UNIVERSITAT DE BARCELONA

Melatonina en maduració i sobremaduració de fruits: de la funció fisiològica a les aplicacions biotecnològiques

Alba Arabia Ferrer

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Melatonina en maduració i sobremaduració de fruits: de la funció fisiològica a les aplicacions biotecnològiques

Memòria presentada per Alba Arabia Ferrer per optar al grau de Doctora per la Universitat de Barcelona

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There is no gate, no lock, no bolt

that you can set upon the freedom of my mind

- Virginia Woolf

Ara ve el temps del gran repòs
i de la pau a les muntanyes;
el primer fred marcí les flors;
fan vels de plata les aranyes.

Ara comença enllà del camp
el tomb august de la natura;
el faig ja té color d'aram
i és el pollanc or que fulgura.

- Felip Graugés

Coll de Pregon. Ascensió al Matagalls

Als meus avis, Andreu i Ramon,

que els porto al cor

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Tot i que probablement aquestes seran les primeres línies que llegireu de la tesi (i per una gran majoria de persones seran les úniques, no ens enganyem), són les últimes que em queden per escriure. Costa de creure que això ja s'acaba, però si he arribat fins aquí és gràcies a tots els que heu estat al meu costat i m'heu ajudat, tant científicament com personalment, al llarg de tots aquests anys. Em sento molt afortunada d'haver pogut compartir aquest camí, que no sempre ha sigut fàcil, amb tots vosaltres, i us ho vull agrair.

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RESUM

Durant el procés de maduració, els fruits adquireixen les propietats organolèptiques i nutricionals apreciades pels consumidors. No obstant això, en el període postcollita sorgeix el repte de mantenir aquesta qualitat, ja que la sobremaduració provoca la degradació progressiva del fruit fins a la senescència. Tant la maduració com la sobremaduració són processos fisiològics complexos regulats per la interacció coordinada de diverses fitohormones i molècules reguladores, que determinen la qualitat final del fruit. Per tant, l'estudi de les possibles molècules involucrades en la seva regulació esdevé essencial. En aquest context, la melatonina ha emergit com una molècula amb un rol dual com a antioxidant i reguladora de processos fisiològics. Tot i els avenços recents per desxifrar la seva funció en el regne vegetal, el seu paper fisiològic i la seva coordinació amb altres fitohormones durant la maduració i sobremaduració continuen presentant moltes incògnites, a causa de resultats divergents que varien en funció de l'espècie, el teixit i els mètodes experimentals utilitzats per a la seva anàlisi. En aquest treball s'han seleccionat diferents models d'alt valor agronòmic per aprofundir en el coneixement sobre l'acumulació i el paper regulador de la melatonina durant la maduració i sobremaduració de fruits, analitzar la seva interacció amb altres fitohormones, i avaluar el seu potencial per a aplicacions biotecnològiques orientades a millorar la qualitat dels fruits. D'acord amb els resultats obtinguts en l'estudi del maduixot, un model clau per als estudis mecanístics sobre la maduració de fruits no climatèrics, la melatonina presenta un patró d'acumulació diferencial en funció de l'òrgan (aqueni i receptacle) i de l'estadi de desenvolupament, corresponent a un rol inhibidor de la maduració. L'aplicació exògena de melatonina indueix una resposta diferencial en el ritme de maduració segons el moment d'aplicació, associada a canvis espaciotemporals en l'acumulació de melatonina i la seva hidroxilació. A més, l'aplicació de melatonina abans de l'inici de la maduració revela que la seva funció inhibidora està, en part, relacionada amb la regulació de gens associats amb les auxines. La melatonina també es presenta com una molècula inhibidora de la sobremaduració dels maduixots, i actua de manera universal endarrerint el deteriorament d'altres fruits vermells altament peribles, com les cireres, els gerds i els nabius. Aquesta tesi també evidencia que la seva funció inhibidora de la sobremaduració, mitjançant l'acumulació d'antioxidants i fitohormones de defensa, és específica del teixit, com es mostra en l'estudi amb la pruna

com a model. Tanmateix, cal considerar que l'ús de la melatonina com a principi actiu en un bioestimulant per millorar la qualitat del maduixot en condicions de camp presenta limitacions quan s'incorpora a un sistema de cultiu. En conclusió, aquesta tesi mostra que la melatonina exerceix una funció inhibidora en la maduració i sobremaduració dels fruits, amb una acció reguladora que depèn no només del tipus de fruit, sinó també del teixit dins del fruit. A més, la melatonina actua de manera coordinada amb altres fitohormones promotores del creixement en la precollita i amb hormones de defensa en la postcollita. Per últim, la melatonina presenta propietats antisenescents que la posicionen com un possible component actiu en formulacions per allargar la vida útil dels fruits, tot i que els seus efectes en la precollita podrien dependre de l'homogeneïtat del cultiu.

PARAULES CLAU:

Fruits, Hormones, Maduració, Melatonina, Postcollita, Qualitat

ABSTRACT

During the ripening process, fruits acquire the organoleptic and nutritional properties valued by consumers. However, during the postharvest period, arises the challenge of maintaining this quality, as overripening leads to the progressive degradation of the fruit until senescence. Both ripening and overripening are complex physiological processes regulated by the coordinated interaction of various phytohormones and regulatory molecules, which ultimately determine the final fruit quality. Therefore, studying the molecules involved in their regulation is crucial. In this context, melatonin has emerged as a molecule with a dual role as both an antioxidant and a regulator of physiological processes. Despite recent progress in understanding its function in the plant kingdom, its physiological role and coordination with other phytohormones during ripening and overripening continue to present many uncertainties, largely due to divergent results that vary depending on the species, tissue, and experimental methods used for its analysis. In this study, different models with high agronomic value were selected to deepen our understanding of melatonin accumulation and its regulatory role during fruit ripening and overripening, to analyze its interaction with other phytohormones, and to evaluate its potential for biotechnological applications to improve fruit quality. Based on results obtained from the study of strawberry, a key model for mechanistic research on the ripening of non-climacteric fruits, melatonin shows a differential accumulation pattern depending on the organ (achene and receptacle) and the developmental stage, corresponding to an inhibitory role in ripening. The exogenous application of melatonin induces a differential response in the ripening rate depending on the timing of application, associated with spatiotemporal changes in melatonin accumulation and its hydroxylation. Moreover, melatonin application before the onset of ripening reveals that its inhibitory function is partially linked to the regulation of auxin-related genes. Melatonin also performs as an overripening inhibitor in strawberries and shows a universal effect on delaying the deterioration of other highly perishable red fruits, such as cherries, raspberries, and blueberries. This thesis further demonstrates that its inhibitory role in overripening, through the accumulation of antioxidants and defense-related phytohormones, is tissue-specific, as shown in the study using the plum as a model. However, it is important to note that using melatonin as an active ingredient in a biostimulant to improve strawberry quality under field conditions has limitations when

incorporated into a cultivation system. In conclusion, this thesis demonstrates that melatonin plays an inhibitory role in fruit ripening and overripening, with regulatory properties that rely not only on the fruit type but also on the tissue within the fruit. Additionally, melatonin works in coordination with other growth-promoting phytohormones during preharvest and with defense-related hormones in postharvest. Finally, the anti-senescent properties of melatonin position the molecule as a potential active ingredient in formulations to extend fruit shelf life, although its effects during preharvest may be influenced by crop homogenity.

KEYWORDS:

Fruits, Hormones, Melatonin, Postharvest, Quality, Ripening

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ABREVIATURES

2-HM: 2-hidroximelatonina

ABA: àcid abscísic

ACC: àcid 1-aminociclopropà-1-carboxílic

ACS: aminociclopropancarboxilat sintasa

ASMT: *N*-acetilserotonina metiltransferasa

CKs: citocinines

COMT: àcid cafeic *O*-metiltransferasa

GAs: gibberel·lines

H_2O_2: peròxid d'hidrogen

IAA: àcid indolacètic

JA: àcid jasmònic

JAs: jasmonats

OPDA: àcid 12-*oxo*-fitodienoic

ROS: espècies reactives de l'oxigen

SA: àcid salicílic

SNAT: serotonina-*N* acetiltransferasa

SST: sòlids solubles totals

TDC: triptòfan descarboxilasa

UHPLC-MS/MS: cromatografia líquida d'alta resolució acoblada a espectrometria de masses en tàndem

INTRODUCCIÓ



INTRODUCCIÓ

1. Desenvolupament i maduració de fruits

1.1. Del quallat del fruit a la maduració comercial

Les angiospermes, o plantes amb flor, dominen la vegetació de la majoria dels ecosistemes terrestres, amb aproximadament 400.000 espècies actuals (Pimm & Joppa, 2015). Aquestes plantes, que van aparèixer fa entre 100 i 125 milions d'anys, representen més del 90 % de les espècies vegetals del planeta. De fet, Darwin va pensar que l'origen recent i la ràpida diversificació de les angiospermes era "un misteri abominable" (Friedman, 2009). L'èxit evolutiu de les angiospermes es fonamenta en dos trets essencials: la formació de flors i el desenvolupament de fruits. Les flors, gràcies a la seva capacitat per atraure pol·linitzadors, han impulsat una reproducció més eficient, mentre que els fruits protegeixen i nodreixen les llavors i en faciliten la dispersió, un procés clau per a la seva supervivència (Crane et al., 1995; Eriksson et al., 2000). Els fruits deriven principalment del gineceu madur fertilitzat, la part reproductiva de la flor composta per un o més pistils (Gillaspy et al., 1993). No obstant això, sovint també es recluten altres components florals per formar el fruit (Fait et al., 2008). Els estudis evolutius indiquen que els fruits carnosos provenen d'espècies amb fruits secs i, per tant, tot i que aparentment siguin molt diferents entre ells, estan formats per teixits similars amb diferents graus de lignificació, nombre de cèl·lules i mida. En general, els fruits estan formats per un pericarpi que es pot dividir en tres parts: exocarpi (la capa exterior que forma la pell del fruit), mesocarpi (la capa intermèdia que, en els fruits carnosos, és la part comestible, també coneguda com a polpa) i endocarpi (la capa més interna, adjacent a la llavor i que la protegeix) (Karlova et al., 2014). Aquestes capes poden variar en gruix i textura, i fins i tot arribar a fusionar-se en determinats fruits. Per exemple, en algunes baies simples com el nabiu, l'exocarpi és molt fi i forma una transició suau amb la polpa, mentre que el mesocarpi i l'endocarpi no presenten una separació clara, constituint una massa carnosa contínua sense presència de teixits lignificats al voltant de les llavors. En canvi, altres fruits considerats carnosos presenten una organització morfològica diferent, com el gerd, un fruit agregat compost per diferents drupes unides, cadascuna amb el seu pericarpi. Així mateix, en fruits com la maduixa, aquesta és classificada com un fruit accessori ja que la part carnosa no deriva del pericarpi sinó del receptacle floral engruixit, mentre que els

veritables fruits són els aquenis situats a la seva superfície, els quals són fruits secs indehiscents (Zhou et al., 2023).

En termes generals, els fruits carnosos han evolucionat per desenvolupar un pericarpi suculent, gustós i suau, amb l'objectiu de resultar atractius per als frugívors i afavorir així la dispersió de les llavors. Paral·lelament, també s'han convertit en una font d'energia molt important per als éssers humans, proporcionant proteïnes i vitamines essencials per a la nostra nutrició (Seymour et al., 2013). Així, els fruits i les llavors són components claus del rendiment en la majoria d'espècies cultivades.

Després de la fecundació, el fruit entra en una etapa en què el seu creixement i maduració estan estretament coordinats amb el desenvolupament de la llavor (Seymour et al., 2013). El desenvolupament del fruit i la llavor es pot dividir en tres estadis principals (**Figura 1**):

- **Quallat**: és el primer pas en el desenvolupament del fruit, que es produeix durant i poc després de la fecundació, i implica la transició de l'òvul i l'ovari a llavor i fruit jove en creixement, respectivament. Aquest esdeveniment de fecundació inicia el desenvolupament de la llavor, la qual desactiva la repressió de la divisió cel·lular i es caracteritza per un desenvolupament coordinat entre els teixits materns i filials (Ruan et al., 2012).
- **Creixement**: en aquesta fase continua la divisió cel·lular i predomina l'expansió cel·lular. La llavor en desenvolupament envia contínuament senyals als teixits circumdants per promoure'n l'expansió. L'embrió madura, la llavor acumula reserves i adquireix tolerància a la dessecació, perdent aigua en el procés (McAtee et al., 2013).
- **Maduració fisiològica**: és l'estat de desenvolupament en què el fruit ha assolit la seva màxima capacitat i s'ha completat el desenvolupament dels seus teixits. En aquest moment, la maduració de la llavor ha finalitzat, i en molts fruits, també coincideix amb el final de l'expansió cel·lular (Fenn & Giovannoni, 2021). Es produeixen canvis en la composició bioquímica del fruit com l'acumulació pigments, sucres i àcids, però el fruit encara no és apta pel seu consum.

  Quan el fruit ja ha assolit la maduració fisiològica, el procés continua amb la **maduració comercial,** una etapa marcada per una sèrie de canvis fisiològics i

bioquímics que permeten al fruit adquirir les propietats organolèptiques desitjades per al consum humà, incloent-hi modificacions en el sabor, el gust, la textura i el color, que el faran més atractiu. A més, és durant aquesta fase quan el fruit adquireix les propietats nutricionals que el converteixen en un component indispensable per a la nostra alimentació (Seymour et al., 2013; Kumar et al., 2014). Tot i que solem pensar en la maduresa comercial com una etapa de desenvolupament específica, en realitat és un punt arbitrari de màxima palatabilitat dins d'un procés continu que condueix a la senescència de tot l'òrgan (Klee & Giovannoni, 2011).

La duració de cada fase, i conseqüentment la mida i la qualitat final del fruit, varia segons l'espècie i la varietat, influïda per factors com el genotip, les condicions ambientals i la regulació exercida per les fitohormones, que actuen com a elements clau en la coordinació de tot el procés de desenvolupament. A més, en el cas dels fruits partenocàrpics, els quals es desenvolupen sense la presència de llavors viables, el fruit es forma completament gràcies a un major contingut endogen de fitohormones als ovaris, estimulant el desenvolupament del pericarpi (Sharif et al., 2022).

1.2. Canvis fisiològics i bioquímics associats a la maduració i qualitat del fruit

El creixement és essencial per aconseguir la mida i el volum del fruit, però és durant la maduració comercial quan la bioquímica, la fisiologia i l'estructura de l'òrgan es transformen, influenciant l'aspecte, la textura, el sabor i l'aroma. A més, és en aquesta etapa que es desenvolupen les característiques organolèptiques i nutricionals que doten el fruit de propietats úniques, convertint els fruits comercials en una part fonamental de la nostra dieta (**Figura 1**).

1.2.1. Modificacions del color

El color del fruit és un tret genètic amb valor ecològic i nutricional, sent un dels principals atributs que condiciona les preferències dels consumidors (Crisosto et al., 2003). Tot i que no tots els fruits experimenten canvis de color durant la maduració, en la gran majoria d'ells aquesta modificació en l'aparença sí que es produeix, servint com a indicador de la seva etapa de maduració i condició fisiològica, a més de representar un atribut clau per determinar la seva qualitat nutricional, donat moltes molècules que proporcionen color tenen propietats antioxidants (Kapoor et al., 2022). Les modificacions en la coloració són el

resultat de la degradació de la clorofil·la, la qual pot estar emmascarant altres pigments com els carotenoides, i la transformació dels cloroplasts en plastidis amb capacitat d'acumulació de molècules antioxidants. Tanmateix, en molts fruits, aquesta pèrdua de clorofil·la va acompanyada de la síntesi i acumulació de nous pigments com les antocianines, responsables de les tonalitats vermelles, porpres i blaves, característiques, per exemple, dels fruits vermells (Seymour et al., 2012; Kapoor et al., 2022). Una de les propietats més remarcable de les antocianines és el seu efecte protector sobre els éssers humans, ja que una ingesta diària adequada s'associa amb la promoció d'un envelliment saludable, la protecció contra malalties degeneratives relacionades amb l'edat i la inhibició del creixement de cèl·lules tumorals (Boeing et al., 2012; Mattioli et al., 2020). Per tant, una major acumulació de compostos fenòlics i d'antocianines es relaciona amb un increment de les propietats antioxidants del fruit i amb una millora del seu valor nutricional (Jian et al., 2023). Les antocianines són compostos flavonoides que se sintetitzen a través de la via fenilpropanoide a partir de l'aminoàcid aromàtic fenilalanina i, estructuralment, estan compostes d'un aglicó d'antocianidina enllaçat a una o més molècules de sucres (Sinopoli et al., 2019). La seva síntesi està regulada per diverses molècules, incloent-hi els factors de transcripció MYB, la sacarosa, hormones vegetals com l'àcid abscísic (ABA) i l'àcid jasmònic (JA), i també per factors ambientals com ara l'exposició a la llum, els quals actuen de manera coordinada per afavorir l'acumulació progressiva d'antocianines durant la maduració (Jaakola et al., 2013).

1.2.2. Modificacions de la textura

L'estovament és una de les característiques més destacades i irreversibles del procés de maduració dels fruits carnosos, on una textura suau i sucosa esdevé fonamental per a una bona qualitat comestible. No obstant això, la taxa d'estovament no només potencia el seu atractiu sensorial pels consumidors sinó que també influeix en altres aspectes econòmicament importants, com la freqüència de collita, els mètodes de manipulació i la distància que es pot transportar la fruita sense que es vegi afectada la seva qualitat (Goulao & Oliveira, 2008). Els canvis en la fermesa del fruit estant estretament relacionats amb un augment de la susceptibilitat a l'atac de patògens, especialment en les últimes etapes de la maduració o després de períodes prolongats d'emmagatzematge. A més, alguns patògens poden induir activament l'estovament com a estratègia d'infecció (Cesarino, 2023). Els canvis

de textura es produeixen per modificacions de la paret cel·lular que afecten la forma, la turgència i la mida de les cèl·lules del fruit, i impliquen la desorganització de la paret cel·lular i la pèrdua d'adherència cel·lular a causa de la dissolució de la làmina mitjana. Això inclou la despolimerització de glicans, la solubilització de pectines i la pèrdua de sucres en les cadenes laterals de les pectines (Shi et al., 2023). Aquestes modificacions debiliten la xarxa cel·lulosa-xiloglucà i augmenten la porositat de la paret, facilitant l'accés dels enzims degradadors (pectinases, xiloglucanases, etc.) amb els seus substrats (Paniagua et al., 2017; Moya-León et al., 2019). Tot i que aquestes modificacions es produeixen en la majoria de fruits carnosos, la seva taxa i intensitat varia d'un fruit a un altre.

1.2.3. Modificacions dels sucres, àcids i perfil volàtil

Les principals modificacions de color i textura observades durant la maduració dels fruits estan estretament vinculades a alteracions en els nivells de sucres, àcids orgànics i compostos volàtils. La percepció del sabor depèn tant del gust com de l'olor, sent els sucres i els àcids orgànics els principals responsables del gust. Durant la maduració, els nivells de sucres tendeixen a augmentar, ja sigui per la degradació del midó acumulat durant els estadis inicials de quallat i creixement en glucosa, fructosa i sacarosa, o per la importació contínua de sucres des de la planta mare cap al fruit (Seymour et al., 2012). Per exemple, en el cas del maduixot, l'acumulació contínua de sucres mentre està unit a la planta determina gran part del seu sabor (Zhang et al., 2011). D'altra banda, els nivells d'àcids orgànics solen disminuir durant la maduració, probablement a causa de la seva utilització com a substrats respiratoris, tot i que aquest comportament pot variar segons l'espècie i la varietat (Batista-Silva et al., 2018). Pel que fa a l'aroma, aquesta és determinada per la síntesi de compostos volàtils específics de cada fruit. El perfil aromàtic és altament complex, i en alguns casos pot incloure més de 200 compostos de naturalesa diversa, com alcohols, aldehids i èsters, que contribueixen a l'aroma característic de cada fruit individual (Defilippi et al., 2009).

1.2.4. Augment de la susceptibilitat a patògens

Durant la maduració, el fruit esdevé més vulnerable als patògens oportunistes, un fenomen probablement relacionat amb la pèrdua de la integritat de la seva paret cel·lular. Així, l'estovament comporta efectes ambivalents: mentre que genera atributs de qualitat desitjats, un excés pot incrementar el dany físic i la susceptibilitat a microorganismes,

accelerant el deteriorament del fruit (Shi et al., 2022). Aquest debilitament, combinat amb l'acumulació de sucres i compostos aromàtics, els canvis de pH, i la presència d'espècies reactives d'oxigen (ROS) associades a la maduració que provoquen danys oxidatius a les cèl·lules, fa augmentar el risc d'infecció per patògens (Li et al., 2024c). Per tant, si bé el procés de maduració millora la qualitat organolèptica del fruit, també en compromet la resistència davant de factors adversos al proporcionar un ambient més favorable pel creixement de patògens.

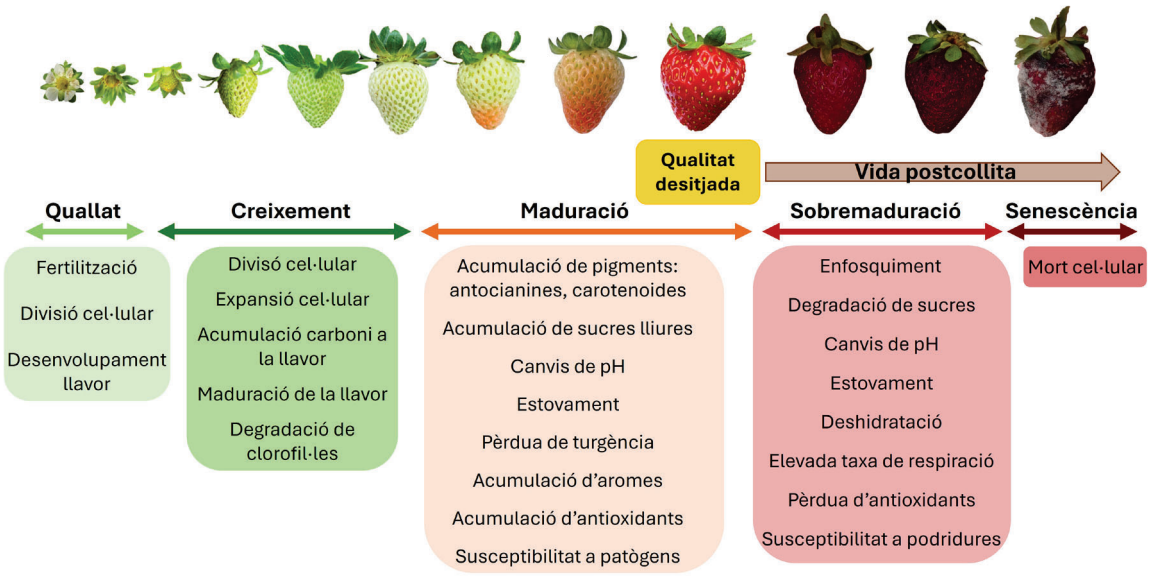


Figura 1. Canvis fisiològics i bioquímics que es produeixen durant les diferents etapes de desenvolupament del fruit, emprant el maduixot com a model, des del quallat fins a la maduració comercial, així com durant el període postcollita fins a la senescència.

1.3. Fitohormones en la regulació de la maduració

La maduració dels fruits implica una sèrie de canvis fisiològics, estructurals i metabòlics que permeten la formació de fruits comestibles. Aquest procés, altament complex, està controlat tant per factors ambientals com per senyals cel·lulars intrínseques a nivell bioquímic i molecular, on les fitohormones i la seva xarxa interactiva exerceixen un paper significatiu en la seva coordinació i regulació (Kou et al., 2021). Les fitohormones són molècules orgàniques que se sintetitzen de forma natural en les plantes i, a concentracions molt baixes, exerceixen un paper clau en la senyalització de diversos processos fisiològics, com la maduració. Donada la importància biològica i el valor comercial dels fruits, comprendre aquesta xarxa reguladora és fonamental no només per aprofundir en el

coneixement del procés de maduració, sinó també per controlar, preservar i millorar la qualitat dels fruits mitjançant diverses estratègies tecnològiques en el sector agroalimentari. Tradicionalment, els fruits s'han classificat en climatèrics i no climatèrics segons la presència o absència d'un augment ràpid de la respiració i de la producció d'etilè durant la maduració, respectivament (Paul et al., 2012). En els fruits climatèrics, com el tomàquet, el plàtan o la poma, l'etilè actua com a senyal central per iniciar i coordinar el procés. En canvi, en els fruits no climatèrics, com la maduixa, el raïm o la cirera, el seu paper és menor, destacant l'ABA com a regulador principal (Pesaresi et al., 2014; Cherian et al., 2014). No obstant, els últims anys s'ha demostrat que la resta d'hormones vegetals, i sobretot, la seva interacció, també tenen una contribució rellevant en la regulació dels processos bioquímics associats a la maduració. Entre aquestes altres fitohormones clàssiques, a més de l'etilè i l'ABA, destaquen les auxines, les citocinines (CKs), les gibberel·lines (GAs), l'àcid salicílic (SA) i els jasmonats (JAs). Es coneix que una sola hormona pot regular diversos processos fisiològics i, alhora, múltiples hormones poden influir en un mateix procés, interaccionant entre elles mitjançant respostes sinèrgiques o antagòniques (Gray, 2004). Així, les hormones exerceixen una funció dinàmica espaciotemporal que depèn de l'estat de desenvolupament, el lloc d'acció i la interacció amb la resta d'hormones. L'auxina natural més abundant és l'àcid indolacètic (IAA), i és considerada l'hormona principal per a l'estimulació de la proliferació cel·lular durant les primeres fases del desenvolupament. Juntament amb les GAs, tenen funcions úniques en el quallat del fruit, així com en la divisió i expansió cel·lular durant les etapes inicials del creixement (Hu et al., 2018). No obstant això, la interacció amb altres hormones també poden influir en aquests estadis primerencs. Per exemple, és necessària la disminució dels nivells d'etilè i ABA en els teixits florals per tal que es produeixi un augment dels nivells d'IAA i GAs, afavorint així la iniciació del quallat dels fruits. A més, els nivells endògens de CKs també augmenten de forma proporcional amb el creixement del fruit en diverses espècies (Fenn & Giovannoni, 2021).

De manera similar, la transició entre el creixement del fruit i la maduració es caracteritza per canvis en el perfil de fitohormones. Els nivells elevats d'auxines, GAs i CKs actuen com a inhibidors de la maduració, de manera que la seva biosíntesi ha de disminuir per permetre el progrés de la maduració (McAtee et al., 2013; Kumar et al., 2014). En fruits no climatèrics com el maduixot, l'avenç de la maduració comporta un increment dels nivells

d'ABA associat a la disminució de l'acidesa, l'acumulació d'antocianines i sucres, així com modificacions de la paret cel·lular (Perotti et al., 2023). A més, els mecanismes moleculars i les vies de transducció de senyal implicats en l'acció promotora de l'ABA han estant descrits (Jia et al., 2011). L'etilè també intervé en la maduració dels fruits no climatèrics, tot i que amb un paper menys central que en els climatèrics. Les auxines, tot i ser considerades inhibidores de la maduració i tenir un paper destacat en les primeres etapes del creixement, poden participar també en etapes posteriors, com la regulació de l'estovament, mitjançant la inhibició de l'expressió dels gens d'expansines, i la regulació positiva de gens relacionats amb la síntesi d'etilè (Figueroa et al., 2009; Merchante et al., 2013; Zhang et al., 2020b; Wang et al., 2021c). Tanmateix, la relació de les auxines amb l'ABA és antagònica (Li et al., 2024a) i en el cas del maduixot, la síntesi d'ABA es regula per una complexa interacció entre ABA, IAA i GA (Fenn & Giovanoni, 2021). Per altra banda, els JAs i el SA són coneguts principalment pel seu paper en la defensa contra estressos abiòtics i biòtics, activant mecanismes de protecció en els fruits (Yao & Tian, 2005). Els JAs inclouen el JA, el seu precursor, l'àcid 12-*oxo*-fitodienoic (OPDA), la seva forma conjugada més activa, el jasmonoil-isoleucina (JA-Ile) i el seu derivat volàtil, el metil jasmonat (MeJA). Tot i la seva associació principal amb la resposta a l'estrès, també tenen un paper significatiu en el creixement dels fruits no climatèrics. En aquest context, la concentració de JA disminueix al final del desenvolupament, exercint un efecte antagònic sobre l'ABA (Fenn & Giovannoni, 2021). A més, s'ha demostrat que el MeJA pot promoure l'acumulació d'antocianines i reduir la síntesi d'ABA (Garrido-Bigotes et al., 2018). No obstant això, el JA també pot promoure la biosíntesi de l'etilè en fruits climatèrics com les pomes (Li et al., 2017c). De manera similar, el SA, tot i estar menys estudiat en relació amb el desenvolupament del fruit, s'ha associat principalment amb la resistència a patògens (Babalar et al., 2007), i pot actuar, a més, com a inhibidor de la maduració en fruits com el plàtan, regulant la producció d'etilè de manera dosi-dependent (Srivastava & Dwivedi, 2000; Shi & Zhang, 2012; Shi & Zhang, 2014).

El paper crucial de les fitohormones en la regulació del creixement i maduració dels fruits, amb uns nivells endògens i una senyalització que canvien de manera dinàmica des del quallat fins a la maduresa comercial, fa que la seva aplicació exògena esdevingui una eina eficaç per a la manipulació d'aquests processos fisiològics amb l'objectiu de potenciar el rendiment i la qualitat dels cultius. En aquest context, s'ha documentat l'ús de reguladors de

creixement vegetals aplicats per via foliar, com per exemple l'àcid 1-naftalenacètic (NAA) i la kinetina, una auxina i CK sintètica, respectivament, i la GA_3, per reduir la caiguda prematura de fruits abans de la collita (Carlomagno et al., 2025; Shahsavar et al., 2024). El NAA també s'ha emprat per incrementar la mida dels fruits i el rendiment del cultiu (Lal et al., 2016), i l'ús combinat de CKs i GAs per promoure el quallat del fruit o millorar-ne la forma i el pes (Koukourikou-Petridou et al., 2007), a més de contribuir a reduir l'aparició de fisiopaties com el to rogenc no desitjat que apareix en fruits com el raïm de taula (Xu et al., 2019). L'aplicació precollita d'etefon, un compost promotor de l'etilè, resulta útil per optimitzar la collita, ja que, en cultius com el tomàquet, pot ajudar a sincronitzar la maduració dels fruits, intensificar la coloració vermella i permetre una recol·lecció mecànica única (Logendra et al., 2004). Paral·lelament, altres fitohormones com el SA o els seus conjugats en el camp també són útils per induir una major resistència dels fruits a patògens, alhora que poden augmentar el contingut antioxidant del fruit (Cui et al., 2020). Així mateix, l'ús de MeJA en aplicacions precollita també ha resultat beneficiós en diversos cultius fructícoles: aplicacions primerenques en préssecs poden alentir la maduració gràcies a la seva capacitat per modular la biosíntesi i percepció de l'etilè i l'expressió dels gens relacionats amb l'ABA, IAA o GAs (Ruiz et al., 2013), mentre que aplicacions més properes a la collita en prunes resulten en fruits més ferms i amb un major contingut d'antioxidants (Karaman et al., 2013). Tot i els beneficis de l'ús de reguladors de creixement en la millora del rendiment i la qualitat dels cultius, així com en la reducció de l'ús de fertilitzants químics i pesticides, la seva aplicació pot variar segons el país, no només en funció dels efectes sobre cultius específics, les condicions ambientals i les pràctiques agrícoles, sinó també segons l'autorització emesa per les autoritats nacionals competents. La seva integració amb bones pràctiques agrícoles i una supervisió tècnica acurada esdevenen essencials per garantir el seu èxit (Trejo et al., 2023). En aquesta línia, els bioestimulants són cada vegada més populars i han emergit com a productes naturals d'origen vegetal, ja siguin substàncies, microorganismes o extractes, que, quan s'apliquen a les plantes, estimulen els processos naturals que afavoreixen l'eficiència nutricional, la tolerància a l'estrès abiòtic i la qualitat i el rendiment dels cultius, de forma respectuosa amb el medi ambient (Mukherjee et al. 2024). Així, els bioestimulants amb perfils hormonals específics esdevenen una opció prometedora per optimitzar de manera sostenible el rendiment i la qualitat dels cultius fructícoles.

2. Sobremaduració i senescència en postcollita

2.1. Canvis fisiològics i bioquímics associats a la sobremaduració

El procés de maduració comercial determina l'adquisició de les característiques que fan el fruit apte per al consum, però el moment òptim de collita varia segons el tipus de fruit. En el cas dels fruits climatèrics, poden ser collits abans d'assolir la maduració comercial, ja que continuen desenvolupant les propietats organolèptiques desitjades després de la collita. Per contra, els fruits no climatèrics han de ser collits gairebé o totalment madurs, ja que només completen la maduració comercial mentre estan units a la planta (Karagiannis, 2024). Després de la collita, passin o no pel procés de maduració, els fruits mantenen la seva activitat biològica, amb processos metabòlics que els condueixen gradualment a la senescència, una fase final caracteritzada per la disfunció, desintegració i mort cel·lular (Gómez et al., 2013). Entre la maduració i la senescència, els fruits experimenten un període de sobremaduració, durant el qual es produeixen transformacions fisiològiques i metabòliques relacionades amb la desconnexió de la planta mare (**Figura 1**). Aquesta fase comporta una pèrdua general de les propietats organolèptiques adquirides, comprometent diversos aspectes de qualitat, com el color, la textura, el sabor, l'aroma i les propietats nutricionals (Brasil & Siddiqui et al., 2018). Aquest deteriorament gradual provoca el rebuig dels consumidors i causa pèrdues econòmiques significatives, sovint abans que els fruits arribin al mercat. El període durant el qual un producte pot ser emmagatzemat sense perdre la seva idoneïtat pel consum humà ni ser rebutjat pels consumidors és el que es coneix com a vida útil. En els productes frescos, la vida útil és especialment dinàmica, ja que depèn de múltiples factors, com ara les característiques intrínseques del fruit, les condicions ambientals abans i després de la collita i les preferències i expectatives dels consumidors.

La manca de subministrament d'aigua i nutrients de la planta mare durant la postcollita, juntament amb els processos mecànics que es produeixen durant la recol·lecta i que indueix danys en la zona d'abscisió, contribueix als diversos canvis metabòlics que acceleren el deteriorament del fruit. Els principals factors implicats en aquest procés són: (1) l'increment de la taxa de respiració per obtenir energia i mantenir el metabolisme, (2) la deshidratació del fruit a causa de la transpiració i la pèrdua de la capacitat de retenció d'aigua per la desintegració de la paret cel·lular, i (3) l'activitat fúngica, que pot accelerar el procés

de deteriorament mitjançant la descomposició de teixits i la producció de toxines que afecten la qualitat i la seguretat del fruit (Pott et al., 2020). L'increment de la taxa de respiració afecta directament les vies metabòliques primàries, modificant negativament els nivells de sucres, àcids orgànics i aminoàcids que actuen com a principals substrats respiratoris (Yu et al., 2022). D'altra banda, com s'ha comentat anteriorment, l'estovament és un procés clau en la maduració del fruit, però la pèrdua excessiva de fermesa durant la sobremaduració esdevé una de les principals causes de deteriorament. A més, la pèrdua de turgència cel·lular a causa de la transpiració pot accelerar aquest procés d'estovament, afavorint la pèrdua de textura. Això provoca danys físics, augmentant també la susceptibilitat a l'atac de patògens i reduint la qualitat del fruit (Alkan & Fortes, 2015).

El color és un altre factor fonamental en la percepció de la qualitat del fruit, i la seva alteració durant la postcollita no només redueix l'atractiu visual, sinó que també afecta les seves propietats nutricionals. Els colors vius i brillants en molts fruits són resultat de l'acumulació de compostos fenòlics, com les antocianines. Tanmateix, l'enfosquiment observat en la majoria de fruits després de la collita sovint és causat per canvis en el perfil de compostos fenòlics i l'activitat de l'enzim polifenol oxidasa (PPO) (Zenoni et al., 2016; Singh et al., 2018). Aquest enzim, en presència d'oxigen, oxida els compostos fenòlics a quinones, que, mitjançant una reacció de polimerització, donen lloc a pigments marrons i a una degradació de les antocianines. Aquest canvi de color es veu agreujat per la pèrdua d'aigua, que provoca una descompartimentació cel·lular, facilitant així el contacte de la PPO amb els seus substrats (Nunes et al., 2005). Per altra banda, la qualitat nutricional dels fruits es veu greument afectada no només pels canvis en l'estructura dels compostos fenòlics, sinó també per la pèrdua d'antioxidants essencials com l'àcid ascòrbic (vitamina C) i l'α-tocoferol (vitamina E) (Arabia et al., 2024; Meitha et al., 2020). Aquest deteriorament es deu a l'increment de la generació de ROS, particularment al mitocondri, associat a l'augment de la respiració durant l'emmagatzematge postcollita i elevant l'estrès oxidatiu. A més, a mesura que el fruit perd aigua o pateix lesions tissulars causades per l'activitat microbiana, la taxa de respiració es veu encara més incrementada, intensificant la producció de ROS. Aquest excés de ROS contribueix a l'acceleració de la senescència mitjançant la producció de radicals derivats del procés de peroxidació lipídica que reaccionaran i danyaran lípids, proteïnes i ADN (Corpas et al., 2023; Meitha et al., 2020). Per últim, cal considerar que tots aquests processos

de deteriorament poden ser accelerats o retardats depenent de diversos factors ambientals i de maneig, com la temperatura d'emmagatzematge, la humitat relativa, les condicions de manipulació física dels fruits i l'exposició a patògens, els quals poden influir significativament en la seva taxa de descomposició i qualitat final.

2.2. Fitohormones en la regulació de la sobremaduració

Al voltant del 14% de la producció mundial d'aliments es perd entre la collita i la venda al detall, i un 17% addicional es malbarata entre la venda i el consumidor. Per tant, més del 30% dels aliments produïts a escala mundial es perden al llarg de la cadena de subministrament, corresponent més del 50% a fruita i verdura (FAO, 2019). Aquesta realitat, accentuada pel creixement demogràfic projectat per als pròxims anys, posa de manifest la urgència de reduir les pèrdues postcollita per garantir la seguretat alimentària i impulsar la sostenibilitat. Identificar i comprendre les causes que contribueixen a la curta vida útil dels fruits és essencial per reduir el malbaratament alimentari i optimitzar l'ús dels recursos disponibles. En aquest context, tant la sobremaduració com la maduració estan regulades per una complexa i dinàmica interacció hormonal que influeix directament en la qualitat del fruit durant aquesta etapa. Diverses fitohormones hi exerceixen un paper clau, modulant processos fisiològics i metabòlics crítics que determinen el comportament del fruit en la postcollita.

La descomposició per l'atac de patògens, els trastorns fisiològics, les lesions mecàniques i la senescència són factors clau en la degradació de la qualitat durant la postcollita. Per afrontar els diversos estressos biòtics i abiòtics que es produeixen durant l'emmagatzematge, el fruit activa mecanismes de defensa en que els JAs tenen un paper rellevant. Aquestes fitohormones promouen la transcripció de gens de defensa i desencadenen respostes protectores. Concretament, la concentració de JA-Ile regula la supressió del factor JAZ, un repressor de diversos factors de transcripció com el MYC2. Aquesta regulació permet activar la transcripció de gens implicats en la producció de fitoalexines, inhibidors de proteases i altres metabòlits que contribueixen a protegir el fruit contra l'atac de patògens (Howe et al., 2018; Guo et al., 2018). De manera complementària, el SA juga un paper fonamental en la resistència a una àmplia gamma de patògens i en la resposta a diferents tipus d'estrès biòtic. Després de la infecció, el SA participa en múltiples processos, des de la defensa basal o mediada per gens de resistència fins al

desenvolupament de la resistència sistèmica adquirida (SAR). Aquest procés inclou una cascada de senyalització en que el SA afavoreix la degradació del factor NPR1, eliminant la inhibició sobre TGA, un activador de gens sensibles al SA. Això permet l'expressió de gens de resistència patogènica, els quals són clau per reforçar les defenses del fruit contra patògens (Fu et al., 2012; Xiang et al., 2021).

D'altra banda, les fitohormones també regulen la senescència del fruit actuant com a factors promotors o inhibidors d'aquesta. L'etilè i l'ABA, dues hormones essencials per a la maduració, exerceixen una influència promotora sobre la senescència, accelerant així el procés de deteriorament del fruit. No obstant això, ambdues també estan implicades en mecanismes de defensa davant condicions adverses. Per exemple, en la postcollita de cireres, s'ha observat que la deshidratació augmenta els nivells d'ABA (Luo et al., 2014). A més, l'etilè s'ha relacionat amb la defensa contra patògens necrotròfics (Romanazzi et al., 2016), i s'ha descrit una interacció entre l'etilè i els JAs, tot i que aquesta relació encara requereix més investigació (Yu et al., 2019; Prusky & Romanazzi, 2023; Zhu et al., 2024). Tanmateix, tant l'ABA com l'etilè poden induir mecanismes de defensa que augmenten la tolerància al fred, afavorint la conservació dels fruits durant l'emmagatzematge postcollita (Yu et al., 2023; Vincent et al., 2020).

