Abscisic acid, a key phytohormone for antioxidant production in sweet cherries

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Abstract

Sweet cherry (*Prunus avium* L.) is a non-climacteric fruit very well-known due to its antioxidant properties, highly appreciated by the consumers. Because abscisic acid (ABA) is a crucial phytohormone in the development and ripening of non-climacteric fruits, the aim of this study was to evaluate the role of this sesquiterpenoid hormone in the synthesis of antioxidants such as vitamin E, anthocyanins and carotenoids of sweet cherries during different developmental stages up to their consumption. For our experiment, we collected sweet cherries var. Prime Giant from orchard trees at eight different stages of ripening during two consecutive years to further measure the endogenous levels of ABA using ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem spectrometry (UPLC/ESI-MS/MS), as well as the concentrations of vitamin E and carotenoids with high performance liquid chromatography (HPLC) at all stages of ripening collected. While our results showed a negative correlation between ABA and carotenoids levels, we could see that ABA endogenous levels positively correlated with vitamin E and anthocyanins concentrations. These results indicate that carotenoids could be present at earlier stages of development as an accessory of photosynthesis, to later act as a precursor of ABA phytohormone during ripening of the fruit. This increment in the ABA levels is also followed by an increment in the vitamin E and anthocyanins concentrations, which arise the relevance of ABA hormone in ripening and enhancing the quality parameters of the fruit.

Keywords: sweet cherry, ABA, ripening, carotenoids, vitamin E, anthocyanins.

Introduction

At present, there is a trend from world population for a healthier lifestyle which requires products with good qualities and properties. Fruits and vegetables could constitute a good option for these consumers due to the benefits of their consumption as a source of vitamins and minerals. In this context, sweet cherries may be considered an excellent alternative because of their low caloric content (only $63 kcal \cdot 100 g^{-1}$) and because they can provide nutrients and phytochemicals with putative health benefits (McCune et al., 2011).

Sweet cherry (*Prunus avium* L.) is a non – climacteric fleshy fruit that undergoes progressive developmental steps (Figure 1) starting with fruit set, followed by fruit growth, maturation and a final ripening stage where the fruit synthesizes bioactive compounds and also continues its growth, constituting a double – sigmoid pattern (Ren et al., 2010).

Abscisic acid (ABA) is known to be an important ripening factor for non – climacteric fruits like sweet cherries that once enters into the ripening process, this cannot be ceased and it usually leads to over – ripening (Ren et al., 2010; Kumar et al., 2014; Leng et al., 2014). This sesquiterpenoid hormone is involved in cell wall modifications,

accumulation of sugars through starch conversion and the biosynthesis of important compounds for human nutrition like flavonoids or vitamins (Miret & Munné, 2016). In fruits, the endogenous ABA content is regulated by an accurate balance between *de novo* biosynthesis through the carotenoid pathway (9 – *cis* – epoxycarotenoid dioxygenase (NCED) conversion), ABA catabolism through hydroxylation reactions by *CYP707A* gene family enzymes, and ABA conjugation (Kumar et al., 2014; Leng et al., 2014).

It has been demonstrated that sweet cherries possess beneficial healthy properties that can be related to the high antioxidant activity of these fruits, being flavonoids and in particular anthocyanins the major antioxidants also responsible for their colour and taste (Serrano et al., 2005). Anthocyanins are water – soluble pigments that accumulate in the vacuoles of plant tissues and give the red to blue colours, very characteristic of many fruits, vegetables and flowers.

Although vitamin C has been widely studied in sweet cherries, little is known about vitamin E in this fruit. Tocopherols and tocotrienols constitute the Vitamin E group of compounds in plants, where these tocochromanols are synthesized (Falk & Munné – Bosch, 2010). Vitamin E also constitutes a high potent antioxidant, essential for human health, which can be found at large amounts in fruits like avocados, raspberries or mangoes (Chun et al., 2006). However, only low levels of α – and β – tocopherol have been reported in sweet cherries (Bastos et al., 2015).

