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Proposal Status: _X_ New Proposal ___Previously funded by SRSFC; has been previously funded for ___ years

Title: Investigation of anthocyanin production in rabbiteye cultivars for commercial market and ornamental pink-fruited varieties.

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

Principal Investigator
Savithri Nambeesan
Asst. Research Scientist/ Faculty
University of Georgia/ CAES
Department of Horticulture
1111 Miller Plant Sciences
Athens, GA 30602
Email: sunamb@uga.edu
Phone: 706-542-0777

Co-Principal Investigator
Dayton Wilde
Professor
University of Georgia/ CAES
Department of Horticulture
1111 Miller Plant Sciences
Athens, GA 30602
Email: dwilde@uga.edu
Phone: 706-542-5816

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Objectives: To determine the ripening characteristics and the association between ethylene, abscisic acid (ABA) and anthocyanin production in rabbiteye cultivars for commercial market in comparison to pink fruited blueberry varieties.

Justification and Description:

Georgia is the leading producer of blueberries with 2018 farm gate value of $300. 4 million dollars and in 2019, the value of fresh fruit production was 119.4 million dollars (UGA Farm Gate Value Report, 2018; USDA-NASS, 2019). Blueberries are considered “super fruits” due to their high antioxidant content. There are several reports that suggest that some of the health benefits of consuming blueberries, are decreased risk of heart diseases and decrease in aging related damage (Neto, 2007; Basu et al., 2010). The nutraceutical capacity of blueberries have been associated with the presence of flavonoids such as anthocyanins, flavonols, phenolic acids and proanthocyanidins (Castrejón et al., 2008). Many of these antioxidants are secondary metabolites, which accumulate during blueberry fruit development, mainly ripening. In spite of the importance of anthocyanins, there is huge gap in our in genetic information available for controlling this trait. **Blueberry fruit with higher anthocyanin content is a valuable trait and information generated about anthocyanin production will aid in the breeding of blueberries with increase anthocyanins. This proposal aims to develop resources and information on endogenous plant hormones, ethylene and ABA, and their effects on anthocyanin production in blueberries.**

Previous work has indicated that ABA application at 1000 ppm can increase anthocyanin accumulation in highbush blueberry (Oh et al., 2018). Previous work in our lab has indicated that ABA application in two rabbiteye cultivars, Premier at 600 ppm and Powderblue at 1000 ppm increased the proportion of pink fruit compared with control treatments (Wang et al., 2018). These results suggest that ABA may play a role in triggering anthocyanin biosynthesis. However, in our study, ABA application did not increase the proportion of ripe fruit as compared with control treatment, suggesting that ABA may not be involved in sustaining anthocyanin production during advanced stages of fruit ripening. Further, our data indicate that another plant hormone ethylene is also important for ripening. Application of ethephon, an ethylene releasing compound in ‘Premier’ and ‘Powderblue’ at 250 ppm can accelerate ripening by increasing the proportion of blue fruit (Wang et al., 2018). These results indicate that ethylene may also be able to activate the anthocyanin biosynthesis pathway, directly or indirectly, in blueberry. It is possible that both ABA and ethylene play a role in anthocyanin accumulation. A role for these two hormones to advance fruit coloration and aid in early harvest has been indicated in grapes (Cantin et al., 2007).

Thus, in this proposal we plan to evaluate three cultivars utilized for fresh fruit consumption and three pink-fruited ornamental cultivars to understand the relationship between ethylene, ABA and anthocyanin production. It is hypothesized that in the three ornamental cultivars, fruit remain pink owing to altered operation of a switch for anthocyanin production. Information about the three ornamental cultivars is presented below.

Pink Lemonade: This cultivar was developed by Dr. Mark K. Ehlenfeldt at USDA, Beltsville, Maryland. It is a hexaploid with half rabbiteye and half highbush genotype. It displays a late ripening phenotype and is emerging as a very popular ornamental cultivar for home growers in Georgia. It is a pink-fruited cultivar with a mild flavor and good firmness (Fig. 1A).

TO-3097: This is new variety developed by Dr. Scott NeSmith. It is a rabbiteye which reportedly has good flavor. As shown in the figure, this has slightly more advanced color compared with ‘Pink Lemonade’ but is not completely blue (Fig. 1B).