Altres fitohormones com les auxines, CKs i GAs, actuen com a factors inhibidors de la senescència, ja que endarrereixen els processos metabòlics associats i poden suprimir la biosíntesi o la sensibilitat a les fitohormones relacionades amb aquest procés (Li et al., 2017b; Aremu et al., 2020; Zhang et al., 2023a). De fet, s'ha descrit que durant la senescència, les CKs poden modular la sensibilitat a l'etilè i inhibir la seva síntesi (Xiang et al., 2021). De manera similar, l'aplicació de GA_3 pot regular la transcripció dels enzims aminociclopropancarboxilat sintasa (ACS) i oxidasa (ACO), modulant així la producció d'etilè (Kashyap & Banu, 2019). No obstant això, la interacció entre les diferents fitohormones durant la sobremaduració del fruit és molt complexa i encara poc compresa. Factors com l'espècie, el cultivar, l'estat fisiològic del fruit, les condicions del cultiu durant la precollita, l'emmagatzematge i les manipulacions mecàniques durant la recol·lecció influencien significativament la regulació hormonal. Els estudis es realitzen en condicions controlades que no poden replicar completament les condicions comercials reals, com ara la constant variabilitat de temperatura, humitat i gasos, o les diferències en els protocols comercials de

postcollita. Aquesta complexitat es veu amplificada per la dificultat de mesurar de manera precisa i a gran escala els paràmetres fisiològics dels fruits, així com per l'impacte combinat de múltiples hormones que interactuen de manera interdependent. Tanmateix, cal considerar que els tractaments amb fitohormones sovint estan subjectes a estrictes regulacions, fet que limita els tipus d'experiments que poden dur-se a terme en condicions comercials.

2.3. Tècniques postcollita per allargar la vida útil

La naturalesa altament perible dels fruits fa que allargar la seva vida útil i conservar la seva qualitat durant tota la cadena de producció sigui un repte constant. Per tant, la implementació de tecnologies precollita que garanteixin la millor qualitat del producte, així com de tecnologies postcollita que n'assegurin la preservació i en redueixin el deteriorament, s'ha convertit en una prioritat. Aquest objectiu ha fomentat nombroses línies d'investigació orientades a desenvolupar solucions innovadores i sostenibles per afrontar els reptes associats a la conservació dels fruits al llarg de tot el procés de subministrament.

Entre les diferents tecnologies implementades, l'emmagatzematge en fred és una de les més utilitzades per alentir la respiració i altres processos metabòlics, alhora que disminueix la incidència de patògens i prolonga la vida útil del fruit (Aghdam & Bodbodak, 2014). Malgrat això, la majoria de fruits són susceptibles a les baixes temperatures, i períodes prolongats d'emmagatzematge en aquestes condicions poden desencadenar una pèrdua d'aromes i desordres fisiològics, com el dany per fred o l'enfosquiment intern, els quals perjudiquen significativament la qualitat del fruit (Zhu et al., 2018; Wu et al., 2024). Moltes vegades, la conservació a baixes temperatures es combina amb altres tecnologies com les atmosferes controlades i dinàmiques, més pobres en oxigen i més riques en diòxid de carboni, per reduir la biosíntesi de l'etilè i la taxa de respiració retardant així la senescència del fruit. No obstant, s'han detectat danys en resposta a aquests sistemes i sabors no desitjats en els fruits, a més de tenir una infraestructura econòmicament elevada i complexa d'aplicar (Mditshwa et al., 2018). Els banys amb fungicides són un dels mètodes químics que han demostrat més eficàcia en el control de les podridures postcollita. Tot i això, la seva aplicació exclusiva s'ha d'evitar dins d'un enfocament sostenible, ja que s'ha reportat l'aparició de resistències en els patògens quan s'utilitzen de manera recurrent (Schirra et al., 2011). En aquest sentit, la combinació amb tractaments com els banys d'aigua calenta, pot

augmentar-ne l'eficàcia i permetre una reducció en la dosi de fungicida aplicada. Aquests tractaments tèrmics (vapor, aigua o aire calent a 40–65 °C), així com també els banys de calci, s'han mostrat com alternatives efectives per reduir el desenvolupament de patògens i mantenir la fermesa del fruit, essent una opció ràpida i relativament senzilla d'implementar. Tot i això, poden comprometre la textura i altres qualitats organolèptiques del fruit com el color (Vicente et al., 2006; Nguyen et al., 2020). Els últims anys també ha agafat força la investigació en el camp dels recobriments comestibles, utilitzant materials com el quitosan, l'alginat de sodi, el clorur de calci, la goma aràbiga, els olis essencial o el gel d'*Aloe vera* (Riva et al., 2020; Chavan et al., 2023). Aquests formen una barrera semipermeable a la superfície dels fruits, que es pot menjar com a part del producte, i regula la transpiració i respiració a més de tenir un cert efecte inhibidor en la proliferació dels microorganismes (Badawy et al., 2017; Nia et al., 2021). Tanmateix, tot i ser prometedores, s'ha descrit que alguns d'aquests recobriments poden alterar les propietats sensorials del fruit perjudicant la seva qualitat (Azarakhsh et al., 2014; Yousuf et al., 2018).

Per altra banda, l'aplicació exògena de molècules de síntesi química inhibidores de l'etilè, com l'1-metilciclopropè (1-MCP), també s'aplica àmpliament en el sector, sobretot en fruits climatèrics, ja que permet allargar la vida útil dels fruits i reduir trastorns fisiològics (Sen et al., 2024; Hasan et al., 2024), tenint un impacte econòmic i social significatiu al permetre l'exportació de productes hortícoles a mercats llunyans. No obstant, l'aplicació d'1-MCP presenta certes limitacions com per exemple la incapacitat d'alguns fruits climatèrics a reprendre la maduració després de l'emmagatzematge (Dias et al., 2021). La seva eficàcia també està subjecte a factors com la quantitat i disponibilitat del receptor de l'etilè segons el fruit i l'estadi de maduració, la concentració utilitzada, la sensibilitat en funció del genotip, el material d'embalatge i el mètode d'aplicació aquós o gasós (Satekge & Magwaza, 2022). L'1-MCP està regulat per la normativa europea, i l'any 2019 l'Autoritat Europea de Seguretat Alimentària (EFSA) va renovar la seva aprovació (EU, 2019a), tot i que sota la premissa de ser utilitzat exclusivament com a regulador del creixement vegetal per a l'emmagatzematge postcollita en magatzems tancats i hermètics. Per tant, hi ha una gran necessitat de desenvolupar tècniques noves i innovadores que s'integrin de manera senzilla, econòmica i sostenible amb el medi ambient en els sistemes actuals. En aquest context, els tractaments amb reguladors vegetals com les fitohormones s'han investigat com una possible eina per

modular els processos de deteriorament, contribuir al manteniment de la qualitat nutricional i prolongar la vida útil dels fruits durant l'emmagatzematge. Aquest enfocament aprofita els mecanismes bioquímics naturals de les plantes, oferint una alternativa sostenible.

Al llarg de l'última dècada, els JAs, i particularment el MeJA, s'han investigat de forma activa per modular els processos de maduració i sobremaduració en fruits (Wang et al., 2021b). S'ha demostrat que el MeJA és eficaç per millorar la resistència contra infeccions per patògens (Pan et al., 2020; El-Mogy et al., 2019), i contribueix a mantenir la integritat de la paret cel·lular, preservant la fermesa del fruit. També millora la seva aparença incrementant el contingut d'antocianines i resulta útil per mitigar desordres fisiològics com el dany per fred (Li et al., 2017a; Moro et al., 2017; García-Pastor et al., 2020). Les aplicacions exògenes de SA redueixen la taxa respiratòria i la producció d'etilè, alhora que ajuden a mantenir atributs de qualitat com la pèrdua de pes, la fermesa i els canvis de color (Kumar et al., 2021; Zhang et al., 2020a). A més, el SA està involucrat en l'estimulació de la via fenilpropanoide, el qual fa incrementar la capacitat antioxidant i és crucial per reduir el dany fúngic (Zhang et al., 2021a; Yang et al., 2025). L'aplicació exògena d'auxines com l'IAA també ha mostrat efectes inhibidors sobre la senescència, regulant negativament la producció d'etilè i contribuint a mantenir la fermesa al modular els enzims degradadors de la paret cel·lular, a més d'incrementar la resistència del fruit (Li et al., 2017b; Chen et al., 2016a; Zhang et al., 2018a). Els tractaments exògens amb CKs, com la bencilaminopurina (BA), també s'han estudiat gràcies a les seves propietats antisenescents. La seva eficàcia està associada a la capacitat de suprimir la producció de ROS i regular els gens relacionats amb la biosíntesi de l'etilè (Zhang et al., 2022c), així com potenciar el sistema antioxidant (Jia et al., 2017). De manera similar, els tractaments exògens amb GAs, principalment GA_3, també han estat efectius per incrementar el contingut d'antocianines, retardar la pèrdua de pes i mantenir la fermesa, a més de millorar la tolerància al fred (Ozturk et al., 2022; Yang et al., 2023; Qu et al., 2021). D'altra banda, tot i que l'ABA té una acció predominant en la promoció de la maduració i sobremaduració, s'ha observat que en alguns casos la seva aplicació exògena pot retardar la senescència de certs fruits, com el maduixot, al reduir la producció d'etilè i alentir la degradació dels components de la paret cel·lular (Zhao et al., 2024). A més, també pot millorar la tolerància a l'estrès per fred (Castro-Cegrí et al., 2023). En resum, la investigació sobre les aplicacions exògenes de fitohormones obre noves possibilitats per millorar la

qualitat postcollita dels fruits, oferint alternatives sostenibles als mètodes tradicionals. No obstant això, tot i els resultats encoratjadors, cal continuar aprofundint en la comprensió dels mecanismes subjacents per optimitzar les aplicacions, garantir un ús estable, eficient i segur, i maximitzar els beneficis per la indústria agroalimentària.

3. La melatonina

3.1. Estructura, síntesi i catabolisme

La melatonina (*N*-acetil-5-metoxitriptamina) és una biomolècula central que es va descobrir en la glàndula pineal bovina al 1958, i durant les següents quatre dècades es va considerar exclusivament una hormona animal (Reiter et al., 1991). No va ser fins al 1995 que la melatonina va ser identificada per primera vegada en diverses espècies de plantes, incloent-hi tomàquets, patates, bananes, tabac o arròs, pels grups d'investigació de Dubbels et al. (1995) i Hattori et al. (1995). Aquell mateix any, el grup de Kolář va demostrar la presència de melatonina en *Chenopodium rubrum* (Kolář et al., 1995). Posteriorment, es va detectar també en herbes medicinals com *Hypericum perforatum*, amb concentracions sorprenentment altes, de l'ordre de micrograms per gram de teixit, molt superiors a les trobades en animals (Murch et al., 1997). A més d'animals i plantes, la melatonina s'ha identificat també en bacteris i algues, consolidant-se com un compost natural altament conservat que se sintetitza en gairebé tots els organismes vius (Hardeland et al., 2016).

Des d'un punt de vista fisicoquímic, la melatonina és una indolamina de baix pes molecular i es caracteritza per la presència d'un grup 3-amida i un grup 5-metoxi, que li confereixen una naturalesa amfipàtica (Tan et al., 2002). En les plantes, la biosíntesi de la melatonina comença amb el triptòfan i implica quatre passos enzimàtics, tot i que es coneix que poden intervenir-hi almenys sis enzims diferents. Així, existeixen diverses vies biosintètiques, com a mínim quatre, les quals requereixen la serotonina com a intermediari essencial (**Figura 2**). La via predominant en plantes comença amb l'enzim triptòfan descarboxilasa (TDC), que converteix el triptòfan en triptamina. Posteriorment, la triptamina-5-hidroxilasa (T5H) catalitza una hidroxilació, donant lloc a la serotonina. No obstant això, també es pot seguir una via alternativa en què primer es produeix la hidroxilació del triptòfan mitjançant l'acció de la triptòfan hidroxilasa (TPH), generant 5-hidroxitriptòfan,

que després és descarboxilat per la TDC per formar serotonina. Posteriorment, la síntesi de la melatonina a partir de la serotonina es produeix en dos passos en els quals intervenen tres enzims: la serotonina-*N*-acetiltransferasa (SNAT), la *N*-acetilserotonina metiltransferasa (ASMT) i l'àcid cafeic *O*-metiltransferasa (COMT). En el primer pas, l'enzim SNAT converteix la serotonina en *N*-acetilserotonina, que després és metilada per ASMT o COMT, donant lloc a la melatonina. No obstant això, el procés també pot seguir una via alternativa: la serotonina és primer convertida en 5-metoxitriptamina per ASMT o COMT i, posteriorment, transformada en melatonina per SNAT. L'ordre d'aquestes reaccions enzimàtiques determina la localització subcel·lular on se sintetitzen la serotonina i la melatonina. Quan la biosíntesi de la serotonina té la triptamina com a intermediari, el procés té lloc al reticle endoplasmàtic en lloc del citoplasma. D'altra banda, la síntesi de melatonina es produeix al cloroplast o mitocondri quan l'últim pas és catalitzat per SNAT, i al citoplasma quan l'encarregat és ASMT o COMT (**Figura 2**) (Back et al., 2016).

La melatonina, un cop formada, pot ser metabolitzada ràpidament a 2-hidroximelatonina (2-HM) i 3-hidroximelatonina cíclica (3-HM) per la melatonina-2-hidroxilasa (M2H) i la melatonina-3-hidroxilasa (M3H) quan la melatonina està present en els cloroplasts i el citoplasma, respectivament (Byeon & Back, 2015; Lee et al., 2016). La 2-HM s'ha proposat com a metabòlit principal del catabolisme de la melatonina en plantes, i anàlisis cinètiques de l'activitat enzimàtica de la M2H han demostrat que és molt més eficient que els enzims de biosíntesi de la melatonina com el SNAT o ASMT (Byeon et al., 2015b). Per tant, la melatonina té un metabolisme ràpid en les plantes. A més de la 2-HM i la 3-HM, s'han trobat altres metabòlits com la 5-metoxitriptamina, la 4-hidroximelatonina (4-HM), la N^1-acetil-N^2-formil-5-metoxiquinuramina (AFMK), la N^1-acetil-5-metoxiquinuramina (AMK), i la *N*-nitrosomelatonina (**Figura 3**) (Byeon et al., 2015b; Mannino et al., 2021).

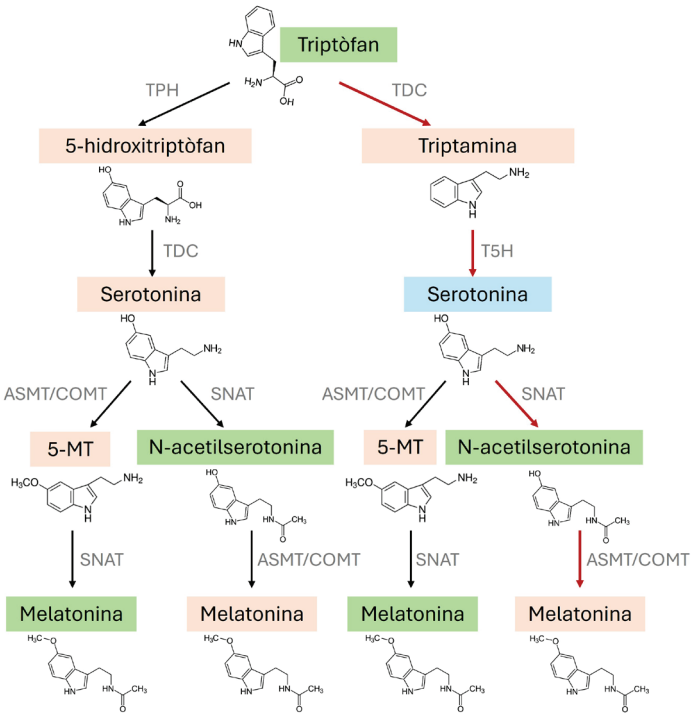


Figura 2. Esquema de les diferents rutes de biosíntesi de melatonina, incloent la localització subcel·lular i els enzims implicats en cada reacció. Color verd indica que la seva síntesi té lloc al cloroplast o mitocondri, taronja al citoplasma i blau al reticle endoplasmàtic. Les fletxes de color vermell indiquen la ruta predominant en plantes. 5-MT: 5-metoxitriptamina; TDC: triptòfan descarboxilasa; TPH: triptòfan hidroxilasa; T5H: triptamina 5-hidroxilasa; SNAT: serotonina-*N*-acetiltransferasa; ASMT: *N*-acetilserotonina metiltransferasa; COMT: àcid cafeic *O*-metiltransferasa.

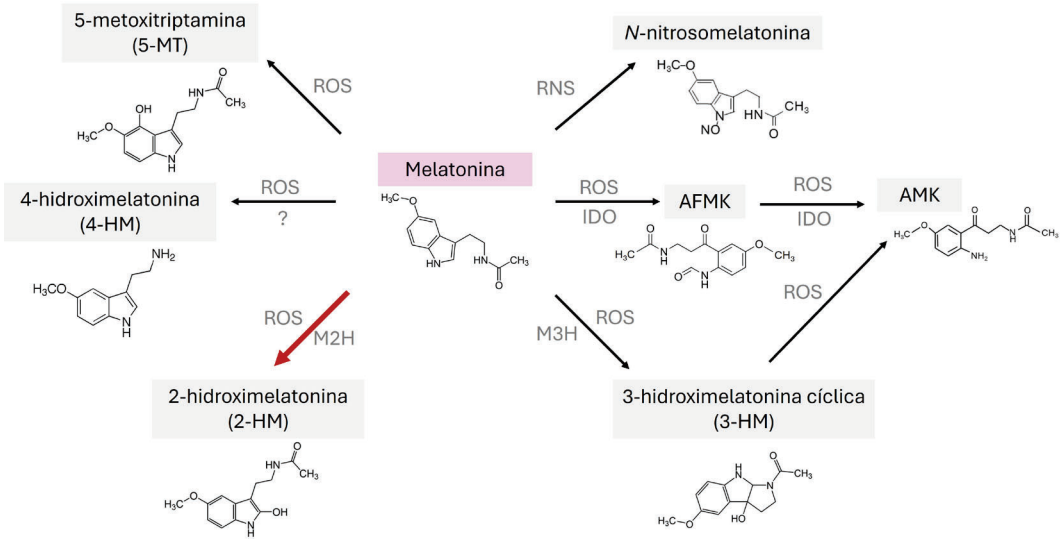


Figura 3. Compostos procedents del catabolisme de la melatonina. La fletxa vermella indica el producte predominant en plantes. AFMK: N^1-acetil-N^2-formil-5-metoxiquinuramina; AMK: N^1-acetil-5-metoxiquinuramina; ROS: espècies reactives de l'oxigen; RNS: espècies reactives del nitrogen; M2H: melatonina-2-hidroxilasa; M3H: melatonina-3-hidroxilasa; IDO: indolamina 2,3-dioxigenasa; ?: desconegut.

3.2. Distribució en el regne vegetal

Diverses espècies de plantes són capaces de produir grans quantitats de melatonina, que s'emmagatzemen en òrgans o teixits especialitzats. Aquesta molècula s'ha identificat en més de 50 espècies vegetals, localitzada en diferents òrgans incloent arrels, llavors, bulbs, fulles, flors i fruits. Bona part d'aquestes espècies pertanyen a famílies com Rosaceae, Vitaceae, Poaceae, Apiaceae, Rubiaceae i Brassicaceae, entre d'altres (Nawaz et al., 2016). Els nivells de melatonina detectats en plantes són altament variables, amb concentracions que poden oscil·lar des de valors gairebé indetectables fins a nivells molt elevats, en rangs de picograms a nanograms (ng) per gram de pes fresc (Hardeland, 2016). En alguns casos, els nivells de melatonina són comparables, o fins i tot superiors, als observats en animals. Per exemple, en llavors del gènere *Coffea* s'han reportat concentracions excepcionalment altes, superant els 5000 ng per gram de pes fresc, seguides de plantes medicinals i oficinals com la menta, la sàlvia i la farigola, que també presenten valors destacats. En altres espècies, com l'arròs, el blat, el tomàquet, la cirera o el nabiu, s'han detectat concentracions mitjanes, situant-se entre 9 i 100 ng per gram de pes fresc. En canvi, en fruits com la poma, la taronja o el plàtan, els continguts mesurats de melatonina són considerablement més baixos, inferiors als 3 ng per gram de pes fresc (Mannino et al., 2021; Nawaz et al., 2016). La variabilitat en la concentració de melatonina depèn de diversos factors, com l'òrgan de la planta i les condicions ambientals. Les concentracions més elevades s'han trobat en flors i llavors, el qual podria estar relacionat amb la seva alta sensibilitat a l'estrès ambiental (Arnao, 2014). A més, s'han trobat alts nivells de melatonina en plantes en condicions desfavorables, inclosos l'estrès per calor o fred, l'exposició a contaminants o a infeccions (Tan et al., 2002)

3.3. Funció fisiològica en les plantes

3.3.1. Funció antioxidant

La melatonina és un compost antioxidant essencial en les plantes. La seva activitat antioxidant es deu principalment a la seva capacitat directa de neutralitzar les ROS i les espècies reactives del nitrogen (RNS), com els radicals hidroxils (·OH), l'anió superòxid (O_2^-), el peròxid d'hidrogen (H_2O_2), o l'òxid nítric (NO). Aquesta acció de neutralització directa contribueix a mitigar els danys oxidatius als components cel·lulars mantenint així la integritat cel·lular. La fracció indòlica de la melatonina és el centre reactiu d'interacció amb les

molècules oxidants per la seva alta estabilitat de ressonància i barrera d'energia d'activació molt baixa cap a les reaccions de radicals lliures. Les cadenes laterals metoxi i amida també contribueixen a la seva capacitat antioxidant (Tan et al., 2012b). S'ha descrit que la seva mida petita i naturalesa amfipàtica li permeten travessar fàcilment els teixits i compartiments subcel·lulars, distribuint-se tant al citosol com a les membranes lipídiques, a diferència d'altres antioxidants que només poden migrar de manera selectiva (Feng et al., 2014). A més, la melatonina actua com un antioxidant "suïcida", ja que no es regenera ni promou reaccions d'oxidació addicionals, fet que li confereix l'avantatge de no tenir activitat prooxidativa (Tan et al., 2002). El mode de vida de les plantes, amb una interacció constant amb el medi ambient, les sotmet a diversos estressos abiòtics i biòtics, la qual cosa incrementa la possibilitat de patir dany oxidatiu. La capacitat de la melatonina per incrementar el seu contingut sota condicions d'estrès ha consolidat el seu paper com a protector cel·lular, gràcies a la seva elevada capacitat antioxidant i la seva habilitat per segrestar les ROS, a més de contribuir a preservar el contingut d'altres antioxidants (Tan et al., 2012a; Wang et al., 2012). Així, se suggereix el paper de la melatonina com un component clau en la primera línia de defensa contra l'estrès oxidatiu en plantes.

3.3.2. Funció reguladora de processos fisiològics

A més de la seva acció directa com a antioxidant capaç de neutralitzar radicals lliures, la melatonina destaca per la seva doble funció, ja que també exerceix un paper com a molècula senyalitzadora. Diversos estudis han destacat les múltiples activitats cel·lulars i fisiològiques de la melatonina en les plantes, confirmant el seu paper com a molècula multifuncional essencial en la regulació de diversos processos fisiològics. La melatonina actua com a estimulador, promovent la germinació de les llavors (Chen et al., 2021; Xiao et al., 2019), el creixement de les arrels (Yang et al., 2021) i el desenvolupament de la part aèria (Sun et al., 2021a; Wei et al., 2015), alhora que retarda la senescència foliar (Arnao & Hernández-Ruiz, 2009; Liu et al., 2022). També exerceix un paper regulador en processos com la floració i el desenvolupament dels fruits (Arnao & Hernández-Ruiz, 2020; Meng et al., 2015; Tijero et al., 2019). A més, la melatonina actua com a reguladora davant l'estrès abiòtic i biòtic, modulant els mecanismes de defensa de les plantes i augmentant la seva tolerància en condicions adverses (Hassan et al., 2022; Ahammed et al., 2019; Zhao et al., 2021; Li et al., 2019a). Diferents autors han proposat que la melatonina exerceix els seus efectes

regulant diversos components del sistema redox, així com interferint amb les activitats de fitohormones com per exemple l'ABA, les auxines, el SA o l'etilè. De fet, s'ha indicat que la melatonina pot influir en l'expressió de gens implicats en la biosíntesi d'hormones vegetals i també d'altres molècules antioxidants, per exemple incrementant el contingut de compostos fenòlics o d'enzims antioxidants com la superòxid dismutasa (SOD), la catalasa (CAT) i l'ascorbat peroxidasa (APX) (Arnao & Hernández, 2020; Zhang et al., 2016; Liu et al., 2020; Li et al., 2022b; Altaf et al., 2022; Nehela & Killimy, 2020). Per tant, la melatonina es revela com una molècula rellevant i versàtil en la regulació de processos fisiològics al llarg de tot el desenvolupament vegetal, així com en les respostes adaptatives a condicions adverses.

4. La melatonina en fruits

La presència de melatonina en fruits està àmpliament documentada a la bibliografia i ha estat descrita en diverses famílies i espècies, consolidant-se com un compost omnipresent a concentracions de ng per gram de pes fresc (Nawaz et al., 2016; Wang et al., 2020b). No obstant això, els estudis que han quantificat melatonina en diverses espècies i varietats de fruits, com per exemple en tomàquet, raïm, cirera, mora i poma, indiquen que les concentracions poden variar notablement depenent de la varietat, de l'estadi de desenvolupament del fruit i fins i tot del tipus de teixit (Okazaki & Ezura, 2009; Vitalini et al., 2011; Zhao et al., 2013; Feng et al., 2014; Wang et al., 2016a; Verde et al., 2022).

4.1. La melatonina en la maduració dels fruits

La melatonina sembla influir directament en el desenvolupament i maduració dels fruits. Diversos estudis han demostrat que els nivells de melatonina fluctuen al llarg dels diferents estadis. Per exemple, en fruits com les cireres, les cireres de cornalina i les mores, s'han detectat els nivells més alts de melatonina durant les primeres fases de creixement, disminuint abans de l'inici de la maduració. Això suggereix un paper clau en els estadis inicials, especialment en el quallat del fruit i en processos com la divisió i l'expansió cel·lular (Tijero et al., 2019; Xia et al., 2020; Wang et al., 2016a; Uğur et al., 2024). Per contra, en fruits com el raïm i la polpa de la poma, la melatonina assoleix el seu màxim durant la fase de canvi de color, destacant la seva funció en les etapes més avançades de la maduració (Xu et al., 2018; Verde et al., 2022).

Les aplicacions exògenes de melatonina han permès aprofundir en els seus efectes durant el desenvolupament dels fruits. En el quallat, el seu paper s'ha vinculat especialment a la partenocàrpia, induint la síntesi de GAs i afavorint la divisió i l'expansió cel·lular del mesocarpi (Liu et al., 2018b). Pel que fa a la maduració, els seus efectes s'han associat principalment amb la interacció amb l'etilè. En fruits climatèrics com els tomàquets, s'ha observat que la melatonina regula positivament l'expressió de l'ACS i de gens implicats en la transducció de senyals d'etilè. A més, també promou l'acumulació de carotenoides com el licopè (Sun et al., 2015; Sun et al., 2020). Tanmateix, la irrigació de tomaqueres amb melatonina millora la qualitat dels fruits en el moment de la collita, obtenint una major concentració de sòlids solubles totals (SST), àcids orgànics i vitamina C (Liu et al., 2016). De manera similar, en pomes s'ha observat que la melatonina estimula la maduració induint l'etilè, alhora que millora aspectes com el pes, la mida i la fermesa del fruit (Verde et al., 2022).

En fruits no climatèrics com el raïm, l'aplicació exògena de melatonina també influeix en la biosíntesi i la senyalització de l'etilè, a més d'afavorir l'acumulació de polifenols i una activitat antioxidant superior. Tanmateix, s'ha associat amb un augment dels nivells d'ABA i H_2O_2, els quals contribueixen a accelerar la maduració (Xu et al., 2017; Xu et al., 2018). En cireres, l'aplicació de melatonina exògena endarrereix la maduració en reduir el contingut d'antocianines i modular els nivells de CKs (Tijero et al., 2019), tot i que també s'ha demostrat que millora la qualitat augmentant el pes, els SST, els compostos fenòlics i la capacitat antioxidant total en el moment de la collita (Xia et al., 2020). Pel que fa al maduixot, la melatonina exògena pot accelerar o retardar la maduració en funció de la dosi aplicada, amb efectes sobre el contingut d'antocianines, SST, àcids, fermesa i l'expressió de gens implicats en la biosíntesi de l'ABA, regulant la seva acumulació (Mansouri et al., 2021; Mansouri et al., 2023). Per tant, tot i els avenços en la comprensió del paper de la melatonina durant la maduració, el seu rol en les diferents etapes del desenvolupament del fruit i la seva interacció amb altres fitohormones és un àmbit d'estudi amb un gran potencial per aprofundir en la seva funció fisiològica i els mecanismes que regulen la fisiologia del fruit.

4.2. La melatonina en la qualitat postcollita de fruits

En fruits, la melatonina no només intervé en el procés de maduració, sinó que també s'ha estudiat el seu potencial per retardar la senescència durant l'emmagatzematge, prolongant la vida útil i preservant-ne la qualitat postcollita (**Figura 4**). Els canvis bioquímics que es produeixen durant la sobremaduració dels fruits, i que comporten una pèrdua general de qualitat, són inevitables, però intentar endarrerir-los per mantenir la qualitat desitjada el màxim temps possible és un gran repte actual. En aquest context, s'està investigant si la melatonina pot ser una estratègia prometedora per aconseguir-ho. En diversos fruits com el maduixot, la cirera, la nectarina, la pera o el mango, s'ha observat que l'aplicació de melatonina pot retardar els canvis en el color, els SST, l'acidesa i la pèrdua de pes. També contribueix a mantenir la fermesa en endarrerir la degradació de la paret cel·lular, reduint l'activitat d'enzims implicats en aquest procés (Liu et al., 2018a; Pang et al., 2023; Cheng et al., 2025; Liu et al., 2020). A més, la melatonina s'ha associat amb una disminució de la taxa respiratòria, un altre factor clau en la preservació de la qualitat del fruit (Wang et al., 2019). Tanmateix, la melatonina no només contribueix a mantenir les propietats físiques dels fruits, sinó que també ajuda a preservar-ne la qualitat nutricional. Això inclou l'augment de la concentració de compostos essencials per a la salut, com els fenols i la vitamina C (Aghdam & Fard, 2017; Luo et al., 2022).

L'efecte inhibidor de la senescència dels tractaments amb melatonina exògena s'ha associat principalment amb la seva acció antioxidant, ja que redueix les concentracions de ROS. D'una banda, l'aplicació de melatonina en fruits indueix un increment en la síntesi de melatonina endògena a través d'una major expressió dels gens relacionats amb la seva biosíntesi (Sharafi et al., 2019; Wang et al., 2020a). De l'altra, s'ha demostrat que durant l'emmagatzematge, el tractament amb melatonina exògena estimula significativament la producció d'antioxidants enzimàtics i no enzimàtics (Zhai et al., 2018; Zhang et al., 2018b; Gao et al., 2016; Cao et al., 2024; Miranda et al., 2020; Sharafi et al., 2021; Shang et al., 2021). Estudis recents també han identificat que la melatonina promou la producció d'altres molècules de senyalització, com l'òxid nítric, que ajuda a eliminar les ROS, i inhibeix hormones promotores de la senescència com l'etilè (Liu et al., 2023; Liu et al., 2019c). També s'ha observat un increment del contingut d'àcid γ-aminobutíric (GABA) després del tractament amb melatonina, associat amb una major capacitat antioxidant dels fruits (Wu et

al., 2023). A més, la melatonina exògena també pot induir canvis en les concentracions d'hormones vegetals durant la postcollita. En plàtans, per exemple, s'ha observat un increment del contingut d'IAA, que contribueix a allargar la vida útil del fruit (Li et al., 2019b). En litxi, el retard de la senescència després del tractament amb melatonina s'ha atribuït a una reducció del contingut d'ABA (Zhang et al., 2021b) i en pomes i peres a una reducció de l'etilè (Onik et al., 2021; Zhai et al., 2018).

Els beneficis de la melatonina per allargar la vida útil postcollita també inclouen una major resistència a les malalties, especialment aquelles causades per infeccions fúngiques. S'ha descrit que la melatonina redueix el deteriorament en diversos fruits i té activitat antifúngica contra organismes com *Penicillium* spp., *Botrytis* spp. i *Alternaria* spp. (Wang et al., 2023; Promyou et al., 2023; Zhang et al., 2022a). Aquesta activitat està relacionada amb la capacitat de la melatonina d'induir la via fenilpropanoide per a la síntesi de compostos fenòlics, com la lignina, que reforcen la resistència a les malalties (Wang et al., 2023). Tanmateix, també actua incrementant el contingut i la senyalització de fitohormones (Liu et al., 2019a; Li et al., 2022b). Finalment, la melatonina exògena s'ha explorat com una estratègia per combatre els desordres fisiològics associats a les condicions d'emmagatzematge, com el dany per fred (Jiao et al., 2022; Molla et al., 2022; Gao et al., 2018).

Tot i els nombrosos estudis que destaquen el potencial de la melatonina per millorar la conservació postcollita dels fruits, la seva eficàcia continua sent inconsistent, ja que depèn en gran mesura de la interacció entre la dosi aplicada, el mètode i el temps d'aplicació (Ze et al., 2021; Aghdam & Arnao, 2024). Per consolidar la melatonina com a principi actiu per formulacions amb aplicacions biotecnològiques, és necessari aprofundir en la comprensió del seu rol fisiològic al llarg de les diferents etapes del desenvolupament, incloent la maduració i la sobremaduració, així com entendre millor la seva influència sobre els nivells i la senyalització d'hormones vegetals implicades en aquests processos fisiològics. Aquesta base de coneixement és clau per establir protocols d'aplicació eficients i transferibles.

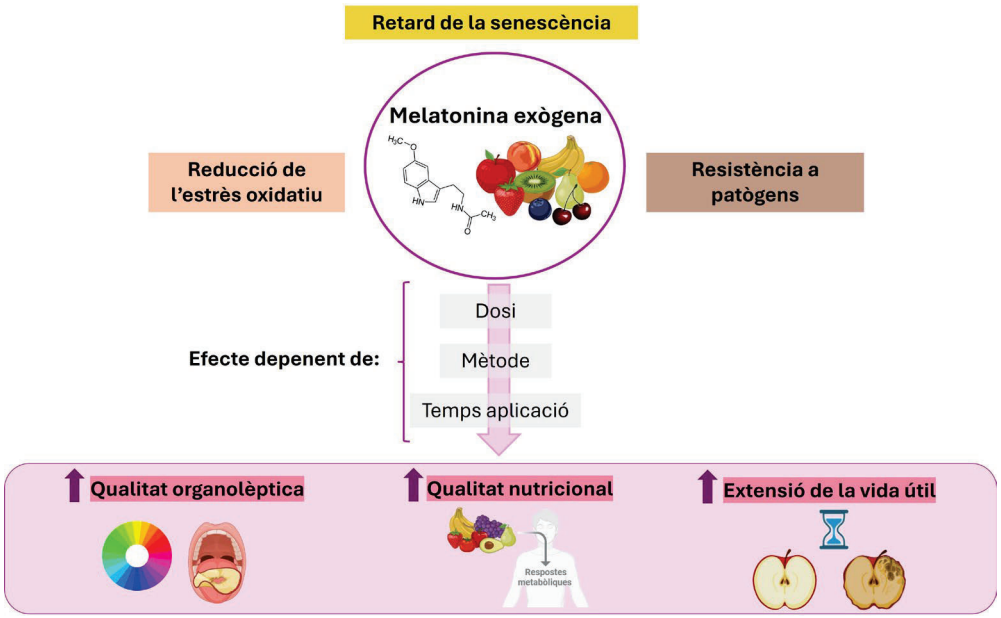


Figura 4. Diferents efectes de l'aplicació de melatonina exògena durant la postcollita dels fruits per mantenir les propietats organolèptiques i nutricionals i prolongar la seva vida útil.

5. Models d'estudi

5.1. Maduixot (*Fragaria* x *ananassa* Duch.)

El maduixot és una de les baies més consumides arreu del món, tant en forma fresca com processada. Es consumeix no només pel seu sabor, textura i atractiu color, sinó també per la seva destacada aportació de compostos bioactius. A més de ser ric en minerals, vitamines i fibra dietètica, el maduixot es caracteritza per la seva elevada concentració en vitamina C, folats i compostos fenòlics, especialment antocianines, fet que el converteix en un aliment molt valorat pels seus beneficis per a la salut i la seva capacitat de prevenir malalties cròniques, cardíaques i inflamatòries (Giampieri et al., 2014; Giampieri et al., 2015). Els maduixots són un dels fruits amb major demanda al mercat, el qual es tradueix amb una producció mundial de més de 10 milions de tones, sent Espanya el 6è productor en l'àmbit mundial amb 329,8 mil tones l'any 2023 i un dels principals exportadors d'Europa (FAO, 2023). En el context mediterrani, marcat per una elevada variabilitat climàtica, el cultiu protegit s'ha convertit en una pràctica clau per garantir l'estabilitat, el rendiment i la qualitat. L'ús de túnels de cultiu alts o baixos ha anat en augment durant les últimes dècades, ja que aquesta espècie s'adapta especialment bé a aquest tipus de sistemes degut a la seva baixa

estatura, l'hàbit de producció i la fenologia radicular. (Singh et al., 2012a) Aquestes estructures permeten crear un microclima més controlat que afavoreix el desenvolupament de la planta i la protegeix davant condicions climàtiques adverses.

El maduixot és una espècie híbrida alo-octaploide que pertany a la família Rosaceae i prové de l'encreuament entre dues espècies de maduixes silvestres, *F. chiloensis* i *F. virginiana*. Aquesta hibridació, juntament amb processos de selecció artificial, va originar plantes amb una capacitat de producció més elevada i fruits més grans i atractius, amb millors propietats organolèptiques, fet que va afavorir la seva consolidació com a cultiu comercial (Edger et al., 2019). El maduixot es desenvolupa a partir d'una única inflorescència i, tècnicament, es considera un fals fruit o fruit accessori. Es tracta d'un fruit agregat format per un receptacle floral engrossit que actua com a estructura principal i conté incrustats els veritables fruits, els aquenis, que deriven dels ovaris i contenen l'embrió (**Figura 5**). Els aquenis estan connectats al receptacle mitjançant feixos vasculars, a través dels quals reben nutrients durant el desenvolupament. Tot i estar altament interconnectats, especialment en les primeres etapes, ambdós òrgans, aqueni i receptacle, difereixen en l'ontogènia cel·lular i la funció. La seva complexa coordinació és fonamental per al correcte desenvolupament i maduració del fals fruit (Fait et al., 2008).

Més enllà del seu valor comercial, el maduixot ha estat àmpliament reconegut com un sistema model per a fruits no climatèrics, ja que no presenta un augment en la respiració i el seu ritme de maduració no es veu afectat per l'aplicació exògena d'etilè (Cherian et al., 2014). A més, la mida del fruit i del cultiu, la facilitat de propagació, la curta etapa vegetativa i el ràpid desenvolupament del fruit el converteixen en un sistema útil per estudiar el procés de maduració (Giovannoni et al., 2001). Tanmateix, les diverses etapes de la maduració del maduixot estan molt ben caracteritzades (Fait et al., 2008).

Per altra banda, al ser un fruit no climatèric, el maduixot s'ha de collir un cop ha assolit la maduració completa a la planta per aconseguir la màxima qualitat comercial. A això s'hi afegeix el seu alt grau de peribilitat, amb una vida útil postcollita limitada a una setmana a 0°C i a aproximadament 2-3 dies en condicions ambientals. A més, el seu elevat perfil nutricional i contingut d'humitat el fan més susceptible a la contaminació microbiana (Priyadarshi et al., 2024). Per tant, és essencial investigar i desenvolupar estratègies que

permetin allargar la seva vida útil, amb l'objectiu de mantenir la qualitat comercial i garantir la seguretat alimentària.

En aquesta tesi s'ha utilitzat el maduixot de la varietat Albion, procedent del Maresme (Catalunya, Espanya), com a model per estudiar tant el paper de la melatonina durant el desenvolupament del fruit com el seu potencial per millorar-ne la qualitat i endarrerir la sobremaduració durant l'emmagatzematge postcollita. Albion és una varietat de maduixot desenvolupada als Estats Units que s'ha introduït en molts països, incloent-hi Espanya. Es tracta d'un cultiu de dia neutre que destaca per la seva gran qualitat organolèptica (color vermell brillant, fermesa i dolçor) i nutricional, gràcies al seu elevat contingut en antocianines i fenols totals (Ornelas-Paz et al., 2013).