As it has already been described by many authors that ABA is the major phytohormone involved in the ripening of non – climacteric fruits such as strawberry or grapes, and that it can regulate the biosynthesis of bioactive compounds present in these fruits (Ren et al., 2010; Kumar et al., 2014; Leng et al., 2014), the aim of this project was to measure the endogenous levels of ABA in sweet cherries at different ripening stages in order to understand the implication of this phytohormone for the biosynthesis of important antioxidants for human health, mainly vitamin E, anthocyanins and carotenoids.

Materials and methods

In order to perform all the analyses, we obtained a variety of sweet cherries (*Prunus avium var.* Prime Giant) from trees growing in an exploited orchard at Partida Vall del Sector III (Lleida, Spain). Cherries were harvested at eight different developmental stages from 15th April to 7th June of 2016, which corresponds to 20 and 73 days after full bloom respectively, being the latest stage the day before harvest for commercialisation. All samples were harvested early in the morning between 9 and 10 a.m. local time with an average temperature of 18 ± 4°C. Six fruits per tree from eight trees were randomly sampled at each time point during the pre-harvest period and immediately frozen in liquid nitrogen and stored at -80°C until analysis. An additional cherry was sampled from each tree at each time point for fruit biomass, transferred in individual bags to avoid desiccation, and dried in an oven for dry weight estimation.

For the analysis of carotenoids, anthocyanins and vitamin E, 200 mg of each sample were taken and extracted with methanol following the protocol described in Figure 2.

For carotenoid quantification by HPLC we followed the protocol described by Munné – Bosch & Alegre (2000) with an Agilent ZORBAX Original 70Å C18 (4.6 x 250 mm, 5 μm, non-endcapped, Teknokroma, St. Cugat, Spain) during 38 min at 30°C.

For the analysis of tocochromanols we followed the protocol of Amaral et al. (2005) in an HPLC system which consisted of an integrated system with a Jasco PU-2089 Plus pump, a Jasco AS- 2055 Plus auto-sampler and a FP-1520 fluorescence detector

(Jasco, Tokyo, Japan). All forms of tocopherols and tocotrienols were separated on a normal – phase column Inertsil 100A (5 mm, 30 x 250 mm, GL Sciences Inc., Tokyo, Japan). Quantification was based on the results obtained from the fluorescence signal and calibration curves made with authentic standards (Sigma–Aldrich, Steinheim, Germany).

Total anthocyanins were determined spectrophotometrically as described by Gitelson et al. (2001) at 530 nm and then calculated by equivalents of cyanidin (MM = 449.2 g · mol^{-1}) and a molar absorption coefficient of 34300 $L \cdot cm^{-1} \cdot mol^{-1}$.

We employed ultrahigh – performance liquid chromatography – electrospray ionisation coupled to tandem mass spectrometry (UHPLC/ESI – MS/MS) in order to quantify the ABA levels of *P. avium var.* Prime Giant as explained by Müller & Munné – Bosch (2011) with a solvent mixture of methanol:isopropanol:glacial acetic acid (50:49:1; v:v:v) and following the protocol described in Figure 2. Internal standard, d6-ABA was added at the beginning of the extraction procedure to a final concentration of 100 $\mu L \cdot L^{-1}$. The UHPLC system consisted of an Aquity UHPLC™ System (Waters, Milford, MA USA) quaternary pump equipped with an autosampler. For the analysis of the extracts, a HALO™ C18 (Advanced Materials Technology, Inc., Wilmington, USA) column (2.1 × 75 mm, 2.7 μm) was used.

Results and discussion

Sweet cherries are fleshy fruits highly appreciated by consumers due to their early appearance in the market and the healthy benefits of their intake (McCune et al., 2011; Ballistreri et al., 2013; Cockchaisawasdee, 2016). ABA has been extensively described as the main phytohormone involved in the ripening of non – climacteric fruits and a support of ethylene for climacteric fruits ripening. For this reason, we wanted to measure the endogenous levels of this plant hormone in sweet cherries at different developmental stages to understand its role on the biosynthesis of bioactive compounds important for human health, such as antioxidants.