Florida Rose: This rabbiteye cultivar has pink to red fruit with good flavor and firmness. It was developed by the Florida blueberry breeding program. This cultivar exhibits an early ripening phenotype (Fig. 1C).

Figure 1: Ornamental cultivars Pink Lemonade (A), TO-3097 (B) and Florida Rose (C); Pictures courtesy Pink Lemonade: “Germplasm release of Pink Lemonade, USDA ARS, Washington, D.C.”; TO-3097: Dr. Scott NeSmith; Florida Rose: http://www.ffsp.net/varieties/blueberry/florida-rose/. The three pictures are not in scale.

<u>Preliminary results:</u> In summer 2020, we developed protocols to measure ethylene, ABA and anthocyanin from rabbiteye cultivar, ‘Powderblue’. As seen in Figure 2, levels of ethylene increase between immature green to more advanced green fruit (where ripening has just initiated) stage and then increases further as fruit begins to ripen. Similarly, ABA increases during initiation of ripening until the pink stage and then declines in ripe fruit. Total anthocyanin concentrations gradually increase during ripening until the pink stage after which it dramatically increases in ripe fruit. It would be further interesting to explore amount of ethylene, ABA and anthocyanin accumulation during ripening in additional rabbiteye cultivars for the commercial market along with the three ornamental cultivars.

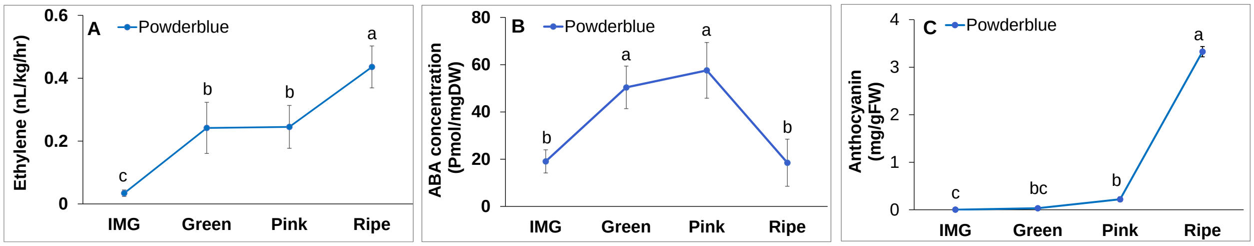

Figure 2: Levels of ethylene (A), ABA (B) and anthocyanin (C) during fruit developmental stages: Immature green (IMG), Advanced green with >10% pink on fruit surface (Green), completely pink fruit (pink), and fully blue fruit (ripe). For each developmental stage, means with the same letter are not significantly different from each other ($\alpha = 0.05$).

Methods

For this work we proposed two objectives. Objective 1 was to determine fruit quality attributes at harvest including fruit weight, texture, titratable acidity, and total soluble solids. Objective 2 included ethylene and ABA measurements in the three ornamental cultivars and compare with some commonly grown cultivars such as 'Premier' and 'Powderblue'.

Fruit sample collection: Pink Lemonade plants were purchased from a local nursery (Cofer's Home & Garden, https://cofers.com/) in 4-Gallon containers. Plants were transplanted in the Durham Horticultural Farm, Athens, GA in February 2021. Due to the first year of establishment of the plants in the field, limited fruits were available. Therefore, in the current year, we quantified ABA and ethylene levels (Objective 2). Objective 1 will be completed in the following year. For cultivar, Florida Rose cuttings were purchased from Agristats (https://www.agristarts.com/) and seedlings have been established in the greenhouse. These seedlings will be ready for transplantation in the field for February 2022. In case of TO-3097, limited fruit were available at the Alapaha Research Farm. Therefore, proposed work for these 2 cultivars will be performed in Summer 2022. We compared hormone levels obtained from Pink Lemonade in Summer 2021 to cultivars, Premier and Powderblue from Summer 2020.