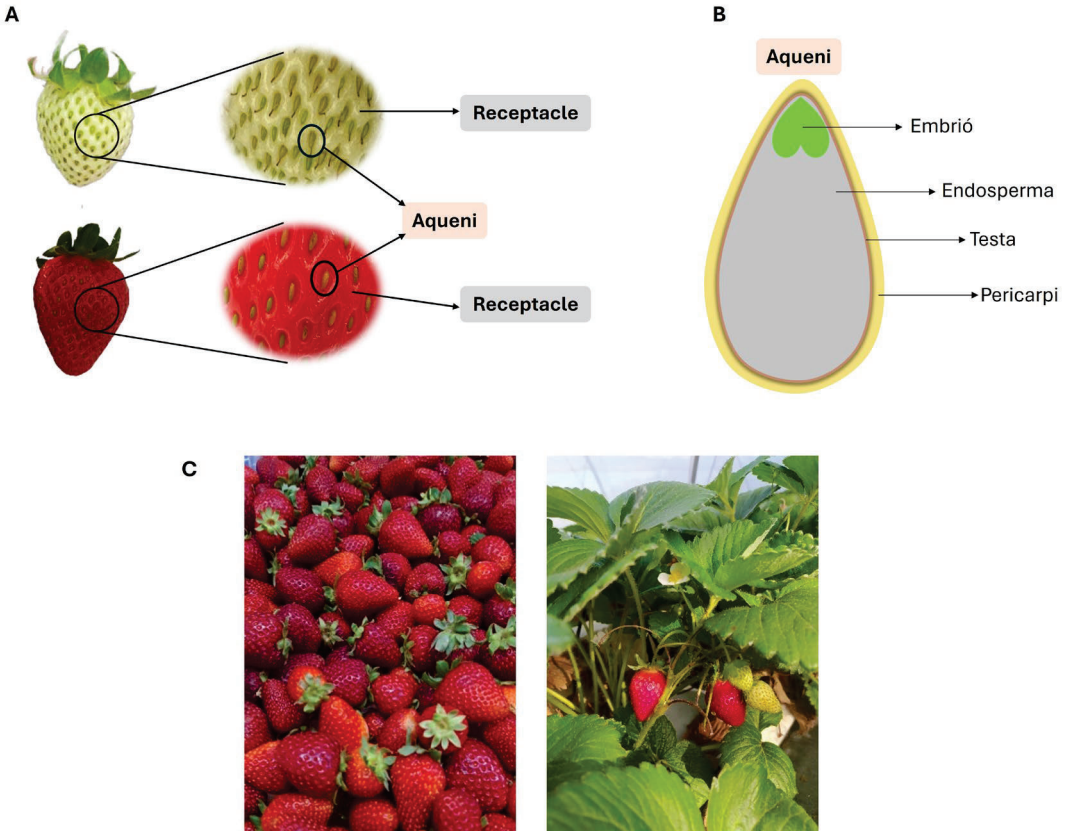


Figura 5. **A)** Diferenciació dels diferents òrgans, aqueni i receptacle, del maduixot (*Fragaria* x *ananassa* cv. Albion) en estadi blanc i totalment vermell. **B)** Esquema de les principals parts que formen l'aqueni. **C)** Fotografies del maduixot Albion després de la collita a la finca Ca l'Estrany, Sant Cebrià de Vallalta (Maresme, Catalunya; esquerra) i al túnel de cultiu de la finca Can Marpons, Arenys de Munt (Maresme, Catalunya; dreta).

5.2. Gerd (*Rubus idaeus* L.), nabiu (*Vaccinium corymbosum* L.) i cirera (*Prunus avium* L.)

Els fruits vermells, que inclouen els gerds, els nabius i les cireres, entre d'altres, són molt valorats pel seu color viu, textura delicada i sabor distintiu (**Figura 6**). Aquests fruits són una part fonamental de la dieta humana gràcies a les seves propietats nutricionals i bioactives i són reconeguts com una font significativa de macronutrients, micronutrients, sucres naturals, vitamines, minerals i fibra (Manganaris et al., 2014; Cosme et al., 2022). A més, són considerats aliments funcionals a causa de les múltiples propietats beneficioses per a la salut humana associades als compostos bioactius que contenen, especialment l'àcid ascòrbic i els compostos fenòlics com les antocianines i els tanins (Cosme et al., 2022; Battino et al., 2009; Gonçalves et al., 2019). L'interès per aquests fruits ha crescut notablement, impulsat pel canvi en les preferències alimentàries cap a una dieta més saludable i equilibrada, en què els fruits vermells es destaquen com una excel·lent opció nutricional (Anil et al., 2022). Aquest interès s'ha traduït en un augment en la seva producció, amb el cultiu de gerds, nabius i cireres que ja supera els 940,98 mil tones, 1,22 milions de tones i 2,96 milions de tones respectivament, el que representa un augment de l'11% en comparació amb el 2019. Països com els Estats Units, Xile, Perú, Mèxic i altres de la conca mediterrània incloent Espanya, Turquia i Itàlia, són els principals productors (FAO, 2023).

Tot i que els fruits vermells comparteixen una sèrie d'atributs, aquests pertanyen a grups botànics diversos. Els gerds, que formen part de la família Rosaceae, són fruits agregats compostos per petites drupes globoses, cadascuna amb una llavor, que es desprenen fàcilment del receptacle blanc quan arriben a la maduresa (Graham et al., 2007). Els nabius, de la família Ericaceae, són fruits simples de tipus baia que es desenvolupen a partir d'un ovari ínfer. Aquestes baies es caracteritzen per una epidermis llisa, de color blau intens o negre, amb una capa cerosa superficial anomenada pruïna, que actua com a barrera protectora. La polpa és carnosa i conté diverses llavors petites distribuïdes uniformement a l'interior (Wan et al., 2024). Finalment, les cireres, també de la família Rosaceae, són fruits simples de tipus drupa, amb un exocarpi llis de color vermell-porpra fosc, un mesocarpi carnós i suculent, i un endocarpi lignificat que protegeix la llavor (Vignati et al., 2022).

Els fruits vermells són cultius de gran valor comercial, però la seva demanda es veu influenciada no només per la seva estacionalitat, sinó també per la seva alta peribilitat (Huynh et al., 2019). Són considerats fruits no climatèrics, de manera que el grau

d'estovament a més del contingut elevat de sucres i aigua en el moment de la recol·lecció els fan extremadament sensibles a lesions mecàniques no només durant la collita sinó també el transport i emmagatzematge. Aquesta fragilitat incrementa la dificultat en el seu maneig i fa que qualsevol dany mecànic al llarg de la cadena de subministrament afavoreixi el desenvolupament de fongs, la pèrdua d'aigua, així com un augment en la respiració i la producció de ROS, factors que acceleren el deteriorament fisiològic dels fruits i limiten la seva vida útil (Kumar et al., 2018). Tot i que aquestes espècies produeixen baixes quantitats d'etilè, en el cas dels gerds i els nabius el deteriorament podria estar influenciat també per la producció d'aquest compost, encara que la relació sembla dependre del cultiu (Graham & Simpson, 2018; Huynh et al., 2019; Kumar et al., 2018). Com a resultat, la seva durabilitat sovint es veu reduïda a menys d'una setmana en condicions ambientals. Malgrat els avenços en tècniques de conservació per a aquests fruits, com l'emmagatzematge a baixa temperatura, irradiació, recobriments comestibles i fumigació, aquestes estratègies sovint requereixen equipaments especialitzats i poden resultar ineficients o inestables (Cao et al., 2024). A més, la major part de les investigacions sobre la conservació de fruits vermells s'han centrat en espècies com el maduixot, mentre que altres fruits amb alt valor econòmic, però menor volum de producció, com els gerds, nabius i cireres, han rebut una menor atenció científica (Huynh et al., 2019).



Figura 6. D'esquerra a dreta: *Rubus Idaeus* cv. Driscoll's, *Vaccinium corymbosum* cv. Duke i *Prunus avium* cv. Lapins, emprats en aquest estudi.

En aquesta tesi, per estudiar la melatonina com a tècnica de conservació alternativa per millorar la vida útil dels fruits vermells, s'han utilitzat gerds d'una varietat propietat de l'empresa Driscoll's, procedents de Huelva (Espanya) i destacada per la seva dolçor. També s'han seleccionats nabius de la varietat "Duke", procedents de Cantàbria (Espanya) i coneguts per la seva gran mida, duresa, textura cruixent i una major resistència a la recol·lecció mecanitzada i al transport. Finalment, s'han emprat cireres de la varietat "Lapins" procedents d'Aragó (Espanya), una de les varietats més cultivades mundialment que es caracteritza per la seva mida mitjana-gran, forma rodona allargada, pell i polpa de color vermell fosc, alta fermesa i sabor dolç.

5.3. Pruna (*Prunus salicina* L.)

La pruna és un fruit d'alt interès en el mercat global que forma part de la família Rosaceae i es caracteritza per un elevat contingut d'aigua, fibra, carbohidrats, vitamines i una àmplia varietat d'antioxidants (Gil et al., 2002; Lozano et al., 2009). Aquest perfil composicional li confereix un alt valor nutricional i que sigui considerat un fruit amb propietats beneficioses per a la salut (Igwe & Charlton, 2016). Les prunes comprenen principalment dues espècies: l'europea (*Prunus domestica* L.), que es consumeix fresca o seca, i la japonesa (*Prunus salicina* L.), que es destina principalment al consum fresc. Morfològicament, es tracta d'un fruit de pinyol o drupa, format per una pell exterior comestible i prima (exocarpi), un mesocarpi carnós i una paret ovàrica gruixuda lignificada (endocarpi) que inclou una llavor, comunament coneguda com a pinyol (Malik et al., 2021; **Figura 7**). Les prunes presenten formes rodones o allargades i una gamma de colors variada segons la varietat. En els darrers anys, el cultiu de pruners ha experimentat un creixement significatiu. El 2023, la producció global de prunes i aranyons va superar els 12 milions de tones, amb Espanya consolidant-se com un dels 15 principals productors a escala mundial (FAO, 2023).

Els fruits de pinyol, com la pruna, es caracteritzen per tenir una vida útil curta al mercat després de la collita a causa de la seva elevada peribilitat. Tradicionalment, les prunes s'han classificat com a fruits climatèrics que pateixen un ràpid deteriorament després de la maduració a causa d'un procés accelerat d'estovament, deshidratació i descomposició.

Aquestes característiques limiten considerablement la seva conservació a temperatura ambient, amb una vida útil estimada de només 8-10 dies (Manganaris & Crisosto, 2020; Menniti et al., 2004). Principalment, s'ha utilitzat l'emmagatzematge en fred per allargar la seva vida útil, i tot i que és una eina apropiada per disminuir la producció d'etilè, la taxa de respiració, els canvis de color i la pèrdua de fermesa, sovint resulta amb grans danys per fred generant translucidesa a la polpa i una descomposició interna que condueix a una pèrdua de qualitat i reducció de l'acceptabilitat del consumidor (Minas et al., 2013). Tanmateix, també s'ha demostrat que existeixen taxes d'estovament i producció d'etilè diferents entre cultivars d'una mateixa espècie. Algunes varietats, com és el cas de l'Angeleno, presenten un patró de maduració climatèrica suprimida caracteritzat per un patró d'estovament/maduració lent per la capacitat reduïda de convertir l'ACC en etilè (Minas et al., 2015; Singh et al., 2012b).

Prunus salicina cv. Angeleno és una varietat de pruna tardana que es distingeix per l'exocarpi llis de color vermell violaci, que en plena maduresa adquireix una tonalitat gairebé negra i un alt contingut de compostos fenòlics, especialment antocianines (Gil et al., 2002). En canvi, el mesocarpi presenta un color groc intens amb un sabor dolç característic. En aquesta tesi doctoral, s'ha utilitzat aquesta varietat de pruna com a model per investigar el paper diferencial de la melatonina en la millora de la qualitat durant l'emmagatzematge en els diferents teixits del fruit, incloent-hi l'exocarpi i el mesocarpi.



Figura 7. *Prunus salicina* cv. Angeleno i els diferents teixits que la constitueixen.

OBJECTIUS



OBJECTIUS

L'objectiu principal d'aquesta tesi doctoral és estudiar el paper de la melatonina en els processos de maduració i sobremaduració dels fruits, determinant si actua com a promotor o inhibidor, i explorar la seva relació amb les fitohormones així com la seva influència en la qualitat final dels fruits.

Per tal d'assolir aquest objectiu general es van plantejar els següents objectius específics:

i. Quantificar els nivells de melatonina, els seus precursors i el seu producte de degradació, en l'aqueni i el receptacle dels diferents estadis de desenvolupament del maduixot.

ii. Avaluar l'impacte del tractament exogen de melatonina aplicat en diferents etapes del desenvolupament sobre el ritme de maduració i qualitat del maduixot.

iii. Determinar els canvis bioquímics i moleculars associats a les fitohormones en l'aqueni i el receptacle de maduixots tractats amb melatonina.

iv. Estudiar els efectes de l'aplicació exògena de melatonina i del seu precursor, el triptòfan, sobre la vida útil i la qualitat postcollita dels maduixots i altres fruits vermells, incloent-hi els gerds, els nabius i les cireres.

v. Analitzar l'impacte dels tractaments exògens de melatonina i triptòfan sobre els nivells d'indolamines derivades i fitohormones durant la sobremaduració dels fruits vermells.

vi. Determinar el mecanisme teixit-específic de la melatonina exògena en la preservació de la qualitat postcollita de la pruna Angeleno.

vii. Avaluar l'eficiència de l'ús de melatonina com a bioestimulant per millorar la qualitat nutricional dels maduixots en condicions de camp al final de la temporada de collita.

INFORME DELS DIRECTORS DE TESI



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El Dr. Sergi Munné Bosch i la Dra. Paula Muñoz Roldán, com a directors de la Tesi Doctoral titulada "**Melatonina en maduració i sobremaduració de fruits: de la funció fisiològica a les aplicacions biotecnològiques**" presentada per la doctoranda Alba Arabia Ferrer,

INFORMEN sobre el factor d'impacte i la participació de la doctoranda en cadascun dels articles inclosos en la memòria d'aquesta Tesi Doctoral

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Capítol 1. Article "**Melatonin delays strawberry ripening by downregulating auxin-related genes** ", enviat a la revista *Journal of Experimental Botany*, amb un índex d'impacte (2023) de 5.8 (Q1, D1, Plant Sciences). En aquest treball es fa un estudi detallat dels efectes de la melatonina en la maduració dels maduixots realitzant un estudi a nivell tissular. El treball demostra que la melatonina té un efecte inhibidor en la maduració dels maduixots a través de la regulació dels nivells d'auxines endògenes mitjançant la modulació de la seva conjugació. La doctoranda ha realitzat tots els mostreigs, la separació dels diferents teixits i anàlisis de les mostres, el tractament estadístic i l'elaboració dels resultats, i a més ha participat en el disseny experimental i discussió dels resultats, constant per tant com a primera autora del treball. El treball es tracta d'una col·laboració amb investigadors de la UC Davis, els quals van contribuir amb suport tècnic i d'anàlisi de dades de la part molecular. La doctoranda ha demostrat una gran capacitat de treball, així com un excel·lent maneig en els mostrejos i una excel·lent predisposició a l'aplicació de tècniques bioquímiques i moleculars. La doctoranda ha adquirit molt bones competències en anàlisis d'hormones per LC-MS/MS i en tècniques de seqüenciació molecular, així com en l'anàlisi d'expressió gènica. Així mateix, la doctoranda mostra bones capacitats de treball en equip amb col·laboradors internacionals i maneig del gran volum de dades. La doctoranda demostra també una gran capacitat d'anàlisi i interpretació dels resultats, realitzant en el seu conjunt un treball molt complert i innovador englobant aspectes fisiològics, bioquímics i moleculars en una espècie de gran interès agronòmic i alimentari.

Capítol 2. Article "**Fruit-specific effects of tryptophan and melatonin as active components to extend the functionality of red fruits during post-harvest processing**", publicat a la revista *Food Chemistry*, amb un índex d'impacte (2023) de 8.5 (Q1, D1, Food Science & Technology). En aquest treball s'examinen els efectes de la melatonina i el seu precursor, el triptòfan, com a inhibidors de la sobremaduració de diversos fruits durant la postcollita, amb un èmfasi especial en els efectes que tenen

aquestes compostos en la qualitat de la fruita i en la seva vida útil. També es fa especial rellevància al mecanisme d'acció de la melatonina en la millora de la qualitat de la fruita durant la postcollita. Cal destacar la importància de l'estudi en quan a la dificultat d'analitzar diferents paràmetres de qualitat de forma complerta i al mateix temps avaluar el possible mecanisme hormonal que expliqui aquests efectes analitzant diverses fitohormones simultàniament per LC-MS/MS en mostres complexes, com ara diversos tipus de fruits que acumulen grans quantitats de pigments i compostos fenòlics. Es descriu per primera vegada la importància de la melatonina com a molècula inhibidora de la maduració en postcollita en aquest tipus de fruits. La doctoranda ha realitzat tots els mostreigs, les anàlisis de les mostres, el tractament estadístic i l'elaboració dels resultats, i a més ha participat molt activament en el disseny experimental i discussió dels resultats, constant per tant com a primera autora del treball. La doctoranda ha demostrat una gran capacitat de treball, així com excel·lents aptituds en l'anàlisi estadístic i interpretació de les dades i elaboració de models de mecanismes de regulació hormonal. La doctoranda sobresurt en el seu coneixement de processos de maduració i sobremaduració de fruita i en el desenvolupament de productes amb aplicacions en fisiologia i tecnologia postcollita.

Capítol 3. Article "**Melatonin triggers tissue-specific changes in anthocyanin and hormonal contents during postharvest decay of Angeleno plums**", publicat a la revista *Plant Science*, amb un índex d'impacte (2022) de 5.2 (Q1, Plant Sciences). En aquest treball es descriu, entre altres aspectes, la importància de la melatonina com a inhibidora de processos de deteriorament de la fruita en postcollita, realitzant importants descobriments de la funció diferencial d'aquest compost a nivell tissular en prunes. Cal destacar l'aproximació experimental, amb un caire molt més enfocat en la biologia cel·lular que en els anteriors capítols, molt original i amb un alt valor científic, ja que es demostra per primera vegada que la melatonina té un efecte diferencial en teixits. La doctoranda ha realitzat tots els mostreigs, la separació dels teixits i anàlisis de les mostres, ha realitzat el tractament estadístic i l'elaboració dels resultats, i a més ha participat molt activament en el disseny experimental i discussió dels resultats, constant per tant com a primera autora del treball. La doctoranda demostra una gran capacitat de treball, així com d'anàlisi i interpretació dels resultats, a més d'un excel·lent maneig al laboratori sent capaç de realitzar treballs de diferent tipologia, des d'aspectes de fisiologia i tecnologia postcollita a estudis bioquímics a nivell tissular.

Capítol 4. Article "**Variability in strawberry tunnels impacts fruit quality and limits melatonin effects**", publicat a la revista *Journal of the Science of Food and Agriculture*, amb un índex d'impacte (2023) de 3.3 (Q2, Food Science & Technology). En aquest darrer treball experimental es descriu la complexitat i importància de la variabilitat en la producció de maduixots en hivernacles en relació als possibles efectes positius de l'aplicació de melatonina en cultius. Aquest treball no només demostra que la variabilitat en el cultiu pot emmascarar els possibles efectes positius de l'aplicació de melatonina com a bioestimulant, sinó que mostra que l'entorn productiu comercial és multifactorial i diversos

estímuls afecten a la qualitat del producte final en el cultiu de maduixots. La doctoranda ha realitzat tots els mostreigs en un camp productiu, les anàlisis de les mostres, el tractament estadístic i l'elaboració dels resultats, i a més ha participat en el disseny experimental i discussió dels resultats, constant per tant com a primera autora del treball. La doctoranda ha demostrat una gran organització gestionant les relacions amb els principals agents del sector productiu, així com capacitat de treball i ha participat també activament en la redacció de l'article com en els altres capítols de la tesi. La doctoranda demostra un excel·lent grau de maduresa científica essent capaç de treballar en àmbits científics molt diferents i a la vegada complementaris i també confirma no només el seu gran coneixement en la fisiologia i tecnologia de fruits en pre- i postcollita i en el desenvolupament de bioestimulants, sinó també una gran capacitat de lideratge.

I, per que així consti als efectes oportuns,

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Dra. Paula Muñoz Roldán

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Dr. Sergi Munné Bosch

RESULTATS



CAPÍTOL 1

La melatonina retarda la maduració del maduixot mitjançant la regulació a la baixa dels gens relacionats amb auxines



Chapter 1. Melatonin delays strawberry ripening by downregulating auxin-related genes

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RESUM

La melatonina ha emergit com un compost similar a les hormones i s'han reportat múltiples efectes en fruits. En el present estudi, s'ha avaluat la melatonina des de la seva biosíntesi fins a la seva localització en els teixits dels maduixots per diferenciar els patrons d'acumulació. Les propietats de la melatonina en la senyalització de la maduració s'han determinat a través de la seva coacumulació amb les fitohormones i de l'expressió diferencial de gens després de la seva aplicació en diferents etapes del desenvolupament, incloent el quallat, el desverdiment i l'inici de la coloració vermella. La melatonina endògena va mostrar una acumulació espaciotemporal específica, assolint el seu màxim en els aquenis del maduixot durant el quallat i disminuint durant la maduració, però l'oposat es va observar en el receptacle carnós, amb els nivells més alts en l'etapa del fruit completament madur. Una tendència similar es va detectar en els precursors de la melatonina i en el seu producte hidroxilat. L'aplicació exògena de melatonina (10^{-4} M) va induir patrons diferencials en l'adquisició del color durant la maduració que estaven directament relacionats amb el moment d'aplicació. El tractament amb melatonina va augmentar el seu contingut i va accelerar el desenvolupament del color quan es va aplicar en el quallat, però el va endarrerir quan es va aplicar durant el desverdiment i a l'inici de la coloració vermella. L'anàlisi de l'expressió gènica ha revelat que la melatonina va induir una reprogramació transcripcional, especialment en els aquenis, on es va detectar un nombre més gran de gens expressats diferencialment. A més, l'aplicació de melatonina en l'etapa de desverdiment va reduir l'expressió del gen Gretchen Hagen 3 (*FaGH3.9*) tant en els aquenis com en el receptacle, 24 hores després de l'aplicació, fet que es va associar a una menor quantitat d'àcid indolacètic conjugat. Els nostres descobriments indiquen que la melatonina exerceix un paper inhibidor en la maduració del fruit, en part a través d'una interacció positiva amb les auxines. Aquests descobriments situen la melatonina com un regulador clau de la maduració de fruits no climatèrics, oferint noves estratègies per millorar la qualitat dels fruits i controlar la seva maduració.

Melatonin delays strawberry ripening by downregulating auxin-related genes

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HIGHLIGHT

Melatonin was found unevenly distributed in strawberry fruits in a time-dependent manner and melatonin applications (10^{-4}M) induced a delay in color acquisition and achenes reprogramming, including downregulation of auxin-related genes.

ABSTRACT

Melatonin has emerged as a hormone-like compound and multiple effects have been reported in fruits. In the present study, melatonin was evaluated from biosynthesis to tissue localization in strawberries to differentiate accumulation patterns. The signaling-ripening properties of melatonin were elucidated by co-accumulation with phytohormones and differential gene expression after application at distinct developmental stages, including fruit set, degreening and initial red. Endogenous melatonin showed specific spatiotemporal accumulation that peaked in strawberry achenes at fruit set and decreased during ripening, but the opposite was found in the fleshy receptacle, with the highest levels found at fully ripe stage. A similar trend was found for melatonin precursors and its hydroxylated product. Exogenous melatonin (10^{-4}M) applications induced differential patterns of color acquisition during ripening that were directly related to the application time. Melatonin treatment increased melatonin content and accelerated color development when applied at fruit set, but delayed color change when applied at degreening and initial red. Gene expression analysis showed that melatonin induced transcriptional reprogramming, particularly in achenes that showed a higher number of differentially expressed genes. Moreover, melatonin application at the degreening stage downregulated *Gretchen Hagen 3* (*FaGH3.9*) in achenes and receptacle 24 h post-application, linked to lower conjugated indole-3-acetic acid. Our findings indicate that melatonin exerts an inhibitory role in fruit ripening partly through positive crosstalk with auxins. These findings position melatonin as a key regulator of non-climacteric fruit ripening, offering targets to improve fruit quality and control ripening.

KEYWORDS: 2-hydroxymelatonin; auxin; melatonin; ripening; strawberry

ABBREVIATIONS

2-HM, 2-hydroxymelatonin;

ABA, abscisic acid;

H_2O_2, hydrogen peroxide;

IAA, indole-3-acetic acid;

Introduction

Fruits are essential for human nutrition and in botanical terms, they were an evolutionary innovation in angiosperms that supported seed formation and ensured effective dispersal (Seymour et al., 2013). Fruit development typically progresses through three main stages: fruit set, where fertilization triggers cell division and subsequent cell expansion; growth, during which the fruit increases in size and the embryo undergoes maturation; and ripening, a meticulously orchestrated phase involving metabolic changes such as sugar accumulation, pigment production, and cell wall softening. These transformations make fruits more appealing, nutritious, and suitable for consumption, fulfilling their role in plant reproduction and human consumption (Klee & Giovannoni, 2011; Kumar *et al*., 2014; Giovannoni *et al*., 2017). Fruit ripening is complexly governed by the coordinated activity of plant hormones, which play a fundamental role and influence multiple aspects of fruit development. Traditionally, fruits have been classified according to their ripening behavior as climacteric or non-climacteric (Paul *et al*., 2012), a distinction that has led to ripening studies focusing primarily on ethylene and abscisic acid (ABA) regulation in climacteric and non-climacteric fruits, respectively. However, current studies propose that the fine-tuning of this process is not driven by the action of a single phytohormone but rather emerges from the coordinated interplay of a complex hormonal network (Garrido-Bigotes *et al*., 2018; Fuentes *et al*., 2019; Fenn and Giovannoni, 2021; Fan *et al*., 2022). For instance, a reduction in auxin levels has been observed in several fruit crops as a prerequisite for ripening to commence. Moreover, the interaction of indole-3-acetic acid (IAA) with ABA or ethylene has been recognized as crucial for regulating the ripening process (Li *et al*., 2024; Gambhir *et al*., 2022). This emphasizes the necessity for further research, as the contribution of other phytohormones and regulatory molecules, particularly in non-climacteric fruits, remains largely undetermined and poorly understood.

Melatonin (N-acetyl-5-methoxytryptamine) is an ancient bioactive molecule first reported in plants 30 years ago (Dubbels *et al*., 1995; Hattori *et al*., 1995). Recent studies have identified this molecule as a new hormone-like compound, owing to its multifunctionality and potential involvement in regulating a wide range of physiological processes as an antioxidant and signal molecule (Arnao and Hernández-Ruiz, 2019; Khan *et al*., 2023). Melatonin is an indolamine synthesized through a series of enzymatic reactions starting from tryptophan, with serotonin as a critical intermediate. Once melatonin is formed, it is rapidly metabolized to 2-hydroxymelatonin (2-HM) through melatonin-2-hydroxylase, with 2-HM accumulation often

exceeding that of melatonin (Byeon, *et al*. 2015). In fruits, numerous studies have confirmed the contribution of melatonin in the regulation of fruit ripening, especially for climacteric fruits in which melatonin can promote ethylene biosynthesis through synergistic crosstalk during ripening (Sun *et al*., 2020; Ma *et al*., 2021; Shan *et al*., 2022; Verde *et al*., 2023). Indeed, melatonin is also associated with the preservation of key quality attributes and the delay of senescence (Arabia *et al*., 2025; Cheng *et al*., 2025). However, there is limited information available about the specific interplay between melatonin and phytohormones in non-climacteric fruits, and contradictory results about its role to delay or promote ripening. For example, Tijero *et al*. (2019) found that during early cherry development, melatonin peaks while ABA remains low, with 10 μM melatonin delaying ripening by reducing anthocyanin content and modulating cytokinin levels. Similarly, Mansouri *et al*. (2023) showed that melatonin (10 μM) in strawberries delayed ripening by decreasing ABA levels and suppressing hydrogen peroxide (H_2O_2). However, a higher concentration (1000 μM) accelerated ripening by enhancing ABA and H_2O_2 signaling (Mansouri *et al*., 2021). Likewise, in grapes, 100 μM melatonin promoted ripening by increasing ABA, H_2O_2, and ethylene levels (Xu *et al*., 2018). Beyond these contradictory findings, the interaction between melatonin and other key hormones, particularly auxins, remains largely unexplored. Melatonin and IAA share a common precursor, tryptophan, and exhibit a similar indole-based structure. Although some studies have demonstrated a positive synergy between IAA and melatonin in root growth (Chen *et al*., 2009; Wen *et al*., 2016) and suggested the potential for similar interactions in fruits (Arabia *et al*., 2023), their role in fruit ripening has, to our knowledge, not yet been investigated. Hence, clear information to define the putative role of this compound in fruit development and ripening as well as its interaction with other plant hormones is necessary because contradictory evidence depending on fruit species, tissue and study condition limit our understanding and potential application.

Cultivated strawberry (*Fragaria* × *ananassa*) is one of the most widely consumed fleshy fruits globally. It is a typical non-climacteric fruit, serving as a model for mechanistic studies on the ripening of non-climacteric fruits. Strawberry is an aggregated false fruit composed of a swollen flower receptacle (fleshy part) that contains the achenes (the true fruits derived from ovaries and enclosing the embryos) (Ariza *et al*., 2016). Both organs are connected through vascular bundles and although the achene and receptacle are closely interconnected during early development, they differ in cell ontogeny and function (Fait *et al*., 2008). Moreover, there is a differential accumulation of bioactive compounds in these tissues (Enomoto *et al*.,

2018) and during strawberry ripening, phytohormone translocation and signaling between achenes and receptacle plays a pivotal role in their crosstalk and ripening regulation (Li *et al*., 2022). Hence, the regulatory processes during development and ripening are likely distinct for each organ. Consequently, studies examining the whole fruit without dissecting its two components may overlook these organ-specific differences, which are essential for drawing accurate and reliable conclusions.

Given the uncertain evidence surrounding the role of melatonin in fruit development and ripening, this study investigates its regulatory function in non-climacteric fruits, using strawberry as a model. We focus specifically on the differential effects of melatonin in the achene and receptacle by analyzing its spatiotemporal distribution, along with its precursors (serotonin and tryptophan) and degradation product (2-HM) throughout fruit development. Additionally, organ-specific effects of exogenous melatonin applied at different developmental stages were assessed, examining both immediate and long-term responses and its interaction with other phytohormones. Through a combination of physiological, biochemical, and molecular analyses, these findings can contribute significantly to clarifying the function of melatonin in non-climacteric fruits, particularly its relationship with auxin. These insights provide valuable implications for developing technological strategies to enhance fruit crop management and control ripening dynamics.

Materials and methods

Plant material, treatments and sampling

Seedlings of the octoploid strawberry cultivar (*Fragaria* x *ananassa* cv Albion) provided by Flors Catalunya (Barcelona, NE Spain) were transferred to 3L pots using a mixture of perlite: peat: vermiculite (2:1:1, by volume) as substrate and grown in a greenhouse (16 h/8 h light conditions at 24 °C and 56% relative humidity) located at the Faculty of Biology of the University of Barcelona (Barcelona, NE Spain). Strawberry plants were subjected to daily, constant irrigation with Hoagland nutritive solution at pot field capacity, according to their evapotranspiration demand. During the productive period (May 2022), five replicates (each consisting of two fruits) were harvested from each developmental stage: Fruit set (FS), immediately after fertilization, Small Green (SG), small, immature green fruit, still growing; Big Green (BG), larger, green fruit near the end of its growth phase; Degreening (DG), the fruit has completed its growth phase, starting to change color from green to white; White (W),

fully white fruit, just before the ripening process begins; Initial Red (IR), early red color acquisition as ripening starts, Full Red (FR), fully commercial ripe fruit, with peak color, flavor, and nutritional content. Samples were frozen in liquid N_2 and stored at −80 °C for subsequent achene separation and analysis of tryptophan, serotonin, melatonin and 2-HM endogenous content.

Moreover, to evaluate the effects of exogenous melatonin depending on the moment of application, strawberries at FS, DG, and IR stages were immersed for 15 seconds in either a 100 μM melatonin aqueous solution or a control solution of Milli-Q water. Both solutions contained 0.1% Tween 20 as a surfactant to enhance absorption. Each group of strawberries was treated exclusively at one of these stages, ensuring that no fruit received more than a single application. To assess the short-term biochemical response, five fruit samples, each consisting of two fruits, were collected 2 h, 4 h, and 24 h after each application. Additionally, five samples, each consisting of two fruits, were collected from all developmental stages following each moment of application, harvesting samples at the SG, BG, DG, W, IR, partial red (PR) and FR stages from fruits treated at FS; samples at W, IR, PR, and FR stages from fruits treated at DG; and samples at PR and FR stages from fruits treated at IR. This approach enabled the study of the long-term effects of melatonin application. All samplings and applications were performed at predawn (1 h before sunrise) and each harvested fruit was immediately frozen in liquid N_2 and stored at −80 °C for subsequent achene separation and biochemical and molecular analysis.

Furthermore, after treatments at FS, DG, and IR stages, the ripening rate of eight fruits was monitored daily until they reached commercial ripening using a colorimeter, as explained in subsequent sections.

Achene and receptacle separation

To separate the achenes from the receptacle of strawberries at different developmental stages, the fruits were kept completely frozen throughout the procedure. Liquid nitrogen was added to the mortar, where the separation was performed manually using tweezers and a scalpel. Afterward, the achenes were carefully collected with a spatula and stored separately from the receptacle (with no achenes) at -80 ºC until further analysis.

Color analysis

Daily monitoring was conducted on 8 fruits treated at each developmental stage (FS, DG, and IR) until harvested at commercial ripening. Colorimetric measurements were taken to assess lightness (L*), red/green coordinates (a*), yellow/blue coordinates (b*), and hue angle (h*), using a colorimeter (Konica Minolta Chroma Meter CR400-410, Tokio, Japan) and taking two measurements per fruit, one on each side of the fruit.

Quality attributes

For the estimation of organoleptic fruit quality, biomass, firmness, total soluble solids (TSS), titratable acidity (TA), and pH were evaluated at harvest (h* < 35). Biomass was determined using a precision balance. Firmness was measured with a PCE-FM200 penetrometer (PCE Group, UK) equipped with 8 mm diameter cone probe. The fruits were penetrated about 5 mm into the surface and the maximum force (N) generated was recorded during measurement. After that, fruits were processed with a mortar and pestle until juice was extracted. The juice was used to determine ºBrix per mL with a refractometer (Hanna Instruments, Italy), as described by Boulton *et al.* (1999). For pH measurement, a 1:10 dilution of the juice was made with Milli-Q water, and pH was evaluated using a pH meter (MicropH 2002, Crison Instruments, S.A., Barcelona, Spain). For TA analysis, a 1:10 dilution of the juice with Milli-Q water was used with 0.1 M NaOH and 1% phenolphthalein as an indicator to estimate the quantity of major acid in the strawberry (g citric acid ·g FW^{-1} strawberry) following the method described by Latimer (2012).

Phytohormones

Tryptophan, serotonin, melatonin, 2-HM, and ripening-related phytohormones, including indole-3-acetic acid (IAA), IAA-Aspartate (IAA-Asp), abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA), were extracted and quantified using UHPLC/ESI-MS/MS, as described by Arabia *et al.* (2025). In summary, 20 mg of ground, frozen achenes and 50 mg of receptacle were extracted with 150 µl of cold 100% methanol containing deuterium-labeled hormones as internal standards. The extracts were ultrasonicated for 30 minutes at 4 °C, followed by centrifugation at 13,000 rpm for 10 minutes at 4 °C. The supernatant was collected, and the pellet was re-extracted. Both supernatants were combined, filtered through 0.22 µm hydrophobic PTFE filters (Phenomenex, Torrance,CA, United States), and analyzed using UHPLC/ESI-MS/MS. The UHPLC system was coupled with a triple quadrupole mass spectrometer (QTRAP 4000, AB Sciex, Concord, Ontario, Canada). Solvent A consisted of

acetonitrile with 0.05% glacial acetic acid, and solvent B was water with 0.05% glacial acetic acid. The flow rate was set at 0.5 mL/min, and a LUNA C18 column (Phenomenex Inc., United States, 1.6 μm, 100 × 2.1 mm) was used. Quantification was based on the recovery rates of each sample, using deuterium-labeled internal standards. Calibration curves for each analyte were generated using MultiQuant™ 3.0.1 software.

RNA extraction

High-quality RNA was isolated from both achenes and receptacle of strawberries to investigate short- and long-term responses to melatonin application. Short responses were evaluated using samples collected 24 h after each melatonin application at the developmental stages FS, DG, and IR, while long-term responses were analyzed in fruits harvested at the PR stage following each treatment. Three biological replicates were used for every condition, with each replicate consisting of a pool of two fruits, and RNA was extracted using a modified fruit extraction protocol (Blanco-Ulate *et al.* 2013). Briefly, 50mg of achene and 200 mg of receptacle were ground in liquid nitrogen and 800uL and 1mL of pre-heated RNA extraction buffer were added to the achenes and receptacle, respectively. The RNA extraction buffer consisted of 2% (v/v) CTAB, 2% (v/v) PVP, 100 mM Tris (pH 8), 5 M NaCl, 25 mM EDTA, and 0.2 g/L spermidine and 100 μL of β-mercaptoethanol per 5 mL of buffer, added just before use. The samples were immediately incubated for 5 min at 65°C. Two extractions with one equal volume of chloroform:isoamyl alchohol (24:1, v/v) followed by centrifugation at 4000 rpm for 30 min at 4°C were performed. The supernatant was recovered and 1/10 volume of 1 M KOAc was added followed by centrifugation at 4000 rpm for 20 min at 4°C. The supernatant was collected and 1/3 volume of 8 M LiCl was added. Samples were incubated 48 h at −20 °C and then centrifuged at 4000 rpm for 45 min at 4 °C. The supernatant was discarded and the RNA pellet was further purified using the Quick-RNA™ Miniprep Kit (Zymo Research, Irvine, CA, USA). DNAse-I treatment was also done in column during the purification step. The RNA was resuspended in 50 μL of nuclease-free water. The RNA concentration and purity were measured using Invitrogen™ Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Inc) and NanoDrop Spectrophotometer.

Transcriptome analyses

RNA samples from achenes and receptacle were sent to Novogene (Novogene Co., Sacramento, CA, USA) for quality control to ensure they met RNA integrity standards, followed by library preparation and assessment. Sequencing was performed using a 150 bp

paired-end strategy on the Illumina NovaSeq platform. Raw data were obtained by sequencing, and the quality was evaluated through FastQC. High-quality clean reads were mapped to the FaRR1 (Royal Royce) reference genome using Salmon for alignment (Patro *et al.*, 2017; Hardigan *et al.*, 2021). To identify genes differentially regulated by melatonin compared to the control in each organ after application, differential expression analysis was conducted using the DESeq2 R package, and the results were visualized with ggplot2 (Love et al., 2014). P_{adj}-value < 0.05 and $|\log_2(\text{fold change})| \geq 1$ were used as threshold values to identify differentially expressed genes (DEGs) using the DESeq R package. The functional annotations of DEGs were based on annotations of the 'Royal Royce' genome (FaRR V1; https://phytozom178e- next.jgi.doe.gov/info/FxananassaRoyalRoyce_v1_0).

GO term molecular enrichment was performed using the library TopGO in R, using GO terms obtained from TAIR to explore relevant biological pathways. We searched for enrichment in each gene set relative to the complete set of genes detected in this experiment and determined enriched GO terms. Moreover, metabolic pathway enrichment analysis (KEGG) was performed to identify genes related to Melatonin Researchponses and to explore their potential functions and mechanisms. The KEGG pathway enrichment was conducted using the ClusterProfiler R package, with annotations based on the KEGG database for *Fragaria vesca* (www.genome.jp/kegg/).

Primer design

Primers for *Fragaria* × *ananassa* cv Albion were designed using the Primer3Plus platform, based on the *GH3.9* nucleotide sequence from the reference genome of *Fragaria* × *ananassa* cv Royal Royce. Before qRT-PCR, each primer was screened using the Phytozome database and BLAST to ensure specificity, and further checked for potential issues such as hairpins, dimer formation, and correct amplicon size. Additionally, the housekeeping DBP (DNA Binding Protein) primer was selected based on the work of Galli *et al.* (2015) and Schaart et al. (2002). The oligonucleotide primer sequences for the target genes are provided in Supplementary Table S1. The primers were synthesized by Integrated DNA Technologies (IDT, San Diego, California, USA).

Quantitative RT-PCR

cDNA was synthesized from RNA extracted from samples collected at 24 h and the PR stage following applications at the DG stage. Synthesis was performed using RevertAid™ RT Kit (Thermo Scientific™) according to the manufacturer's instructions. qRT-PCR was performed

on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystem™) using PowerUP™ SYBR™ Green Master mix (Applied Biosystems, Thermo Fischer Scientifics). All qRT-PCR reactions were performed with the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min with a final reaction volume of 10 µl. DBP was used as reference gene and process in parallel with the genes of interest. Primer efficiencies were calculated using 4-fold cDNA dilutions (1:8, 1:64, 1:256, and 1:2048) in duplicate as well as checking for amplification in a negative control without DNA. A 1:256 cDNA dilution was established. Specificity of the primers was checked by analyzing dissociation curves ranging from 60 to 95°C. The $2^{-\Delta\Delta CT}$ was used to normalize and calibrate transcript values relative to the endogenous constitutive gene. Data presented is from 3 three biological replicates consisting of a pool of two fruits. All samples were run per triplicate (three technical replicates were performed for each biological replicate).

Statistical analysis

A two-way analysis of variance (ANOVA) with interaction was performed for biochemical analyzed parameters, considering time and treatment as factors. Multiple comparisons were conducted using a post-hoc LSD test. Data were transformed for statistical analyses whenever necessary to achieve normal distribution and homoscedasticity of residuals; and if any transformation worked, individual nonparametric ArtAnova tests were performed. In all cases, differences were considered significant at a probability level of $p < 0.05$. All analyses were performed using RStudio (RStudio Team, 2020).

