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Short communication

# Melatonin as an inhibitor of sweet cherries ripening in orchard trees

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ABSTRACT

Although melatonin effects on postharvest fruit ripening have been studied in some detail, information is still scarce during pre-harvest. Here, we examined whether or not melatonin may exert a regulatory role during sweet cherries ripening in orchard trees. We evaluated (i) the endogenous variations in melatonin contents, in comparison to those of well-known phytohormones such as ABA, salicylic acid and jsamonic acid, by ultrahigh performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) during fruit ripening over two consecutive years, and (ii) to what extent melatonin treatments at low and high concentrations (at $10^{-4}$ M and $10^{-5}$ M, respectively) influence fruit ripening on the tree. Endogenous melatonin contents decreased in parallel to those of salicylic acid and jasmonic acid, while ABA contents increased as fruit ripening progressed, thus suggesting an inhibitory role for melatonin in fruit ripening. Furthermore, melatonin treatment at $10^{-5}$ M, which transiently increased endogenous melatonin contents at physiological concentrations, delayed anthocyanin accumulation, thus confirming an inhibitory regulatory role for melatonin in fruit ripening. We also found that the endogenous contents of cytokinins, but not those of ABA were transiently affected by melatonin treatment at $10^{-5}$ M. It is concluded that melatonin may delay sweet cherries ripening in orchard trees, probably exerting a modulatory role through a hormonal cross-talk. These results have important implications for the use of melatonin in the control of the timing of sweet cherries ripening in orchard trees.

## 1. Introduction

Fleshy fruit ripening is a complex process where many physiological and biochemical changes take place. This process involves colour modification, in which chloroplasts turn into chromoplasts, and carotenoids and anthocyanins start to accumulate, in addition to organic acids, sugars, vitamins and volatiles that ultimately determine fruit quality (Giovannoni, 2004). Fleshy fruits are classified as climacteric, when an increase in respiration rate regulated by ethylene is needed to start the ripening process, and non-climacteric, when fruits do not exhibit an increase in the respiration rate (Chai et al., 2011; Symons et al., 2012).

Many studies have shown how abscisic acid (ABA) plays an important role in non-climacteric fruit ripening, by regulating fruit softening (Castellarin et al., 2016), colour change (the accumulation of anthocyanins and/or carotenoids, Deytieux et al., 2005; Jia et al., 2011), and the contents of sugars and organic acids (Kondo and Gemma, 1993; Kondo and Inoue, 1997; Luo et al., 2013), along with vitamins and antioxidants (Tijero et al., 2016). Other phytohormones like auxins, gibberellins (GAs), cytokinins (CKs), jasmonic acid (JA) and salicylic acid (SA) are also involved in non-climacteric fruit ripening, working in coordination with ABA, but increasing fruit size by promoting cell expansion and cell division, and delaying or inhibiting fruit ripening (Lenahan et al., 2006; Zhang and Whiting, 2013; Kumar et al., 2014; Teribia et al., 2016).

Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine synthesized from tryptophan, widely studied in mammals and, more recently, in plants. Melatonin is a ubiquitous compound in plants, predominantly occurring in the *Rosaceae*, *Poaceae*, *Vitaceae*, *Apiaceae* and *Brassicaceae* plant families, the richest sources of melatonin found in roots, seeds, leaves, bulbs, flowers and fruits (Nawaz et al., 2016). Since the recent application of liquid chromatography (LC) coupled to mass spectrometry (MS) for an accurate identification and quantification of melatonin in plant tissues (Chen et al., 2008), the interest on the possible roles of melatonin and its effects on plant systems has recently increased. Although some recent studies have indicated an important modulatory role for melatonin during fruit ripening during postharvest in climacteric fruits (Sun et al., 2015, 2016; Hu et al., 2017; Zhai et al., 2007), information is still scarce on the putative role of this compound on the ripening of non-climacteric fruits. In a recent study, measurements of endogenous melatonin by HPLC in two sweet cherry varieties showed an increase of this compound during early stages of fruit