Our results showed an increased accumulation of ABA over the preselected developmental stages (Figure 3), being very low during the first stages (under 700 ng·g DW $^{-1}$) and with the first peak at stage IV, 51 days after full bloom. This first peak of ABA corresponds with the first visual effects of the ripening process with an increase in the percentage of red colour in the fruit. The highest peak of ABA was achieved at stage VI with an accumulation of 4777.64 ± 369.65 ng·g DW $^{-1}$, after which the levels of this phytohormone decreased progressively.

Contrary to other fleshy fruits such as tomatoes, sweet cherries do not accumulate carotenoids during fruit ripening, as our results showed a decrease in the levels of these lipophilic pigments until stage IV, 51 days after full bloom, where the last carotenoids remaining were lutein, β – carotene and violaxanthin (Figure 4). After this stage, no carotenoids were detected with HPLC. I could be that carotenoids were playing a protective role as accessory pigments for photosynthesis at first stages of development of sweet cherries, as we found high levels of violaxanthin (5191.94 ± 662.78 μg·g DW^{-1}), antheraxanthin (1271.85 ± 283.72 μg·g DW^{-1}) and zeaxanthin (922.62 ± 241.53 μg·g DW^{-1}), involved in the chlorophyll protection through the xanthophyll cycle (Moise et al., 2014). We performed Pearson linear correlations between ABA and violaxanthin, along with ABA and neoxanthin, the main ABA precursors, and we found negative correlations which were statistically significant (Table 1). This data suggests that carotenoid degradation in cherry fruits could be explained for their function as ABA precursors, although the accumulation of this phytohormone in the fruit is a tight regulation between its biosynthesis and degradation (Leng et al., 2014).

Because sweet cherries are specially valued due to their antioxidant properties, we analysed total anthocyanin levels of our cherries variety Prime Giant. Our results showed an exponential accumulation of anthocyanins, beginning at stage V of development, 59 days after full bloom (Figure 5a). Besides, the highest yield of anthocyanins was achieved at stage VIII, the day before fruit harvesting, with 1134.73 ± 41.45 µg cyanidin·g DW^{-1}, 15 fold higher than at the first stage selected. We performed a Pearson linear correlation between anthocyanin accumulation and ABA levels and we could see that there was a statistically significant positive correlation with a *P – value* = 0.005 (Figure 5b). However, the correlation coefficient was not very strong, with a value of 0.344 and this could be explained because fruit ripening is a complex set of events that need the regulation and interaction of different hormones involved in this process (Teribia et al., 2016). In the case of anthocyanin biosynthesis, it has been described that ABA plays a major role in the regulation of its pathway for the formation of these compounds, but other hormones such as jasmonates and gibberellins have been found to exert also a pivotal role in their biosynthesis (Jaakola, 2003).

For vitamin E, we identified that tocopherols were the natural form of this group of compounds in these fruits. Moreover, α – tocopherol was the main form of tocopherols found and, into a lower extent, γ – tocopherols. These forms of vitamin E had a major increase at stage IV of development in both cases (Figure 6). Then, α – tocopherol values had a 30% decrease until stage VIII where sweet cherry fruits contained 18,71 ± 0,98µg α – tocopherol·100g DW^{-1} (Figure 6a). A similar pattern was found for γ – tocopherol, which showed a decrease of 38% from stage IV to the last stage, a day before its harvesting, with levels of 14,42 ± 1,22 µg γ – tocopherol·100g DW^{-1} (Figure 6b). Total amounts of tocopherols were very low compared with anthocyanin amounts, which were 97% fold higher. Moreover, sweet cherries have 86% lower values than other fruits with high levels of anthocyanins, such as blueberries or raspberries (Chun et al., 2006). Nevertheless, while anthocyanins are a group of hydrophylic pigments, vitamin E is a potent lipophilic antioxidant that can prevent lipid peroxidation, thus protecting cell membranes and associated diseases (Brigelius-Flohé et al., 2002). We also performed Pearson linear correlations and we found strong positive correlation coefficients between ABA and both forms of tocopherols detected (Figure 7), which were statistically significant (*P – value* < 0.001). Therefore, these results could indicate that ABA is driving vitamin E accumulation in sweet cherries.