Results

Endogenous melatonin, its precursors and 2-HM exhibit temporal and organ-specific accumulation patterns

To assess variation in endogenous melatonin, its precursors, and oxidation product during strawberry fruit development, their contents in the achene and receptacle were quantified by ultrahigh-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UHPLC/ESI-MS/MS). We selected different stages across the strawberry development including fruit set (FS), small green (SG), big green (BG), degreening (DG), white (W), initial red (IR) and full red (FR) based on the size and color of the receptacle (Fig. 1). UHPLC/ESI-MS/MS analysis clearly detected distinct peaks for each compound related

to melatonin, confirming their presence and quantifiable concentrations in strawberries (Fig. 1A). Tryptophan, melatonin, and 2-HM predominantly accumulated in the receptacle, with concentrations significantly surpassing those in the achenes (Fig. 1B). In the receptacle, melatonin and 2-HM levels were three times higher than in the achenes, while tryptophan was remarkably higher, by more than 20-fold, compared to achenes. In contrast, serotonin was more abundant in the achenes, where its levels were twice as high as those found in the receptacle. Furthermore, melatonin accumulation showed a differential pattern dependent on both the developmental stage and the organ analyzed (Fig. 1B). In achenes, the highest melatonin concentrations were found in the early developmental stages, FS and SG, with levels progressively decreasing as ripening advanced. The lowest concentrations, approximately 2 ng/g DW, were observed at the onset of ripening, and these reduced levels were maintained through harvest, which were more than two-fold lower compared to the early stages. In contrast to achenes, the lowest melatonin levels were found in the early developmental stages in the receptacle. Melatonin levels increased gradually during ripening, peaking at 17 ng/g DW in the FR stage and representing a 6-fold rise compared to the FS stage.

The melatonin precursors, tryptophan and serotonin, exhibited patterns similar to melatonin (Fig. 1B). Tryptophan levels in achenes decreased significantly from the DG stage to W and remained low until FR, but increased sharply in the receptacle at the FR stage, reaching 28 times the levels in FS and 3 times those in IR. Similarly, serotonin levels mirrored melatonin with maximum levels in achenes at FS, declining thereafter, and showed a similar pattern to melatonin and tryptophan in the receptacle. Likewise, the melatonin oxidation product, 2-HM, followed the same accumulation trend as its precursors, but its levels were three times higher than those of melatonin in both achenes and receptacles (Fig. 1B).

Exogenous melatonin regulates the rate of color development during ripening in a stage-dependent manner

To determine potential roles of melatonin in strawberry ripening, we assessed the effect of exogenous melatonin application, using fruit color as a proxy for overall ripening progress. In order to quantify changes in color during ripening, we measured chromatic properties of hue angle (h*), red-green (a*), and yellow-blue (b*) of fruits treated at either the early FS stage or later in the ripening, at DG and IR stages. Melatonin treatment at the FS stage led to a general shift in the h* value, with the hue approaching red-associated angles starting 14

days after application, when the control group was at W stage. In fact, control fruits had not yet begun this transition, with the color hue approaching red occurring only after day 15. Additionally, increases in the a* component related to redness were observed in melatonin-treated fruits starting on day 14 relative to day 0. Control fruits, on the other hand, showed a significant change in redness on day 15 (Fig. 2, A-B). However, this pattern contrasted to when melatonin was applied at more advanced developmental stages, such as DG and IR, where a slower progression in color development was observed (Fig. 2, A-B). When applied at DG stage, the increase in the a* component and the shift in h* towards red hues were slower in melatonin-treated fruits compared to controls. While control fruits began to show color changes within three days after treatment, melatonin-treated fruits required two additional days to initiate the transition to red. In fact, this was the only case where a significant change in the days to transition to advanced stages of development was delayed (Fig. S1), melatonin-treated strawberries treated at DG requiring up to 2 extra days to reach IR (Fig. S1, C). Similarly, a delayed color change was observed when melatonin was applied at the IR stage. Control fruits displayed a significant increase in redness and reached the PR stage just one day after treatment, while melatonin-treated fruits required two days after application to get to the same level of development. Complementary to these findings, in fruits treated at the FS stage the L* value-drop associated with ripening onset started two days earlier in melatonin-treated fruits, while at the DG and IR stages, it was delayed by one day compared to control (Fig. S2). Additionally, higher b* values were observed in melatonin-treated fruits at both the DG and IR stages, indicating a shift towards yellower hues (Fig. S2).

At harvest, no significant changes were observed in the sugar-acid ratio (TSS/TA), pH, firmness, or biomass (Fig. 2C). Additionally, although melatonin treatment at the DG stage led to a decrease in TA, this did not impact on the TSS/TA ratio. Therefore, the progression of ripening was altered after melatonin treatments with no significant effects on the final fruit quality.

The differential response of melatonin is associated with its endogenous accumulation and its hydroxylation rate

To determine whether exogenous melatonin application at the FS, DG, and IR stages significantly increased endogenous melatonin levels in both achenes and receptacle and discern whether this could be sustained over time or immediately degraded, both melatonin and 2-HM were analyzed following the application. In all cases, the exogenous application of

melatonin increased the endogenous contents of the hormone, while a specific dynamic was found for 2-HM. Within 24 h after FS treatment, melatonin levels in achenes rose more than 200-fold compared to control fruits, reaching over 2000 ng/g DW, while in the receptacle, melatonin increased by 70-fold. After the first 24 h, melatonin concentrations in achenes decreased significantly, with a 93% reduction at the SG stage, reaching 100 ng/g DW, and further declining to 30 ng/g DW at the W stage, just before color change. In the receptacle, melatonin decreased from 300 ng/g DW to 10-40 ng/g DW at later stages, with levels similar to the control group at the color change stages. At the same time, when melatonin was applied at the FS stage, 2-HM levels in achenes slightly increased at 4- and 24-h post-application, from 15 to 25 ng/g DW, before gradually declining as ripening progressed. (Fig 3A). However, no significant 2-HM accumulation was observed in the receptacle after treatment at FS (Fig 3A).

After melatonin application at DG, endogenous melatonin increased approximately 100-fold within 4 h in achenes, surpassing 1500 ng/g DW, but slightly declined to 600 ng/g DW by 24 h. In the receptacle, melatonin increased by 17-fold. Unlike FS treatment, melatonin concentrations in achenes and receptacle remained elevated throughout the later stages, lasting until harvest, with achenes maintaining around 600 ng/g DW and receptacle concentrations of approximately 400 ng/g DW. Likewise, IR treatment resulted in a 60-fold increase in achenes at both 4 and 24 h after application, maintaining levels above 1700 ng/g DW, while melatonin in the receptacle increased by 13-fold. As observed with DG treatment, in IR-treated fruits melatonin levels remained elevated in both achenes and receptacles up until harvest, with achenes maintaining around 600 ng/g DW and receptacle concentrations ranging from 1000 to 2000 ng/g DW during the later ripening stages. Moreover, DG application resulted in a 2- to 4-fold increase in 2-HM levels in achenes, lasting from 24 h post-treatment through the FR stage, coinciding with a slight decline in melatonin levels (Fig 3B). In the receptacle, 2-HM accumulation began at the W stage (Fig 3B). In a similar trend, in fruits treated at IR stage, 2-HM levels in achenes increased progressively, peaking 20-fold higher than controls at 24 h post-application, and remained elevated through the FR stage. A less pronounced 24-h increase was also seen in the receptacle (Fig 3C).

To assess the relationship between melatonin accumulation and hydroxylation, the ratio between melatonin and 2-HM (Mel/2-HM) was evaluated. Melatonin application at the FS stage enhanced the Mel/2-HM ratio in both achenes and receptacle during the first 24 h, effectively reducing melatonin degradation (Fig.4, S3A). However, from the SG stage

onwards, the ratio declined, reflecting increased melatonin degradation in both organs, with receptacle levels eventually resembling the control (Fig.4, S3A). After application of fruits at DG stage, the Mel/2-HM ratio increased significantly, particularly at 2- and 4-h post-application (Fig.4, S3B). In achenes, the ratio decreased 8-fold by 24 h but remained stable, higher than control fruits, until harvest. No significant differences were observed in the receptacle beyond 24 h (Fig.4, S3B). After application of fruits at IR, melatonin degradation slowed at 2 and 4 h in achene and receptacle, with a higher Mel/2-HM ratio, but degradation increased afterward, returning to control levels (Fig.4, S3C).

In addition to melatonin and 2-HM, we measured the changes of plant hormones ABA, IAA, SA, and JA, which are critical for regulating fruit ripening and stress responses during development. A slight increase in JA levels was observed in the receptacle at the SG stage after application at FS, as well as an increase in SA during the first 24 h in the control fruits (Fig. S4). However, no consistent and significant changes were observed in the contents of these hormones following melatonin application at the FS, SG, or IR stages (Fig. S4, S5). In fact, rather than total hormonal increases, a shift of the endogenous balance in IAA composition was found as further detailed in the following section.

Transcriptional reprogramming upon melatonin application in both achene and receptacle

To identify the short- and long-term transcriptional changes induced by melatonin treatment at FS, DG, and IR stages, differentially expressed genes (DEGs) were analyzed relative to control fruits at two key time points: 24 h post-application and a later ripening stage, PR (Fig. 5A, S6). At FS, 20 DEGs were upregulated and 16 downregulated in achenes, while only 1 DEG was upregulated and 4 downregulated in the receptacle. At 24 h, no DEGs were detected in the receptacle, while in achenes, 8 DEGs were upregulated and 5 downregulated. (Fig. S6A). In contrast, melatonin application at the DG stage induced an immediate response, with 81 upregulated and 9 downregulated DEGs in achenes, and 61 upregulated and 78 downregulated DEGs in the receptacle at 24 h (Fig. 5, B-C). At the PR stage, changes were minimal, with only 4 DEGs in receptacles and 1 in achenes. Similarly, application at IR showed immediate effects, particularly in receptacle at 24 h, with 13 upregulated and 45 downregulated DEGs (Fig. S6B)

When melatonin was applied at the DG stage, which showed the most pronounced response with the highest number of DEGs, we identified *Gretchen Hagen 3* (*FaGH3.9*) as a key gene

downregulated in both achenes and receptacle 24 h post-application (Fig. 5, C-D). *GH3.9* encodes an indole-3-acetic acid-amido synthetase involved in IAA conjugation with amino acids. It was located on chromosome 2C and downregulated 3.29-fold in achenes and 3.34-fold in receptacle (Fig. 5D). Furthermore, KEGG pathway analysis revealed significant enrichment of DEGs in the 'Plant hormone signal transduction' pathway, while GO analysis highlighted the enrichment of molecular functions related to 'auxin efflux' and 'transmembrane transporter activity' in the receptacle 24h after melatonin application at DG. This is consistent with the downregulation of an auxin efflux carrier gene in the receptacle 24 h post-application, alongside the previously identified *FaGH3.9* (Fig. 5D). Expression analyses by RT-qPCR further confirmed the downregulation of *FaGH3.9* in the achene and receptacle (Fig. 6A and S7). These molecular findings align with the biochemical observations, where a 15% reduction in IAA-Asp levels was detected in the receptacle compared to the control, accompanied by an increase in melatonin content 24 h after application at the DG stage. While no significant changes were observed in free IAA levels, this alteration in IAA-Asp levels resulted in a 30% higher IAA/IAA-Asp ratio in melatonin-treated strawberries (Fig. 6B and 7).

Discussion

In this study, the developmental and organ-specific distribution of melatonin in a non-climacteric fruit model like strawberry was characterized by analyzing its accumulation patterns, along with those of its precursors and hydroxylation product. Besides, melatonin fruit-organ functionality and action window were dissected, highlighting a strong relationship with the physiological developmental stage and auxin-, thereby updating the role of melatonin in fruit ripening at the molecular level.

Melatonin endogenous spatiotemporal distribution gives us hints of its role as a ripening inhibitor molecule

The analysis of biomolecules at endogenous tissular level is useful to determine their temporality and whether their mode-of-action requires specific environmental conditions to trigger developmental processes. Melatonin studies in fruit ripening have focused on whole fruit analysis after exogenous applications (Hu *et al.*, 2017; Wang *et al.*, 2020; Mansouri *et al.*, 2023) but little is known about the endogenous distribution of this molecule over time, and even less during *in planta* ripening, which could directly affect interaction with other

biomolecules and its putative signaling role. In achenes, melatonin levels were highest during the early green stages, from FS to BG, before rapidly declining before ripening onset. In contrast, melatonin levels in the receptacle showed a marked increase during the last stage of development, peaking at fruit commercial ripening (Fig. 1). These two organs are very different in terms of their origin, physiological role and associated metabolic network; the relative contribution of each to the whole berry is highly dependent on the development stage of the fruit (Cherian *et al.*, 2014). The achene's metabolic program prioritizes the accumulation of storage and protective compounds, along with precursors for plant hormones and defense compounds, to maintain seed quiescence, support extended pre-germination periods and regulate seed development. Indeed, correlation network analysis reveals that metabolism in the achenes is well-coordinated, particularly in early development, while metabolic processes in the receptacle show dynamic fluctuations either in early or late stages (Fait *et al.*, 2008). The high melatonin levels observed in the early stages of development suggest a key role in regulating the growth processes during these initial phases. Its accumulation pattern in the achenes is like that previously described for IAA (Jang *et al.*, 2024). Consistent with this pattern, we observed that levels of tryptophan increased during the early stages of achene development but rapidly decreased before ripening (Fig. 1). Therefore, considering that both melatonin and IAA are derived from tryptophan, their structural similarity, and the parallel accumulation pattern of melatonin to tryptophan and IAA in the achenes, it is likely that endogenous melatonin is playing a role in negatively regulating ripening. On the other hand, considering the pattern of its essential precursor, serotonin, is probably responsible for the decrease observed in melatonin in the late stages of development, which could be linked to substrate limitations. To our knowledge, only one study evaluated the temporal accumulation of melatonin in grape seeds during on-tree development for a brief period (just 5 days after ripening onset) and while there was a slight variation in accumulation increasing over time, the variation range was of less than 2 ng/gFW (Gao *et al.*, 2024). Taken together, it appears that there could be limitations in melatonin accumulation in seeds after the fruit ripening onset.

We observed great spatiotemporal differences in endogenous melatonin accumulation during fruit ripening. While melatonin content decreased during strawberry ripening in achenes, a significant rise in melatonin levels was observed towards the end of receptacle development. Reduction in endogenous melatonin during fruit ripening in the achenes suggest an inhibitory role for this compound, while increases in the receptacle could be linked to the growing redox

demands and increased metabolic activity at later ripening stages associated with fruit respiration, softening, and cell wall loosening (Kumar *et al.*, 2016; Lombardo *et al.*, 2011). In fact, melatonin levels were 4-times higher in the receptacle at FR than in the achenes at FS, suggesting a physiological specialization. These results partly contrast with the findings of Mansouri *et al.* (2021) where a peak of melatonin was reported at the IR stage, with levels gradually decreasing as the fruit ripened. Nevertheless, it is important to notice that their study was conducted on whole strawberries, and the inherent heterogeneity between the achenes and receptacle may have implied a bias. Furthermore, their analysis was based on fresh weight, whereas this study utilized dry weight to minimize potential errors caused by water content variations across different organs and developmental stages, which could otherwise lead to inaccurate estimations of melatonin levels. Taken together, the results of the present study suggest that melatonin is required for the normal development of strawberry acting organ-specifically to stimulate growth in the early stages of achenes and to provide protection in the fully ripened red receptacle, thereby implying an organ-specific functionality.

At the same time, related to differential accumulation of melatonin and total contents, this study has, for the first time, reported the accumulation pattern of 2-HM throughout strawberry development. In fact, although melatonin has been detected in fruits and its function based on content accumulation discussed in multiple studies, the balance between synthesis and degradation is largely unexplored. In the present experiment, 2-HM accumulation in strawberry fruits was highly dependent on melatonin concentration-dependent dynamics and organ-specific distribution. Interestingly, its levels were higher in both achene and receptacle compared to those of melatonin, consistent with the findings of Byeon *et al.* (2015) and Back *et al.* (2021), which demonstrated the predominant accumulation of 2-HM in plants. These results raise important questions about the potential biological role of 2-HM in fruit development and ripening, which remains to be fully understood. Likewise, the balance between biosynthesis and hydroxylation may be key to fully understanding the pleiotropic effects associated with melatonin in fruits.

Melatonin dependency on fruit color acquisition is directly related to the physiological developmental stage and fine-tuned hydroxylation

Exogenous melatonin induced distinct phenotypes depending on the strawberry developmental stage at the time of application. While application at the FS stage accelerated color acquisition, at later growth stages, such as DG (prior to color change) and IR (at the

onset of ripening), melatonin exhibited the opposite effect, slowing ripening progression (Fig. 2). In comparison to our study, Mansouri *et al.* (2021) found no significant effects with 100 µM melatonin at BG stage, but 10 µM delayed ripening. However, their application method involved injection, while in our study, 100 µM melatonin was applied via dipping, which may have triggered differential pathways. Furthermore, the same research also stated that 1000 µM melatonin promoted strawberry ripening, exhibiting dose-dependent effects, likely due to high concentrations inducing a cellular stress response, such as an antioxidant reaction, rather than a direct hormonal effect. Likewise, Ruiz-Arcacil *et al.* (2024) demonstrated that the effect of melatonin on color hue acquisition was highly dependent on seasonality. In our study, the difference in phenotypes was associated with a differential accumulation of melatonin and its conversion to 2-HM (Fig. 3 and 4). When melatonin was applied at the FS stage, its levels showed a sharp increase, especially in the achenes. However, within 24 h after application in FS, melatonin levels began to decline significantly and continued to decrease until the end of ripening. The progressive decline was not accompanied by a corresponding accumulation of 2-HM, suggesting that melatonin was not being converted into the hydroxylated product. In contrast, when melatonin was applied at the DG or IR stages, its levels remained consistently higher before the onset of ripening and stayed elevated throughout color change and advanced ripening, compared to the lower levels observed after application at the FS stage. This difference could also explain the delayed color acquisition observed with DG and IR applications but not in FS, potentially reflecting melatonin's role as an endogenous ripening inhibitor. Moreover, after DG and IR applications, a slight melatonin decline was observed after 24 h, which, unlike in the FS stage, was accompanied by a significant accumulation of 2-HM, particularly in the achenes. This suggests that during these later stages, melatonin metabolism shifts towards oxidation producing 2-HM. Notably, 2-HM has been shown to perform melatonin-like functions, such as mitigating oxidative stress caused by abiotic factors (Lee and back, 2019; Shah *et al.*, 2020, Korkmaz *et al.*, 2023). However, 2-HM has also been suggested to promote the production of reactive oxygen species (ROS) and inducing senescence in Arabidopsis (Lee and Back, 2021). The physiological function of 2-HM has only begun to be explored and clearly more remains to be discovered. This study demonstrates 2-HM detection across the various organs of the whole strawberry and it is proposed that the rate of hydroxylation could have an impact in ripening, underscoring the intricate, stage-specific and fine-tuned balance between melatonin and its metabolites in regulating fruit physiology and development.

Melatonin delays ripening through an interplay with auxin in strawberries

The interplay of melatonin with developmental and ripening-related phytohormones has been extensively revised (Arnao and Hernández-Ruiz, 2018; Sun *et al.*, 2021). In the present experiment, melatonin did not induce any significant variation in the content of the main ripening-related phytohormones controlling strawberry ripening, contrasting with prior results where exogenous applications of melatonin induced ABA or JA production (Xu *et al.*, 2018; Mansouri *et al.*, 2021; Arabia *et al.*, 2022), but induced a control in the hormonal balance of IAA through amino acid conjugation. Indeed, the results obtained in strawberries are consistent with melatonin being a fruit ripening inhibitor relying on the activation of an additional regulator to exert its function.

To gain a deeper understanding of molecular mechanisms underlying melatonin effects on strawberry development and ripening, a transcriptomic analysis was conducted to identify gene expression changes. In this study, at the FS stage, melatonin application led to long-term transcriptomic changes in the achenes, which were consistent with the accelerated ripening observed up to 15 days after treatment (Fig. S6A). In contrast, melatonin application at the DG and IR stages resulted in short-term responses, with most significant changes occurring 24 h post-application rather than at later stages like PR (Fig. 5B and S6B). Melatonin application at the DG stage resulted in the highest number of DEGs in achenes and receptacle, compared to the IR stage, which exhibited greater phenotypic variability, likely due to its short duration and fast metabolic changes. Likewise, DG seems to represent a turning point at which melatonin levels may be particularly influential since applications at this stage increased and maintained melatonin contents with a differential metabolization of exogenous melatonin. In fact, when applications were performed at DG stage, melatonin downregulated the *FaGH3.9* gene in both achenes and receptacle, which encodes IAA-amido synthetase, further emphasizing its impact on auxin metabolism and signaling before ripening onset (Fig. 5, C-E). It has long been known that achene-derived auxin is the key phytohormone controlling the growth and ripening of strawberry receptacles. Auxin promotes receptacle expansion during development, and as the fruit matures, a decrease in its levels helps initiate ripening by activating related genes (Aharoni *et al.*, 2002). Regulating hormone levels involves not only biosynthesis but also catabolism and conjugation. The GH3 protein family plays a crucial role in maintaining auxin homeostasis by conjugating auxins, providing a negative feedback mechanism that ensures low free IAA concentrations during ripening. These proteins have been found at high levels at fruit maturity in several fruit-bearing species (Böttcher *et al.*,

2010), and high levels of IAA-Asp and IAA-Glu have been detected specifically in achenes at IR and FR stages (Gu *et al.*, 2019). A direct biological function for IAA-Asp and IAA-Glu has not been clearly demonstrated, and these conjugates are rather considered as the starting point of auxin catabolism. In our study, the downregulation of the *FaGH3.9* gene was associated with a decrease in IAA-Asp levels. Although no increase in free IAA was observed, the IAA/IAA-Asp ratio was enhanced (Fig. 6), which might modulate auxin signaling pathways and further influence delaying ripening. In line with our findings, other studies have also shown that melatonin influences *GH3* gene expression. For instance, exogenous melatonin enriched and upregulated auxin biosynthesis and signaling pathways, including GH3-like genes, in soybean and Arabidopsis (Weeda *et al.*, 2014; Wei *et al.*, 2022). Additionally, melatonin has been shown to alter *GH3* expression in response to abiotic stress, downregulating it in soybean under alkaline stress (Duan *et al.*, 2024) and upregulating it in rice and grapes under root hypoxia and salt stress, respectively (Liu *et al.*, 2023; Zhang *et al.*, 2024). Furthermore, while *GH3* expression has been shown to be influenced by hormones like ethylene and ABA during the ripening of capsicum fruits, grapes and tomatoes (Liu *et al.*, 2005; Böttcher *et al.*, 2010; Kumar *et al.*, 2012), this study is the first to reveal that melatonin can also induce downregulation of *GH3* expression during strawberry ripening (Fig. 7).

Conclusions

Regulation of fruit ripening constitutes an important tool in fruit production, in particular color acquisition since it conditions temporality to reach the commercial market. By taking an integrated approach, the present study demonstrated that melatonin can actively function as a ripening regulator to delay color acquisition in strawberry fruits during *in planta* development and after exogenous applications. Melatonin effects were dependent on its balanced accumulation and specific localization within the fruit, which reinforces the idea that melatonin exerts its effects in a context-dependent manner that illustrates the contrasted effects reported in literature and provides powerful information for biotechnological purposes. It is also demonstrated that one must consider that an external melatonin supply, especially at specific developmental stages, may disrupt the plant's metabolic balance. This is concluded after showing particular dynamics between melatonin and its hydroxylated product during fruit development, as well as the re-programming of achenes after melatonin applications. Finally, we conclude that melatonin exerts an inhibitory ripening function in

strawberry fruits by regulation of auxin-related genes, such as *GH3.9*. Taking all together, we conclude that melatonin can modulate ripening dynamics, this is dependent on the regulation of auxin-related genes and it is subject to the physiological stage of the fruit. Therefore, fruit pre-condition is essential to determine biotechnological applications of melatonin and should be considered in agricultural practices.

Supplementary data

Materials on primers used for qPCR analysis and complementary data on days for ripening transition as well as phytohormonal changes after melatonin applications.

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Author contributions

AA, SMB and PM were responsible for conceptualization, writing the original draft, reviewing and editing. AA, SMB, MJ.F and SJK were responsible for funding acquisition. AA, PM, MB, MJ.F and SJ.K were responsible for methodology. AA, PM and MB were responsible for formal analysis. SMB was responsible for project administration. All authors revised the final version of the manuscript.

Conflict of Interest

All authors declare no conflicts of interest related to the work presented in the manuscript.

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Figure legends

Figure 1. (A) Chromatogram of tryptophan, serotonin, melatonin, and 2-HM (from top to bottom) detected via UHPLC-MS in strawberry achenes at the SG stage, showing their respective retention times. (B) Endogenous concentrations of tryptophan, serotonin, melatonin, and 2-HM in achenes and receptacle at different developmental stages of strawberries. Data are presented as the mean ± SE of n = 5. Letters denote statistical differences within each plot (p <_0.05), from one-way ANOVA followed by LSD post-hoc test. FS, Fruit Set; SG, Small Green; BG, Big Green; DG, Degreening; W, White; IR, Initial Red; FR, Full Red.

Figure 2. (A) Effect of melatonin treatment at FS, DG, and IR stages on strawberry ripening, showing changes in ripening rate and color over time post-treatment. (B) Redness (a*) and hue angle (h*) of strawberries treated with control or melatonin- at FS (green arrow), DG (violet arrow) and IR (orange arrow) during fruit growth and ripening. Data are presented as the mean ± SE of n = 8. NS indicates no significant differences (two-way ANOVA, $P > 0.05$). Upward arrows indicate the days at which significant differences were observed in the control (blue) and melatonin-treated fruits (red) compared to day 0 of each treatment. The developmental stage corresponding to the days after application was determined in relation to the control. (C) Quality parameters at harvest of control and melatonin-treated strawberries at FS, DG and IR stages. Individual nonparametric ArtAnova tests were performed. DAA, Days After Treatment; FS, Fruit Set; SG, Small Green; BG, Big Green; DG, Degreening; W, White; IR, Initial Red; PR, Partial Red; FR, Full Red; Total soluble solids, TSS; Total Acidity, TA.

Figure 3. Endogenous levels of melatonin and 2-hydroxymelatonin in strawberries following melatonin application at (A) FS, (B) DG, and (C) IR stages, measured during the first 2h, 4h, and 24h post-application to assess the short-term response, and at subsequent developmental stages until commercial maturity to evaluate the long-term response. Data are presented as mean ± SE of n = 5. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences by LSD posthoc test. FS, Fruit Set; SG, Small Green; BG, Big Green; DG, Degreening; W, White; IR, Initial Red; PR, Partial Red; FR, Full Red

Figure 4. (A) The ratio Mel/2-HM serves as an indirect indicator of melatonin degradation, where lower values suggest higher degradation and higher values suggest lower degradation. (B) Degradation of melatonin expressed as the Mel/2-HM ratio in strawberries after each melatonin application at FS, DG, and IR stages, relative to control fruits. Data reflect

measurements during the first 2h, 4h, and 24h post-application to assess the short-term response, and across subsequent developmental stages until reaching commercial maturity to evaluate the long-term response. Orange colors indicate lower degradation levels (higher Mel/2-HM ratio), while blue shades represent higher degradation levels (lower Mel/2-HM ratio). Asterisk (*) indicates significant differences by LSD posthoc test. The numerical values corresponding to the data in this figure are provided in the supplementary material (Figure S2). Melatonin, Mel; 2-hydroxymelatonin, 2-HM.

Figure 5. (A) Schematic representation of the sampling time points for transcriptomic analysis following melatonin application at the DG stage, showing short-term (24 h post-application) and long-term (PR stage) responses in both achene and receptacle. (B) Volcano plots showing DEGs in the achenes and receptacle at 24 h post-application and at the PR stage. (C) Venn diagram illustrating the overlap of DEGs in the achene and receptacle at 24 h post-application and at the PR stage. (D) Table summarizing downregulated auxin-related genes in the achene and receptacle 24 h post-application. (E) KEGG metabolic pathway and GO molecular function analysis of all DEGs in the receptacle 24 h post-application.

Figure 6. (A) Expression analysis of *FaGH3.9* in the receptacle of strawberries 24 h after melatonin application at the DG stage. Asterisk (*) indicates significant differences between control and melatonin-treated fruits at $P < 0.05$ (two-sided t-test). (B) Content of melatonin, indole-3-acetic acid (IAA), IAA-Aspartate (IAA-Asp), and the IAA/IAA-Asp ratio in the receptacle of strawberries 24 h after melatonin application at the DG stage. NS indicates no significant differences ($P > 0.05$, two-sided t-test)

Figure 7. Exogenous melatonin application at the DG stage delays fruit ripening by slowing the color acquisition process. It is suggested that exogenous melatonin increases endogenous melatonin levels in both achenes and receptacle, which downregulates auxin-related genes, including *FaGH3.9*, thereby acting as a ripening inhibitor.

Figure 1

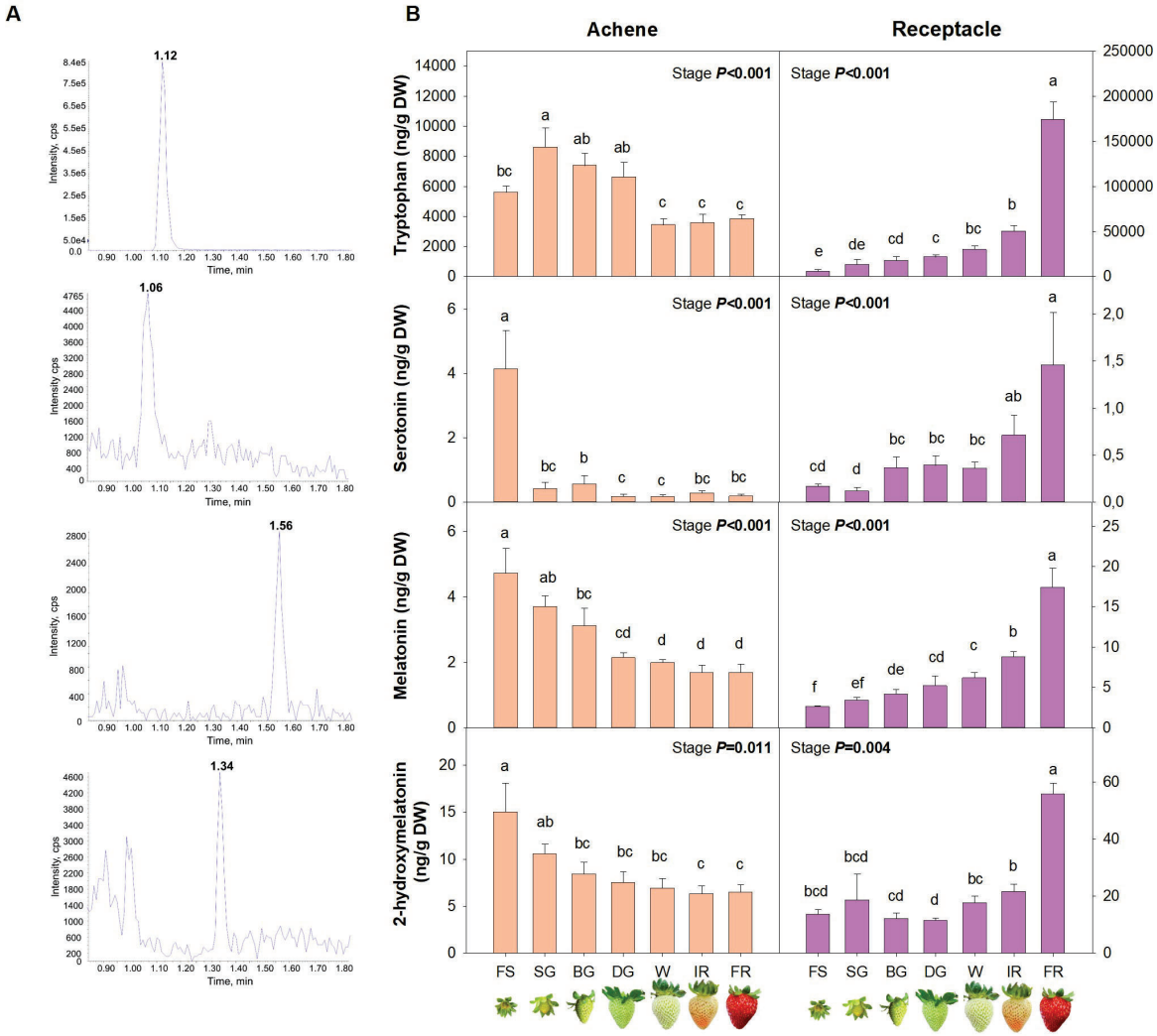

A
1.12
1.06
1.56
1.34
Intensity, cps
Time, min
B
Achene
Receptacle
Tryptophan (ng/g DW)
Serotonin (ng/g DW)
Melatonin (ng/g DW)
2-hydroxymelatonin (ng/g DW)
Stage P<0.001
Stage P=0.011
Stage P=0.004
FS
SG
BG
DG
W
IR
FR

Figure 2

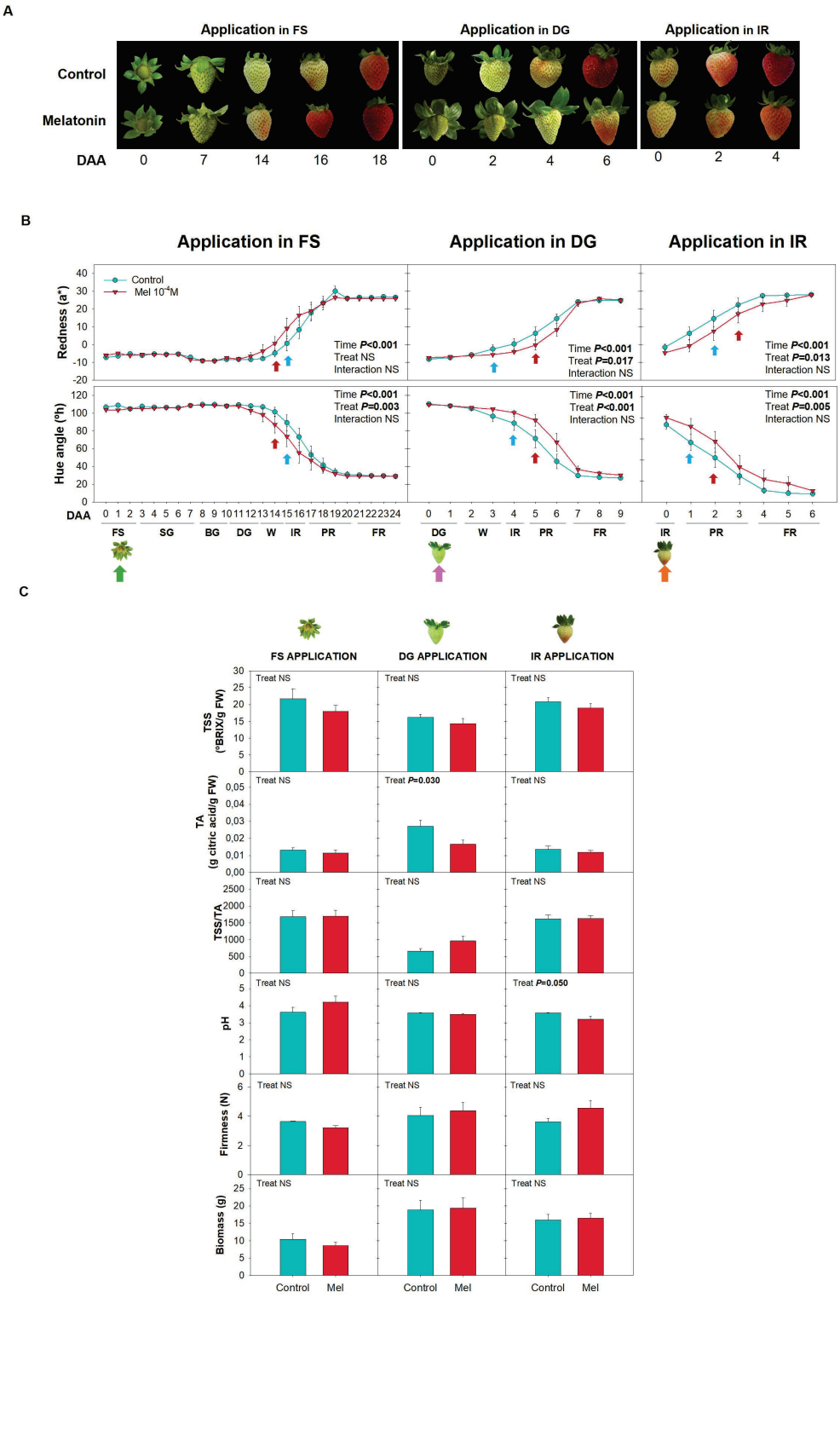

A
Application in FS
Application in DG
Application in IR
Control
Melatonin
DAA
0 7 14 16 18
0 2 4 6
0 2 4
B
Application in FS
Application in DG
Application in IR
Control
Mel 10⁻⁴M
Redness (a*)
Hue angle (°h)
Time P<0.001
Treat NS
Interaction NS
Time P<0.001
Treat P=0.017
Interaction NS
Time P<0.001
Treat P=0.013
Interaction NS
Time P<0.001
Treat P=0.003
Interaction NS
Time P<0.001
Treat P<0.001
Interaction NS
Time P<0.001
Treat P=0.005
Interaction NS
DAA
FS SG BG DG W IR PR FR
DG W IR PR FR
IR PR FR
C
FS APPLICATION
DG APPLICATION
IR APPLICATION
TSS (°BRIX/g FW)
TA (g citric acid/g FW)
TSS/TA
pH
Firmness (N)
Biomass (g)
Treat NS
Treat P=0.030
Treat P=0.050
Control
Mel

Figure 3

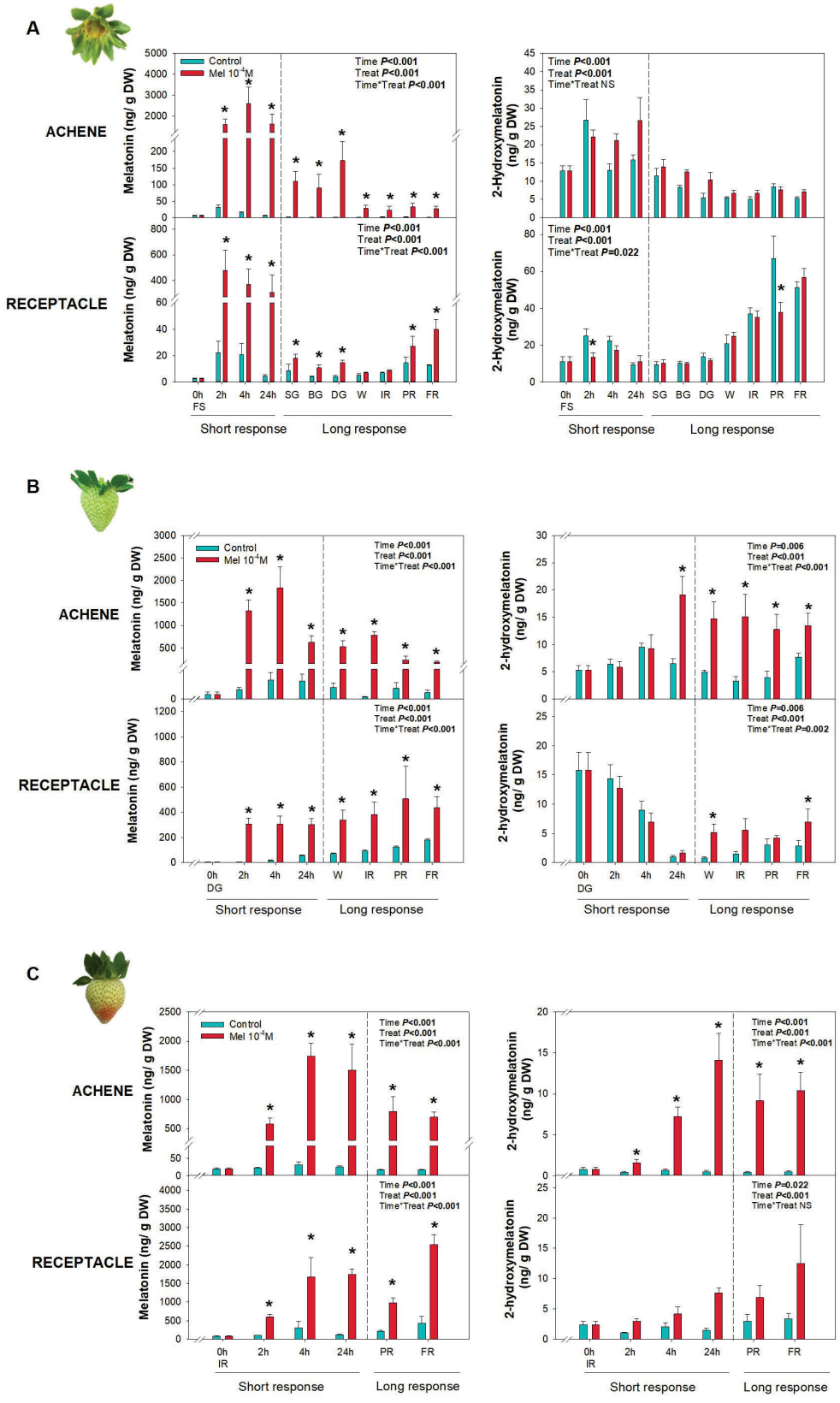

A
ACHENE
RECEPTACLE
Control
Mel 10-4M
Melatonin (ng/ g DW)
2-Hydroxymelatonin (ng/ g DW)
Time P<0.001
Treat P<0.001
Time*Treat P<0.001
Time*Treat NS
Time*Treat P=0.022
0h FS
2h
4h
24h
SG
BG
DG
W
IR
PR
FR
Short response
Long response
B
ACHENE
RECEPTACLE
Time P=0.006
Time*Treat P=0.002
0h DG
C
ACHENE
RECEPTACLE
Time P=0.022
0h IR