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development, followed by a decrease during ripening, which was associated with a possible role of melatonin as an antioxidant, since the contents of melatonin inversely correlated with those of malondialdehyde, an indicator of membrane lipid peroxidation (Zhao et al., 2012). In this study, however, the contents of melatonin ranged between 7 and 35 ng/g fresh matter, which might be considered too low for a direct antioxidant function and is more consistent with a putative hormonal/regulatory role (Arnao and Hernández-Ruiz, 2018). In another non-climacteric fruit, grape berries, melatonin contents were higher on the skin during pre-*veraison* to later decrease at the *veraison* stage, which is also consistent with an inhibitory role for melatonin in fruit ripening (Vitalini et al., 2011). Furthermore, exogenous melatonin treatment at pre-*veraison* increased berry size and improved the aroma of wines (Meng et al., 2015). In contrast, other studies have shown that exogenous melatonin can promote ripening of grape fruits through increases in ABA (Xu et al., 2007). Therefore, more studies are needed to unravel the role of melatonin in the ripening of non-climacteric fruits. The aim of the present study was not only to evaluate the possible role of melatonin during the ripening process of a non-climacteric fruit, such as sweet cherry, in the orchard tree, but also its possible cross-talk with other hormones. In particular, we focused on the possible cross-talk of melatonin with ABA and auxin, known to be important in the control of ripening in non-climacteric fruits, but also with salicylic acid, jasmonic acid, cytokinins and gibberellins, less studied thus far but also with a putative role in ripening. We hypothesized that melatonin might exert an inhibitory role in the ripening of sweet cherry on the tree by modulating the contents of endogenous hormones, mainly ABA and auxin.

## 2. Materials and methods

### 2.1. Plant material, treatments and sampling

We carried out two different experiments for this work. For the first one, we studied the sweet cherry (*Prunus avium* L. *var* Prime Giant) ripening process on the tree in commercial orchards in Lleida (Partida Vall del Sector II, Lleida, NE Spain). The fruits where harvested at seven different developmental and ripening stages during the spring of 2015 and 2016, using the developmental stages described in Teribia et al. (2016). Experiments were performed between April 30$^{th}$, 2015 (stage I) and May 22nd, 2015 (stage VII), and between April 15th, 2016 (stage I) and May 31st, 2016 (stage VII). Supplementary Fig. 1 shows the prevailing climatologic conditions at each developmental stage over the two consecutive seasons studied.

For the second experiment, we applied three different treatments to the fruits: melatonin at either $10^{-4}$ M or $10^{-5}$ M, which were compared to a control (water), containing each solution 0,1% Tween-20. The solutions were sprayed on the surface of fruits at stage II (for a description of stages see Teribia et al., 2016). Samplings were performed at 0 h before treatments, and at 4 h, 1d, 5d, 11d and 19d of treatments. Treatments started on May 5th, 2016.

A pool of six fruits per tree was used as one replicate, and eight trees (eight replicates) were used for each development stage and treatment at each sampling time point. Samplings for each experiment were performed between 9 and 10 a.m. local time. After being collected, samples were immediately frozen in liquid nitrogen, transported to the laboratory and then stored at −80 °C until analyses.

### 2.2. Fruit quality parameters

Total anthocyanins were determined as described (Gitelson et al., 2001). In short, 200 mg per sample were extracted in 2 mL methanol using ultrasonication and vortexing. The extracts were centrifuged at 13000 rpm during 10 min at 4 °C. The pellet was re-extracted following the same procedure. Supernatants were collected and pooled in order to acidify the extracts by adding 1% HCl. Then total anthocyanins were measured spectrophotometrically at 530 nm using the molar extinction coefficient of cyanidin-3- glucoside as a reference.

Total acidity (TA) was estimated with 5 g of each sample homogenized in 25 mL of distilled water, using a vortex (Branson 2510 ultrasonic cleaner, Bransonic, USA). Ten mL of the mix were diluted in 100 mL of distilled water and used for titratable acidity determination with 0,1M NaOH and 1% phenolphthalein as an indicator to estimate malic acid content as described (Latimer, 2012).

We used a refractometer (Hannah Instruments, Italy) to obtain the °Brix in 1 mL of sweet cherries juice. Total soluble solids (TSS) were then calculated as described (Boulton et al., 1999).

### 2.3. Hormone profiling

The plant hormones, including melatonin, ABA, salicylic acid (SA), jasmonic acid (JA), the auxin indole-3-acetic acid (IAA), the cytokinins *trans*-zeatin (*t*-Z), its riboside (*t*-ZR) and isopentenyl adenosine (IPA), and the gibberellins $GA_1$, $GA_3$, $GA_4$ and $GA_7$, were extracted and quantified by UHPLC/ESI-MS/MS as described (Müller and Munné-Bosch, 2011). Deuterium-labelled compounds were used as internal standards.

### 2.4. Statistical analyses

Data from the fruit ripening process on the tree and its quality were analysed by one-way factorial analysis of variance (ANOVA), while the effects of the exogenous treatments were analysed by two-way ANOVAs. Multiple comparisons tests were carried out using Tukey's HSD posthoc tests. Differences were considered significant at a probability level of $P < 0.05$. All statistical tests and Spearman correlations were carried out using the SPSS 20.0 statistical package. In all cases, differences or correlations were considered significant at a probability level of $P < 0.05$.