Quantification of ethylene and hormones: For determination of ethylene evolution, 25 g of fruit were placed into a 125 mL glass jar and capped with a lid fitted with a septum. After a 4 h incubation, 1 mL of the head space gas was withdrawn using a needle fitted to a syringe and injected into a gas-chromatograph (GC-17A, Shimadzu, MD). The peak area of the sample chromatograph was quantified using ethylene standards.

For hormones quantification we shipped samples to the University of North Texas (UNT) BioAnalytical Facility. The protocol used by UNT was based on Forcat et al. (2008). Briefly, samples were lyophilized, and 10 milligrams of sample was extracted with 400 µl of 10% methanol containing 1% acetic acid with an internal standard, ^{13}C-benzoic acid. Subsequently samples were disrupted using a Tissuelyzer bead mill, and then incubated in ice for 30 min. Samples were centrifuged at 17,000 x *g* for 10 min at 4 °C and the supernatant collected. The pellet was reextracted in methanol and acetic acid and the two supernatants were pooled together. Samples were filtered using ultra-centrifugal filter units from Amicon (Florida, USA).

Finally, hormones were quantified by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Results

Phenology: Fruit at different developmental stages were collected based on their size (S5: < 8 mm) and peel color (S6, S7, S8) (Figs. 3, 4). Fruit collected at S6 stage was >30% light pink, S7 fruit were 80-100% light pink, and S8 fruit were dark pink (equivalent to fully ripe). Fruit diameter of ‘Pink Lemonade’ was lower than ‘Premier’ and ‘Powderblue’, however fruit from ‘Pink Lemonade’ were from young plantings in comparison to the other two well established rabbiteye cultivars.

Ethylene production: In ‘Pink Lemonade’, the rate of ethylene production was lowest at the S5 stage, then it increased steadily and reached its maximal value at the S7 stage. Similar increase in ethylene production during ripening initiation was also observed in ‘Premier’ and ‘Powderblue’. ‘Pink Lemonade’ produced less ethylene than ‘Premier’, however, the rate of ethylene production was similar to Powderblue (Fig. 5).

Hormone production: Eight different hormones were quantified at all four developmental stages in ‘Pink Lemonade’ (Figs. 6, 7). Among these, ABA was the most abundant. The level of ABA was lowest at the S5 stage and reached a maximum at S6 and S7 stages. At S5 stage, ABA level was 2.8-fold less than the average present at the S6 and S7 stages and by 2-fold less than the S8 stage (Fig. 4). The level of ABA-glucose ester (ABA-GE) steadily increased and became maximum at the S8 stage. ABA-GE levels increased by 2.6 and 1.7-fold at the S8 stage compared with S5 and S6 stages, respectively. Indole-3-acetic acid (IAA) showed a decrease during ripening and IAA-Aspartate (IAA-ASP) increased during ripening, however the levels were not significant (Fig. 5). Jasmonic acid (JA) was maximum at the S5 stage and became minimum at S6-S8 stages (Fig. 6). Overall, JA was higher by 4.2-fold at the S5 stage compared with an average of S6, S7, and S8 stages. A small quantity of Methyl-JA was found and was not significantly different at various developmental stages. Ile-JA-1 was not detected, and Ile-JA-2 was only detected at S5 stages (Fig. 7). The pattern as well as the amount of ABA content in ‘Pink Lemonade’ is similar to the pattern of ABA content in ‘Powderblue’ (Figs. 6-8). Overall patterns of other hormones during fruit development were also comparable between ‘Pink Lemonade’ and ‘Powderblue’.

Conclusion: Ethylene and ABA play an important role in the ripening and color development of many fruits. In this study, similar levels of production in ethylene and ABA in ‘Pink Lemonade’ and ‘Powderblue’ suggested that ethylene and ABA (and other hormones) may not play an important role in altering anthocyanin production in ‘Pink Lemonade’ blueberry. A recent study found a positive correlation between MYB transcription factor/ basic-loop-helix (bHLH) domain protein/ WD-repeat (MYB-bHLH-WD40) complex and anthocyanin accumulation in ‘Pink Lemonade’ (Die et al., 2020). This study suggested the switch of anthocyanin production in pink fruited cultivar, ‘Pink Lemonade’ is possibly due to the *MYB1* transcription factor. Future studies will focus on hormone content and transcription factor abundance of the other pink fruited cultivars.