Figure 4

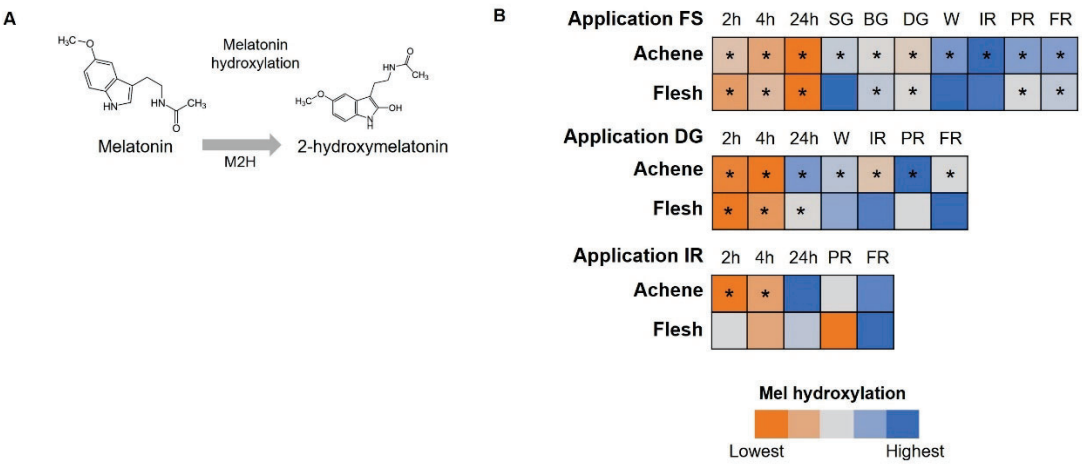
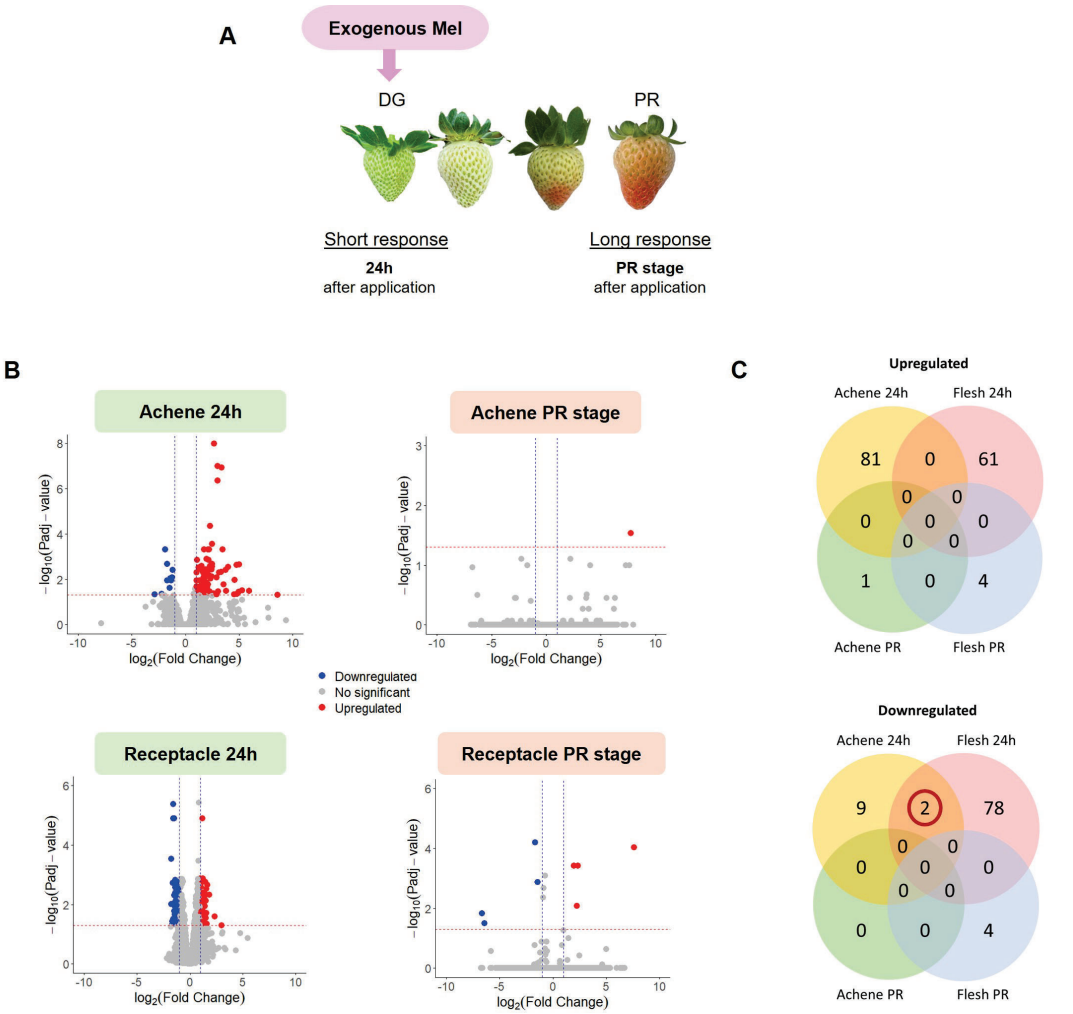

A
Melatonin hydroxylation
Melatonin
M2H
2-hydroxymelatonin
B
Application FS
2h 4h 24h SG BG DG W IR PR FR
Achene
Flesh
Application DG
2h 4h 24h W IR PR FR
Achene
Flesh
Application IR
2h 4h 24h PR FR
Achene
Flesh
Mel hydroxylation
Lowest
Highest

Figure 5



D

	Organ	Gene ID	Function	Locus	Normalized Control counts	SE Control counts	Normalized Mel counts	SE Mel counts	Log_2 Fold Change	P adj
24h after application	ACHENE	**Fxa2Cg203283**	Indole-3-acetic acid-amido synthetase GH3.9	chr_2C:25402507-25409467	135.7	29.9	40.7	6.4	-1.72	0.011
	FLESH	**Fxa2Cg203283**	Indole-3-acetic acid-amido synthetase GH3.9	chr_2C:25402507-25409467	315.3	64.0	94.0	22.8	-1.74	<0.001
		Fxa5Dg201430	Auxin efflux carrier	chr_5D:9443239-9450322	215.9	41.7	94.5	11.1	-1.19	0.008

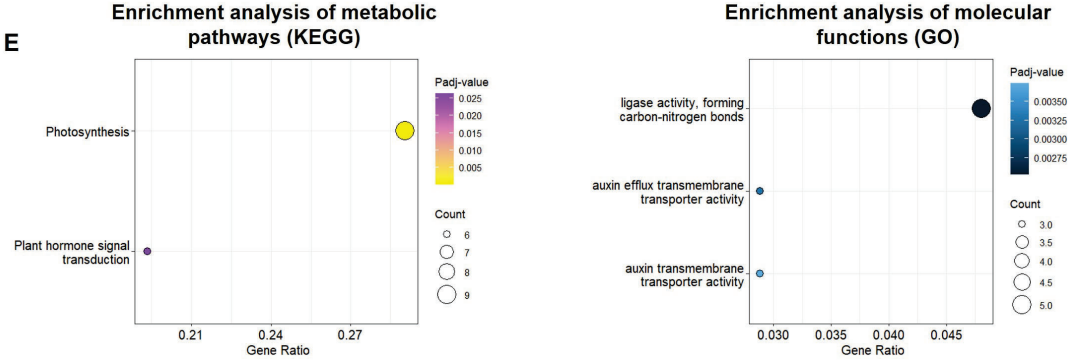


Figure 6

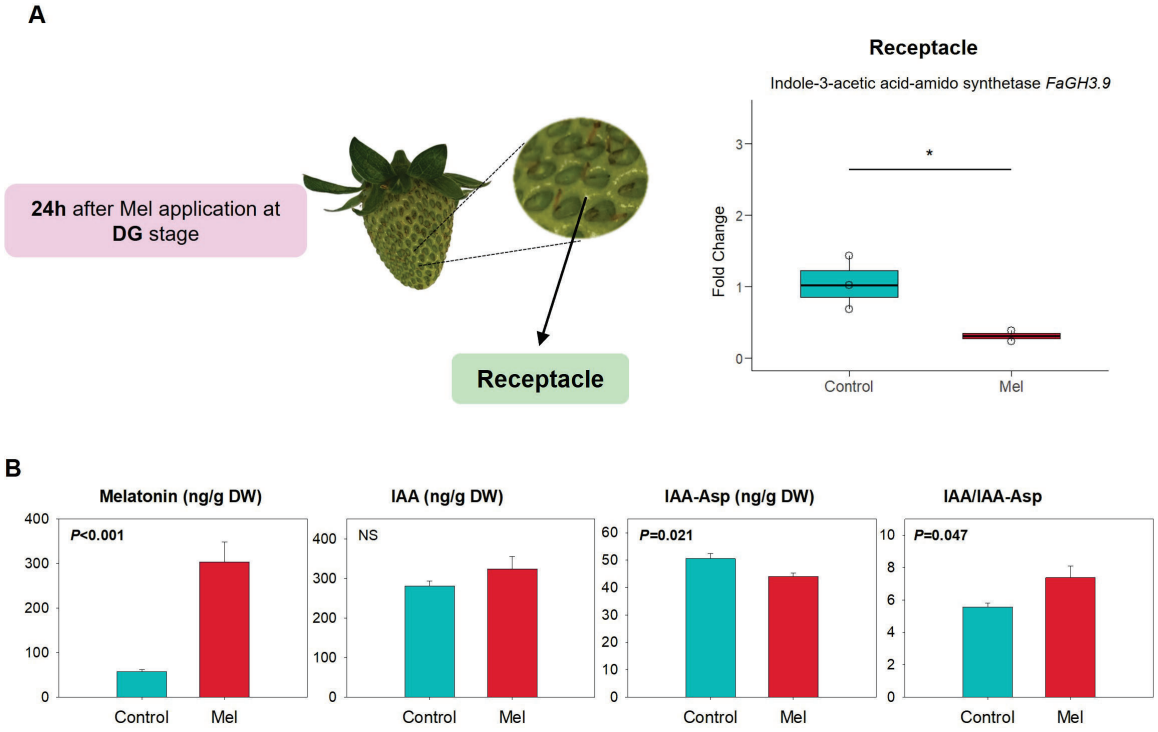

A
24h after Mel application at DG stage
Receptacle
Receptacle
Indole-3-acetic acid-amido synthetase FaGH3.9
Fold Change
*
Control
Mel
B
Melatonin (ng/g DW)
P<0.001
IAA (ng/g DW)
NS
IAA-Asp (ng/g DW)
P=0.021
IAA/IAA-Asp
P=0.047
Control
Mel

Figure 7

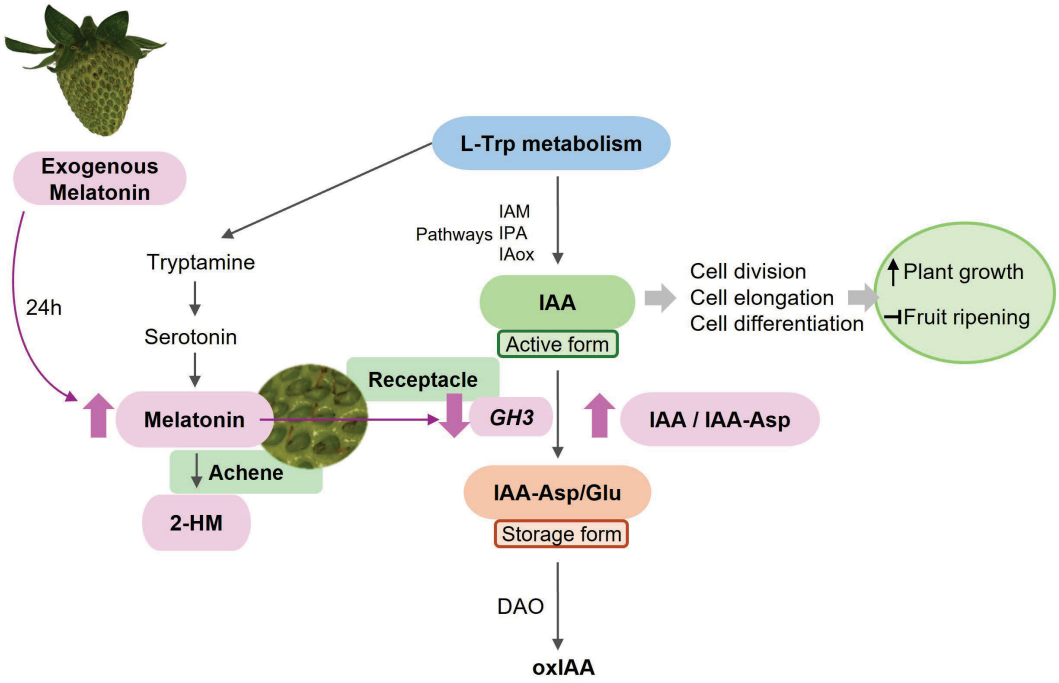

Exogenous Melatonin
L-Trp metabolism
IAM
Pathways IPA
IAox
Tryptamine
24h
Serotonin
IAA
Active form
Cell division
Cell elongation
Cell differentiation
Plant growth
Fruit ripening
Receptacle
Melatonin
GH3
IAA / IAA-Asp
Achene
2-HM
IAA-Asp/Glu
Storage form
DAO
oxIAA

Table S1. Primers used for qPCR analysis.

Gene name	Sequence	Tm (ºC)	Product size	Reference
FaGH3.9 Fxa2Cg203283_Fw (5'-3')	CCGAAAATCCATGGCTAAAA	59.9	213	-
FaGH3.9 Fxa2Cg203283_Rv (3'-5')	ATGGTGCCGACTAATGGAAG	60		-
*FaDBP*_Fw	TTGGCAGCGGGACTTTACC	60	72	Galli et al., 2015; Schaart et al., 2002
FaDBP_Rv	CGGTTGTGTGACGCTGTCAT	60	72	Galli et al., 2015; Schaart et al., 2002

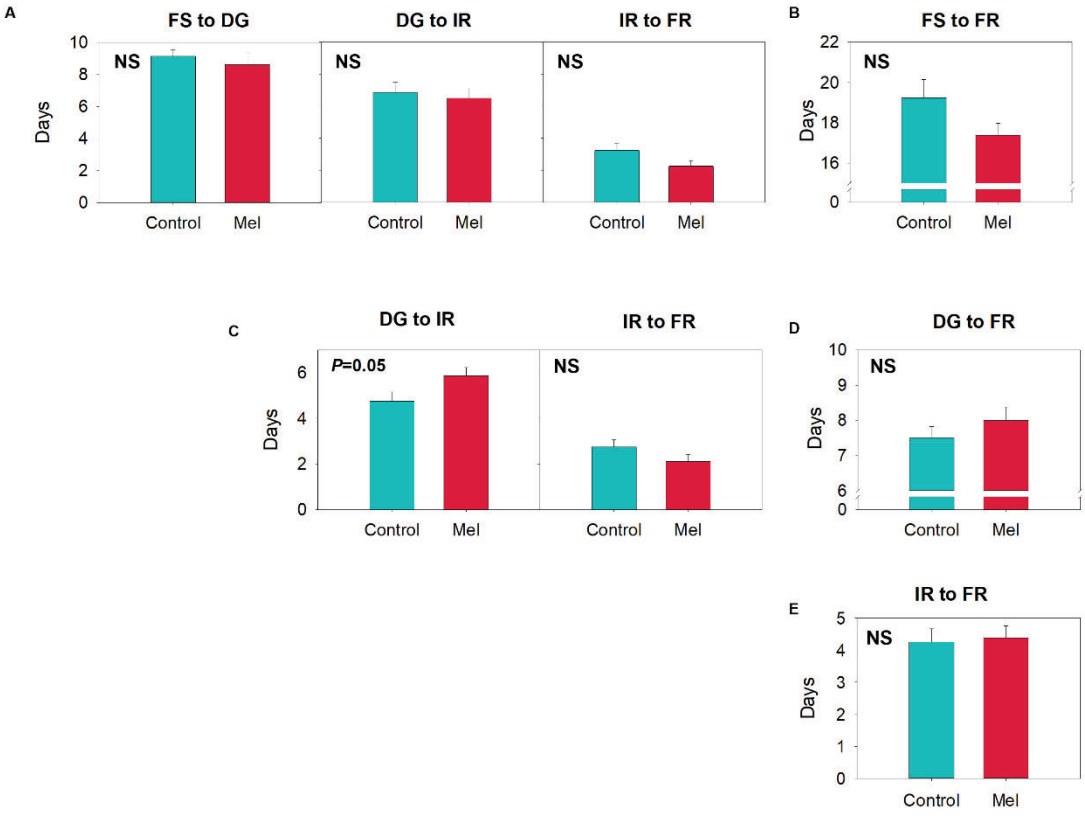


Fig. S1. Days to transition through developmental stages after melatonin application in fruit set (FS) (A-B), degreening (DG) (C-D) and initial red (IR) (E). Data are presented as the mean ± SE of n = 8. NS indicates no significant differences (P > 0.05). The developmental stage linked to the days after application was established in comparison to the control.

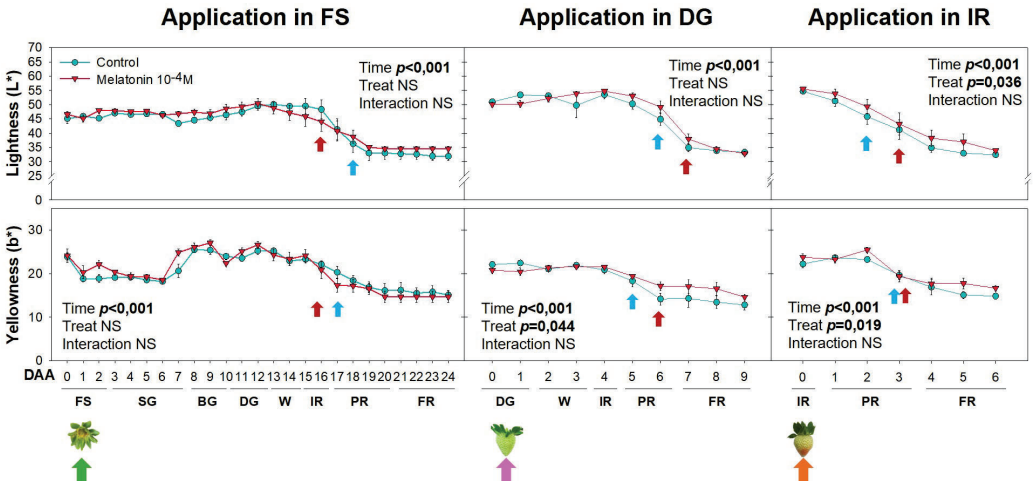


Fig. S2. Lightness (L*) and yellowness (b*) of control and melatonin-treated strawberries at FS (green arrow), DG (violet arrow) and IR (orange arrow) during fruit growth and ripening. Data are presented as the mean ± SE of n = 8. NS indicates no significant differences (P > 0.05). Upward arrows indicate the days from which significant differences were observed in the control (blue) and melatonin-treated fruits (red) compared to day 0 of each treatment. The developmental stage linked to the days after application was established in comparison to the control. FS, Fruit Set; SG, Small Green; BG, Big Green; DG, Degreening; W, White; IR, Initial Red; PR, Partial Red; FR, Full Red.

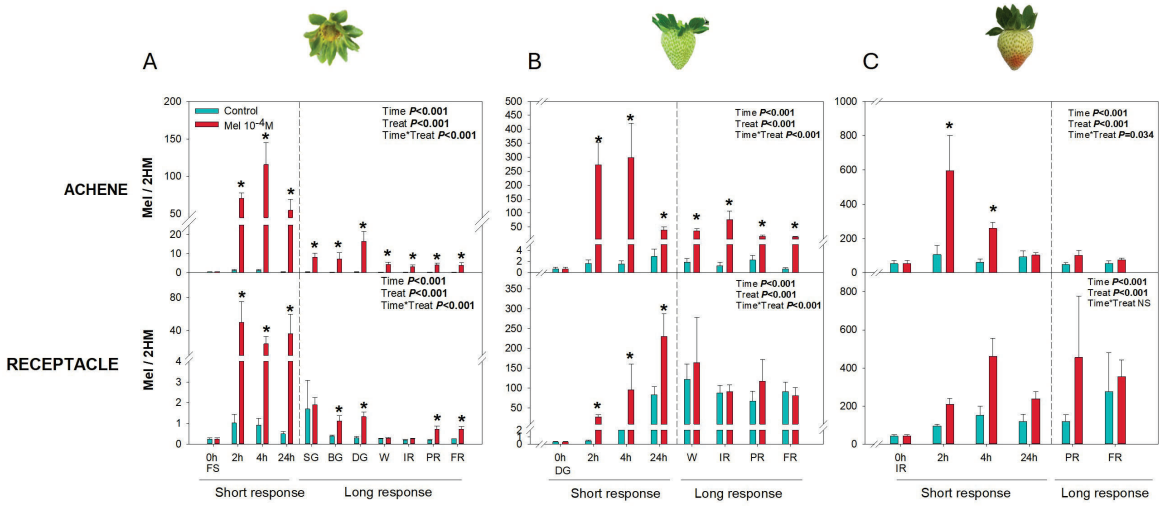


Fig. S3. Melatonin/2-hydroxymelatonin ratio (Mel/2HM) in strawberries following melatonin application at (A) FS, (B) DG, and (C) IR stages, measured during the first 2h, 4h, and 24h post-application to assess the short-term response, and at subsequent developmental stages until commercial maturity to evaluate the long-term response. Data are presented as mean ± SE of $n = 5$. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences at $P < 0.05$ in the posthoc test. FS, Fruit Set; SG, Small Green; BG, Big Green; DG, Degreening; W, White; IR, Initial Red; PR, Partial Red; FR, Full Red.

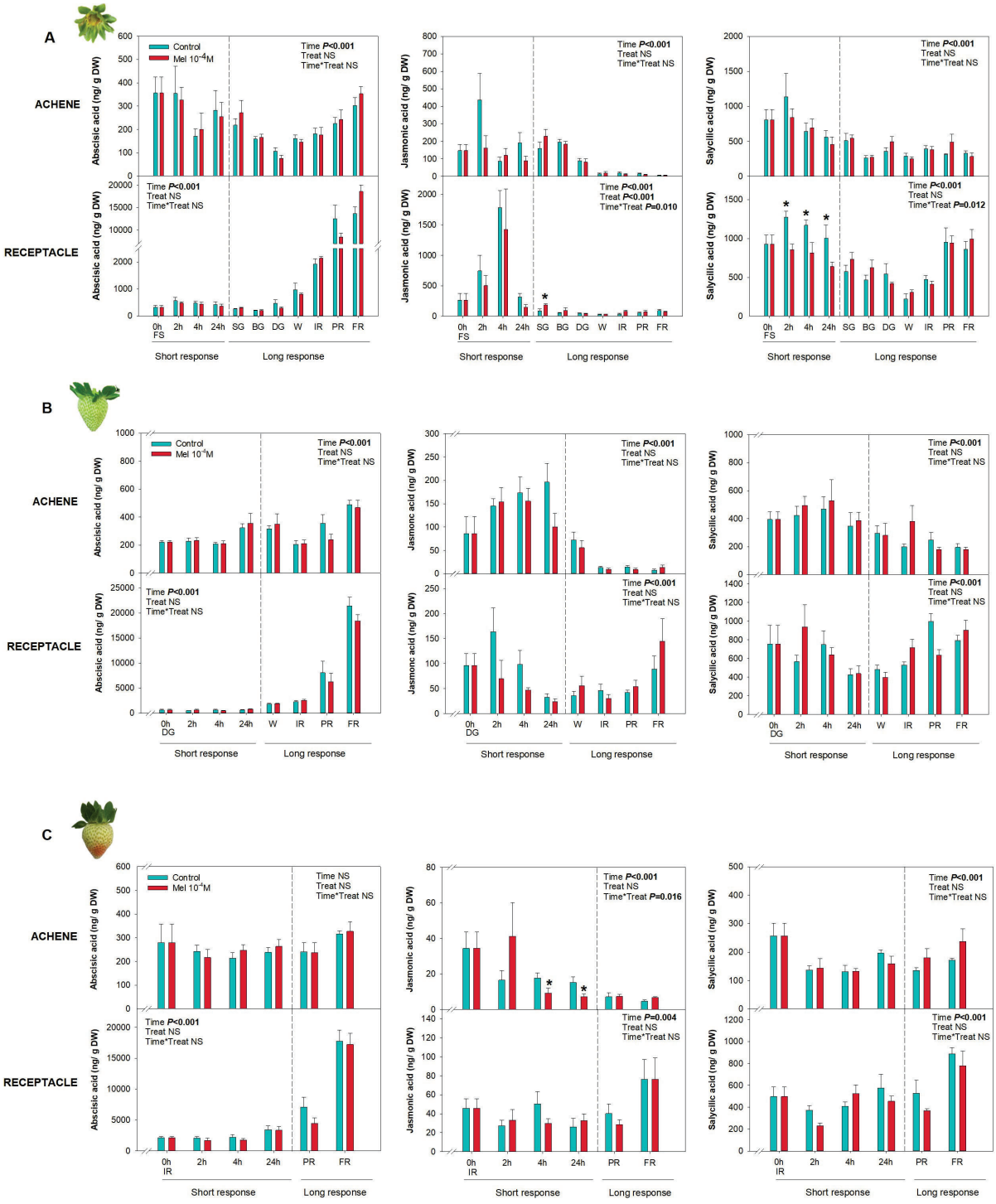


Fig. S4. Abscisic acid (ABA), Jasmonic acid (JA) and Salicylic acid (SA) in strawberries following melatonin application at (A) FS, (B) DG, and (C) IR stages, measured during the first 2h, 4h, and 24h post-application to assess the short-term response, and at subsequent developmental stages until commercial maturity to evaluate the long-term response. Data are presented as mean ± SE of $n = 5$. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences at $P < 0.05$ in the posthoc test. FS, Fruit Set; SG, Small Green; BG, Big Green; DG, Degreening; W, White; IR, Initial Red; PR, Partial Red; FR, Full Red.

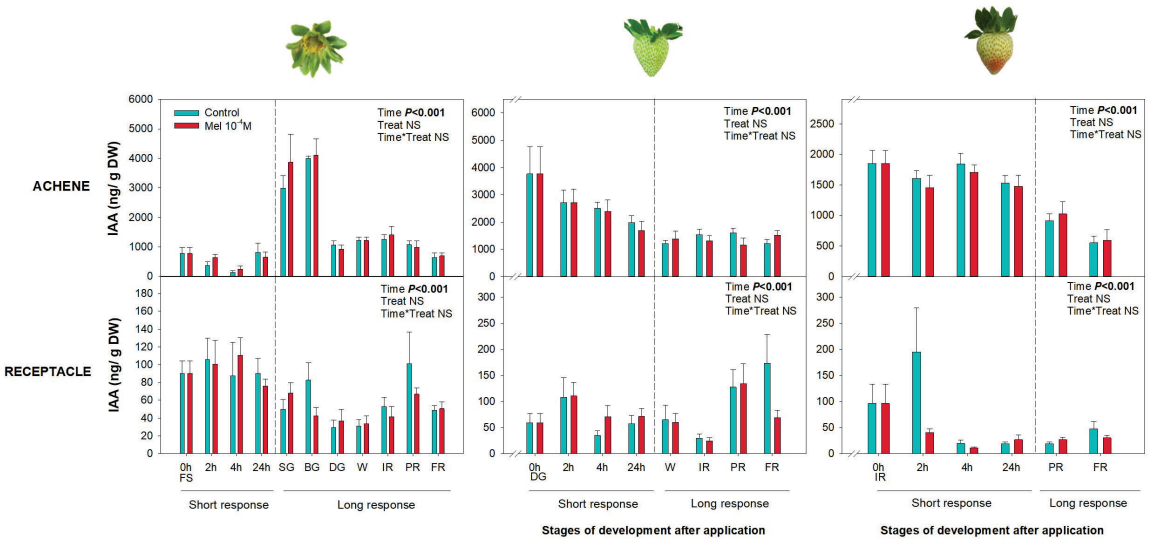


Fig. S5. Indole-3-acetic acid (IAA) in strawberries following melatonin application at FS, DG, and IR stages, measured during the first 2h, 4h, and 24h post-application to assess the short-term response, and at subsequent developmental stages until commercial maturity to evaluate the long-term response. Data are presented as mean ± SE of $n = 5$. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences at $P < 0.05$ in the posthoc test. FS, Fruit Set; SG, Small Green; BG, Big Green; DG, Degreening; W, White; IR, Initial Red; PR, Partial Red; FR, Full Red.

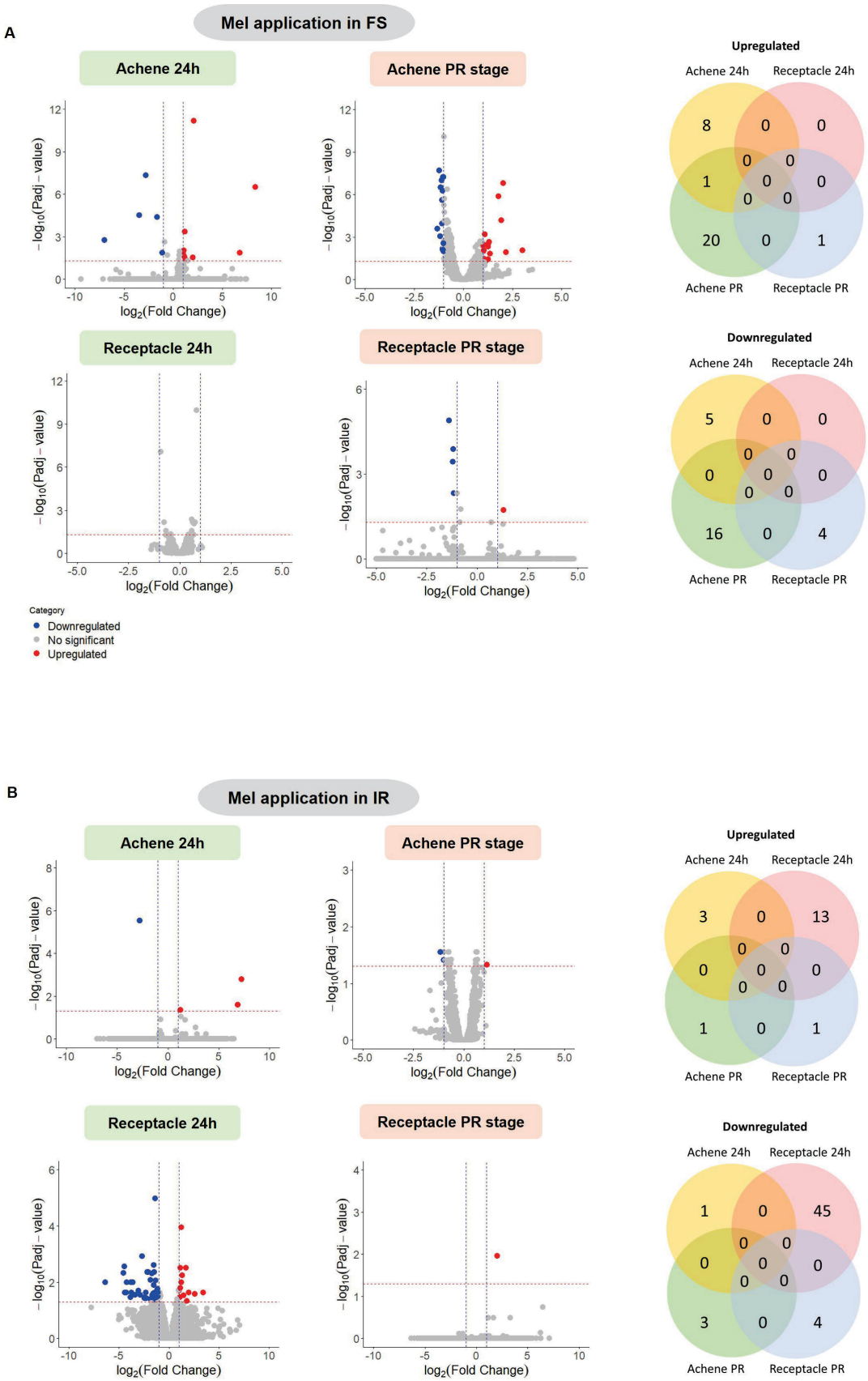


Fig. S6. Volcano plots and Venn diagram showing DEGs in the achene and receptacle at 24 hours post-application and at the PR stage following melatonin treatment at the FS (A) and IR (B) stages.

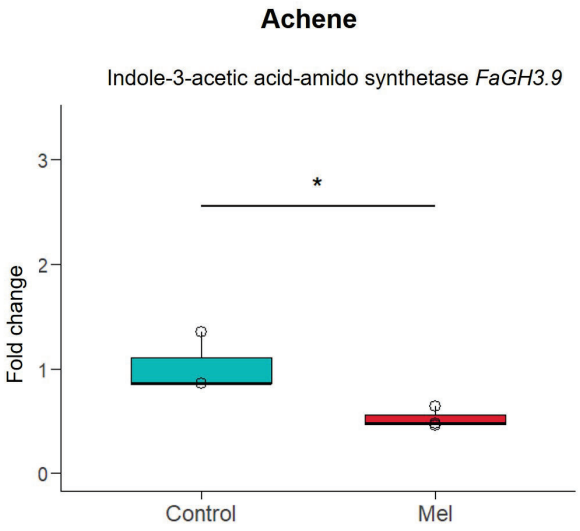


Fig. S7. Expression analysis of FaGH3.9 in the achenes of strawberries 24 hours after melatonin application at the DG stage. Asterisks (*) indicate significant differences between control and melatonin-treated fruits at $P < 0.05$.

CAPÍTOL 2

Efectes específics del triptòfan i la melatonina en funció del fruit com a components actius per prolongar la funcionalitat dels fruits vermells durant el processament postcollita



Chapter 2. Fruit-specific effects of tryptophan and melatonin as active components to extend the functionality of red fruits during post-harvest processing

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RESUM

Preservar els atributs de qualitat en la cadena de distribució és un gran repte, especialment en fruits amb una breu vida útil. Es va avaluar l'aplicació de melatonina en cireres, gerds, maduixots i nabius emmagatzemats a temperatura ambient, així com els efectes del seu precursor (triptòfan) per determinar la seva especificitat i viabilitat intercanviable per a aplicacions postcollita. Els resultats van demostrar que la melatonina és eficaç en tots els fruits testats, reduint la taxa de deteriorament i la seva severitat, preservant la fermesa i reduint l'enfosquiment i la pèrdua de pes. A més, les aplicacions de triptòfan van incrementar els nivells de melatonina en maduixots i nabius i van retardar la descomposició en ambdós fruits. La melatonina va reduir les pèrdues postcollita en tots els fruits estudiats gràcies a les seves propietats antisenescents, mentre que l'impacte beneficiós del triptòfan per allargar la vida útil va ser específic de cada fruit i sembla estar en part mediat per la melatonina. La melatonina i el triptòfan s'han de considerar com a components actius de noves formulacions per allargar la vida útil dels fruits vermells durant el processament postcollita.

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Fruit-specific effects of tryptophan and melatonin as active components to extend the functionality of red fruits during post-harvest processing

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ABSTRACT

Preserving quality attributes in the distribution chain is a challenging task, particularly in fruits with a brief shelf life. The application of melatonin in cherries, raspberries, strawberries and blueberries stored at room temperature was evaluated, as well as the effects of its precursor (tryptophan) to determine their specificity and interchangeable feasibility for post-harvest applications. The results demonstrated that melatonin is effective in all tested fruits, reducing deterioration rate and its severity, preserving fruit firmness and reducing darkening and weight loss. Furthermore, tryptophan applications incremented melatonin contents in strawberries and blueberries and delayed decay in both fruits. Melatonin reduced postharvest losses in all studied fruits related to its antisenescent properties, while the beneficial impact of tryptophan in extending shelf life was fruit-specific and appeared to be partly mediated by melatonin. Melatonin and tryptophan must be considered as active components of new formulations for extending the shelf life of red fruits during post-harvest processing.

1. Introduction

Red fruits include, among others, strawberries (*Fragaria x ananassa*), raspberries (*Rubus idaeus*), blueberries (*Vaccinium* spp.), and sweet cherries (*Prunus avium*). These fruits are considered important sources of micro- and macronutrients, sugars, dietary fiber, vitamins and minerals (Kumar et al., 2018). However, most of their health-promoting properties have been largely associated with their high levels of bioactive compounds (ascorbic acid, flavonoids, anthocyanins, and tannins) with known antioxidant capacity, making them functional foods (Battino et al., 2009; Giampieri et al., 2013; McCune et al., 2011). Nevertheless, the perishability of red fruits is a striking drawback for commercialization amid they are highly prone to mechanical damage during harvesting and transportation, with a shelf life under ambient conditions limited to just 2 to 7 days (Piljac-Žegarac & Šamec, 2011; Shah et al., 2024). Likewise, they are easily spoiled and do not store very well for long periods, challenging their handling and distribution (Huynh et al., 2019; Vu et al., 2011). Moreover, it is not always possible to preserve fruit under cold conditions, such as during transport from fields to processing plants or storage chambers, at distribution sites in warehouses, and at various stages of technological fruit processing (Shoji et al., 2022). An additional challenge to this equation is that red fruits must be harvested at their optimum ripening point to obtain the best eating quality, which should be preserved during the postharvest period. After harvest, overripening is one of the main causes of red fruit decay inducing short shelf life and senescence. It encompasses different physiological changes such as water loss, excessive softening, color darkening and rot susceptibility (Kumar et al., 2018). That, in turn, will negatively affect the quality parameters of fruits and result in a general loss of nutritional and organoleptic attributes, leading to products being discarded (Pott et al., 2020). Although none of the changes that occur during overripening can be stopped, it is urgently needed the development of new sustainable postharvest technologies for a wide variety of fruits to delay these events at different points of the supply chain. In this regard, overripening is well-regulated by a synergistic and dynamic hormonal interplay that determines postharvest fruit quality (Kumar et al., 2014; Perotti et al., 2023). Therefore, the modulation of contents and activity of distinct plant hormones and regulatory molecules throughout postharvest might be crucial for controlling and prolonging fruit shelf life.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a natural, multifunctional and ubiquitous indolamine in plant species, including fruits. Melatonin is synthesized from the amino acid tryptophan, one of the least abundant amino acids in plant cells. This limited presence is

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explained because tryptophan is essential as a N source for synthesizing proteins and N compounds and acts as a biosynthesis precursor of phytohormones and secondary metabolites (Mukherjee, 2018). Even since the melatonin discovery in plants, there has been a great interest in the functional, biotechnological, nutraceutical, and evolutionary aspects of this bioactive molecule. It is found in varying amounts in a wide range of species in all plant organs including fruits (Arabia et al., 2023). Moreover, its pathway is highly coordinated and well-regulated (Mukherjee, 2018). Tryptophan is converted into tryptamine by tryptophan decarboxylase (TDC) and then into serotonin through the enzyme tryptamine 5-hydroxylase (T5H). Further, melatonin is synthesized from serotonin in two reactions including 3 enzymes: serotonin *N*-acetyltransferase (SNAT), *N*-acetylserotonin *O*-methyltransferase (ASMT), and caffeic acid *O*-methyltransferase (COMT) (Back et al., 2016; Liu et al., 2022; Negri et al., 2021). Additionally, melatonin can also be a precursor for metabolites being rapidly converted to 2-hydroxymelatonin (2-HM) by melatonin 2-hydroxylase (M2H) (Byeon et al., 2015). Besides being an excellent antioxidant, there is increasing evidence that melatonin can act as a signaling molecule performing a multitude of regulatory functions in plants (Arnao & Hernández-Ruiz, 2019; Chen et al., 2022; Verde et al., 2023; Yang et al., 2021).