## 3. Results and discussion

### 3.1. Melatonin contents in ripe cherries

A growing number of studies describe various techniques to detect melatonin in a large variety of vegetables, seeds, herbs and fruits (Reiter et al., 2007), as melatonin is found in different plant families, such as the *Rosaceae*, to which sweet cherries belong to. Supplementary Table 1, which includes a summary of melatonin contents found in various sweet cherry varieties at harvest, shows melatonin ranging between non-detectable values and 20 ng/g fresh matter, depending on the variety, method of extraction and analytical procedure. Among the various separation techniques used thus far, LC-MS methods appear to be the most accurate for identification of this compound, giving as well high sensitivity and specificity (Feng et al., 2014). Irrespective of the varietal and methodological differences, all studies suggest that melatonin is found at relatively low contents in sweet cherries, which is more likely associated with a hormonal than an antioxidant role (Arnao and Hernández-Ruiz, 2018). However, more studies are needed to unravel the tissue-specific distribution of melatonin in sweet cherries, since bulk melatonin contents in the fruit may hinder high concentrations of this compound in specific tissues.

### 3.2. Endogenous melatonin contents decrease during sweet cherries ripening

The ripening process involves some characteristic physiological and biochemical changes in fruits, as well as changes in their organoleptic properties. In the present study, a progressive increase of the fruit mass was observed during fruit development from stage I to stage VII over the two seasons studied during 2015 and 2016 (Suppl. Fig. 2). Anthocyanin content showed a similar trend between both years of study, with an exponential increase between stages IV and VII, reaching maximum amounts of 0,5 mg/g DW and 0,8 mg/g DW (during 2015 and