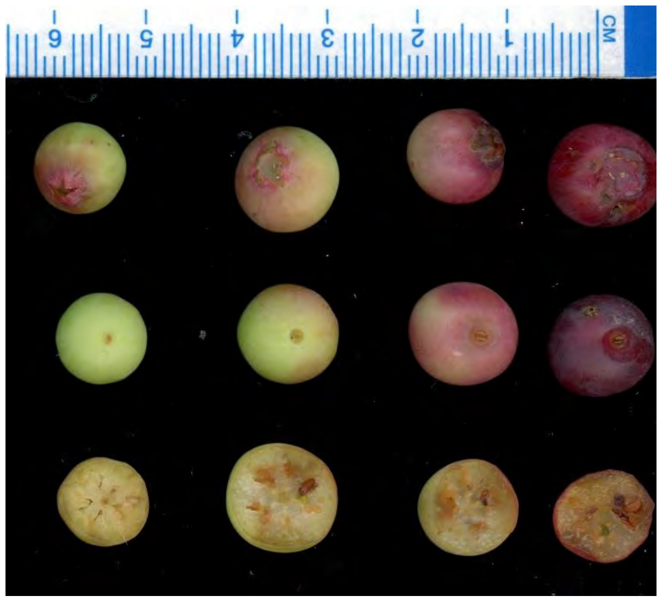

Figure 3: Charecterization of fruit developmental stages in the Pink Lemonade.