The multiple effects of melatonin in fruits have been extensively reviewed over the past 10 years. It has been reported that melatonin can be involved in fruit ripening enhancing the efficiency and biosynthesis of other antioxidants, regulatory molecules and phytohormones that will determine the fruit defense system and final quality (Liu et al., 2019; Mansouri et al., 2021; Qu et al., 2022; Shan et al., 2022; Sun et al., 2015; Wang et al., 2020). The treatment of exogenous melatonin to extend postharvest shelf life has been studied in a wide variety of fruits (Arabia et al., 2022; Onik et al., 2021; Shah et al., 2023; Wang et al., 2019). Moreover, in the case of red fruits, melatonin maintains quality in cold-stored blueberries (Magri & Petriccione, 2022), delays senescence in sweet cherries stored at both 4 °C and 20 °C (Miranda et al., 2020; Pang et al., 2023), and prevents decay in strawberries by enhancing antioxidant systems and specific metabolic pathways both at cold and room temperature (Liu et al., 2018; Promyou et al., 2023). For red raspberries, pre-harvest melatonin treatment helps preserving nutritional quality and reducing postharvest oxidative stress (Shah et al., 2024). Therefore, melatonin treatment can be a worthwhile and health-conscious alternative to consider for alleviating postharvest deterioration. Nonetheless, despite all the previous reports on melatonin, there is a lack of consistency on doses, methods of application and experimental conditions in the literature that lead to very different results and therefore, making it difficult to recommend its implementation as a universal postharvest strategy for a wide range of fruits. In this study, we aimed to evaluate the effectivity and mode of action of applying exogenous melatonin and tryptophan (as a highly cost-effective alternative) on four different red fruits under the same postharvest conditions to determine the uniformity of their effects on postharvest shelf life. The specific objective was to determine the impact of exogenous applications of melatonin and tryptophan on the shelf life and organoleptic and nutritional quality of red fruits, while assessing how these treatments influence the content of various derived-indolamines and ripening-related phytohormones.

2. Materials and methods

2.1. Fruit materials, treatments and samplings

Different red fruits with botanical differences according to origin and composition, being simple or aggregate fruits, and true or false fruits were used to understand the effects of melatonin and tryptophan for broad spectrum applications. Red raspberries (*Rubus idaeus* Driscoll's variety) were harvested from an orchard in Huelva (Spain, provided by Driscoll's) at the end of May 2023, blueberries (*Vaccinium corymbosum* cv. Duke) were harvested from an orchard in Cantabria (Spain) in the middle of June 2023, strawberries (*Fragaria x ananassa* cv. Albion) from an orchard in Sant Cebrià de Vallalta (Catalonia, Spain) at the end of June 2023 and cherries (*Prunus avium* cv. Lapins) from an orchard in Zaragoza (Spain) in July 2023 (Fig. S1). All of them were collected at commercial harvest maturity and were immediately transported to a laboratory at the University of Barcelona (Barcelona, Spain). Fruits of uniform size that were free from mechanical damage, plant diseases, and insect pests were selected for the experiment. Then, fruits were randomly divided in three groups and were sprayed by nebulization with melatonin solution (100 μM), L-tryptophan solution (100 μM) and distilled water (control). To facilitate proper solution absorption, 0.1 % Tween 20 was used as a surfactant in treatments and control solutions. Subsequently, fruits were allowed to dry naturally at room temperature (25 °C). Finally, fruits were distributed in 20 commercial plastic boxes of 125 g for raspberries and blueberries (28 and 50 fruits in each box, respectively) and in 0.5 kg boxes for strawberries (40 fruits in each) and 0.4 kg boxes for cherries (50 fruits in each). Fruits remained at room temperature, in facilities equipped with temperature controllers to minimize fluctuations, until control fruit boxes reached 70 % decay. For each species, 10 boxes were used as replicates for daily monitoring of fruit decay and weight loss. On the other hand, the other 10 boxes were used as replicates for each day's analysis of firmness, hydration and color. Moreover, 3 fruits of each replicate were frozen in liquid N_2 at 0 % decay (start point) and when control boxes reached 25 % decay and 70 % decay (end point). Fruits were kept at −80 °C until biochemistry analysis.

2.2. Determination of decay incidence, severity and weight loss

Fruit decay was monitored daily until control fruits reached 70 % deterioration. The incidence of fruit decay was determined by the number of fruits showing decay symptoms (such as lesions, overripening symptoms or visible fungal growth) relative to the total number of fruits in each replicate and expressed in percentage (%). The severity of the decay was scored as follows: 0: no decay, 1 = 0–25 % surface damage, 2 = 25–50 % surface damage, 3 = 50–75 % surface damage, 4 = 75–100 % surface damage. The decay rate was calculated with the following equation:

$$decay\ rate(\%) = \sum \frac{AxB}{CxD}\ x\ 100$$

where A is the decay scale, B is the number of fruits in that scale, C is the number of total fruits (varies according to fruit type) and D is the highest scale of decay (D = 4). The weight loss rate of the fruits in each plastic box was calculated by the percentage of difference between initial weight and final weight compared to the initial weight.

2.3. Analysis of color, firmness and fruit hydration

Fruit color, firmness and hydration were measured daily on 3 fruits from each replicate (n = 10). Peel color was determined by measurements of the basic parameters of L*(lightness), a* (redness), b*(yellowness), C* (chroma) and Hue angle using a colorimeter (Konica Minolta Chroma Meter CR400–410) and taking two measurements per fruit, one on each side of the fruit. Firmness (N) was assessed in strawberries and cherries using a PCE-FM200 penetrometer (PCE Group, UK) with a motorized force test stand (PCE-VTS 50) equipped with 8 mm diameter cone probe under the condition of 5 mm depth and at a speed of 500 mm·min^{-1}. In the case of blueberries and raspberries, the penetrometer was equipped with a compression probe of 24 mm diameter under the same conditions. Finally, the hydration of the fruits was analyzed using the formula [(Fresh Weight-Dry Weight / Dry Weight] after incubating the samples at 70 °C for two weeks.

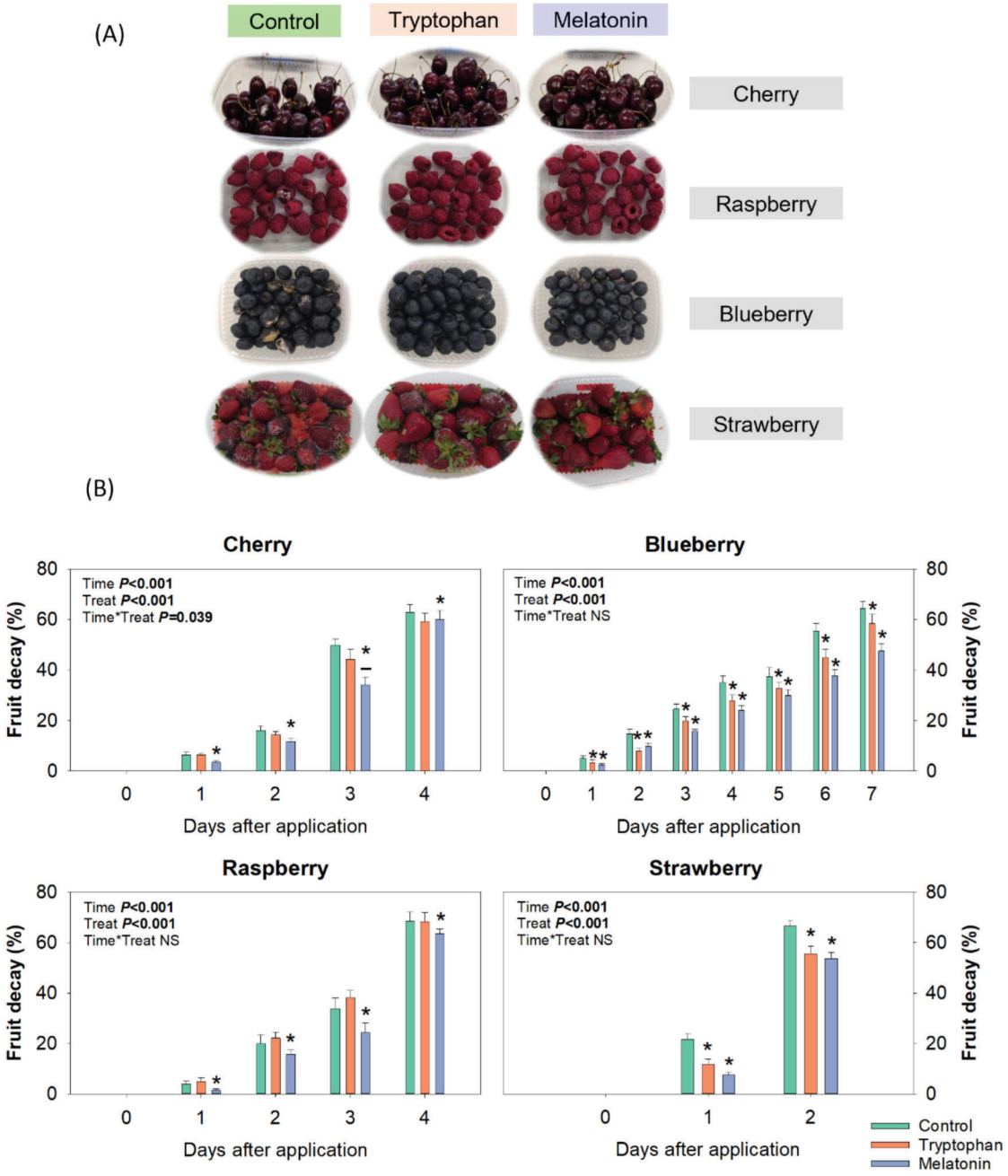


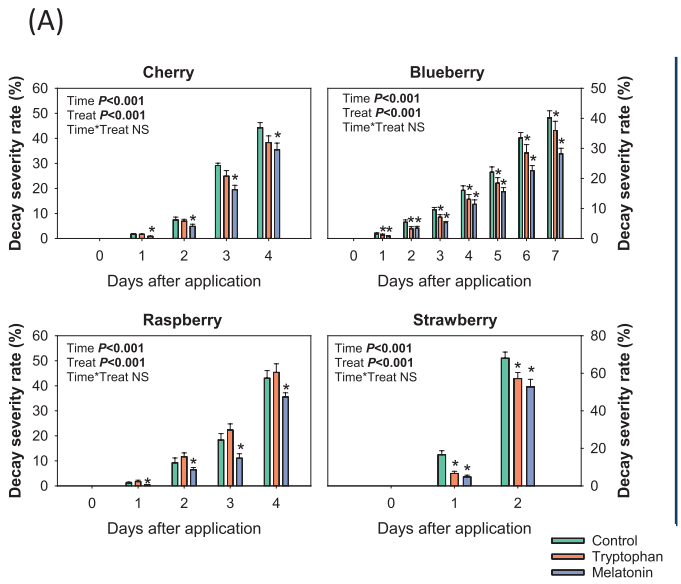
Fig. 1. (A) Appearance of cherries, raspberries, blueberries and strawberries at the end of the storage after non-treatment (control) or 100 μM melatonin and 100 μM tryptophan application. (B) Fruit decay (%) of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of $n = 10$ boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that day ($P < 0.05$).

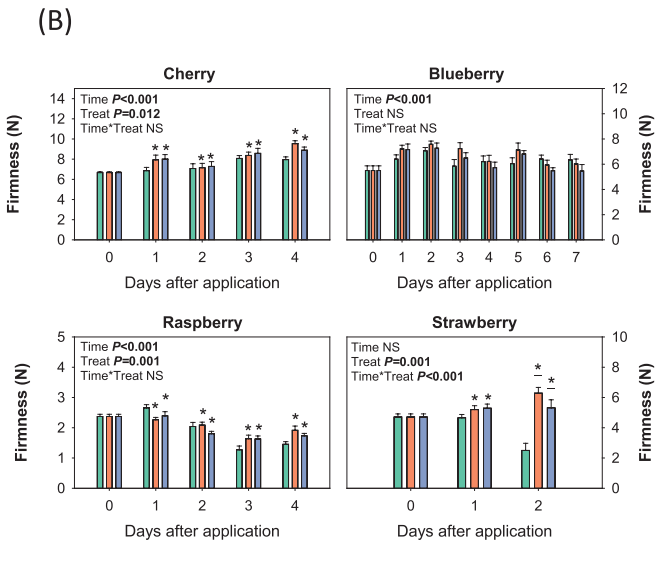
2.4. Determination of total soluble solids, titratable acidity and pH

For estimating organoleptic fruit quality, total soluble solids (TSS), titratable acidity (TA) and pH were evaluated. For the analysis of TSS, 2 g of powdered frozen samples were homogenized with 10 ml of H_2O MilliQ and the obtained juice was used to determine °Brix in 1 mL using a refractometer (Hannah Instruments, Italy), as described by Boulton et al. (1999). Furthermore, pH was evaluated using a pH meter (Hanna instruments, Woonsocket, RI, USA). For TA measures, a 1:10 dilution of the juice with H_2O MilliQ was used with 0.1 M NaOH and 1 % phenolphthalein as an indicator, to estimate the quantity of major acid in each fruit following the method described by Latimer (2012).

2.5. Analysis of anthocyanins, total phenols, and total antioxidant capacity

To determine total anthocyanin, phenol content, and antioxidant capacity, methanolic extracts were used. In summary, 100 mg of each frozen sample were extracted in 750 μl 100 % (*v*/v) cold methanol. After vortexing, the extracts were ultrasonicated (Branson 3800 ultrasonic cleaner, Bransonic, United States) for 30 min in an ice bath at 4 °C and centrifuged (Labnet International Inc.) at 13000 rpm for 10 min and 4 °C. The supernatants were recovered in a new tube and the remaining pellet underwent one additional re-extraction with 750 μl of cold 100 % (v/v) methanol, repeating the previously described procedure. For





(C)

Melatonin application	Cherry	Raspberry	Blueberry	Strawberry
Fruit Decay	↓	↓	↓	↓
Decay severity rate	↓	↓	↓	↓
Fruit weight loss	↓	-	↓	↓
Hydration	-	-	-	-
Firmness	↑	↑	-	↑
L*	-	↑	-	-
a*	-	↓	↑	↑
b*	-	↓	-	↑
C*	-	-	-	↑
Hue	-	↑ Red	↑ Violet	-
TSS	-	-	-	-
TA	-	-	-	↓
TSS/TA	-	-	-	-
pH	-	-	-	-

Tryptophan application	Cherry	Raspberry	Blueberry	Strawberry
Fruit Decay	-	-	↓	↓
Decay severity rate	-	-	↓	↓
Fruit weight loss	↓	↑	↓	↓
Hydration	-	-	-	-
Firmness	↑	↑	-	↑
L*	-	-	-	↑
a*	-	-	↑	-
b*	-	↓	↓	-
C*	-	-	↑	-
Hue	-	↑ Red	↑ Violet	-
TSS	-	-	-	↓
TA	-	-	-	-
TSS/TA	-	-	-	↓
pH	-	-	-	-

Fig. 2. (A) Decay severity rate (%) and (B) firmness of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). (C) Summary table on the changes in the different physicochemical quality parameters of the fruits (cherries, raspberries, blueberries and strawberries) after melatonin and tryptophan application. Up and down arrows indicate an increase or a decrease of that parameter, respectively. Dashes (-) indicate no change. L* = Lightness, a* = redness, b* = yellowness, C* = Chroma, Hue = Hue angle, TSS = Total Soluble Solids, TA = Titratable acidity.

anthocyanins, acidification was carried out with 10 N HCl (~37 %), and absorbance was measured at 530 nm using a spectrophotometer (CE Aquarius UV/Visible, Cecil Instruments Ltd., Cambridge, United Kingdom). The anthocyanin content was calculated using cyanidin-3-glucoside equivalents, following the method described by Siegelman and Hendricks (1958).

To estimate the total phenolic content (TPC), a volume of 100 µl of the prior extract was taken, and 1 ml of Folin-Ciocalteu reagent (diluted 1:10 with H_2O MilliQ) was added. This mixture was incubated for 4 min and 800 µl Na_2CO_3 5 % were added. Absorbances were measured at 765 nm spectrophotometrically (CE Aquarius UV/Visible, Cecil Instruments Ltd., Cambridge, United Kingdom). TPC was calculated using a gallic acid standard curve, as described by Singleton and Rossi (1965) and Li et al. (2007) expressed in mg gallic acid equivalents (GAE) / 100 g FW. The total antioxidant capacity (Radical Scavenging Activity, RSA) was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as was described in Barros et al. (2008) and Poojary et al. (2015). RSA was quantified with a gallic acid standard curve and indicated as mg GAE / 100 g FW.

2.6. Determination of melatonin, its precursors, 2-hydroxymelatonin and plant hormones content

Tryptophan, serotonin, melatonin, 2-hydroxymelatonin and ripening-related phytohormones including abscisic acid (ABA), jasmonates (12-*oxo*-phytodienoic acid (OPDA), jasmonic acid (JA) and the conjugated form JA-Ile), salicylic acid (SA), cytokinins (CKs) including trans-zeatin (t-Z), its riboside trans-zeatin (t-ZR), 2-isopentenyl adenine (2iP) and its riboside isopentenyl adenosine (IPA), indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) and gibberellic acid (GA_3) were extracted and quantified by ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC/ESI-MS/MS). Ground and frozen fruits (100 mg) were extracted with 200 µl cold 100 % (*v*/v) methanol containing deuterium-labeled hormones (Olchemim Ltd., Olomouc, Czech Republic), which were used as internal standards. The extracts were subjected to ultrasonication (Branson 3800 ultrasonic cleaner, Bransonic) in an ice bath at 4 °C for 30 min, followed by a 10 min centrifugation at 13000 rpm and 4 °C (Labnet International Inc.). The supernatant was

collected, and the pellet was reextracted. Both supernatants were merged and filtered with hydrophobic PTFE filters of 0.22 μm (Phenomenex, Torrance, CA, United States) prior to UHPLC/ESI-MS/MS analysis. High-performance liquid chromatography was coupled to a triple quadrupole mass spectrometer (QTRAP 4000, AB Sciex, Concord, Ontario, Canada) for analyses as described by Müller and Munné-Bosch (2011) but with some modifications. Solvent A was acetonitrile with 0.05 % glacial acetic acid and solvent B was water with 0.05 % glacial acetic acid. The flow rate was set at 0.5 mL/min and a LUNA C18 column (Phenomenex Inc., United States [1,6 μm, 100 × 2,1 mm]) was used. In addition, tryptophan, serotonin, melatonin and 2-hydroxymelatonin were included in the method. To detect these compounds, positive mode (ESI+) was used. For tryptophan, the monitored product ion was 188 *m/z* and the collision energy (CE) 15 V, for serotonin it was 160 m/z and 13.5 V, for melatonin it was 174 m/z and 20.6 V and finally, for 2-hydroxymelatonin 189.3 m/z and 18 V. Quantification was made considering recovery rates for each sample by using the deuterium-labeled internal standards. For tryptophan, serotonin, melatonin and 2-hydroxymelatonin, d_4-Mel (178 m/z, 21.8 V CE) was used. Finally, calibration curves for each analyte were generated using MultiQuantTM 3.0.1 software.

2.7. Statistical analysis

For all analyzed parameters, a two-way analysis of variance (ANOVA) with interaction was performed considering time and treatment as factors. Multiple comparisons were conducted using a post-hoc Tukey test. Data were transformed for statistical analyses whenever necessary to achieve normal distribution and homoscedasticity of residuals; and if any transformation worked, individual nonparametric ArtAnova tests were performed. In all cases, differences were considered significant at a probability level of $p < 0.05$. All analyses were performed using RStudio (RStudio Team, 2020).

3. Results and discussion

3.1. Impact of melatonin and tryptophan post-harvest treatments on fruit decay and quality attributes

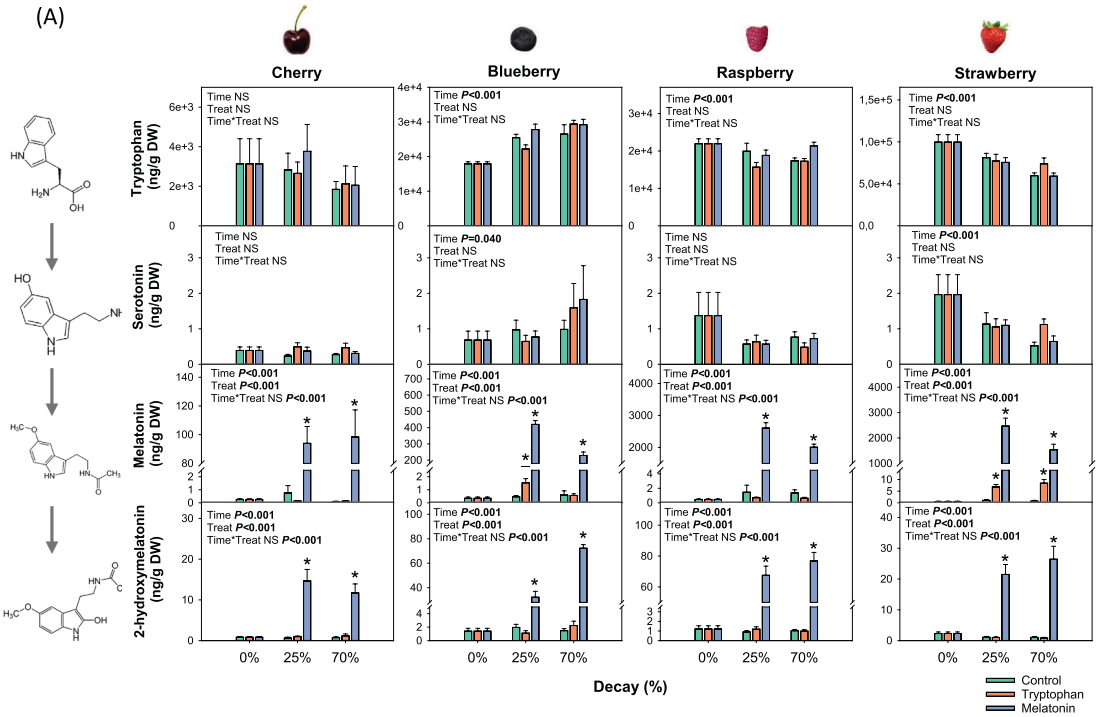
The storage of red fruits constitutes an important challenge, mainly because this type of fruits has a very thin epidermis that is sensitive to bruising and make them highly susceptible to deterioration and fungal infection. Consistently, the four fruits studied here had a very short, although still fruit-specific variable postharvest shelf life (Fig. 1). Blueberries had the greatest durability, up to 7 days at room temperature, and strawberries were the most perishable, only lasting 2 days after harvest. As indicated in Fig. S1, Duke blueberry is an early variety harvested at the beginning of the season, while Albion strawberry was already at the end of the season, which might have reduced its postharvest quality and greater fruit susceptibility (Yahia et al., 2019). As shown in Fig. 1A, strawberries became softer and lacked consistency, losing more than 50 % of water content (Fig. S2A) and promoting fungal appearance (Fig. 1A). Cherries and raspberries mean shelf life was up to four days after harvest, with their deterioration mainly marked by a decrease in firmness, hydration, and pathogen spoilage (Fig. 1, 2B, S2A).

Despite their short shelf life, melatonin treatment was effective in reducing the percentage of deterioration in all four studied fruits (Fig. 1B). For cherries and raspberries, melatonin was most effective in the first few days, reducing deterioration by up to 30 % compared with control fruits towards the third day of postharvest. Similarly, in the case of blueberries, melatonin reduced their deterioration by 30 % regarding control fruits throughout the entire storage period, with treated fruits showing less than 50 % decay on the last day. This positive effect was also very pronounced in strawberries, where deterioration was reduced by 65 % the first day after harvest and 20 % the second day in comparison to control fruits. Similar results were previously found in strawberries immersed in 100 μM melatonin but stored at 4 °C (Liu et al., 2018; Pang et al., 2020). In agreement with our results, Pang et al. (2023) also showed that soaked sweet cherries in 100 μM melatonin delayed their deterioration during storage at 20 °C.

Tryptophan, a cheaper and more cost-effective ingredient that was not investigated previously for the postharvest treatment of red fruits, also showed very positive results, but they were fruit specific (Fig. 1B). Tryptophan was effective for strawberries, reducing the percentage of deterioration by 45 % on the first day and by 16 % on the second day. Similarly, it achieved a 30 % reduction in blueberries decay during the first days and 15–20 % for the last few days. Nevertheless, it had no effect on the decay percentage in cherries and raspberries (Fig. 1B). Tryptophan is an aromatic amino acid that serves as a precursor of indolamines such as auxin, melatonin, phytoalexins, glucosinolates and both indole-and anthranilate-derived alkaloids. In this sense, it has been widely applied to plants in different ways to improve growth and productivity of various crops (Mosa et al., 2021; Mustafa et al., 2018), fruit quality through prebloom sprays in apples (Wójcik et al., 2019) and inhibit senescence in broccoli (Sohail et al., 2021), but to our knowledge, this is the first study showing positive effects for tryptophan during postharvest handling of fruits.

Melatonin also reduced the severity rate of fruit decay, particularly at the beginning of the postharvest period, showing reductions of 70 % in strawberries and raspberries, and 50 % in blueberries and cherries. Indeed, at the end of the storage, all melatonin-treated fruits had 20–30 % less severity of fruit deterioration (Fig. 2A). Melatonin also preserved fruit firmness during postharvest, with the most prominent effects in strawberries (Fig. 2B). This is consistent with a previous study in blueberries stored at 4 °C showing that 50 μM melatonin delayed pectin and cellulose degradation by inhibiting cell wall degrading enzymes, which is related to firmness maintenance (Liu, Shang, et al., 2023). Similarly to melatonin, tryptophan also attenuated the decay severity in blueberries and strawberries, as well as maintained fruit firmness in strawberries, raspberries, and cherries by more than 100 %, 30 %, and 20 % respectively (Fig. 2A, B, C). Both melatonin and tryptophan also led to less weight loss, with reductions of up to 20 % in blueberries, as well as positively affected surface color retention in blueberries, strawberries, and raspberries (Fig. S2B, S3, S4). These findings align with Miranda et al. (2020), who observed that the reduction in weight loss of sweet cherries after melatonin treatment was associated with the up-regulation of aquaporin and cuticle-related genes, and consequently to an improvement of water relations. Finally, no change in TSS/TA ratio was observed in red fruits after treatments, except in tryptophan-treated strawberries where a slight reduction in TSS occurred (Fig. S5). TSS and TA are important quality attributes for postharvest red fruits. El Sayed et al. (2014) reported an increase in TSS in pomegranate after preharvest tryptophan application, but there are no further reports on how tryptophan may influence sugar content during fruit overripening, which warrants further investigations.

Red fruits are renowned for their abundance of bioactive compounds, such as phenolic compounds which possess important biological activities and play an essential role in fruit protection and controlling senescence (Liu, Wei, et al., 2023; Valdés et al., 2015). Melatonin and tryptophan treatments did not result in an increase in total anthocyanin contents or total antioxidant capacity in any of the four fruits studied (Fig. S6, S7). However, exogenous applications of melatonin increased TPC in strawberries and blueberries by 36 % and 17 % at the end of storage, respectively (Fig. S6). Previous studies have demonstrated that melatonin can increase TPC during the postharvest period of various fruits such as plums, mulberries, peaches, and bananas alleviating the deterioration and extending the shelf life (Al-Qurashi et al., 2024; Arabia et al., 2022; Gao et al., 2018; Kakaei et al., 2024). Moreover, Magri and Petriccione (2022) found that melatonin increased polyphenols, flavonoids, and anthocyanins in blueberries after 2 weeks of cold storage, which mirrors our findings at room temperature. Similarly, Pang et al. (2020) reported melatonin up-regulating phenolic metabolism genes in



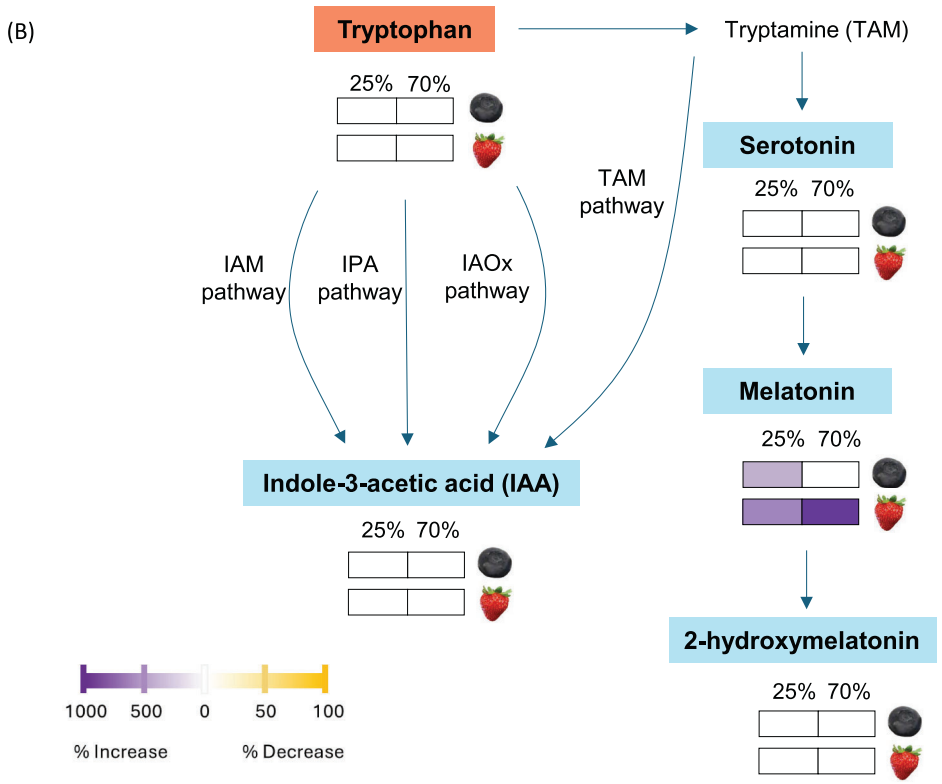


Fig. 3. (A) Endogenous content of tryptophan, serotonin, melatonin and 2-hydroxymelatonin of cherries, raspberries, blueberries and strawberries at 25 % decay and 70 % decay after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that decay percentage ($P < 0.05$). (B) Schematic representation of the changes in tryptophan, serotonin, melatonin, and indole-3-acetic acid contents after tryptophan application in blueberries and strawberries at 25 % and 70 % decay. An increase in their content is represented in purple colors, while a decrease in their levels is indicated in yellow colors. IAM = Indole-3- acetamide, IPA = Indole-3-pyruvic acid, IAOx = Indole-3-acetaldoxime, TAM = Tryptamine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

strawberries under cold storage, while Aghdam and Fard (2017) observed related effects in non-cold conditions. However, no differences were observed in cherries and raspberries in terms of TPC (Fig. S6) and the positive results on strawberries and blueberries occurred at the end of storage, too late to have a positive nutritional impact since deterioration was very advanced. Similarly, tryptophan treatment increased TPC at the end of storage in blueberries (Fig. S6). However, it slight decreased TPC at 25 % deterioration in the same fruit, which might reduce health benefits. It is interesting to note that in this fruit the dynamics in tryptophan-induced melatonin accumulation and the associated changes in TPC suggest that melatonin, rather than tryptophan, may be the substance directly modulating TPC in blueberries. Unfortunately, there is no previous evidence of other studies showing TPC variations upon tryptophan application in postharvest red fruits, thus further research is needed.

In summary, melatonin has proven to be effective in reducing decay and maintaining the quality of red fruits, even under challenging conditions for fruits, where extending shelf life is critical. Despite the botanical differences among the studied fruits, melatonin consistently showed positive results. Moreover, melatonin's efficacy was evident even when the postharvest life was already compromised, as seen with strawberries. Additionally, this study provides the first evidence that postharvest application of 100 μM tryptophan can reduce decay in red fruits, marking a promising new approach.

3.2. Melatonin and tryptophan application alter derived-indolamines during postharvest of red fruits

In the four fruits studied, endogenous melatonin was found at low concentrations in the range of 0.5–1.5 ng/g DW. After its exogenous application, its content increased significantly, reaching values of 100 ng/g DW in cherries, 400 ng/g DW in blueberries, and up to more than 2000 ng/g DW in strawberries and raspberries (Fig. 3A). However, despite the significant increase in melatonin in all the fruits, the extent of this increase varied depending on the fruit. This variation may be related to the activation capacity of its biosynthetic pathway as well as each fruit's ability to absorb the exogenous solution, which will be influenced by its cuticle. Nonetheless, in the case of strawberries, blueberries and raspberries, melatonin increments slightly diminished throughout the postharvest period. Furthermore, beyond increasing endogenous melatonin contents, which had already been described in other studies (Arabia et al., 2022; Liu et al., 2018; Liu, Shang, et al., 2023), the content of 2-HM also increased by more than 1000 % in all four red fruits (Fig. 3A). The endogenous contents of 2-HM found in control fruits were very similar to those of melatonin, around 1–2 ng/g DW in the different fruits. Here, exogenous melatonin increased 2-HM content to 26 ng/g DW in strawberries, 14 ng/g DW in cherries, and more than 70 ng/g DW in blueberries and raspberries. Melatonin can be metabolized to 2-HM through the enzyme M2H, which has a much higher catalytic activity compared to other melatonin biosynthetic enzymes (Byeon & Back, 2015). Moreover, Byeon et al. (2015) demonstrated that melatonin is metabolized rapidly to 2-HM and its accumulation predominates over that of melatonin, being the average 2-HM content 368-fold higher than the melatonin content. In this sense, other studies have also highlighted the role of 2-HM in plants. 2-HM can upregulate defense genes in plants, including *pathogenesis-related gene 1* (*PR1*) and *isochorismate synthase 1* (*ICS1*), suggesting it may play a partial role in defense against biotic stress (Back, 2021). Additionally, exogenous 2-HM treatment has been shown to alleviate oxidative stress in response to combined cold and drought stress (Lee & Back, 2019) and cadmium treatment (Shah et al., 2020). Recently, Korkmaz et al. (2023) also indicated that 2-HM confers multiple stress tolerance in pepper at seed germination stage having melatonin-like functions in enhancing abiotic stress tolerance. Moreover, Back (2021) suggested that the higher catalytic activity of melatonin catabolic enzymes amply demonstrates that melatonin's role (even in exogenous application) results from the combined effects of melatonin and its various metabolites, which possess unique features that differ from its parent molecule. Therefore, after melatonin application, part of the synthesized melatonin is rapidly metabolized into 2-HM, and it is possible that this compound also plays a role in fruit protection during postharvest. However, its specific physiological function in fruit overripening and senescence still needs to be clarified.

Regarding tryptophan treatment, it did not lead to an increase in the tryptophan content in any of the fruits studied, nor in serotonin levels (Fig. 3A). However, it raised the melatonin content throughout the postharvest period in strawberries, up to 11-fold compared to control fruits, rising from 1 ng/g DW in the control fruits to 8 ng/g DW in strawberries treated with tryptophan. Similarly, it also increased the endogenous melatonin content up to 3-fold in blueberries at the beginning of the postharvest period when they were at 25 % decay. Additionally, the application of tryptophan led to a reduction in the TRP/MEL ratio in blueberries and strawberries (Fig. S8), indicating an increase in melatonin levels relative to tryptophan. Nonetheless, there were no changes in the amount of melatonin in cherries and raspberries after tryptophan application. These results are consistent with the effectiveness of tryptophan in prolonging the shelf life of strawberries and blueberries, but not cherries and raspberries. Furthermore, in contrast to melatonin application, exogenous tryptophan did not lead to any changes in the 2-HM content. In plants, the relevance of tryptophan resides as a precursor of secondary metabolites such as IAA, serotonin and melatonin (Corpas et al., 2021). Generally, the signaling effect on plant growth and defense response following a tryptophan application has been associated with an actively metabolization to the phytohormone IAA (Jiang et al., 2022; Naveed et al., 2015). However, other studies also describe that tryptophan application in response to pathogenic infection in rice triggered defense responses via serotonin production (Ishihara et al., 2008). Similarly, a study on strawberry plants under short photoperiods and low temperatures showed that tryptophan application resulted in green foliage and fruit-setting flowers, linked to increased levels of serotonin and 2-HM in the flowers (Ayyanath et al., 2024). Contrary to these studies, in this case the beneficial effect of tryptophan in delaying overripening of strawberries and blueberries is not associated with an accumulation of serotonin or IAA (Fig. 3B). Therefore, these results indicate that tryptophan can extend the shelf life of red fruits when its metabolization to melatonin takes place. This finding aligns with previous research, such as the study by Xiang et al. (2024), indicating that applying tryptophan to grape berries before harvest enhances the melatonin content upon harvest.

Thus, in this study, we demonstrate that the positive effects of exogenous melatonin in prolonging fruit shelf life are mediated by the increase in its endogenous content and that of 2-HM, highlighting their potential physiological function. Indeed, melatonin application did not alter the levels of upstream molecules involved in its synthesis. Furthermore, we can also establish that tryptophan application leads to an increase in melatonin levels in fruits where it effectively delays overripening. Therefore, the beneficial effect of tryptophan in strawberries and blueberries is primarily associated with the accumulation of melatonin, rather than other indolamines such as IAA, serotonin or 2-HM.

