Hormonal cross-talk in the regulation of ripening and over-ripening in sweet cherries

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Abstract

Currently, sweet cherry fruits (*Prunus avium* L.) are highly appreciated by consumers worldwide because of their visual and organoleptic characteristics. As a non-climacteric fleshy fruit, the biochemical and physical changes that occur during the ripening process are known to be regulated by abscisic acid (ABA). To better understand the interplay between ABA and the other phytohormones in this complex process, sweet cherries were harvested from an exploited orchard at different developmental stages, from green stages to harvest time, and during over-ripening at two temperatures (4ºC and 23ºC). We measured the endogenous levels of ABA, indole-3-acetic acid (IAA), GA_1, GA_3 and GA_4, and the cytokinins, *trans*-zeatin (*t*Z), *trans*-zeatin riboside (*t*ZR), and isopentenyl adenosine (IPA), by using UHPLC-ESI-MS/MS. Furthermore, we analysed fruit quality parameters, such as fruit mass, anthocyanin accumulation and sugar contents both during pre- and post-harvest. Results revealed high concentrations of IAA, GA_3, *t*Z and *t*ZR, at first stages of growth, which decreased during ripening. In contrast, ABA and IPA concentrations increased during ripening on the tree. ABA, as well as GA_4 and IPA, positively correlated with anthocyanins accumulation and sugar levels, while *t*Z showed a negative correlation with these quality parameters, thus suggesting a possible hormonal cross-talk in the regulation of quality parameters during ripening. During postharvest, ABA concentrations decreased at 23ºC, while they increased at 4ºC, thus suggesting ABA may inhibit the over-ripening process in sweet cherries, an aspect that warrants further investigations. It is concluded that (i) not only ABA, but also other phytohormones may control ripening of sweet cherries, (ii) the hormonal balance, rather than a single phytohormone, regulates fruit ripening, and (iii) the same hormone can exert different roles during pre- and post-harvest, depending whether the fruit is attached or detached, consequently receiving or not, other signals from the tree.

Keywords: ABA, phytohormones, pre-harvest, post-harvest, *Prunus avium* L.

Introduction

Some biochemical and physiological modifications occur during fleshy fruit ripening, leading to colour, flavour and texture changes. In some cases, these changes are regulated by ethylene, which increments before the respiration burst at the onset of ripening in climacteric fruits (Barry & Giovannoni, 2007). Contrary, non-climacteric fruits do not depend on ethylene to start this process. Indeed, several studies suggest that abscisic acid (ABA) regulates ripening in these fruits, as it is known to be involved in sugar and anthocyanin accumulation (Kumar et al., 2014; Leng et al., 2014; Miret et al., 2014)

Over the past decade, sweet cherries (*Prunus avium* L.) have become a fruit highly appreciated by consumers, due to its organoleptic properties, like colour, sweetness or acidity, along with their nutrient and vitamin content (Serrano et al., 2005), with a

significant worldwide distribution (FAO, 2015). As a non-climacteric fruit, their ripening process in the tree require a high concentration of ABA (Luo et al., 2013; Wang et al., 2015), thus, improving its quality from fruit set to harvest time. After their harvest, sweet cherries are usually cold stored to prevent over-ripening and a loss of quality (Meheriuk et al., 1995), although little is known about the role of ABA during this process.

It has been described that other phytohormones such as auxins, gibberellins (GAs) and cytokinins (CKs) are involved in the ripening process of non-climacteric fruit like strawberries, grapes or sweet oranges (El-Otmani et al., 1995; Symons et al., 2012). However, further investigation is needed to unravel the possible interplay of these hormones during the growth and ripening process in sweet cherry fruits.

The aim of this study was to get some insights in the possible role of auxins, GAs and CKs in growth and ripening of sweet cherries, focusing on the endogenous levels during its development in orchard trees, as well as the role of ABA during ripening and over-ripening under two different postharvest conditions. In addition, we simultaneously analysed different parameters associated to the ripening process and fruit quality, such as fruit biomass, anthocyanins accumulation and total soluble sugars to total acidity ratio (TSS/TA), as a flavour indicator determining consumer's acceptance (Crisosto et al., 2003).