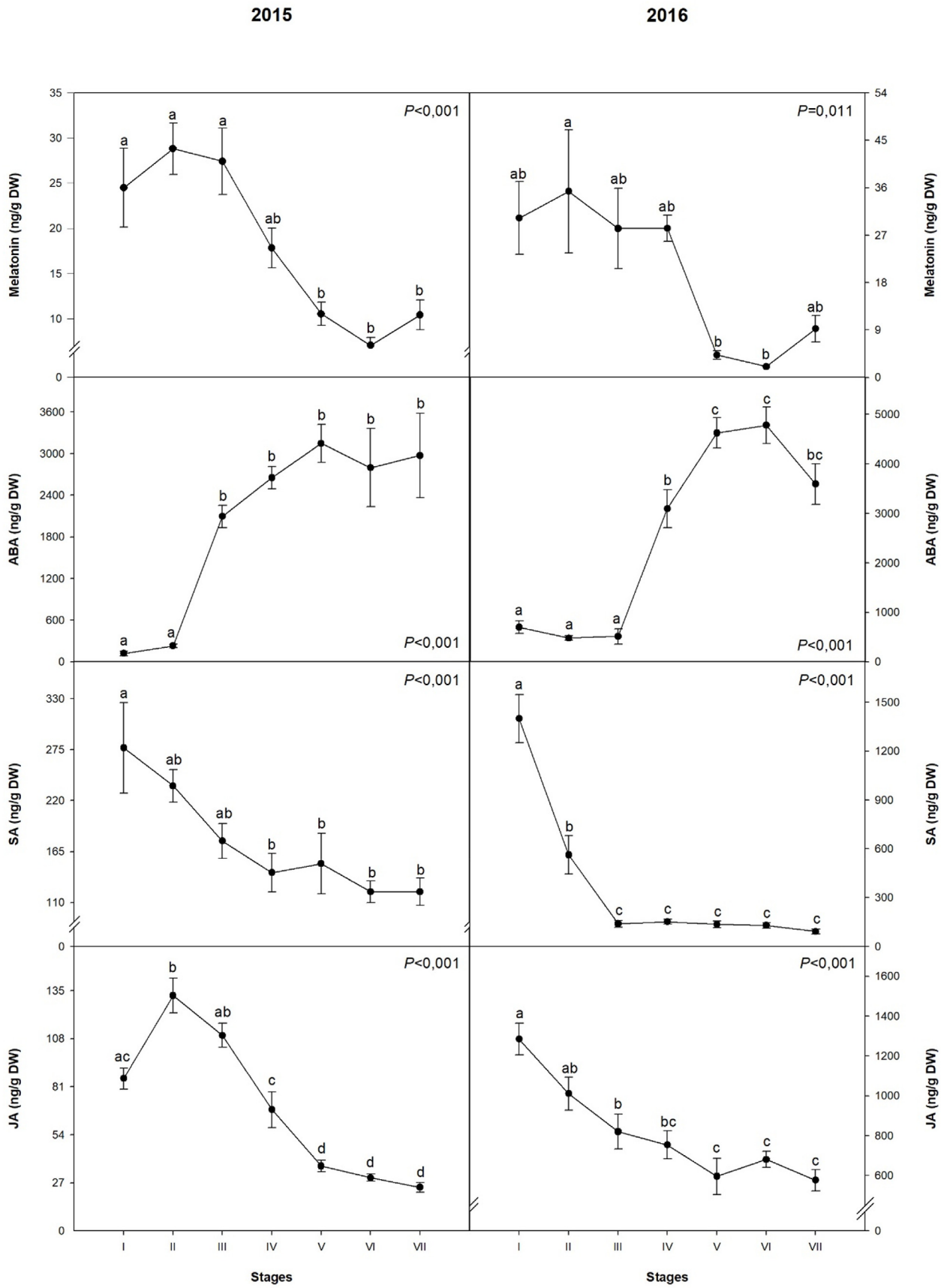


**Fig. 1.** Melatonin, abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) content during natural fruit development over two consecutive seasons (during 2015 and 2016). Data are the mean ± SE of n = 8. Statistical comparisons were performed by one-way ANOVA followed by a posthoc Tukey test. Different letters indicate significant differences between stages at $P < 0.05$.

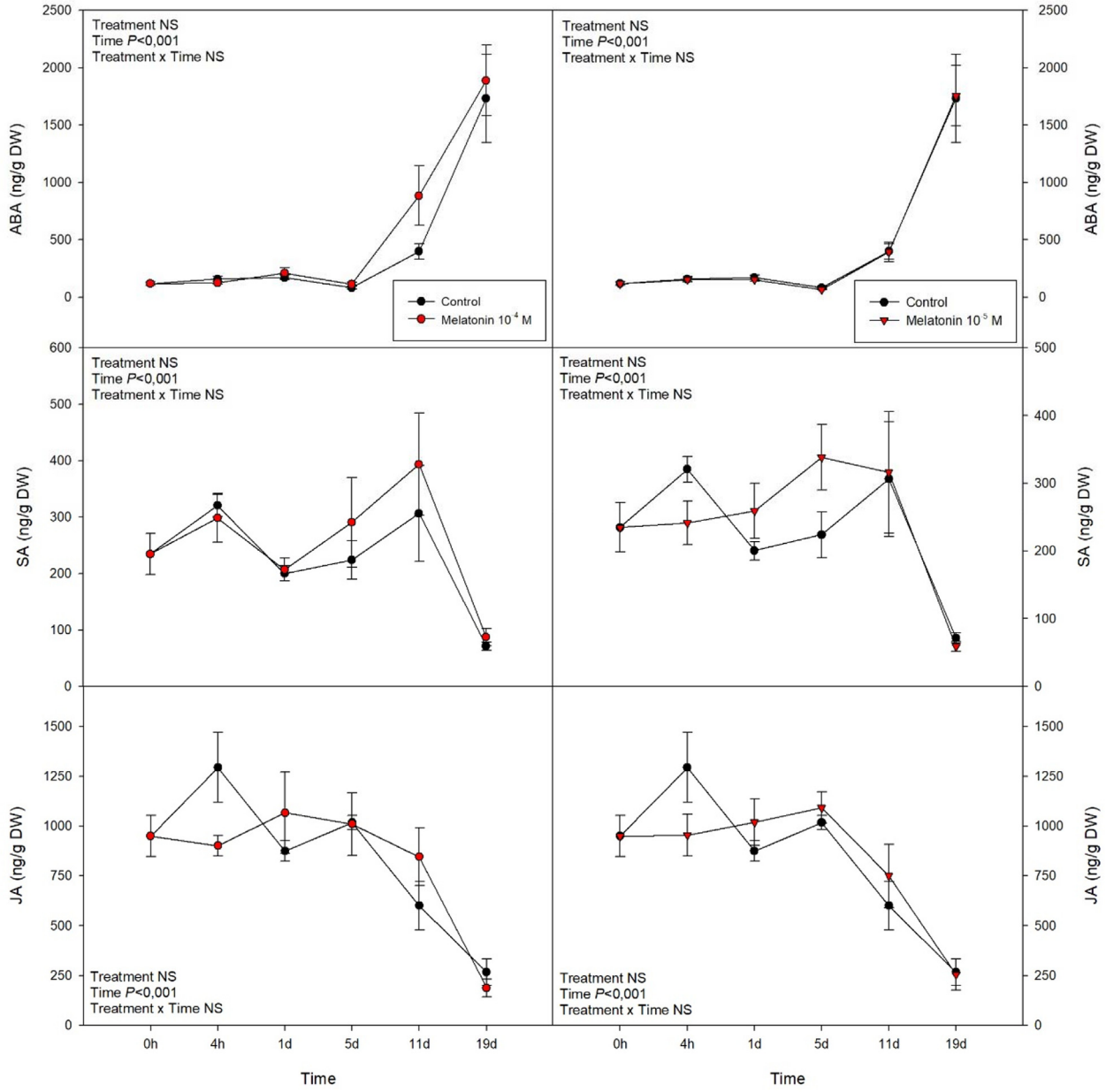


**Fig. 2.** Effects of melatonin treatments on the endogenous contents of abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA). Melatonin was applied at either $10^{-4}$ M or $10^{-5}$ M and compared to a control, and hormones were measured at 0 h (before treatments) and at 4 h, 1d, 5d, 11d and 19d of treatments. Data are the mean ± SE of n = 8. Statistical comparisons were performed by two-way ANOVAs followed by a posthoc Tukey test. Results of statistics are shown in the inlets. Differences were considered significant when $P \leq 0.05$. NS, not significant.