3.3. Variations in the contents of ripening-related phytohormones upon melatonin and tryptophan treatments during postharvest of red fruits

The intricate signaling network between phytohormones is pivotal in regulating fruit senescence. This regulatory process encompasses both promoter and inhibitor signaling molecules, and their content throughout overripening plays an essential role in prolonging fruit shelf life (Xiang et al., 2021). In this study, the content of different plant hormones was analyzed to evaluate their relationship with the application of melatonin and tryptophan. In cherries and raspberries, no differences were found in the content of any of the studied plant

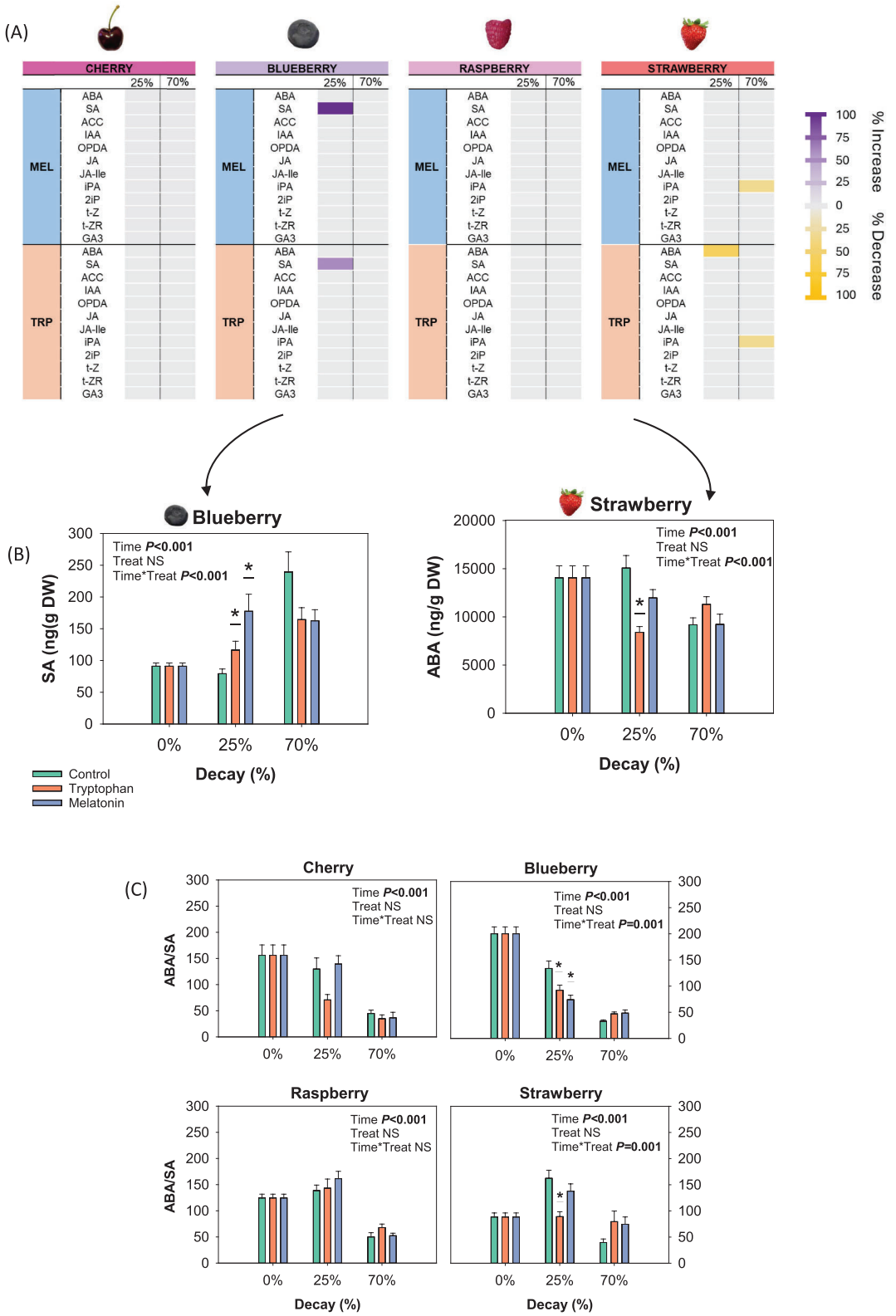


Fig. 4. (A) Changes in hormonal content due to melatonin (MEL) or tryptophan (TRP) treatment in cherries, raspberries, blueberries and strawberries. An increase in their content is represented in purple colors, while a decrease in their levels is indicated in yellow colors. (B) Endogenous content of salicylic acid in blueberries and abscisic acid in strawberries at 25 % decay and 70 % decay after melatonin and tryptophan applications. Control refers to untreated fruits. (C) ABA/SA ratio in cherries, raspberries, blueberries and strawberries at 25 % decay and 70 % decay after melatonin and tryptophan applications. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that decay percentage ($P < 0.05$). ABA = abscisic acid, SA = salicylic acid, ACC = 1-aminocyclopropane-1-carboxylate, IAA = indole −3-acetic acid, OPDA = 12-*oxo*-phytodienoic acid, JA = jasmonic acid, JA-Ile = jasmonoyl isoleucine, iPA = isopentenyl adenosine, 2iP = 2-isopentenyl adenine, t-ZR = trans-zeatine riboside, t-Z = trans-zeatin, GA3 = gibberellic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

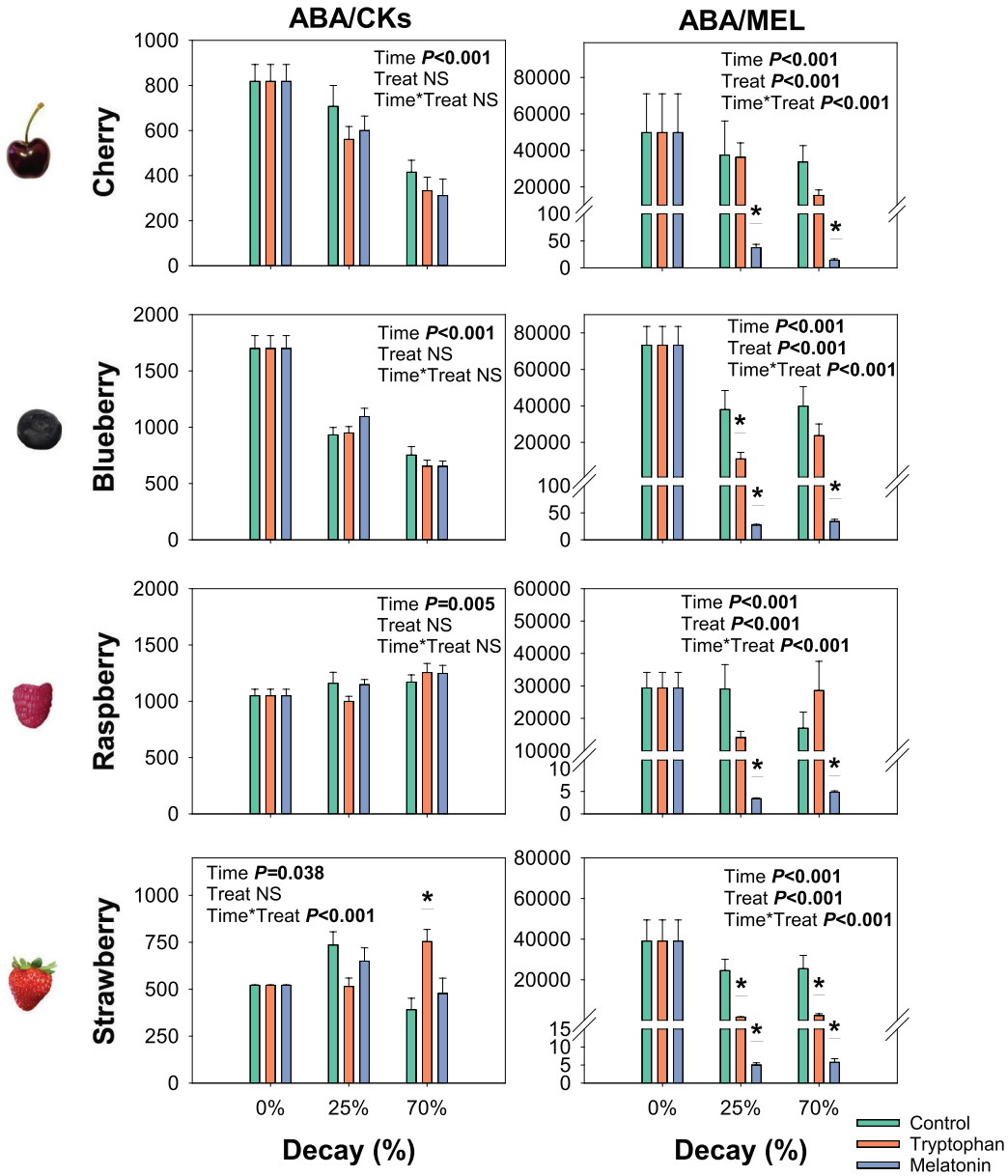


Fig. 5. ABA/CKs and ABA/MEL ratio in cherries, raspberries, blueberries and strawberries at 25 % decay and 70 % decay after melatonin and tryptophan applications. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that decay percentage ($P < 0.05$). ABA = abscisic acid; CKs = cytokinins (including the content of 2ip, iPA, Z and ZR); MEL = melatonin.

hormones (ABA, SA, ACC, JAs, CKs, and GA3) after melatonin and tryptophan application (Fig. 4A, S9, S10, S11, S12, S13, S14). Considering that both melatonin and IAA are derived from tryptophan, and the established relationship between the levels of IAA and melatonin treatment (Liu, Wei, et al., 2023; Wen et al., 2016), it was expected that melatonin or tryptophan application would interfere with the IAA content. However, neither application led to differences in terms of IAA levels (Fig.S9C).

Nevertheless, in the case of blueberries, an increase in the SA content was observed when fruits had 25 % deterioration after the application of both melatonin and tryptophan (Fig. 4B). The content of SA increased at the end of the postharvest period (70 % decay) in all untreated red fruits (Fig. 4B, S9B), but in blueberries the exogenous applications induced an increase of this phytohormone when fruits had 25 % decay, thereby accelerating SA boost. This increment in SA content was more notable with the application of melatonin, which resulted in a 125 % increase in SA content, while exogenous tryptophan led to a 46 % increase compared with untreated fruits. Moreover, it is known that SA alters the fruit physiology by increasing secondary metabolites in plant tissues which in turn increases the resistance level of plant organs (Prasad & Sharma, 2018). Molecular evidence also indicates that melatonin can up-regulate the expression genes of SA pathway decreasing pathogen susceptibility in Arabidopsis plants (Lee et al., 2014; Weeda et al., 2014). In fruits, Gao et al. (2018) reported that melatonin application enhances PAL activity stimulating the accumulation of TPC as well as delaying the loss of endogenous SA. Moreover, SA signaling pathway and phenylpropanoid metabolism were also involved in melatonin-induced disease resistance in tomato (Li et al., 2022). Therefore, it is suggested that the increase in SA may be related to the subsequent increase in phenols observed in blueberries, thus increasing the protective mechanisms of the fruit during postharvest. After tryptophan application in blueberries, SA content also increased. Zhang et al. (2024) showed that tryptophan

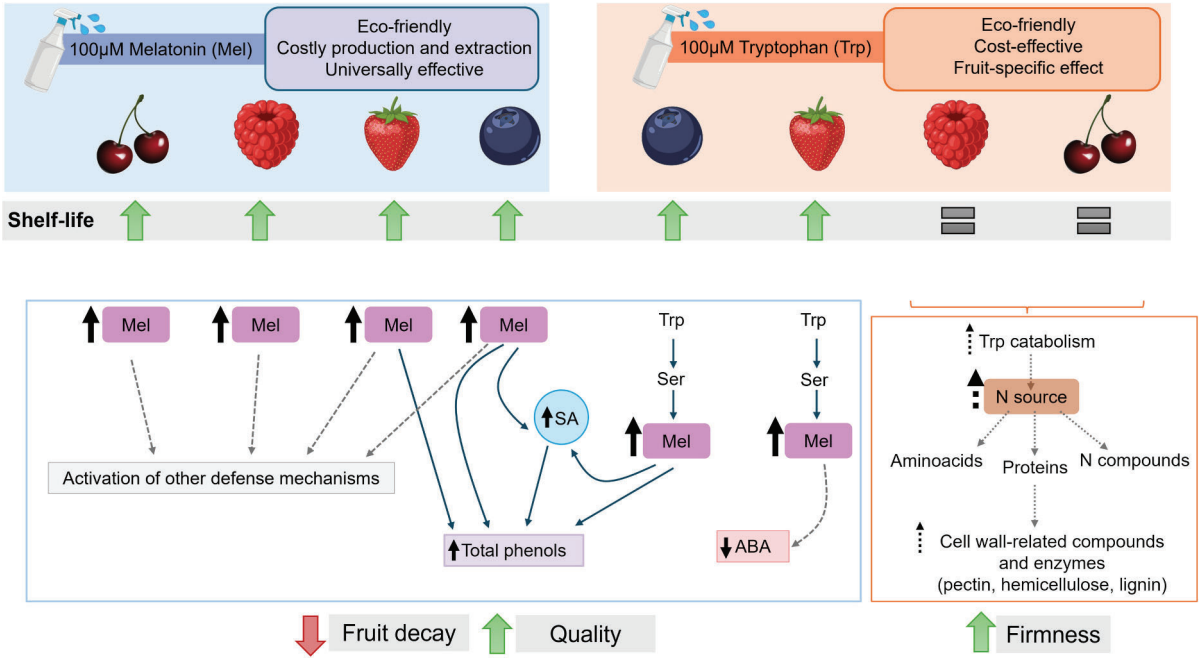


Fig. 6. Graphical representation of the effect and biochemical changes following exogenous melatonin and tryptophan application in different red fruits during postharvest. Trp = tryptophan, Ser = serotonin, Mel = melatonin, SA = salicylic acid, ABA = abscisic acid, N = nitrogen. Dashed lines indicate suggested mechanisms. This figure was created with BioRender.com. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

improved disease resistance in stored citrus fruits and slowed decay by upregulating genes involved in SA biosynthesis and transduction pathways. Here, it is probably that the SA increase in tryptophan-treated blueberries is mediated by the enhanced melatonin content, since the observed physiological response aligns with that observed in the exogenous melatonin application.

In strawberries, the tryptophan application also decreased ABA content by 45 % compared to control fruits at 25 % decay (Fig. 4B). ABA is widely known as one of the main plant hormones promoting fruit ripening, stimulating physiological changes such as softening or anthocyanins accumulation as well as inducing fruit senescence (Liu et al., 2020; Luo et al., 2014). For that, ABA levels decrease throughout postharvest fruit life (Tijero et al., 2019), which could also be observed in the red fruits of this study, where the lowest ABA levels are found at 70 % decay (Fig. 4, S9A). Therefore, considering its role in promoting ripening and senescence, a greater decrease in ABA content in strawberries at 25 % deterioration may be related to an overripening delay. In addition to the observed changes in content of SA and ABA, the ABA/SA ratio was also significantly affected (Fig. 4C). This ratio decreased in blueberries following treatments with melatonin and tryptophan, and it also showed a reduction in strawberries treated with tryptophan. However, considering the changes in individual hormone content, it is likely that in blueberries, the effect is primarily due to the increase in SA, whereas in strawberries, the reduction is more attributable to a decrease in ABA. The reduction in the ABA/SA ratio is particularly important in the postharvest context, as lower ABA/SA ratio may reflect an enhanced balance between stress response and senescence promotion. Beyond the hormonal changes already mentioned, melatonin did not lead to alterations in the balance between senescence-inhibiting hormones, such as cytokinins or IAA, and senescence-promoting hormones, like ABA and ACC. Therefore, the ABA/CKs, ABA/IAA and ACC/CKs ratios remained unchanged after the treatments (Fig. 5, S15, S16). However, melatonin application did reduce the ABA/MEL and ACC/MEL ratios in all fruits, as did the application of tryptophan in blueberries and strawberries (Fig. 5, S16). This suggests that the antisenescence effect of the postharvest treatments is not primarily due to changes in the content or balance of the major plant hormones, but rather to the increased melatonin levels, which may play a key role in delaying senescence and extending shelf life.

Therefore, these results indicate that the positive effects of the treatments are primarily driven by melatonin's antisenescence properties, showing that its effect is universal in red fruits. However, its mode of action varies depending on the specific fruit, either by regulating phytohormone content or through other defense mechanisms (Fig. 6). Moreover, tryptophan treatment followed the same pattern as exogenous melatonin, increasing SA content in blueberries. Tryptophan has only been effective in strawberries and blueberries, where part of exogenous tryptophan was converted into melatonin. Additionally, in cherries and raspberries, where tryptophan had no effect on shelf life extension but maintained firmness, no increase in melatonin, serotonin or the phytohormones analyzed was observed. Given that tryptophan, in addition to being a precursor of bioactive molecules, also serves as a N source through its catabolism (Shende et al., 2024; Tzin & Galili, 2010), it is plausible that these fruits have prioritized its use for increasing N availability. A greater source of N is essential for the synthesis of proteins, amino acids and nitrogenous compounds as well as metabolic regulation. Indeed, N is a fundamental component for the synthesis of enzymes and precursors necessary for the formation of compounds such as pectins, hemicelluloses, or lignin which enhance cell wall integrity and fruit firmness (Rivai et al., 2021; Vanholme et al., 2010; Wang et al., 2018). Thus, it may be that depending on the requirements and fruit needs at time of application, tryptophan is used for melatonin synthesis or not (Fig. 6). Nevertheless, it is suggested that it is the increase of endogenous melatonin that determines, in part, the success of its application.

4. Conclusions

In this study, melatonin application extended the shelf life at room temperature of all studied red fruits, including cherries, raspberries, blueberries, and strawberries, while tryptophan was effective in reducing post-harvest over-ripening in blueberries and strawberries.

These later effects were fruit-specific and appeared to be mediated, at least in part, by melatonin. Therefore, tryptophan treatment emerges both as an eco-friendly and highly cost-effective choice for the post-harvest treatment of some red fruits. Additionally, we found that exogenous melatonin increased endogenous SA content in blueberries (an effect that was also observed upon tryptophan application), thus showing that melatonin may exert direct antisenescence effects in some fruits, but also indirect effects mediated by ripening-related hormones. Results also indicate that part of the beneficial effects of tryptophan delaying fruit decay are subject to its conversion to melatonin rather than to other indolamines. Overall, this study shows that melatonin treatment, and in some cases also its precursor tryptophan, is a sustainable and economical option to be used in the postharvest treatment of red fruits to extend their shelf life. This could contribute to reducing food waste, and minimize its impact on society, the economy, and the environment.

CRediT authorship contribution statement

Alba Arabia: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paula Muñoz:** Validation, Supervision, Methodology, Investigation, Conceptualization. **Sergi Munné-Bosch:** Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141487.

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Figure S1. Raspberry, blueberry, strawberry and sweet cherry season calendar in Spain. The red triangle indicates the time of the season in which the varieties used for the experiment were harvested.

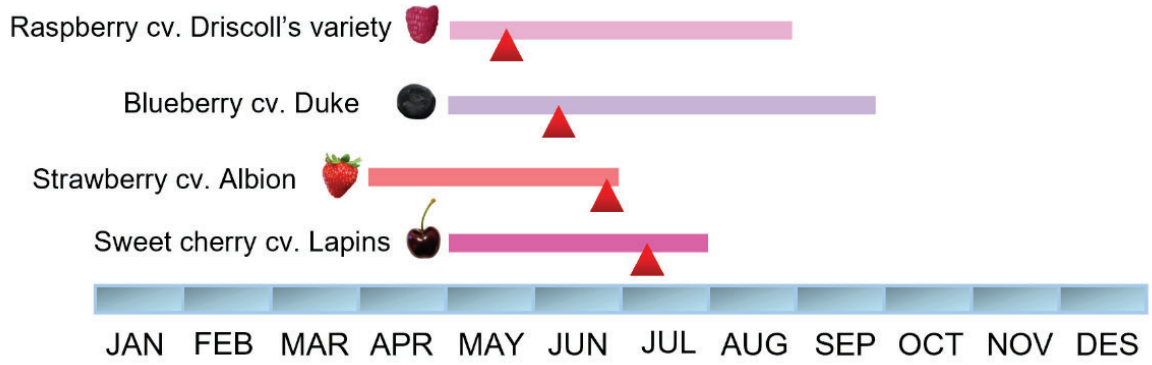


Figure S2. (A) Hydration and (B) Fruit weight loss (%) of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that day ($P < 0.05$).

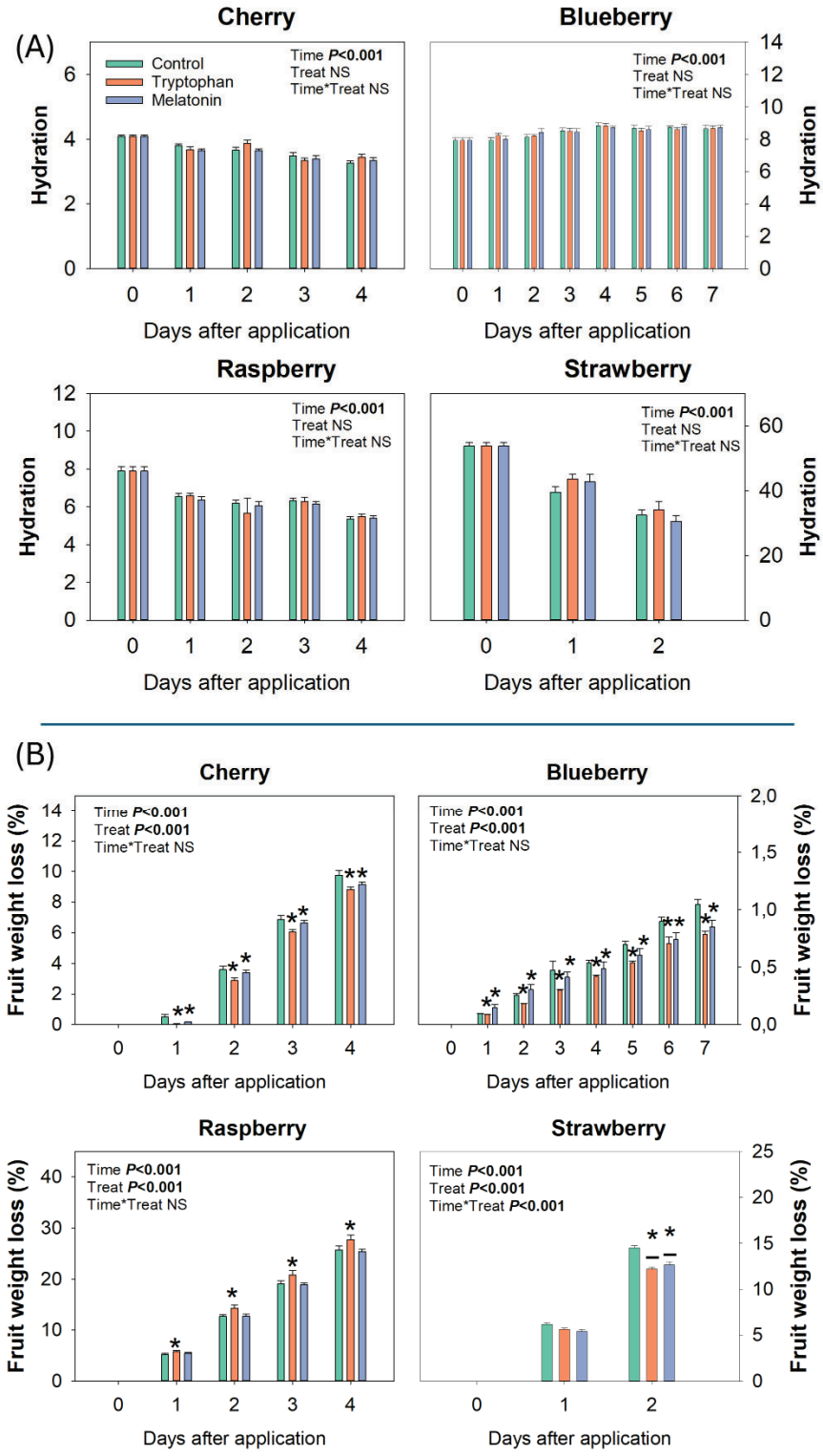


Figure S3. Lightness (L*), redness (a*) and yellowness (b*) of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that day ($P < 0.05$).

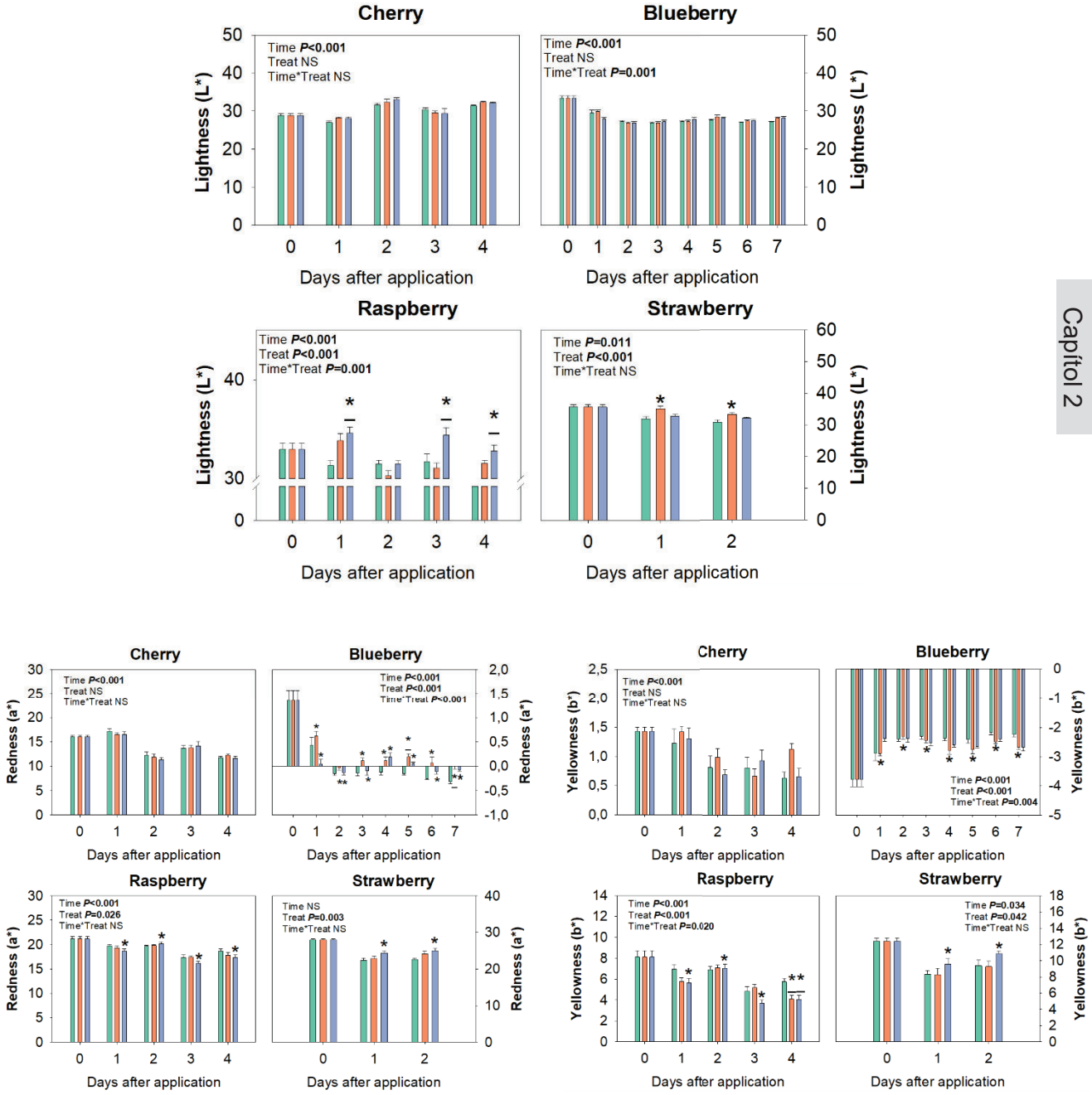


Figure S4. Chroma (C*) and hue angle of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significant differences between the control and that treatment during storage ($P < 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that day ($P < 0.05$).

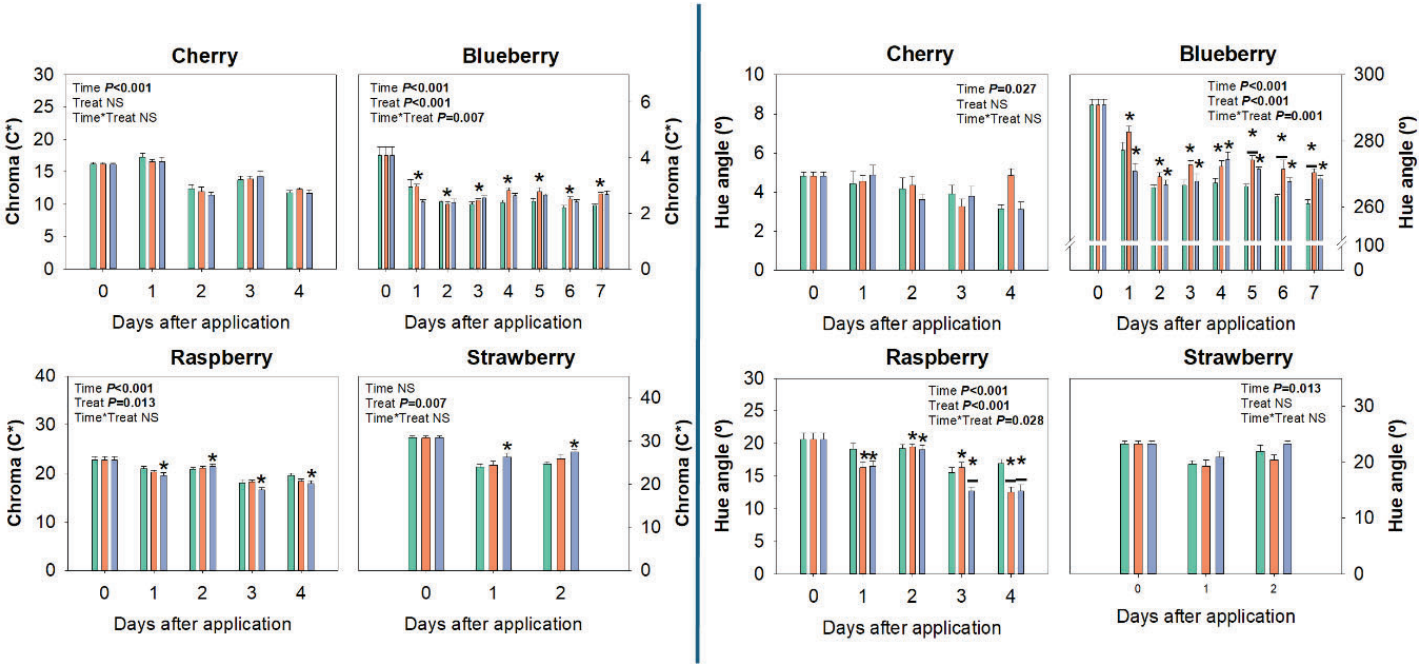


Figure S5. Organoleptic quality parameters of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that decay ($P < 0.05$). TSS = Total soluble solids, TA = Total acidity.

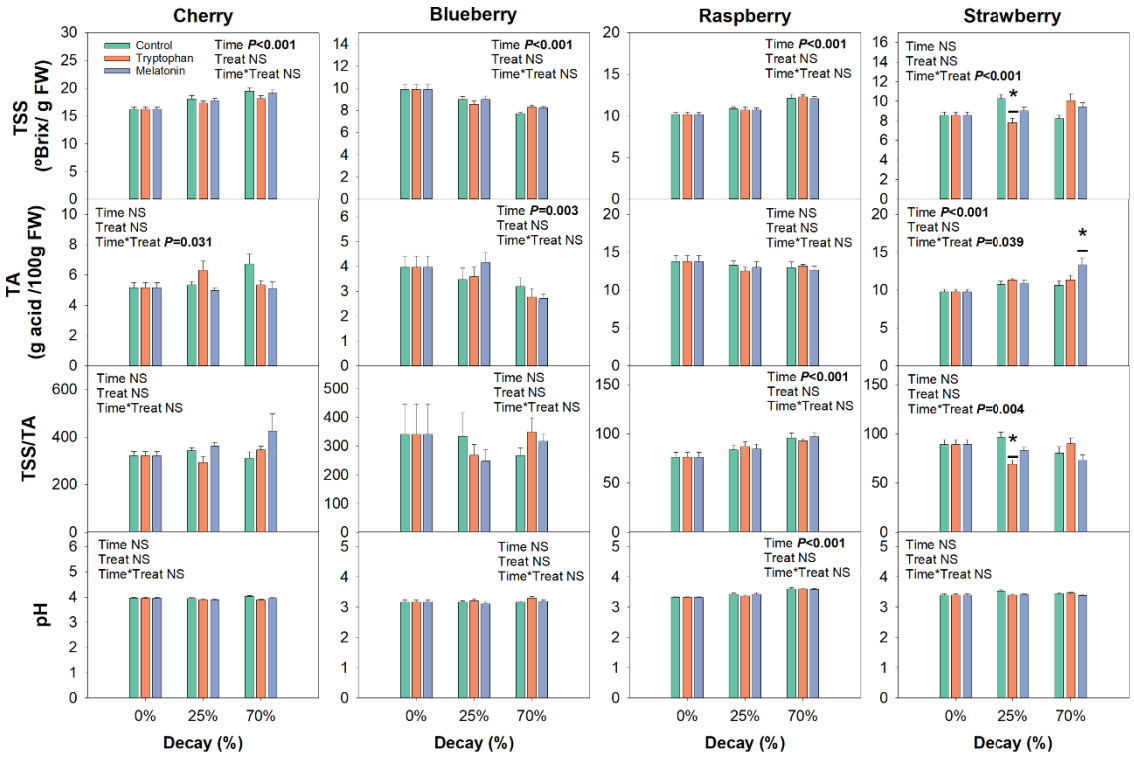


Figure S6. Total Phenol Content (TPC) and Radical Scavenging Activity (RSA) of cherries, raspberries, blueberries and strawberries at 25% decay and 70% decay after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences (P > 0.05). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that decay percentage (P < 0.05).

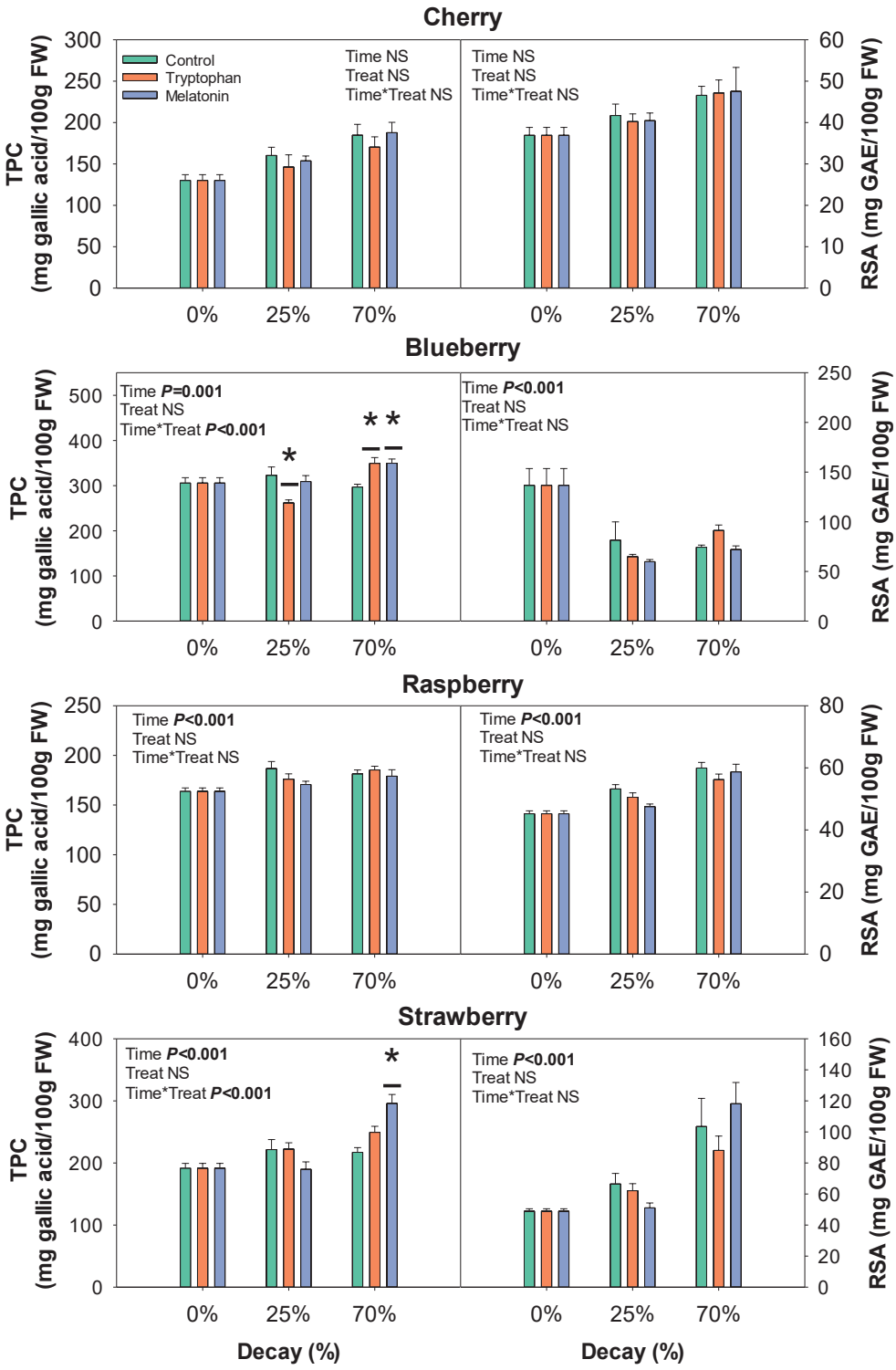


Figure S7. Total anthocyanin content of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$).

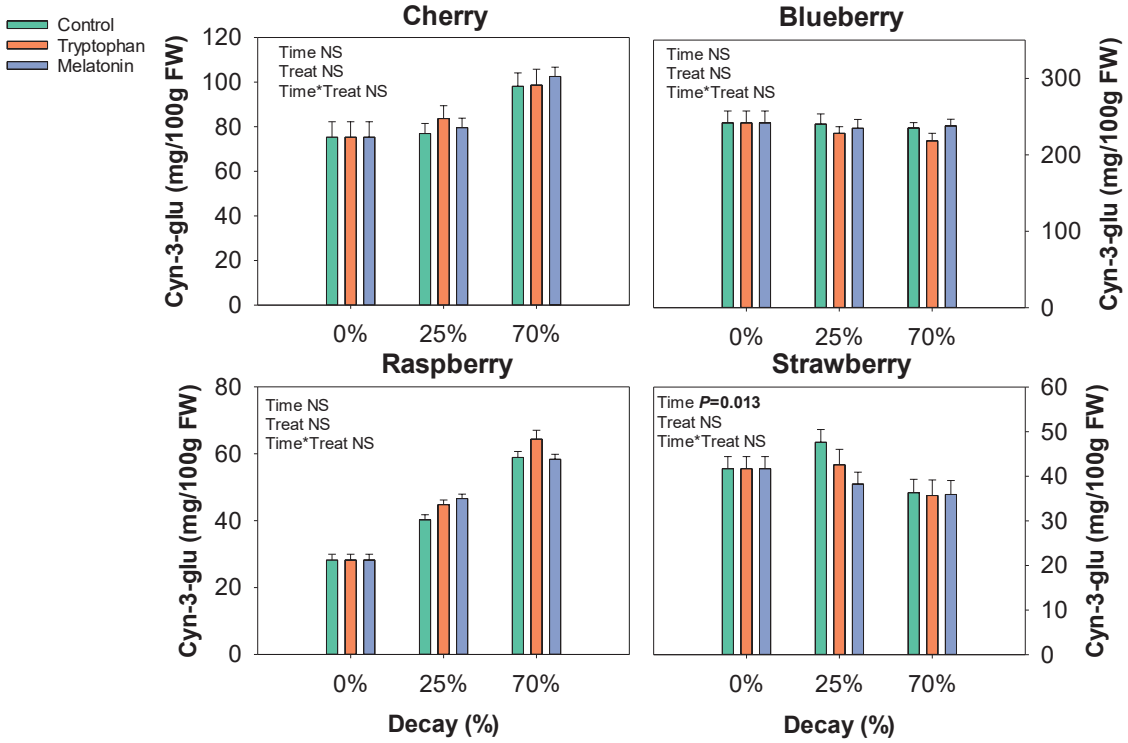


Figure S8. TRP/MEL ratio of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significantly different values between the control and treated fruit on that decay. TRP = Tryptophan; MEL = Melatonin.

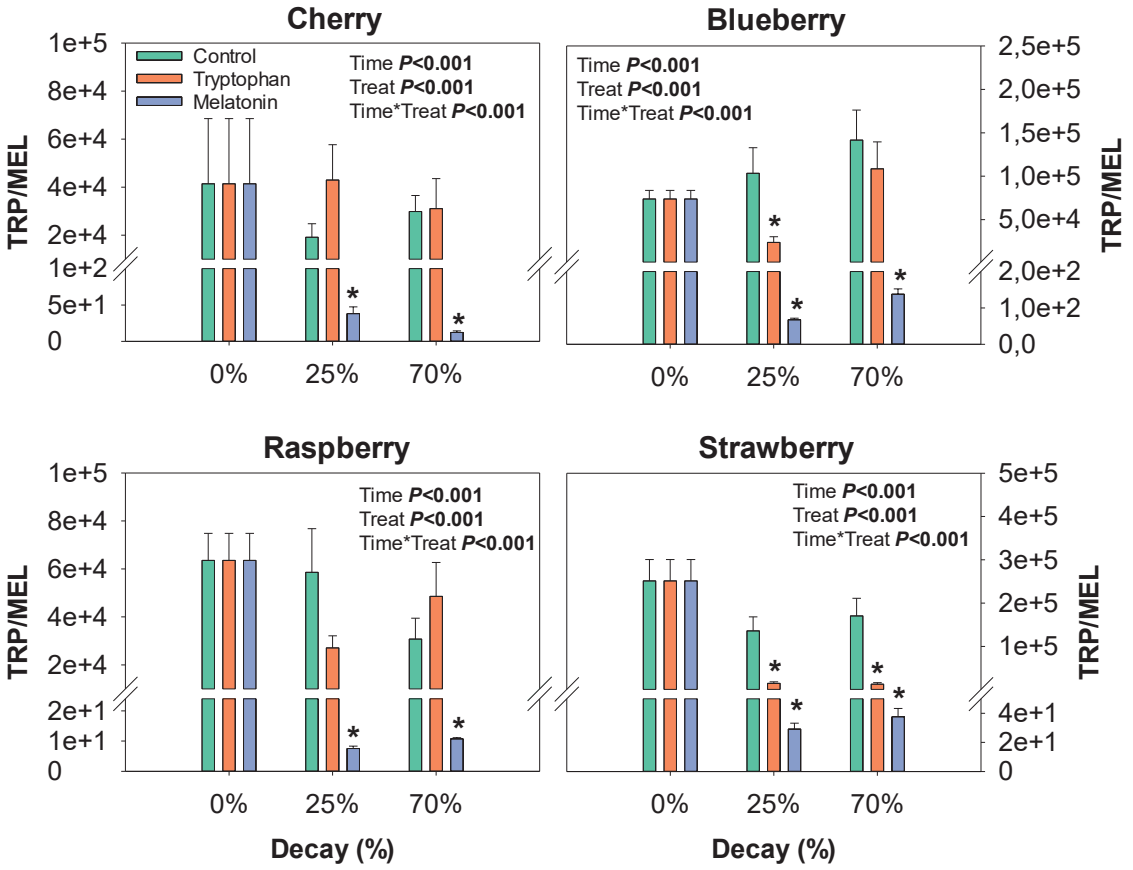


Figure S9. (A) Abscisic acid (ABA) content of cherries, raspberries and blueberries during storage at room temperature after melatonin and tryptophan applications. (B) Salicylic acid (SA) content of cherries, raspberries and strawberries during storage at room temperature after melatonin and tryptophan applications. (C) Indole-3-acetic acid (IAA) content of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$).