Material and methods

Experimental design and sampling

For the ripening on the tree study, we obtained samples of sweet cherries (*Prunus avium* L. *var*. Prime Giant) from an exploited orchard at Lleida, NE Spain. Six fruits per tree from eight trees were randomly harvested at seven developmental stages, from 26 and 4 days before its harvest time, starting from 30th April in 2015. All samplings were performed between 9 and 10 a.m., local time. A second experiment was performed with 10 kg of sweet cherries from the same fruit cultivar and orchard. These cherries were brought to the laboratories 3 days after its commercial harvest. The ones with no visual defects were used for the experiment. Sampling was held in darkness, half of the fruits were kept under room temperature conditions (23±2º C), and the other half were stored in a cold chamber (4±1º C). In both cases, six fruits from each postharvest temperature were randomly sampled daily during one week. For all experiments, sweet cherry samples were immediately frozen in liquid nitrogen and stored at -80º C until analyses.

Fruit quality parameters

Fruit biomass was estimated by weighing sweet cherry fruits immediately at each sampling time, after transferring them to the laboratory in bags, with high humidity to avoid desiccation. Anthocyanin content was determined as described in Gitelson et al., 2001. Briefly, 200 mg per sample were extracted in 1 mL methanol twice and 1% HCl was added for anthocyanin measurement at 530 nm spectrophotometrically. Total anthocyanins were calculated using the molar extinction coefficient of cyaniding-3-glucoside as a reference (Siegelman & Hendricks, 1958). For the TSS/TA ratio, we considered TSS as glucose and fructose content analysed with HPLC. Samples were extracted with 1 mL ethanol 80%, and were detected with a differential refractometer (adapted from Gerrits et al., 2001). TA were calculated using equivalents of malic acid after the determination of titratable acidity with 0,1 N NaOH, to neutralise all titratable protons (Latimer, 2012).

Hormone profiling

Hormones endogenous levels were determined by ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) as previously described (Müller & Munné-Bosch, 2011). In short, 100 mg per sample were extracted in

200 µL of methanol:isopropanol:acetic acid (50:49:1, v/v/v), using ultrasonication and vortexing. Deuterium-labelled internal standards of each hormone analysed were added and after centrifugation at 600 *g* during 10 min, the pellet was re-extracted using the same procedure. The supernatants were collected and filtered through a 0,22 µm PTFE filter. Phytohormones levels were analysed by UHPLC-ESI-MS/MS. Quantification was made considering recovery rates for each sample by using the deuterium-labelled internal standard.

Statistical analysis

Data were analysed by using one-way and two-way factorial analysis of variance (ANOVA). Multiple comparison tests were carried out using Tukey HSD post-hoc test and Bonferroni post hoc test. In addition, correlation analysis using the Spearman's rank correlation were made. In all cases, differences were considered significant at a probability level of $P<0,05$. All statistical tests were performed by SPSS 22.0 statistical package.

Results and discussion

Sweet cherries are highly appreciated by consumers due to their quality and properties. We measured quality parameters of sweet cherries *var*. Prime Giant at different points of development, like fruit biomass (Fig. 1), which increased 18,5-fold from 26 days before harvest (DBH) to 3 days after harvest (DAH), achieving the maximum of 20,74 g at that point. After a week of postharvest storage, there was a fruit biomass reduction of 20% in sweet cherries stored at 23º C, while biomass was maintained in fruits kept at 4º C. During preharvest, anthocyanins began to accumulate visually at 19 DBH, but continued increasing further up during over-ripening, reaching its highest level (773,3 µg/g) after 5 days of postharvest at 23º C. Anthocyanin content remained low at 4º C during over-ripening (Fig. 1). We measured TSS/TA (Fig. 1) as a flavour indicator and it showed an increment during ripening on the tree, due to sugars accumulation. During postharvest, no significant differences between room temperature and cold storage were observed.