2016, respectively, when cherries were ready to harvest, Suppl. Fig. 2). This is in agreement with other studies in sweet cherry revealing that anthocyanins start to accumulate at the onset of the ripening process (Luo et al., 2013).

ABA is a well-known ripening promoter, not only in sweet cherries but also in different non-climacteric fruits such as strawberries or grapes, being involved in colour modulation (through the regulation of anthocyanin biosynthesis) and sugar accumulation (at the onset of fruit ripening, Kumar et al., 2014; Wang et al., 2015). In contrast, very little is known about the putative role of melatonin during fruit ripening on the tree, and how the endogenous contents of melatonin are altered by the fruit developmental stage in sweet cherries, except for a study using HPLC showing that melatonin contents inversely correlated with malondialdehyde amounts in two varieties (Zhao et al., 2012). Our results showed that endogenous melatonin decreased during fruit ripening in sweet cherries, obtaining similar results between the two consecutive seasons studied (Fig. 1). Melatonin contents reached their maximum, while ABA contents remained at their lowest at early stages of development. Later on development, ABA contents increased sharply at the start of ripening, while melatonin contents decreased (Fig. 1). This increment of ABA started just before anthocyanins accumulated in sweet cherries (Suppl. Fig. 2), which is in accordance with several studies pointing out ABA as a key phytohormone in non-climacteric fruit ripening (Shen et al., 2014). Other studies have shown that various hormones, such as CKs, GAs and auxins, in addition to ABA, are indeed involved in the modulation of fruit ripening in sweet cherries (Teribia et al., 2016). In the present study, results suggest that melatonin should be added to this group of endogenous regulators, results being consistent with an inhibitory role for this compound in fruit ripening. Variations in the endogenous contents of SA and JA were compared to those of melatonin, showing a contrasting dynamic, with a drastic decrease of melatonin content from maximum levels at stage I of sweet

cherry development, to minimum levels at stage VII, when cherry fruits were ready to harvest (Fig. 1). Although JA and SA are commonly associated with plant defence responses, these hormones affect the ripening by inhibiting or delaying this process (Ziosi et al., 2008; Wang and Zheng, 2005; Garrido-Bigotes et al., 2017). JA is known as a promotor of cell division and fruit set at the first developmental stages in grapevine (Böttcher et al., 2015), while SA has an important role delaying sweet cherry ripening as a postharvest treatment (Valero et at., 2011). Similar to JA and SA, melatonin was found at high concentration during the first stages of development, being constant until stage III during 2015, and stage IV during 2016, to decrease later as ripening progressed (Fig. 1). This result supports the contention of an inhibitory role of melatonin during sweet cherries ripening, as melatonin remained higher during the first stages of development until the onset of ripening, when it started to drop to minimum levels, as it happens with JA and SA. These results are indicative of the fact that a delicate hormonal balance is needed to modulate sweet cherries ripening, as melatonin, JA and SA are kept at low levels, while ABA contents increase to start the ripening process.

### 3.3. Exogenous melatonin effects

To confirm the putative role of melatonin as an inhibitor of fruit ripening on the tree, the effects of melatonin treatments were evaluated using two different melatonin concentrations at $10^{-4}$ M and $10^{-5}$ M, which were compared to a control (treated with water), all of them applied on the surface of fruits at stage II. A huge increase in the endogenous melatonin content was observed 4 h after $10^{-4}$ M treatment was applied (Fig. 3). Endogenous melatonin increases were more moderate after applying the $10^{-5}$ M treatment, not as sharp as $10^{-4}$ M treatment, but significantly different after 1d relative to controls (Fig. 3). After reaching a maximum after treatments, endogenous melatonin contents progressively decreased in sweet cherries, reaching similar values as the control treatment by the end of the experiment (Fig. 3).