Conclusions

In this work we revised the role of ABA hormone in the ripening regulation of sweet cherry fruits and we could see that this hormone might be regulating the biosynthesis of important antioxidants like anthocyanins and vitamin E, as we found positive correlations between ABA and the major forms of these compounds in sweet cherry fruits. Vitamin E in sweet cherries was mainly found in the forms of α – and γ – tocopherol, being the first one the most abundant homologue, although this vitamin was found at low levels, even at the time of fruit harvesting. In the case of sweet cherries, carotenoids are not accumulated in chromoplasts during fruit ripening and are degraded during developmental stages serving as precursors for ABA biosynthesis.

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Tables and Figures

Table 1: Pearson linear correlation results between ABA and violaxanthin and ABA against neoxanthin.

		Violaxanthin	Neoxanthin
ABA	Pearson correlation coefficient	-0.495**	-0.535**
	P – value	0.001	0.001
	N	64	64

Asterisks show a significant correlation.

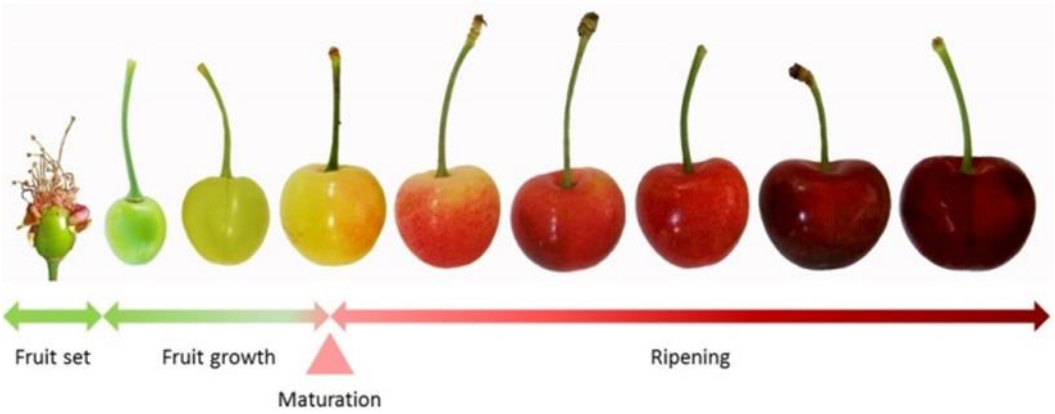

Figure 1: Developmental stages in sweet cherry fruit

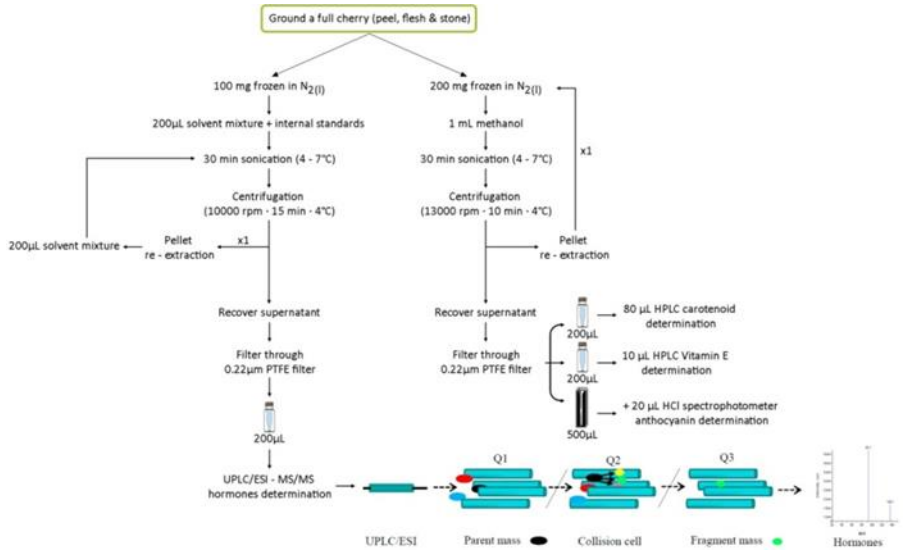

Figure 2: Brief summary of the extraction protocols for hormones and antioxidants determination [Image adapted from Müller & Munné – Bosch, 2011].

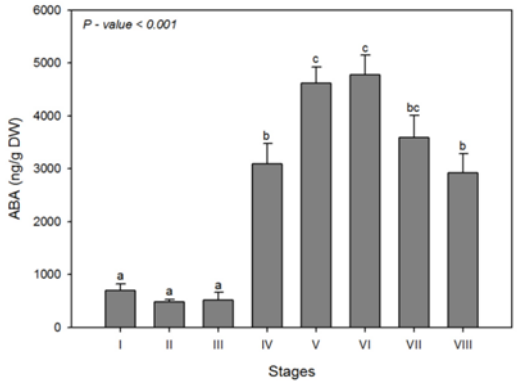

Figure 3: ABA levels at different stages expressed as nanograms of the hormone per gram of dry weight (ng·g DW -1). Data shown is the mean ± standard error of n = 8. Different letters show a P – value < 0.001 between the different stages, result of the ANOVA and post – hoc Tukey test.

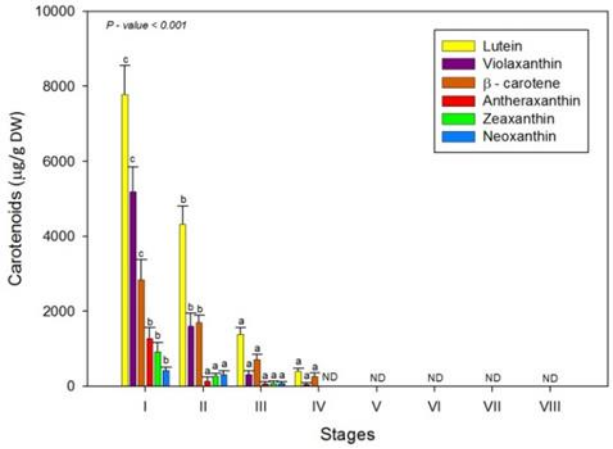

Figure 4: Carotenoids levels at preselected developmental stages expressed as micrograms per gram of dry weight (µg·g DW -1). Data shown is the mean ± standard error of n = 8. Different letters show a P – value < 0.001 between stages for each carotenoid, result of the ANOVA and post – hoc Tukey test. ND means non – detected values.