Sweet cherry, as a non-climacteric fruit, depend on ABA endogenous levels to determine its fruit quality, regulating anthocyanins and sugars accumulation (Luo et al., 2013). In our experiment, ABA content (Fig. 2) incremented from 15 ng/g to 580 ng/g during ripening on the tree. Moreover, we observed a strong-positive correlation with quality parameters (Table 1) during preharvest, which is in agreement with previous studies. However, we found that ABA was negative correlated to anthocyanin levels during postharvest (Table 1). In storage at 23º C, ABA concentration decreased during sweet cherries over-ripening (Fig. 2). In contrast, ABA levels at 4º C were enhanced 5 days after sweet cherry harvest, and then decreased further, although they remained higher compared to ABA levels at 23º C. Thus, this data suggest that ABA has an inhibitory role in over-ripening during cold storing of sweet cherry fruits.

The role of other phytohormones during development and ripening of non-climacteric fruits has been described. However, in sweet cherries, little is known about the possible implication of auxins, GAs and CKs during this process. Hormonal profiling results showed auxin indole-3-acetic acid (IAA) high concentration at 26 DBH, when fruit was small and green (10 days after fruit set, approximately), but a sharply decrease at 23 DBH, remaining constant through the ripening process (Fig 3). In strawberries and grapes, auxins are growth regulators, increasing fruit size by stimulating cell expansion, and are inhibitors of ripening (Symons et al., 2012; Kumar et al., 2014; Fortes et al., 2015). Hence, our results suggested a similar role in sweet cherry fruits. Among gibberellins measured, GA_3 levels peaked at 19 DBH, to decrease thereafter (Fig 3) when

anthocyanins started to accumulate, hinting a possible function as a promoter of cell expansion (Lenahan & Whiting, 2006). *trans*-Zeatin (*t*Z) and *trans*-zeatin riboside (*t*ZR) concentration was higher at first stages of fruit development (Fig. 3), but remained at lower levels through sweet cherries ripening process, acting as a fruit growth regulator, and suggesting a possible role as a ripening inhibitor (Kumar et al., 2014), as we found a negative correlation between *t*Z and quality parameters (Table 1). In contrast, isopentenyl adenosine (IPA) showed a positive correlation with anthocyanin content and TSS/TA ratio (Table 1), but its levels were low (<0,5 ng/g) (Fig. 3).

Conclusion

ABA plays an important role in the regulation of sweet cherry fruit ripening process, positively promoting anthocyanins and sugars accumulation, quality parameters appreciated by consumers. In contrast, ABA can act as an inhibitor of over-ripening during postharvest conditions. In addition, our results suggest that other phytohormones, such as auxins, GAs and CKs, may be controlling fruit development and ripening on the tree, and not only ABA regulates this process. Although, endogenous hormonal profiles hint an interplay with quality parameters, further research is needed to better understand the whole engine of ripening in sweet cherries and other non-climacteric fruits

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Tables and Figures

Table 1. Spearman's rank correlation analyses between endogenous levels of phytohormones and quality parameters during pre and postharvest. Correlations coefficients with significant *P*-values are given after one or two asterisks (*P*<0,05 and *P*<0,01, respectively). No asterisk indicates correlation was not significant.

	Preharvest		*Postharvest*	
Hormones	Anthocyanins (µg/g FW)	TSS/TA	Anthocyanins (µg/g FW)	TSS/TA
ABA	**0,446****	**0,656****	**-0,531****	-0,103
IAA	0,19	-0,143	0,022	-0,081
GA_1	0,002	0,016	0,048	0,047
GA_3	0,073	0,112	-0,154	-0,239
GA_4	**0,341***	**0,314***	0,038	-0,050
*t*Z	**-0,307***	**-0,633****	-0,279	-0,281
*t*ZR	0,027	-0,222	**-0,309***	-0,207
IPA	**0,390****	**0,489****	**-0,556****	**-0,369****

