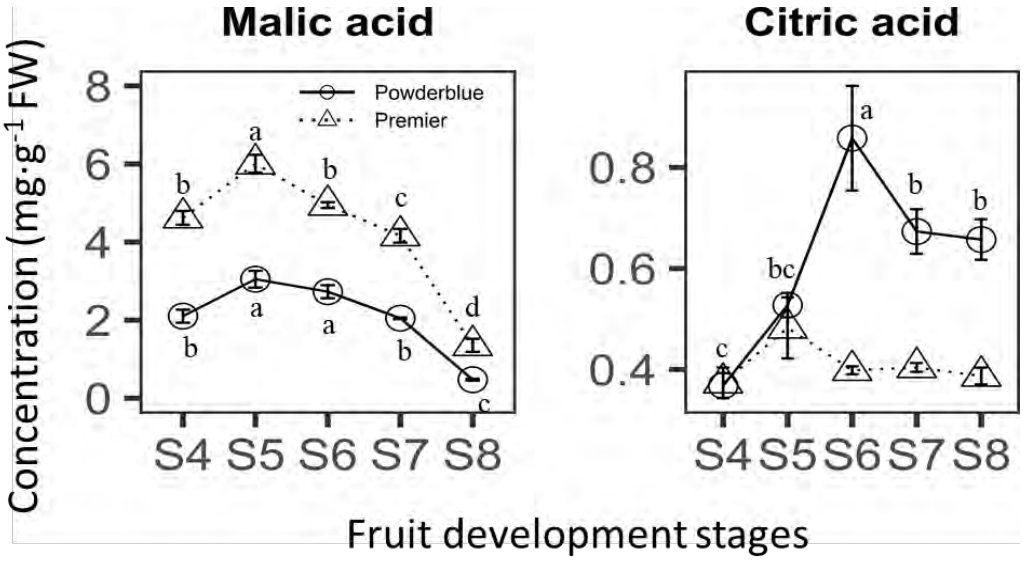

Figure 5: Major Primary acid metabolism at different stages of fruit ripening. Means at each cultivar fruit developmental stages followed by different letters are significantly different according to ANOVA and Fischer's LSD (α=0.05).

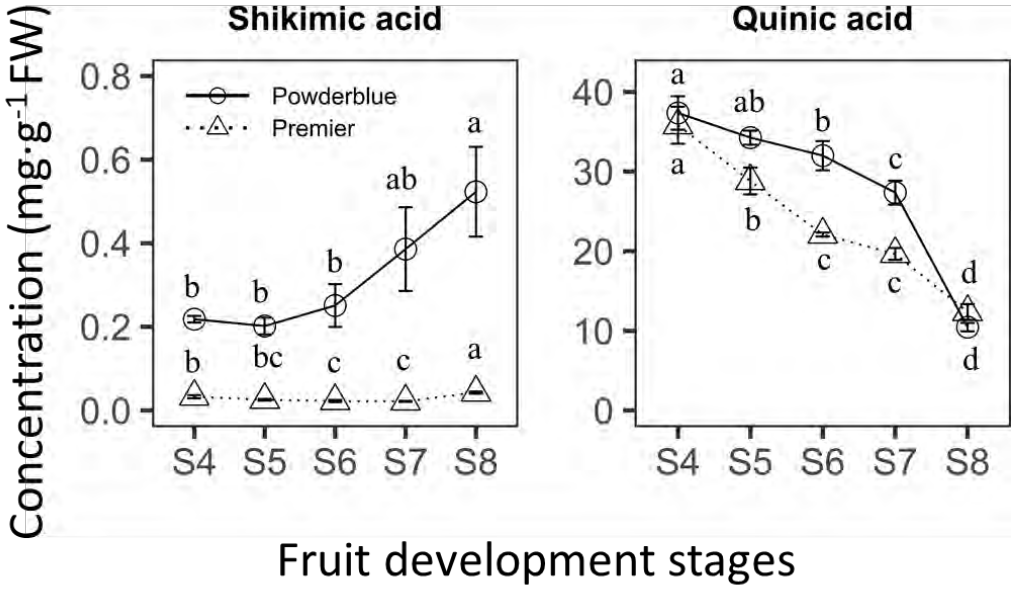

Figure 6: Major Secondary acid metabolism at different stages of fruit ripening. Means at each cultivar fruit developmental stages followed by different letters are significantly different according to ANOVA and Fischer's LSD (α=0.05).