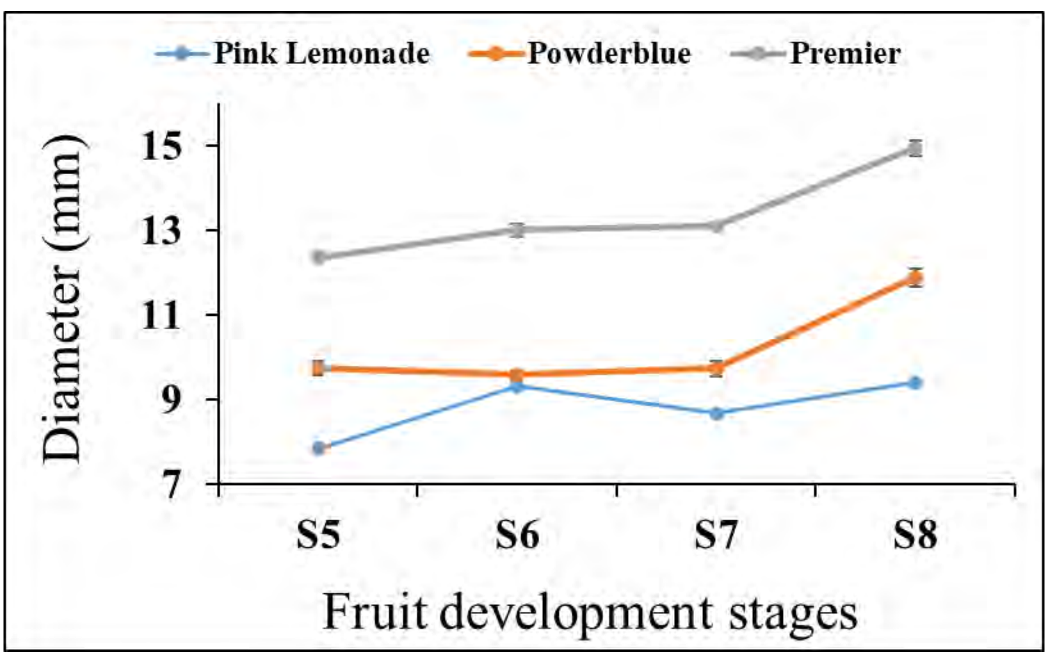

Figure 4: Fruit diameter at various developmental stages

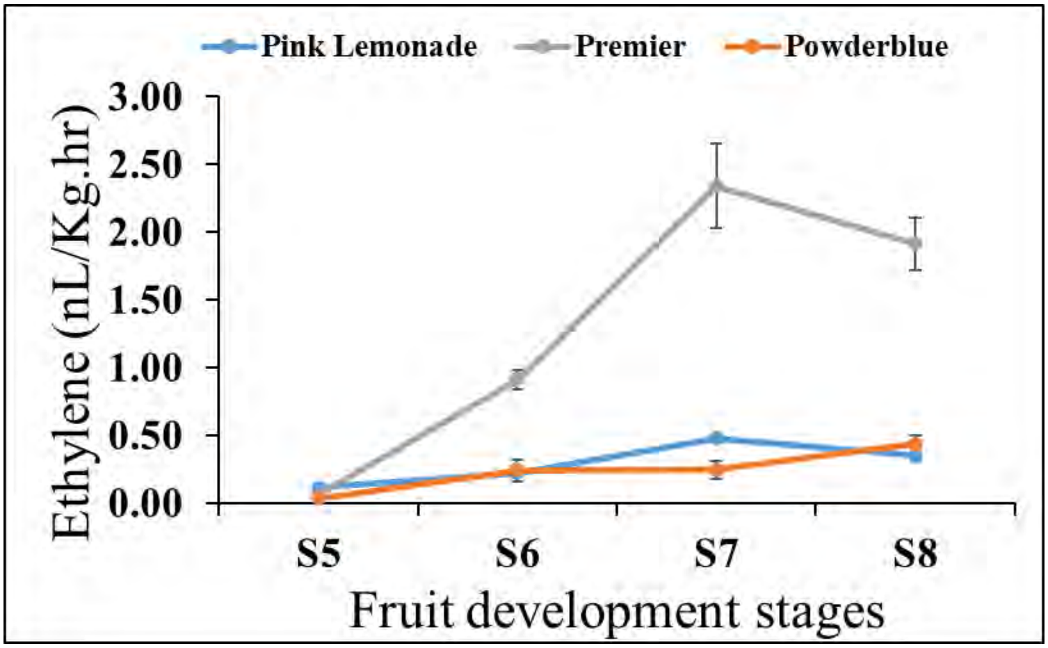

Figure 5: Ethylene production