Cherry fruit quality was assessed to evaluate the effects of exogenous melatonin treatments. Table 1 shows the results of fruit mass, anthocyanin contents, TA, TSS and the TSS/TA ratio at 11d and 19d of treatments. No significant differences were observed between treatments for fruit biomass, TSS and the TSS/TA ratio. However, sweet cherries treated with melatonin at $10^{-4}$ M presented higher levels of TA. In addition, results showed a lower accumulation of anthocyanin in cherry fruits treated with $10^{-5}$ M melatonin (Table 1). Given that melatonin has an indole-based structure and is considered a plant growth regulator similar to auxins (Arnao and Hernández-Ruiz, 2006, 2018), our results confirm melatonin may play an inhibitory role of fruit ripening, as indicated by reduced anthocyanin contents when melatonin was applied at low concentrations. Interestingly, this effect was not observed when applied at higher concentrations. In contrast, huge increases in endogenous melatonin, as a result of the melatonin application at $10^{-5}$ M, resulted in significant changes in TA but not in anthocyanins.

### 3.4. Hormone cross-talk

A complete phytohormone profiling was carried out after exogenous melatonin applications to better understand the possible effects of melatonin treatments during the hormonal regulation of cherry fruits ripening. While melatonin did not affect endogenous ABA, JA and SA contents (Fig. 2), the endogenous amounts of GAs (Suppl. Fig. 3) and cytokinins (Fig. 4) were strongly, and differentially, influenced by melatonin treatments, depending on the concentration applied. Results of $GA_1$, $GA_3$, $GA_4$ and $GA_7$ showed no significant differences between sweet cherry fruits treated with $10^{-5}$ M and fruits treated with control solution (Suppl. Fig. 3). However, fruits treated with $10^{-4}$ M melatonin showed significant differences for $GA_1$, $GA_3$ and $GA_7$ compared to

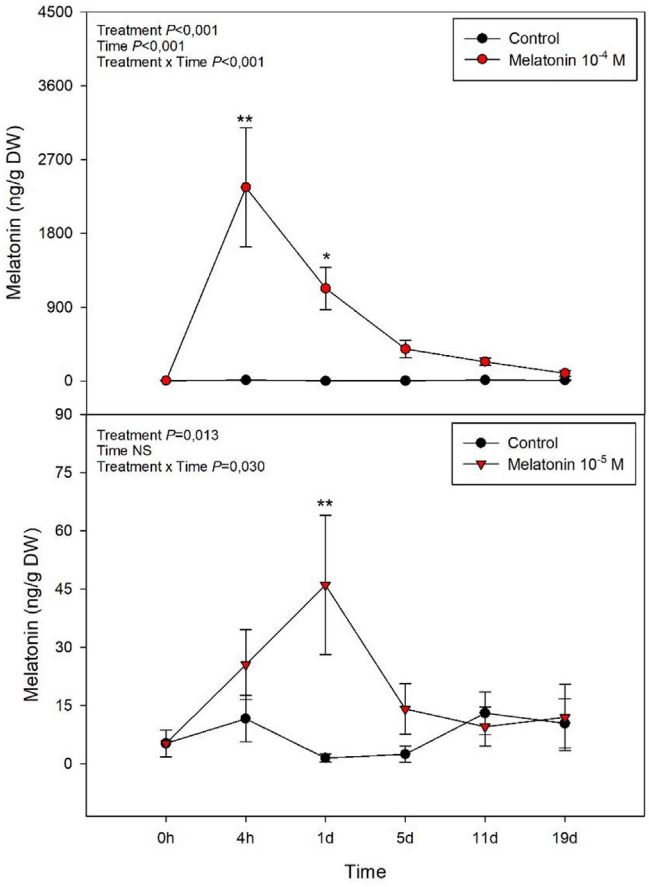


**Fig. 3.** Endogenous melatonin contents after melatonin applications at either $10^{-4}$ M or $10^{-5}$ M relative to controls. Measurements were performed at 0 h (just before treatments) and 4 h, 1d, 5d, 11d and 19d of treatments. Data are the mean ± SE of n = 8. Statistical comparisons were performed by two-way ANOVAs followed by a posthoc Tukey test. Results of statistics are shown in the inlets. Two asterisks indicate significant differences at $P \leq 0{,}001$ (one asterisk at $P < 0{,}05$) in the posthoc test. NS, not significant.

controls (Suppl. Fig. 3). Endogenous contents of $GA_1$ after 4 h of $10^{-4}$ M treatment were higher than the control, and remained constant throughout the entire experiment. Contrary to $GA_1$, cherries treated with $10^{-4}$ M presented lower levels of $GA_3$ and $GA_7$ compared to the control treatment (Suppl. Fig. 3). Some studies suggest that GAs may act as a signal to increase sink demand in fruits, stimulating fruit growth and sugar accumulation, thus leading to changes in carbon metabolism (Zhang et al., 2007). Although fruit biomass was not affected in our study, we observed an increase in TA with $10^{-4}$ M melatonin (Table 1), suggesting that changes in GAs might be associated with the TA increase. It is noteworthy, however, that huge (non-physiological) endogenous contents of melatonin resulted from $10^{-4}$ M melatonin treatments, thus indicating that the changes in TA caused by this melatonin treatment do not reflect indeed any physiological relevant effect.