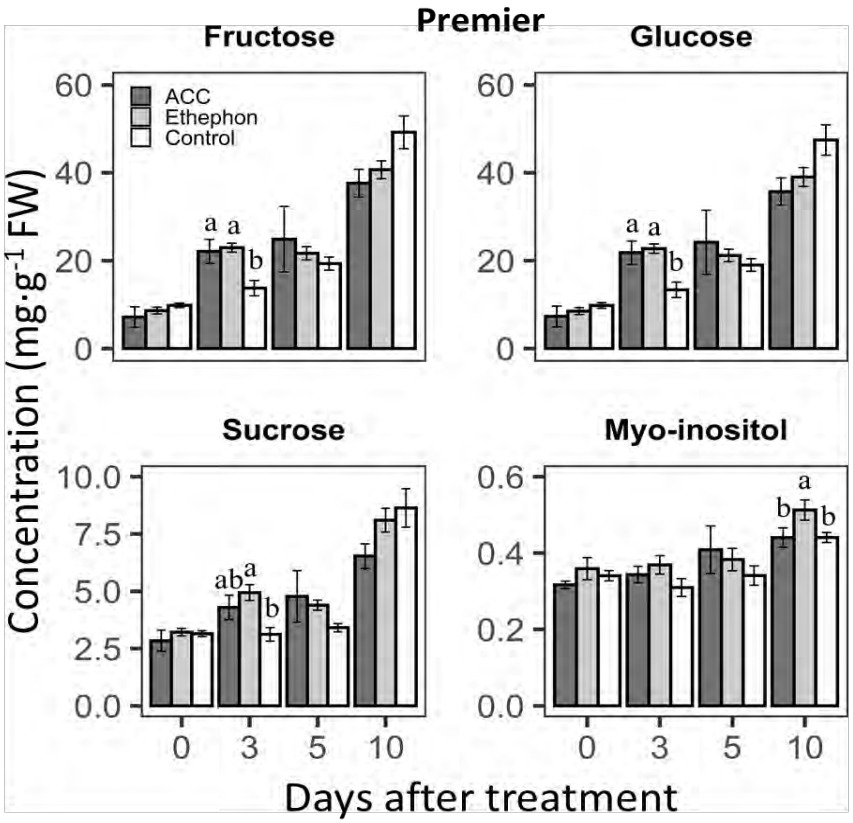

Figure 7: Effect of pre-harvest application of Ethephon, ACC, and Control (Latron B-1956; control) on the sugar and sugar alcohol concentration in 'Premier'. Means at days after treatment, separated by the different letters are significantly different according to ANOVA and Fischer's LSD (α=0.05).

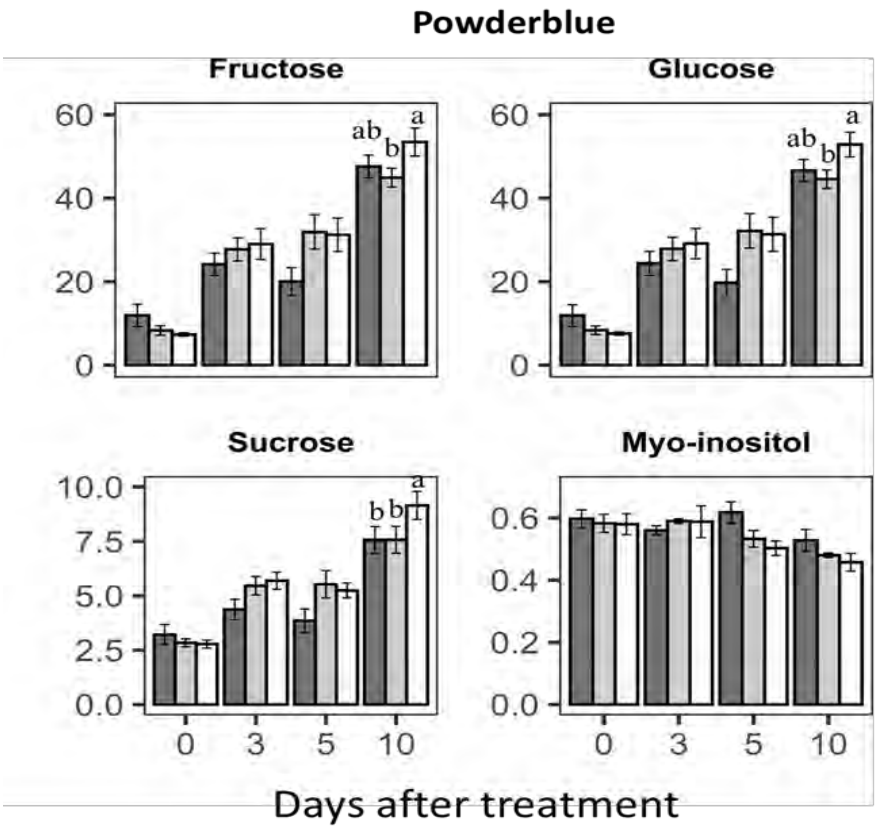

Figure 8: Effect of pre-harvest application of Ethephon, ACC, and Control (Latron B-1956; control) on the sugar and sugar alcohol concentration in 'Powderblue'. Means at days after treatment, separated by the different letters are significantly different according to ANOVA and Fischer's LSD (α=0.05).