at various developmental stages

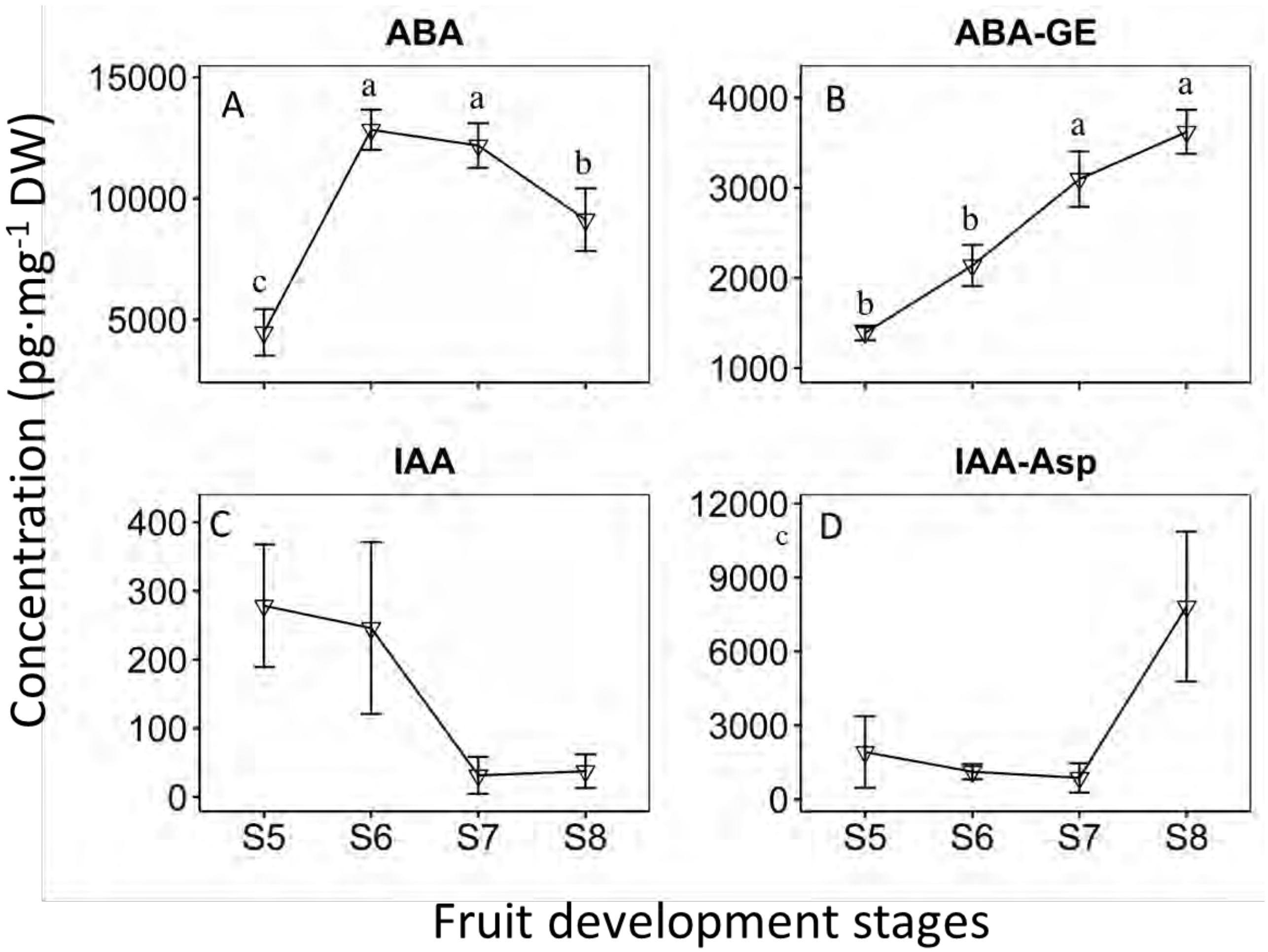

Figure 6: Concentration of key hormone in Pink Lemonade at various developmental stages.