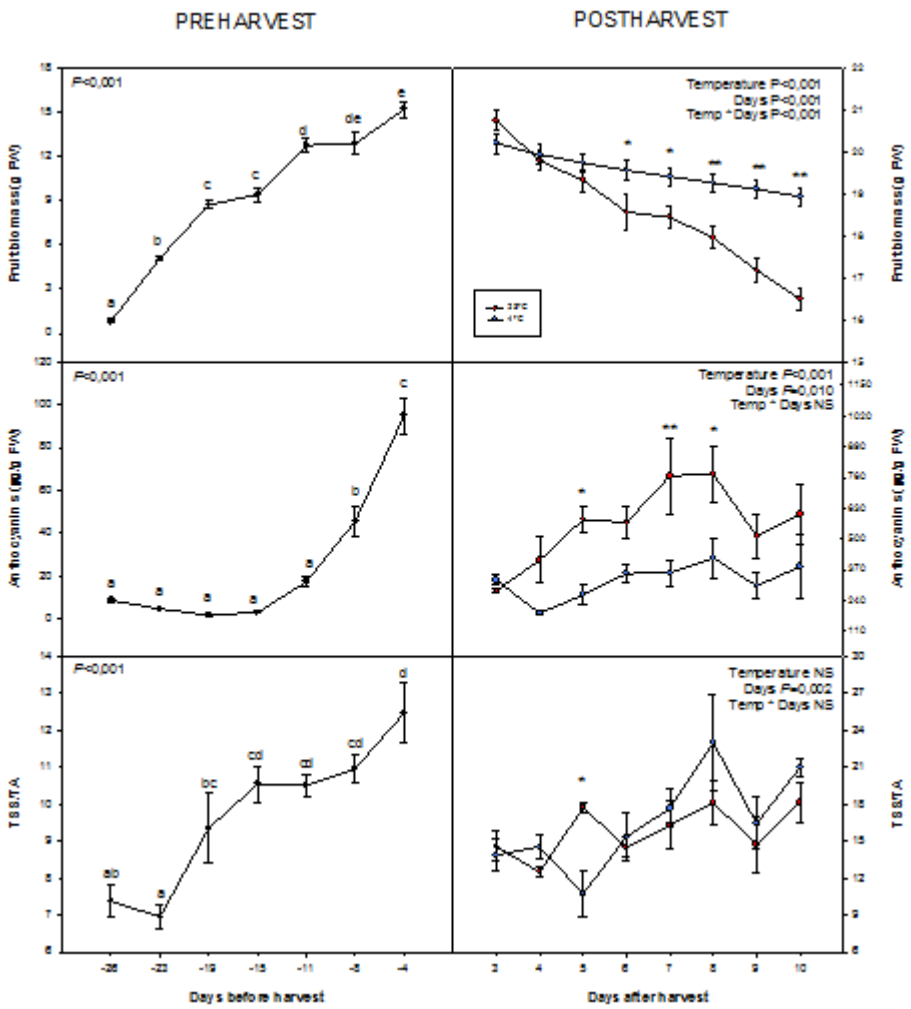

Figure 1. Quality parameters during pre and postharvest. Data are the mean±SE of n=8 (preharvest) and n=3 (postharvest). Statistical analyses were performed by one and two-way ANOVA follow by a Bonferroni post hoc test. Results of statistics are shown in the inlets. Letters indicate significant differences between DBH, and one or two asterisks are shown when differences between temperature at any given time point are significant or highly significant (P<0,05 and P<0,01, respectively). NS, not significant.

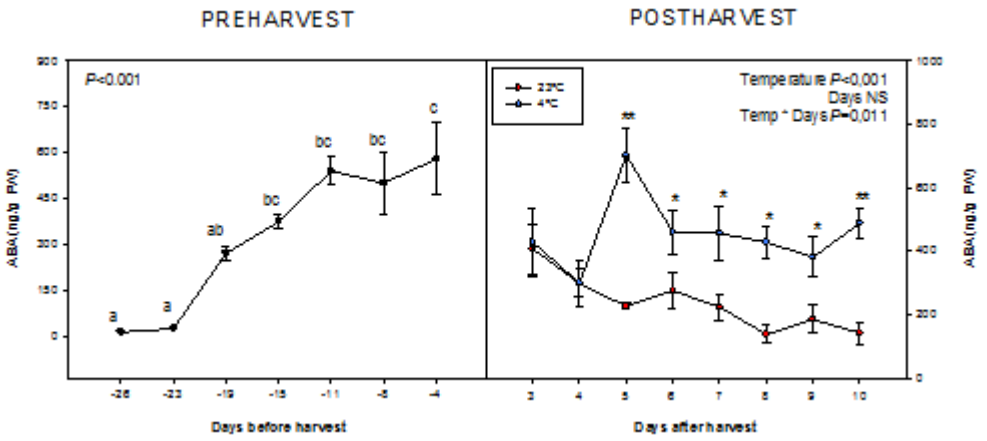

Figure 2. Endogenous ABA concentration during pre and postharvest. Data are the mean±SE of n=8 (preharvest) and n=3 (postharvest). Statistical analyses were performed by one and two-way ANOVA follow by a Bonferroni post hoc test. Results of statistics are shown in the inlets. Letters indicate significant differences between DBH, and one or two asterisks are shown when differences between temperature at any given time point are significant or highly significant (P<0,05 and P<0,01, respectively). NS, not significant.