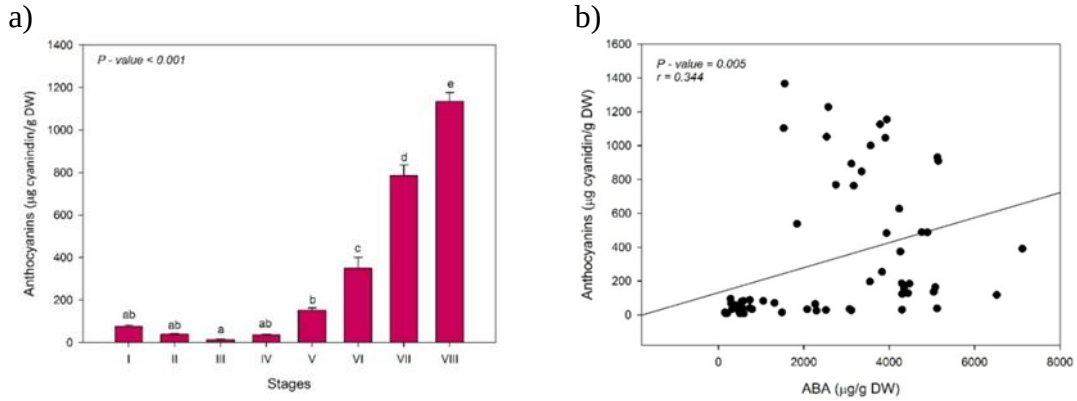

Figure 5: a) Anthocyanin yields measured as micrograms of cyanidin per gram of dry weight (µg cyanidin·g DW-1) over the different developmental stages selected. Data shown is the mean ± standard error of n = 8. Letters show a *P – value* < 0.001 between stages, result of the ANOVA and post – hoc Tukey test. b) Pearson linear correlation between ABA and anthocyanins. P – value and Pearson correlation coefficient (r) are given inside panels.

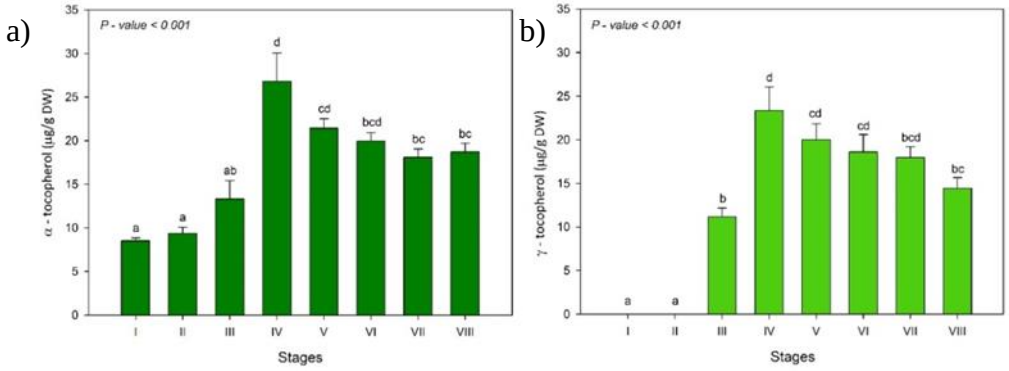

Figure 6: Tocopherols levels measured as micrograms per gram of dry weight (µg·g DW^{-1}) during the different developmental stages selected. a) α – tocopherol and b) γ – tocopherol. Data shown is the mean ± standard error of n = 8. Letters show a *P – value* < 0.001 between stages, result of the ANOVA and post – hoc Tukey test.

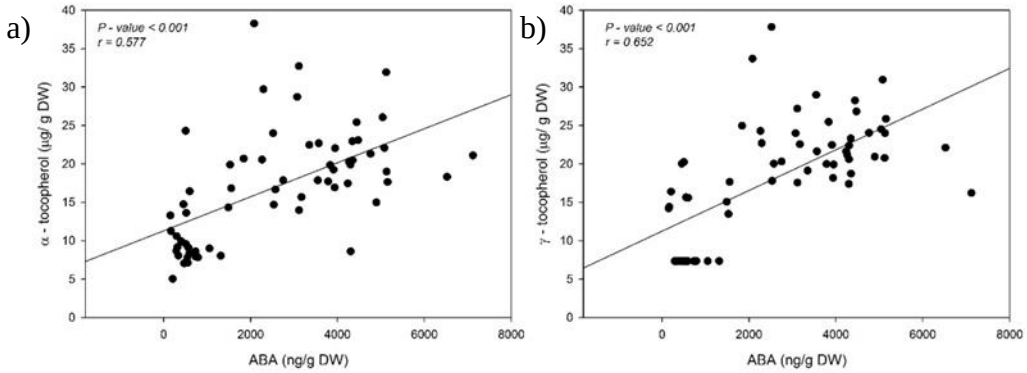

Figure 7: Pearson linear correlations between endogenous levels of a) ABA and α – tocopherol; and b) ABA and γ – tocopherol. *P – value* and Pearson correlation coefficient (*r*) are given inside panels.