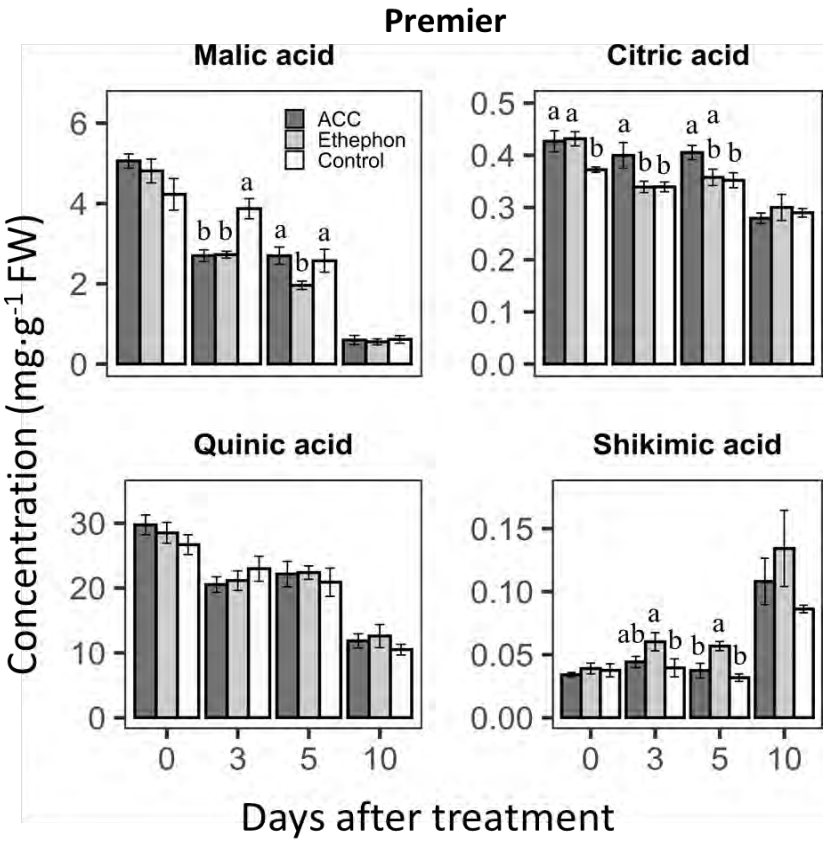

Figure 9: Effect of pre-harvest application of Ethephon, ACC, and Control (Latron B-1956; control) on the acid concentration in 'Premier'. Means at days after treatment, separated by the different letters are significantly different according to ANOVA and Fischer's LSD (α=0.05).

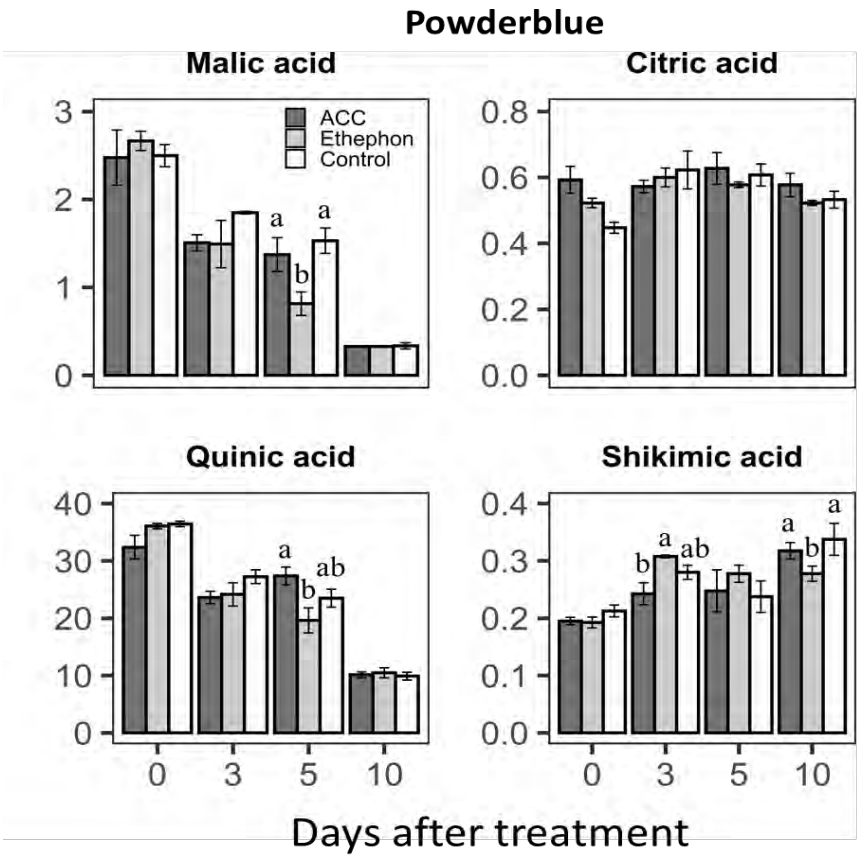

Figure 10: Effect of pre-harvest application of Ethephon, ACC, and Control (Latron B-1956; control) on the acid concentration in 'Powderblue'. Means at days after treatment, separated by the different letters are significantly different according to ANOVA and Fischer's LSD (α=0.05).

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