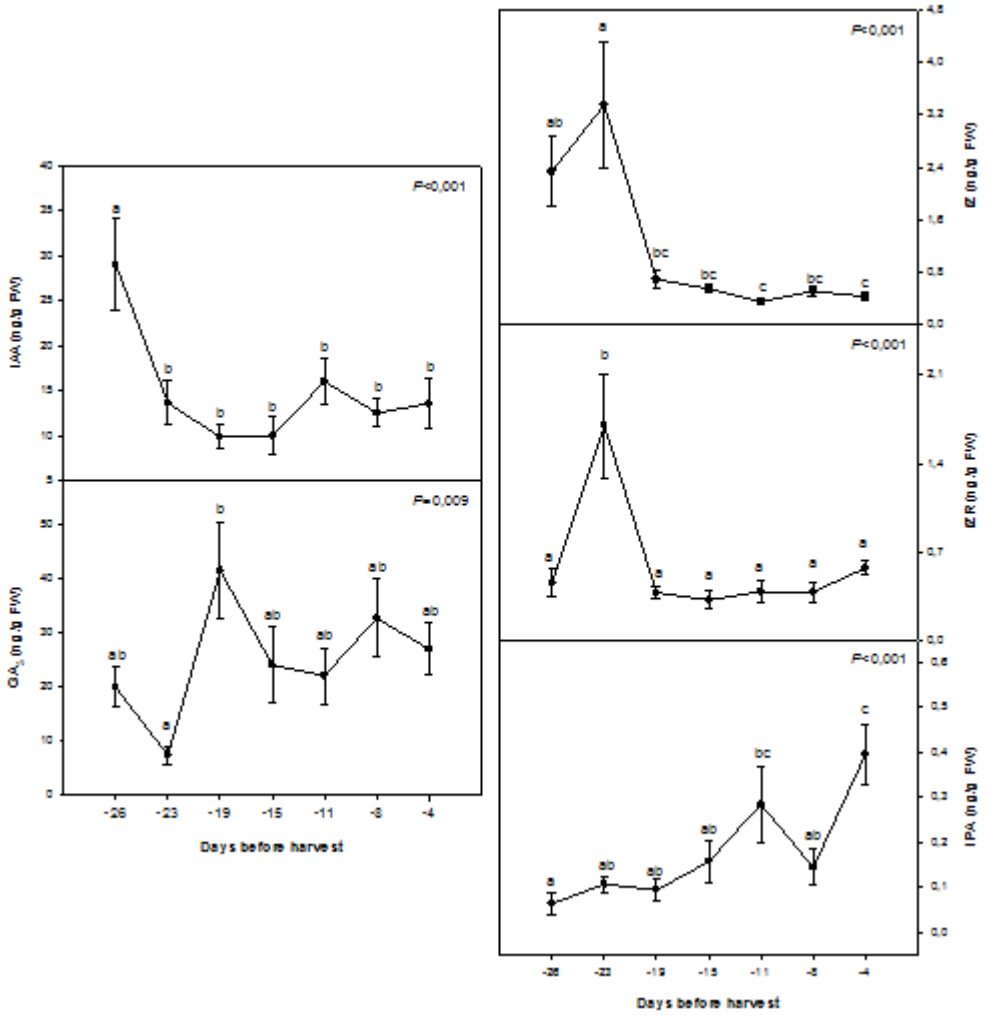

Figure 3. Endogenous phytohormones concentration during ripening process. Data are the mean±SE of n=8. Statistical analyses were performed by one-way ANOVA follow by a post hoc Tukey test. Results of statistics are shown in the inlets. Letters indicate significant differences between DBH (P<0,05). NS, not significant.