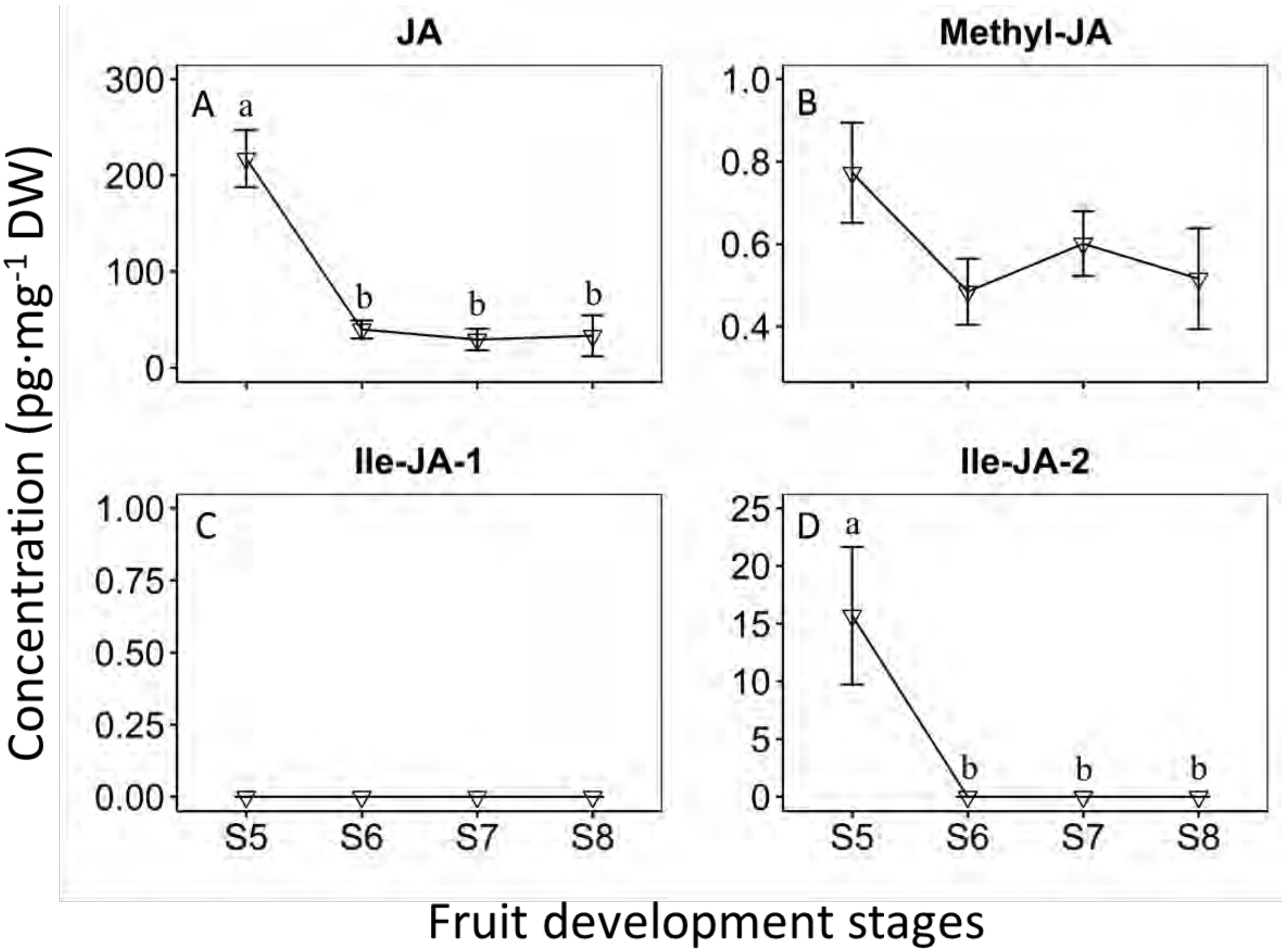

Figure 7: Concentration of key hormone in Pink Lemonade at various developmental stages.

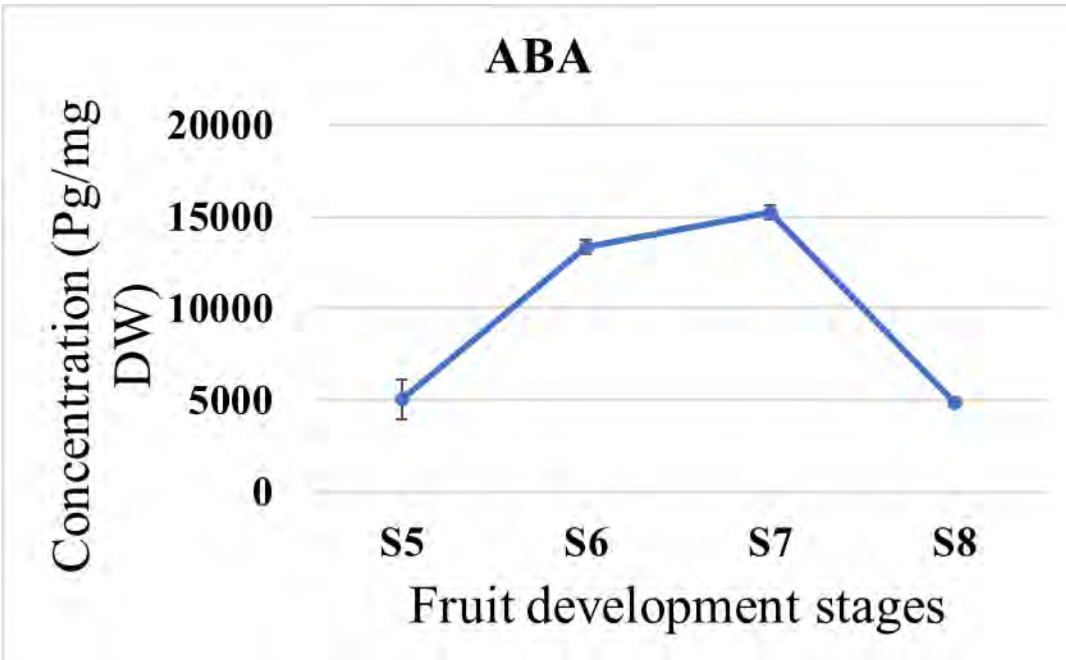

Figure 8: ABA concentration at various developmental stages in Powderblue.

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