In contrast, endogenous cytokinin, particularly zeatin contents, were altered with the $10^{-5}$ M melatonin treatment, achieving maximum values of 27 ng/g DW at 24 h of treatment, to later decrease drastically after 5d ($P = 0.013$ for the interaction effect in the two-way ANOVA, Fig. 4). At the onset of ripening, there is a dramatic fruit colour change, so that green fruit turns into a dark red colour by the accumulation of anthocyanins, while chlorophylls start its degradation and chloroplasts are dismantled (Muñoz and Munné-Bosch, 2018). Melatonin-induced cytokinin increases might lead to a delay in sweet cherries ripening, causing a lower anthocyanin accumulation after 19d

**Table 1**
Sweet cherries quality parameters at 11 and 19 days of treatments. Data are the mean of n = 8. An asterisk indicates significant difference between the melatonin and control treatments (Student's *t*-test, $P \leq 0{,}05$).

| Quality parameters | 11 days | | | 19 days | | |
|---|---|---|---|---|---|---|
| | Control | $10^{-4}$M | $10^{-5}$M | Control | $10^{-4}$M | $10^{-5}$M |
| Fruit biomass (g FW) | 6,28 ± 0,44 | 6,19 ± 0,43 | 6,21 ± 0,29 | 8,82 ± 0,52 | 8,74 ± 0,59 | 9,62 ± 0,52 |
| Anthocyanins (μg/g DW) | 19,87 ± 5,92 | 15,04 ± 2,75 | 15,01 ± 3,04 | 77,70 ± 7,70 | 155,99 ± 49,23 | **34,28 ± 11,57*** |
| TA (mg/g DW) | 5,97 ± 0,16 | **6,69 ± 0,30*** | 5,98 ± 0,21 | 5,41 ± 0,42 | **6,56 ± 0,21*** | 5,46 ± 0,26 |
| TSS (mg/g DW) | 9,87 ± 0,91 | 11,12 ± 0,73 | 9,27 ± 0,29 | 9,41 ± 0,38 | 10,49 ± 0,60 | 8,36 ± 0,42 |
| TSS/TA ratio | 1,65 ± 0,14 | 1,68 ± 0,12 | 1,55 ± 0,05 | 1,75 ± 0,07 | 1,60 ± 0,09 | 1,53 ± 0,08 |

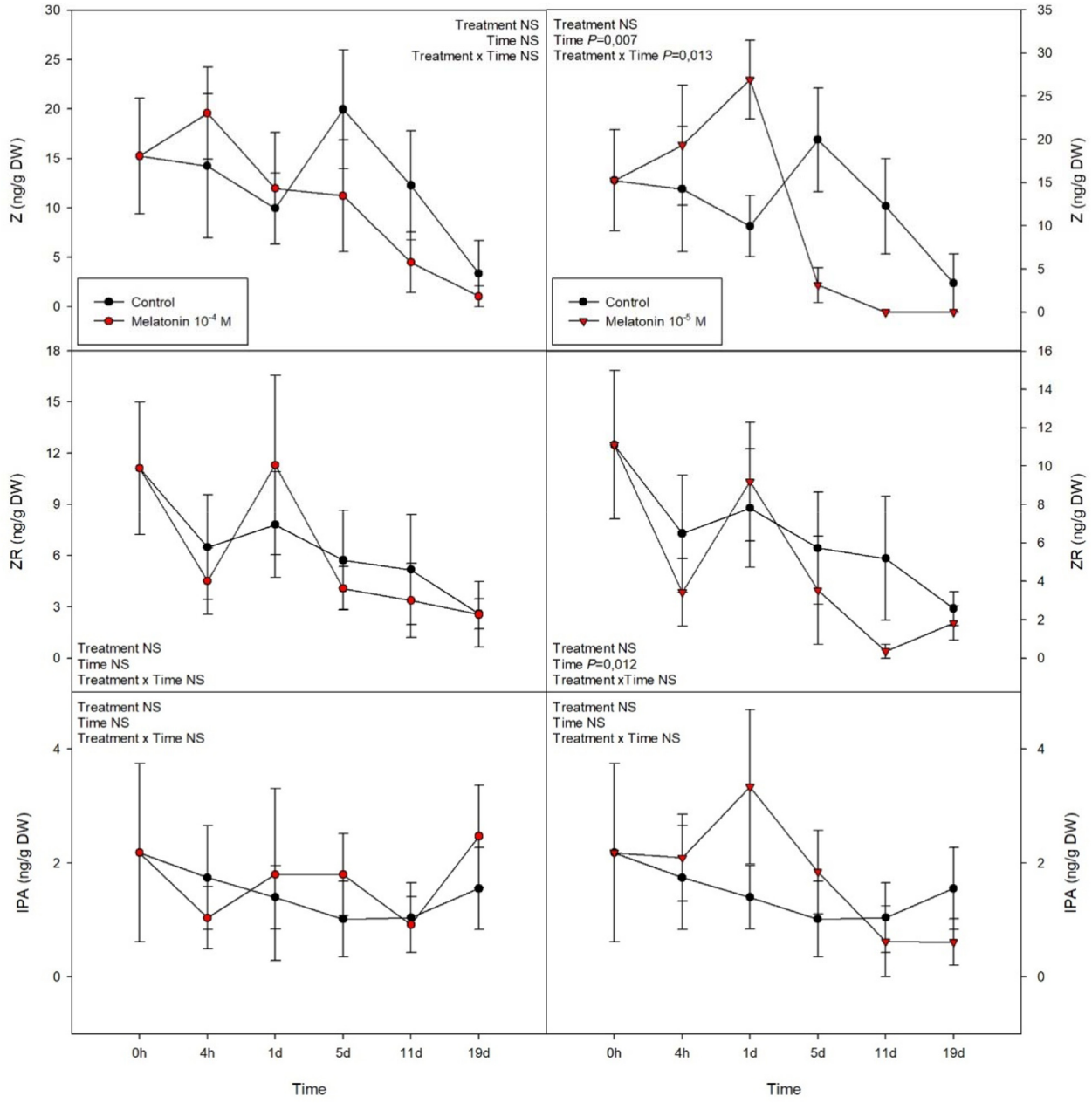


**Fig. 4.** Effects of melatonin treatments on the endogenous contents of cytokinins, including *trans*-zeatin (Z), *trans*-zeatin riboside (ZR) and isopentenyl adenosine (IPA). Melatonin was applied at either $10^{-4}$ M or $10^{-5}$ M and compared to a control, and hormones were measured at 0 h (before treatments) and at 4 h, 1d, 5d, 11d and 19d of treatments. Data are the mean ± SE of n = 8. Statistical comparisons were performed by two-way ANOVAs followed by a posthoc Tukey test. Results of statistics are shown in the inlets. Differences were considered significant when $P \leq 0.05$. NS, not significant.

of application (Table 1). Indeed, a strong correlation between endogenous melatonin and zeatin was observed when all data were pooled together in a correlation analysis (Suppl. Fig. 4). Interestingly, the positive correlation between melatonin and cytokinins has also been observed in other studies (Arnao and Hérnandez-Ruiz, 2009; Ma et al., 2018), evidence obtained thus far therefore indicating that melatonin effects on fruit ripening might be exerted, at least in part, through a cross-talk with cytokinins in sweet cherries. It is noteworthy, however, that posthoc analyses did not reveal differences in zeatin contents in any time point of measurement after exogenous melatonin treatments (Fig. 4), thus indicating that the strong inter-individual variability in the field might be partly masking the putative melatonin-cytokinin interaction. Interestingly, as it occurred with ABA, endogenous contents of auxin were not influenced by melatonin treatments (Suppl. Fig. 5), thus suggesting that melatonin effects on fruit ripening may be more related to cytokinins than to auxin and ABA in sweet cherries.

## 4. Conclusions

The ripening of non-climacteric fruits has been classically considered to be mainly regulated by ABA, which is involved in the accumulation of anthocyanins and sugars, and also partly by auxin, with an inhibitory effect on ripening. Results obtained in the present study suggest that melatonin may add to the compounds involved in the control of the hormonal balance exerting a role on the control of the timing of fruit ripening. Endogenous melatonin showed the same dynamics as JA and SA, hormones that inhibit or delay ripening together with auxin and cytokinins. In contrast, ABA increases may trigger sweet cherry ripening. In addition, results from our experiments with exogenous melatonin support a delaying effect for melatonin in fruit ripening, melatonin applied at low concentration possibly exerting an inhibitory role on fruit ripening through a cross-talk with cytokinins. As melatonin is a non-harmful compound, our results have an important implication in the agri-food biotechnology sector since melatonin applications could be used to modulate the timing of sweet cherries ripening. Our results support the contention that endogenous melatonin may be present at low concentration in sweet cherries while ABA promotes fruit ripening, whereas exogenous melatonin can be applied at initial stages of development to delay fruit ripening in orchard trees. The effectiveness of these applications will however be notably influenced by the concentration of melatonin applied, among other possible factors. Yet, further research is required to better understand the underlying mechanisms governing the role of melatonin in sweet cherries ripening.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.007.

## Author Contribution

SMB designed the experiments with the help of VT and PM. VT and PM performed the experiments. VT prepared figures and performed statistical analyses. VT wrote the manuscript with the help of SMB. All authors approved final submission.

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