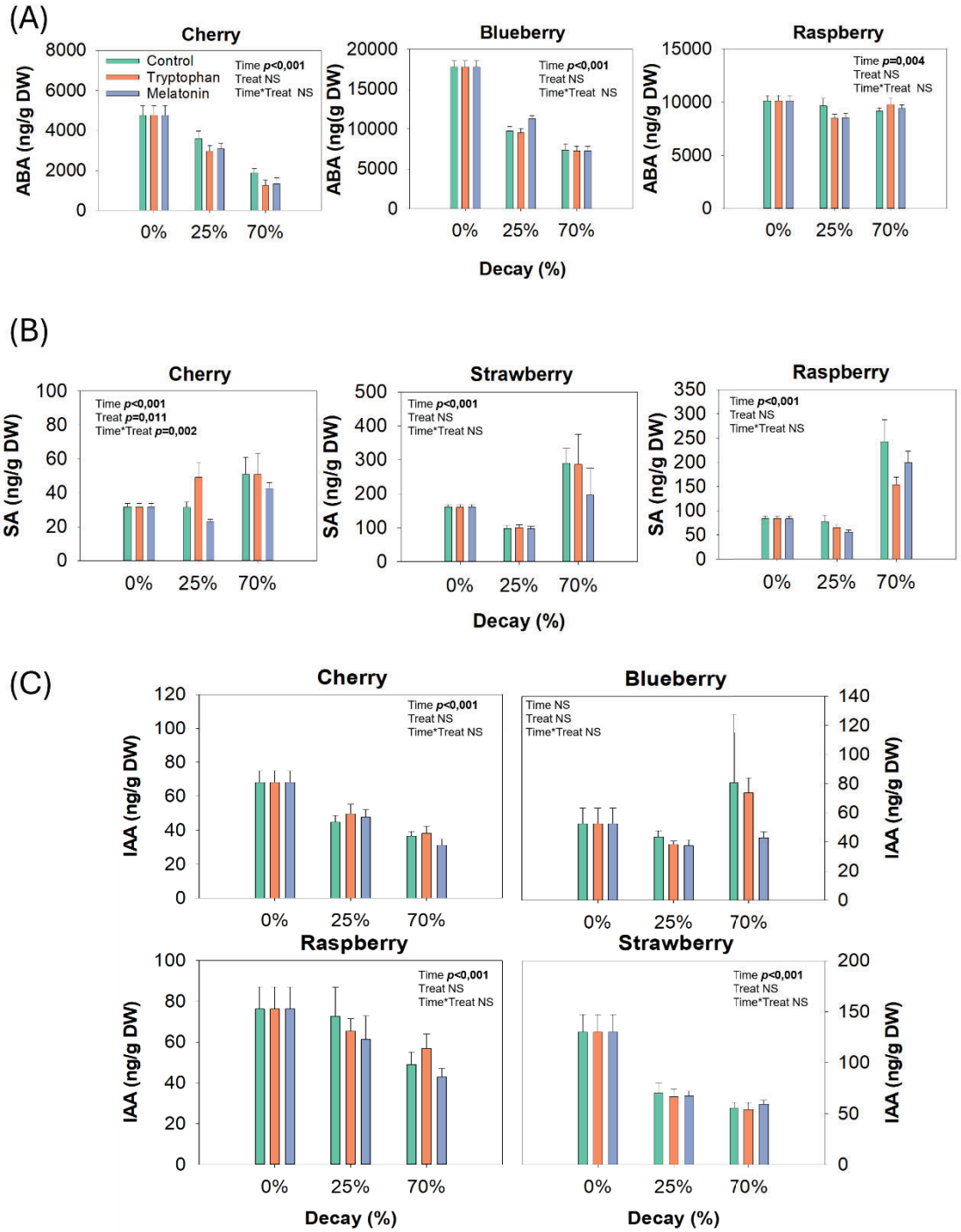


Figure S10. Content of OPDA, JA and JA-Ile of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). OPDA = 12-*oxo*-phytodienoic acid, JA = jasmonic acid, JA-Ile = jasmonoyl isoleucine.

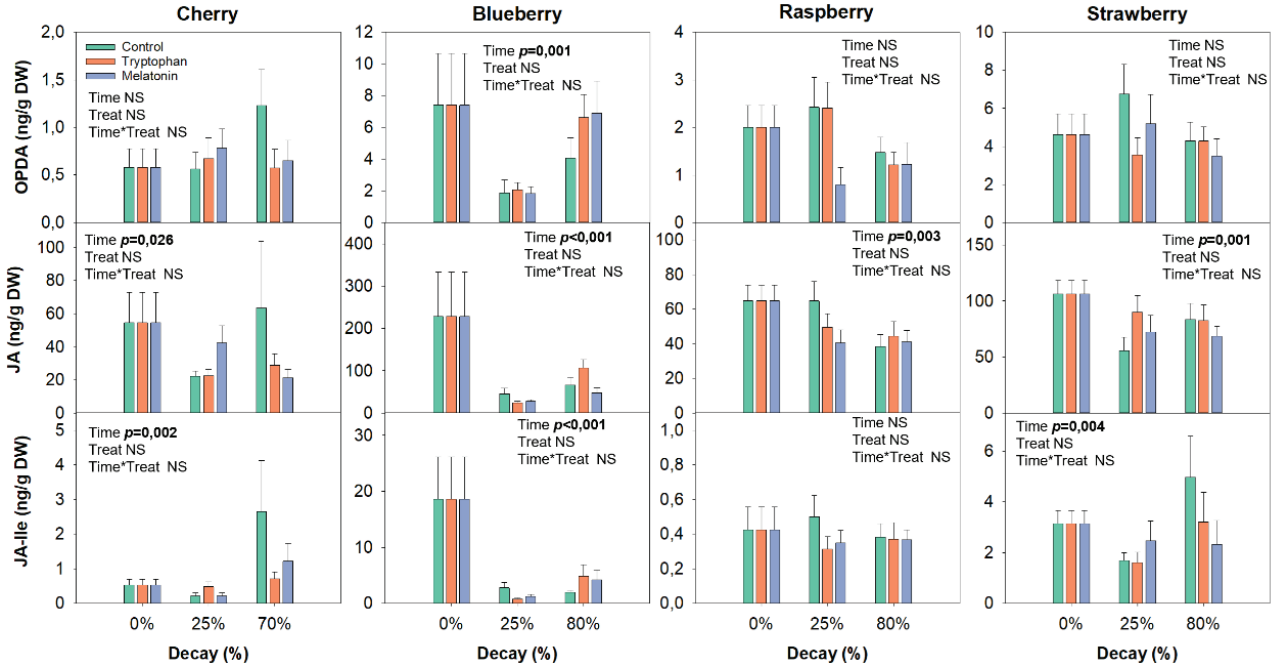


Figure S11. Content of gibberellic acid (GA3) of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$).

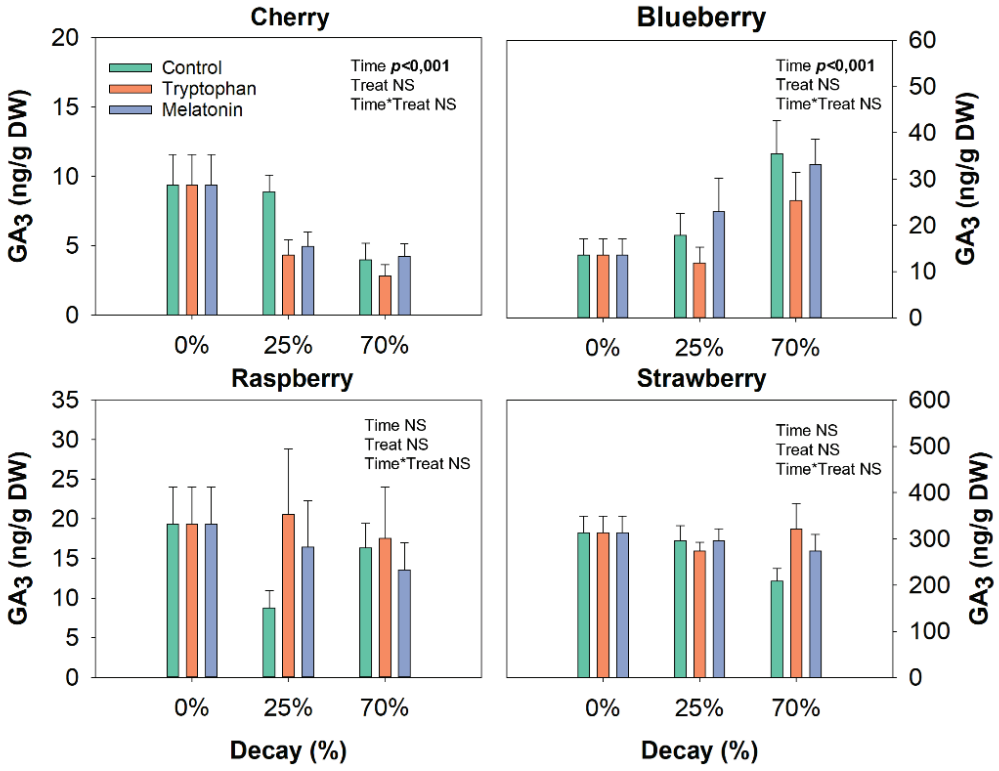


Figure S12. Content of isopentenyl adenosine (iPA), 2-isopentenyl adenine (2iP), trans-zeatine riboside t-ZR and trans-zeatin (t-Z) in cherries (A) and raspberries (B) during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$).

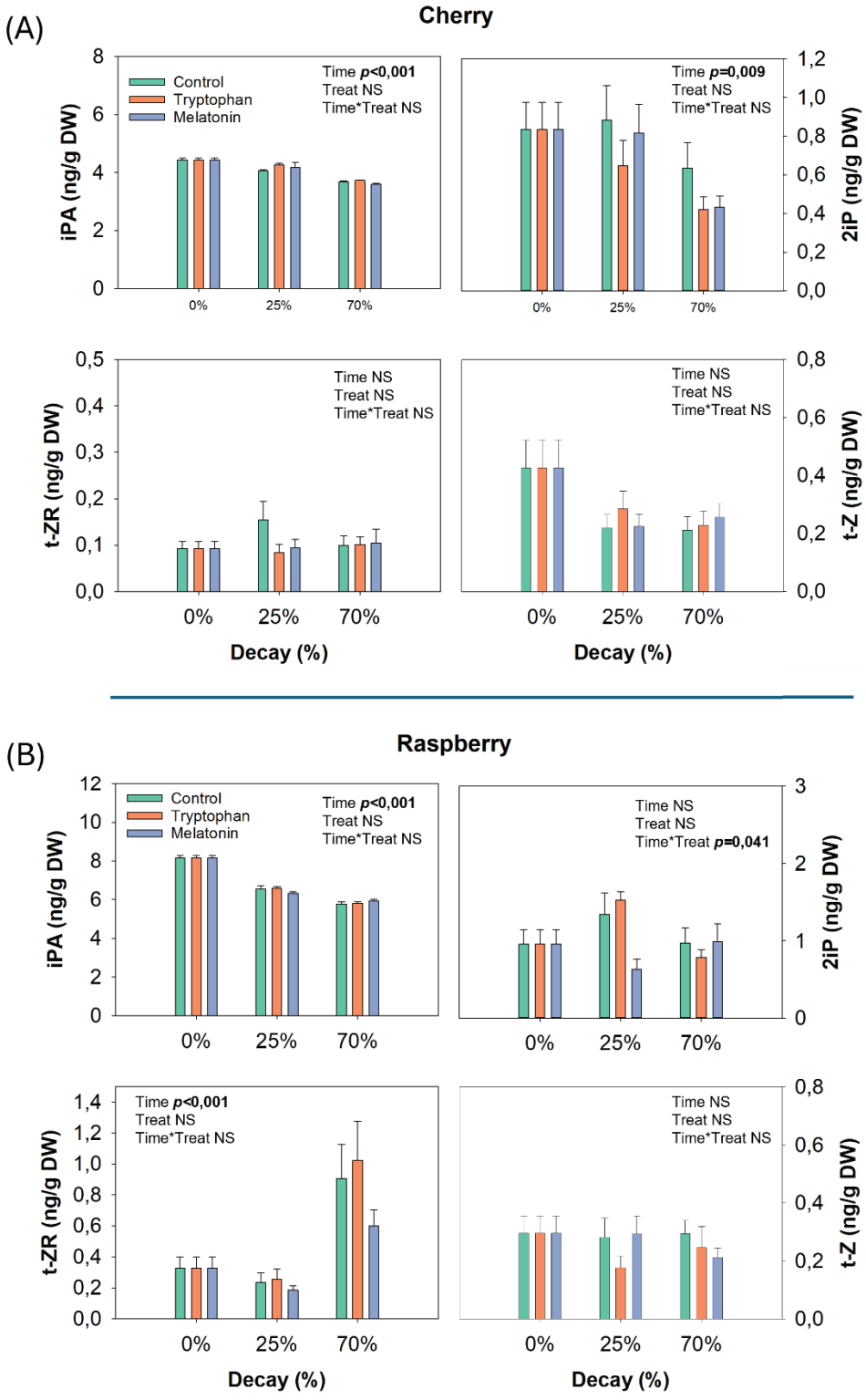


Figure S13. Content of isopentenyl adenosine (iPA), 2-isopentenyl adenine (2iP), trans-zeatine riboside t-ZR and trans-zeatin (t-Z) in blueberries (A) and strawberries (B) during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Underlined asterisk (*) indicates significantly different values between the control and treated fruit on that decay ($P < 0.05$).

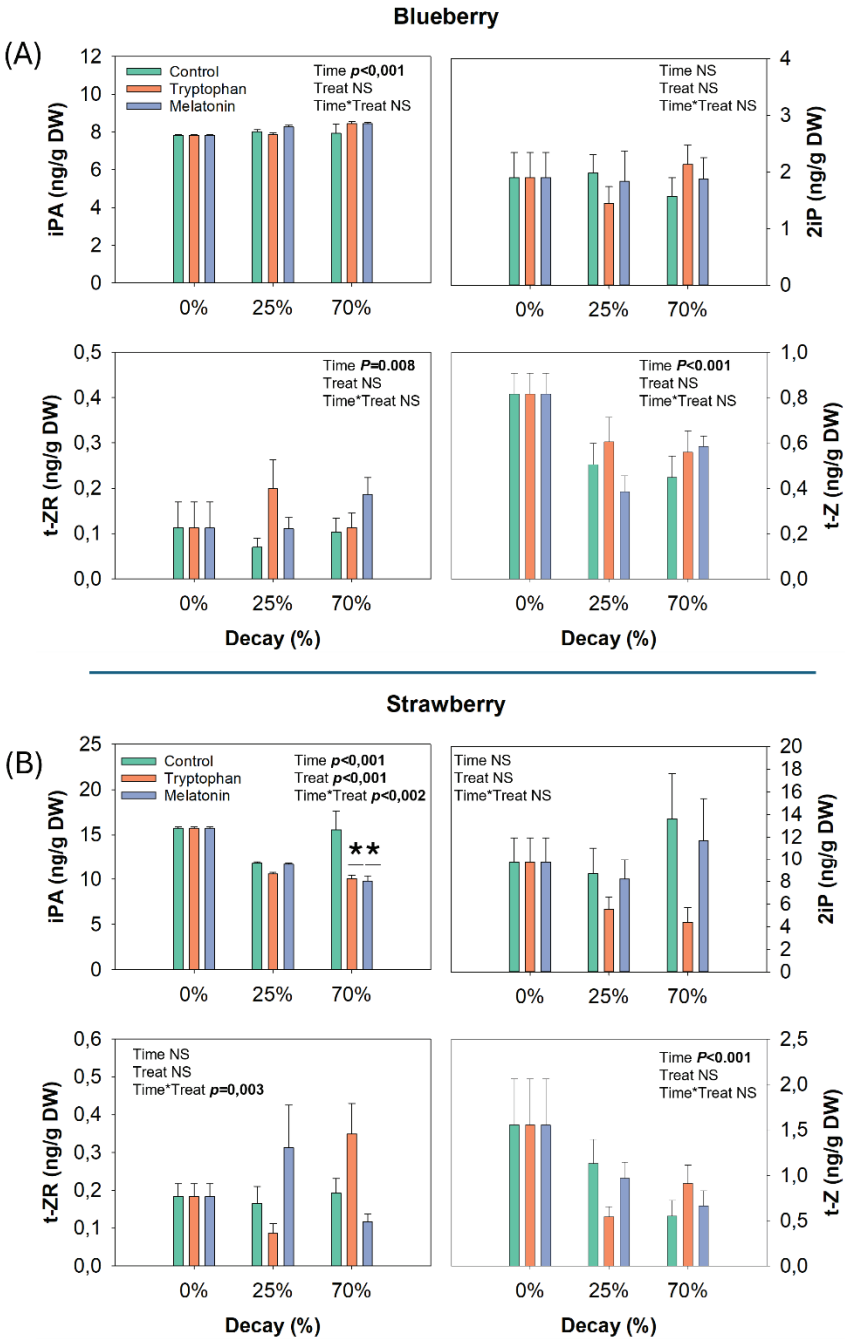


Figure S14. 1-Aminocyclopropane-1-carboxylic acid (ACC) content of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$).

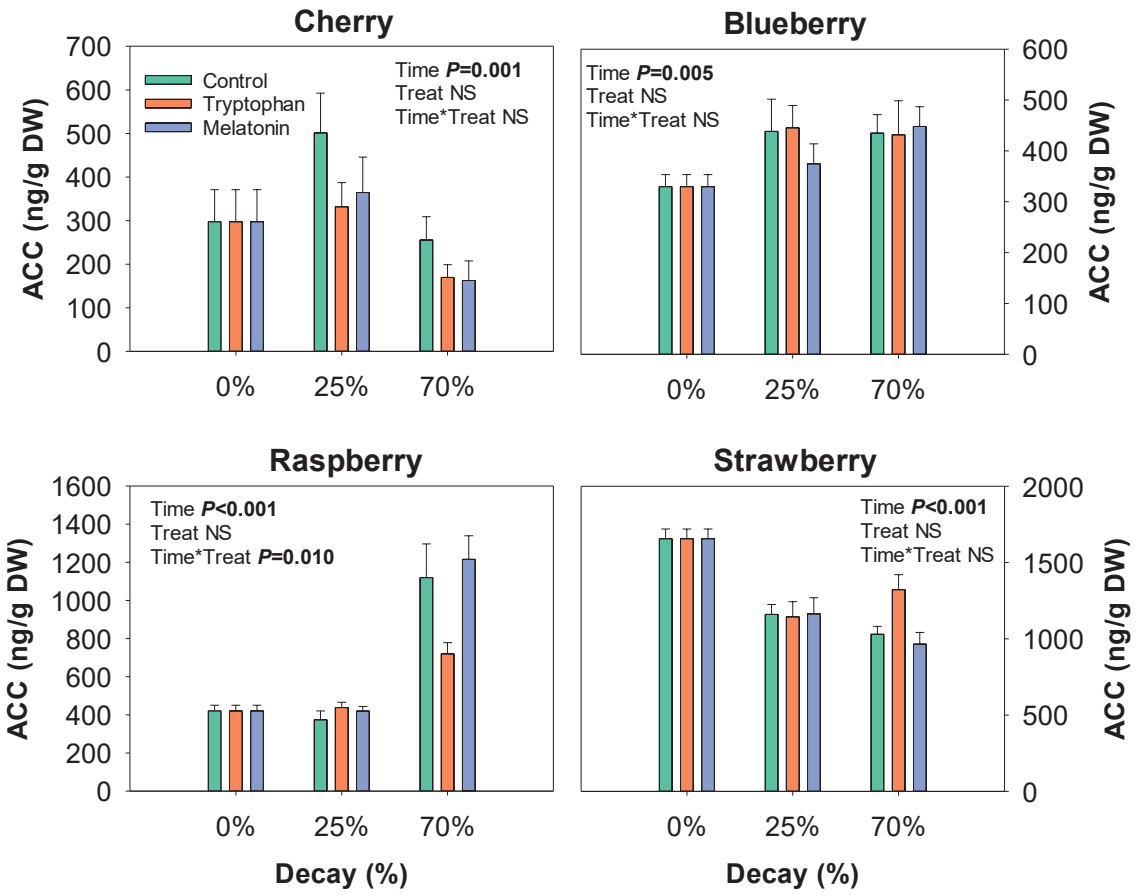


Figure S15. ABA/IAA ratio of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). ABA = abscisic acid; IAA = indole-3-acetic acid

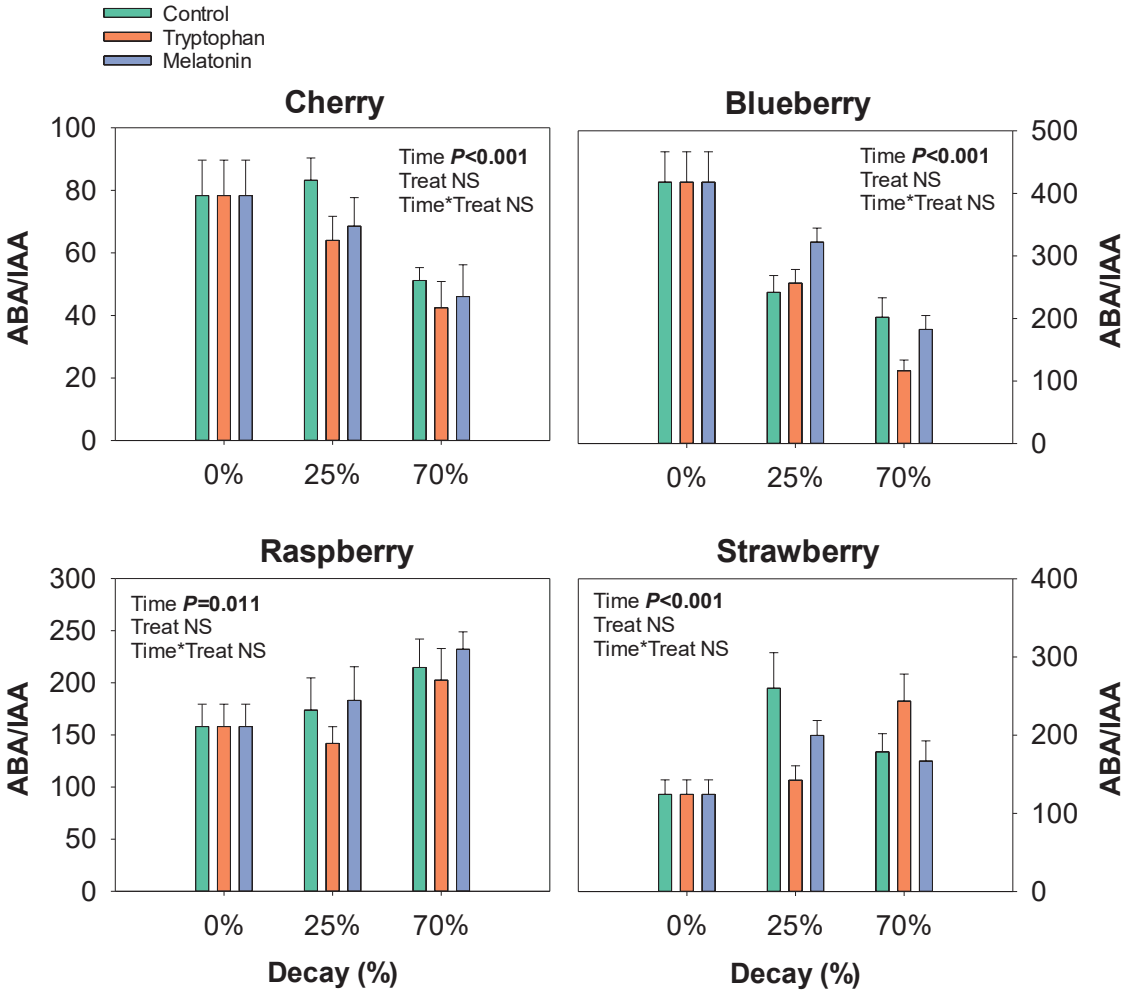
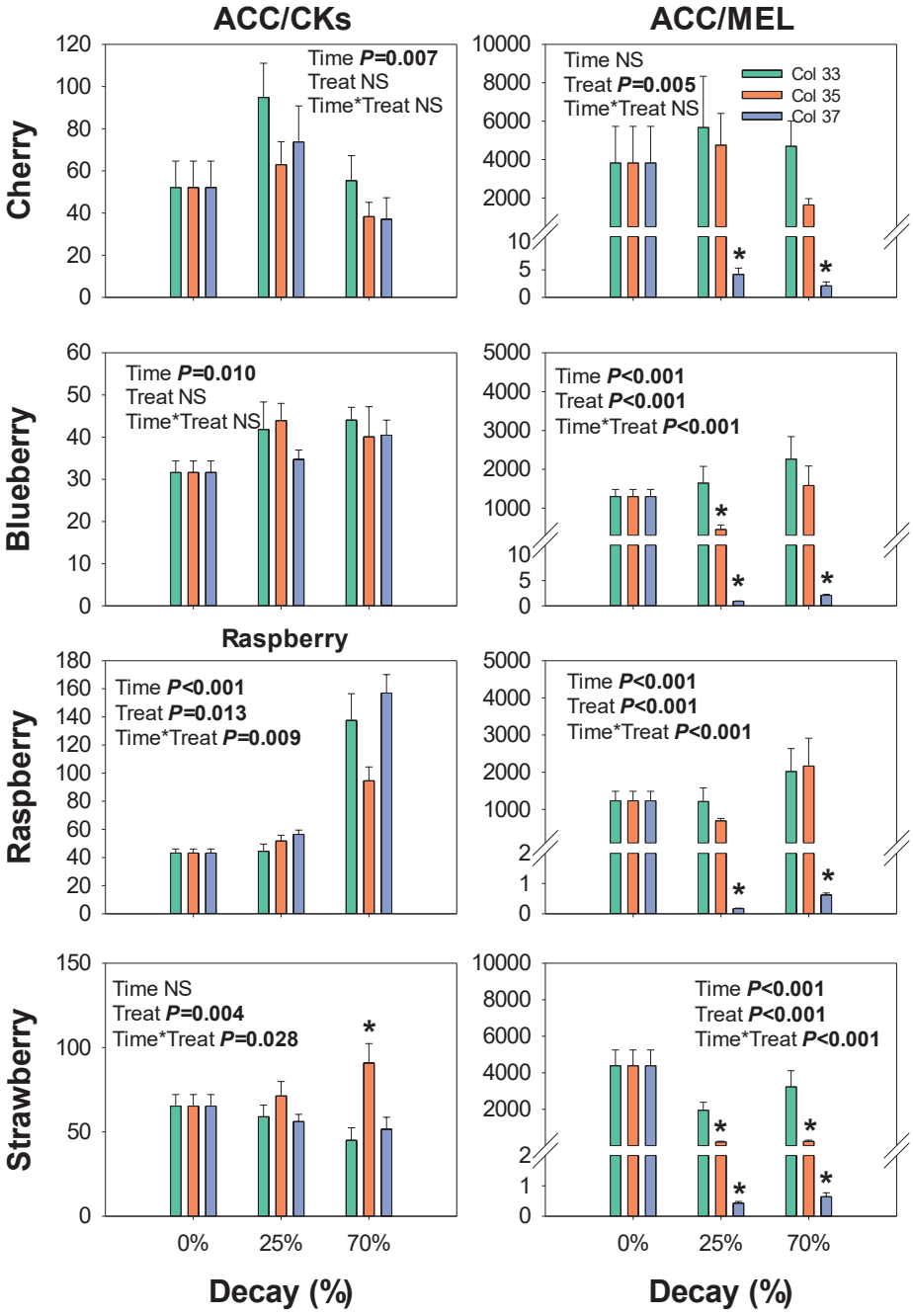


Figure S16. ACC/CKs and ACC/MEL ratio of cherries, raspberries, blueberries and strawberries during storage at room temperature after melatonin and tryptophan applications. Control refers to untreated fruits. Data represent the mean ± standard error of n = 10 boxes. NS indicates no significant differences ($P > 0.05$). Asterisk (*) indicates significantly different values between the control and treated fruit on that decay ($P < 0.05$). ACC = 1-Aminocyclopropane-1-carboxylic acid; CKs = cytokinins (including the content of 2ip, iPA, Z and ZR); MEL = melatonin.



CAPÍTOL 3

La melatonina desencadena canvis en el contingut d'antocianines i fitohormones específics del teixit durant el deteriorament postcollita de les prunes Angeleno



Chapter 3. Melatonin triggers tissue-specific changes in anthocyanin and hormonal contents during postharvest decay of Angeleno plums

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RESUM

La pruna és un fruit de pinyol que destaca per la seva curta vida útil a causa de la seva alta susceptibilitat a un ràpid deteriorament. Part d'aquesta deterioració és explicada per la sobremaduració del fruit. Recentment, s'ha investigat el paper de la melatonina en l'endarreriment de la descomposició postcollita, però la seva funció reguladora durant la sobremaduració encara està en debat. En aquest estudi, per entendre els esdeveniments fisiològics que tenen lloc durant la sobremaduració de la pruna i dilucidar el paper de la melatonina en la qualitat postcollita d'aquest fruit i la seva relació amb altres fitohormones, es va aplicar una solució de melatonina a 10^{-4} M immediatament després de la collita a les prunes Angeleno. Es va dur a terme una anàlisi específica dels teixits (mesocarpi i exocarpi) de fenols totals i d'antocianines, així com l'avaluació de diferents fitohormones mitjançant LC-MS/MS i paràmetres de qualitat del fruit. Els resultats van mostrar que durant la postcollita, els nivells endògens de melatonina van disminuir tant en el mesocarpi com en l'exocarpi de les prunes Angeleno. Així mateix, la fermesa de la pruna també va disminuir i es va trobar una forta correlació entre aquest paràmetre i l'àcid jasmònic (JA) i les citocinines. Per contra, després de l'aplicació exògena de melatonina, els nivells endògens de melatonina van augmentar tant en el mesocarpi com en l'exocarpi, però va tenir un efecte diferencial segons el teixit de la pruna. De fet, els continguts de fenols totals i antocianines van augmentar un 21% i un 58%, respectivament, en el mesocarpi després del tractament amb melatonina, però no es van trobar variacions en l'exocarpi de les prunes Angeleno. L'anàlisi hormonal del mesocarpi també va revelar un augment del JA i el seu precursor, l'àcid 12-*oxo*-fitodienoic (OPDA), el quart dia després de l'aplicació de melatonina, així com una correlació positiva entre la melatonina i la gibberel·lina 1 (GA_1). Aquests resultats suggereixen que la melatonina podria actuar com a molècula senyal, augmentant els continguts de compostos fenòlics mitjançant una regulació directa i també a través de la senyalització d'altres fitohormones. Per tant, aquesta recerca aporta informació valuosa per entendre el paper regulador de la melatonina i la seva relació amb les fitohormones durant la sobremaduració per contribuir a millorar la qualitat postcollita de les prunes.

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Melatonin triggers tissue-specific changes in anthocyanin and hormonal contents during postharvest decay of Angeleno plums

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ABSTRACT

Plum is a stone fruit that stands out for having a short shelf-life because of its high susceptibility to rapid deterioration. Part of this deterioration is explained by fruit overripening. Recently, the role of melatonin in delaying postharvest decay has been investigated but its regulatory function during overripening is still under extensive debate. In this study, to understand physiological events taking place in plums overripening and elucidate the role of melatonin on the postharvest quality of these fruits and its relationship to other plant hormones, Angeleno plums were sprayed with 10^{-4} M of melatonin solution immediately after harvest. We carried out tissue-specific (mesocarp and exocarp) analysis of total phenols and anthocyanin quantification, as well as the evaluation of different phytohormones by LC-MS/MS and fruit quality parameters. Results showed that during postharvest, endogenous melatonin contents decreased both in the mesocarp and the exocarp of Angeleno plums. Likewise, plum firmness also decreased and a strong correlation was found for this parameter with jasmonic acid (JA) and cytokinins. Conversely, after exogenous melatonin application, endogenous melatonin content increased both in mesocarp and exocarp but it had a differential effect depending on the plum tissue. Indeed, total phenol and anthocyanin contents arose by 21% and 58%, respectively, in the mesocarp after melatonin treatment but no variations were found in the exocarp of Angeleno plums. Hormonal analysis of Angeleno mesocarp also revealed an increase in the JA and its precursor, 12-*oxo*-phytodienoic acid (OPDA), on the fourth day after melatonin application as well as a positive correlation between melatonin and gibberellin 1 (GA_1). These results suggest that melatonin may be acting as a signal molecule increasing phenolic compounds contents through direct regulation and by signaling with other phytohormones. Therefore, this research provides valuable information for understanding the regulatory role of melatonin and its relationship with plant hormones during overripening to contribute to improve the postharvest quality of plums.

1. Introduction

Plum (*Prunus salicina* L.) is a popular fruit worldwide belonging to the genus *Prunus* and the Rosaceae family. Plums are stone fruits, as their morphology consists of an outer exocarp (skin), a fleshy mesocarp (pulp) and a lignified endocarp (pit) in the middle of the seed-containing fruit (Kader and Mitchell, 1989). Consumers appreciate plum fruits for their organoleptic properties (color, aroma, sweetness) as well as nutritional properties. Likewise, its richness in water, fiber, carbohydrates, vitamins (C, E, A, K) and antioxidants (phenolic compounds, anthocyanins and carotenoids) along with very low-fat contents provides great benefits for human health (Kim et al., 2003a; Lozano et al., 2009). However, stone fruits have a very short shelf-life during storage, being one of the most perishable foods (Kader, 1993). After fruit harvest, a shortage of water and nutrition from tree occurs, resulting in rapid postharvest deterioration that leads to greater waste.

Fruit overripening is one of the main causes of postharvest decay and entails a general loss of organoleptic properties, including flavor deterioration, peel darkness, firmness loss, dehydration and high pathogen susceptibility (Pott et al., 2020; Alkan and Fortes, 2015). Moreover, overripening is a complex process regulated by various endogenous signals, where an intricate interactive hormonal network determines the final quality of fruits and thus, commercial value (Xiang et al., 2021). Hence, elucidate the main causes that contribute to the short shelf-life once the fruit has been harvested has a high impact on both economics and environmental resources because extending fruit quality

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would prevent from important food losses (Riva et al., 2020). In this sense, several phytohormones have been described to modulate fruit postharvest performance. For instance, postharvest ripening is dependent upon abscisic acid (ABA) and ethylene contents in fruits, both known to fine-tune quality-trait attributes that strongly influence fruits shelf-life (Jia et al., 2016; Farcuh et al., 2018; Siebeneichler et al., 2020; García-Pastor et al., 2021). At the same time, anti-senescence properties have been attributed to auxins, cytokinins or gibberellins (Figueroa et al., 2012a; Li et al., 2016a; do Amarante et al., 2020), even though these phytohormones are usually at very low contents in fruit postharvest (Tijero et al., 2019). Other phytohormones, such as jasmonic acid and salicylic acid have also been described to participate in biotic stress tolerance during postharvest (Yao and Tian, 2005; Babalar et al., 2007) and, in the case of jasmonic acid, several authors have also highlighted its role in sugar and phenolic acid accumulation (Flores et al., 2017; Liu et al., 2017; Zhao et al., 2021). In plums, there has been a huge effort to elucidate ripening elicitors during fruit development, especially because of the presence of both climacteric and non-climacteric cultivars (Minas et al., 2015; Farcuh et al., 2019), but there is scarce information about the involvement of other phytohormones in plum postharvest performance.

Melatonin (N-acetyl-5-methoxytryptamine) is a natural indolamine synthesized from tryptophan (Back et al., 2016). Since its identification in vascular plants in 1995 (Dubbels et al., 1995), it has been found in a wide number of species being ubiquitous in plants highlighting Rosaceae, Poaceae, Vitaceae, Apiaceae and Brassicaceae families (Nawaz et al., 2016). In recent years, melatonin has been considered both an excellent antioxidant and a signal molecule (Hu et al., 2016) involved in a wide range of physiological processes such as germination (Xiao et al., 2019), plant development and growth (Sun et al., 2021), fruit development, ripening (S.Y. Wang et al., 2020), senescence (S. Li et al., 2019) and responses to biotic and abiotic stresses (Nawaz et al., 2021). In fruits, endogenous melatonin contents differ between different species and varieties, even between different tissues of the same fruit (S.Y. Wang et al., 2020). Additionally, it has been reported that melatonin is involved in delaying postharvest decay and senescence during fruit storage and contributes to quality maintenance (X. Wang et al., 2019). Previous studies found that exogenous melatonin kept firmness, delayed cell wall degradation, reduced weight loss and respiration rate in different fruits such as pear (J. Liu et al., 2019), sweet cherry (F. Wang et al., 2019), peach (Gao et al., 2016), plum (Bal, 2019) and tomato (Aghdam et al., 2019). Moreover, it has been described that melatonin application increases the antioxidant system during postharvest fruits both by itself and by increasing the content of other antioxidants such as ascorbic acid, glutathione, phenols and anthocyanins as well as enzymatic antioxidants (Zhang et al., 2020).

Nevertheless, the regulatory role of melatonin together with its interaction with other phytohormones during overripening is still poorly understood and there is no information about its tissue-specific effects in delaying postharvest decay and maintaining the quality of fruit. Therefore, the aim of this study was (i) to understand the physiological events taking place in Angeleno plums overripening, which have been described as suppressed-climacteric cultivars (Minas et al., 2015), (ii) to evaluate the tissue-specific role of exogenous melatonin on postharvest quality as well as (iii) to elucidate its relationship to other phytohormones during plum postharvest shelf-life.

2. Materials and methods

2.1. Plant material, treatment and sampling

Plums (cv. 'Angeleno') were collected at commercial harvest maturity from a commercial orchard in Extremadura (Spain) and they were immediately brought to the laboratory at the University of Barcelona (Barcelona, Spain). Plums were selected for uniformity according to their size and color and were excluded those with mechanical damage or pathogen symptoms. Then, fruits were distributed randomly into two groups (i) control and (ii) melatonin consisting of 5 replicates each (10 fruits/replicate) and were stored at room temperature (25 °C) for 8 days. For melatonin treatments, plums were sprayed immediately with 10^{-4} M melatonin, whereas the control group was not subjected to any treatment.

Samplings were performed at 0, 2, 4, and 8 days after treatment application. For each sampling time, the firmness of one fruit for replicate was measured and then, the fruit was cut into two pieces. In both pieces, the endocarp was removed and in one of them, the peel and pulp were separated for subsequent tissular analysis. Then, both the entire fruit, the mesocarp and exocarp were immediately separately frozen in liquid nitrogen and kept at −80 °C until analysis.

2.2. Plum quality parameters

Fruit firmness was determined by using a FT 327 penetrometer (QA Supplies, Norfolk, VA, USA) and pushing the cone into fresh plum mesocarp avoiding seed contact.

For total soluble solids (TSS) and total acidity (TA) analysis, 5 g of entire fruit samples were ground to a fine powder using a bead mill (Qiagen TissuLyzer II) and homogenized with MilliQ water using a vortex. TSS estimation was measured from ºBrix in 1 mL of the mix using a refractometer (Hannah Instruments, Italy) following the methodology described by Boulton et al. (1999). For TA measures, 10 mL of the mix were diluted in 100 mL of MilliQ water and used for its determination with 0.1 M NaOH and 1% phenolphthalein as an indicator to estimate predominant plum acid content (g malic acid·g fresh weight^{-1}) as described in Latimer (2012). Finally, pH was estimated using a pH-meter (MicropH 2002, Crison Instruments, S.A, Alella (Barcelona), España).

Total anthocyanins were determined as described by Gitelson et al. (2001). Mesocarp and exocarp samples (100 mg) were ground to a fine powder using a bead mill (Qiagen TissuLyzer II) and extracted with 750 µl methanol (100%, v/v) using vortex and ultrasonication (Branson 2510 ultrasonic cleaner, Bransonic, USA). Extracts were centrifuged at 13,000 rpm for 10 min at 4 °C and then, supernatants were collected and the pellets were re-extracted following the same procedure. Supernatants were pooled followed by acidification with 1% HCL. Total anthocyanins were measured spectrophotometrically (CE Aquarius UV/Visible, Cecil Instruments Ltd, Cambridge, United Kingdom) at 530 nm and they were calculated using cyanidin-3-glucoside as a reference as described in Siegelman and Hendricks (1958).

In order to estimate total phenols content (TPC), methanolic extracts were performed following the same procedure as in total anthocyanins. Subsequently, methanolic extracts were mixed with pure methanol (1:2 dilution, v/v) and 1 mL Folin-Ciocalteu reagent (1:10 diluted with MilliQ water) was added. Then, the mix was incubated for four minutes and 800 µl Na_2CO_3 5% (75 g/L) were added. After 30 min at room temperature and darkness, absorbances were measured spectrophotometrically (CE Aquarius UV/Visible, Cecil Instruments Ltd, Cambridge, United Kingdom) at 765 nm. TPC was calculated as described in Singleton and Rossi (1965) and Li et al. (2007) expressed in mg gallic acid equivalents (GAE)·g dry weight^{-1}.

2.3. Analysis of regulatory cis-elements of chalcone synthase (CHS) and phenylalanine ammonia lyase (PAL) from Prunus salicina

To elucidate putative cis-elements on the CHS and PAL sequences of *Prunus salicina* from NCBI accessions CM035341.1:289590–290885 and CM035342.1:10256063–10259379. Melatonin responsive elements were retrieved based on work by Liang et al. (2017), excluding regions related to ARFs and non-fruit related elements. Likewise, jasmonic acid (JA) responsive elements were also analysed according to Fernández--Calvo et al. (2011) and Moore et al. (2022).

2.4. Hormonal profiling

Endogenous hormones content, including melatonin, abscisic acid (ABA), salicylic acid (SA), indoleacetic acid (IAA), the gibberellins GA_1, GA_3, GA_4 and GA_7, JA, its precursor 12-*oxo*-phytodienoic acid (OPDA) and its conjugates (JA-Ile, JA-Met, JA-Val, JA-Leu, JA-Phe), the cytokinins *trans*-zeatin (*t*-Z), its riboside *trans*-zeatin (*t*-ZR), 2-isopentenyl adenine (2iP) and its riboside isopentenyl adenosine (IPA), and the ethylene precursor 1-amynocyclopropane-1-carboxylic acid (ACC), were determined by ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) as described previously in Müller and Munné-Bosch (2011). In short, 100 mg of each mesocarp and exocarp samples were extracted with 250 µl methanol: isopropanol:acetic acid, 50:49:1 (v/v/v) and deuterium-labeled internal standards (d4-Mel, d6-ABA, d4-SA, d5-IAA, d2-GA_1, d2-GA_4, d2-GA_7 d5-JA, d5-tZ, d6–2iP, and d6-IPA) using vortex and ultrasonication (Branson 2510 ultrasonic cleaner, Bransonic, USA). Extracts were centrifuged at 13,000 rpm for 10 min at 4 °C, supernatants were collected and the pellets were re-extracted with 250 µl of the same solvent. Then, collected supernatants were merged and filtered through

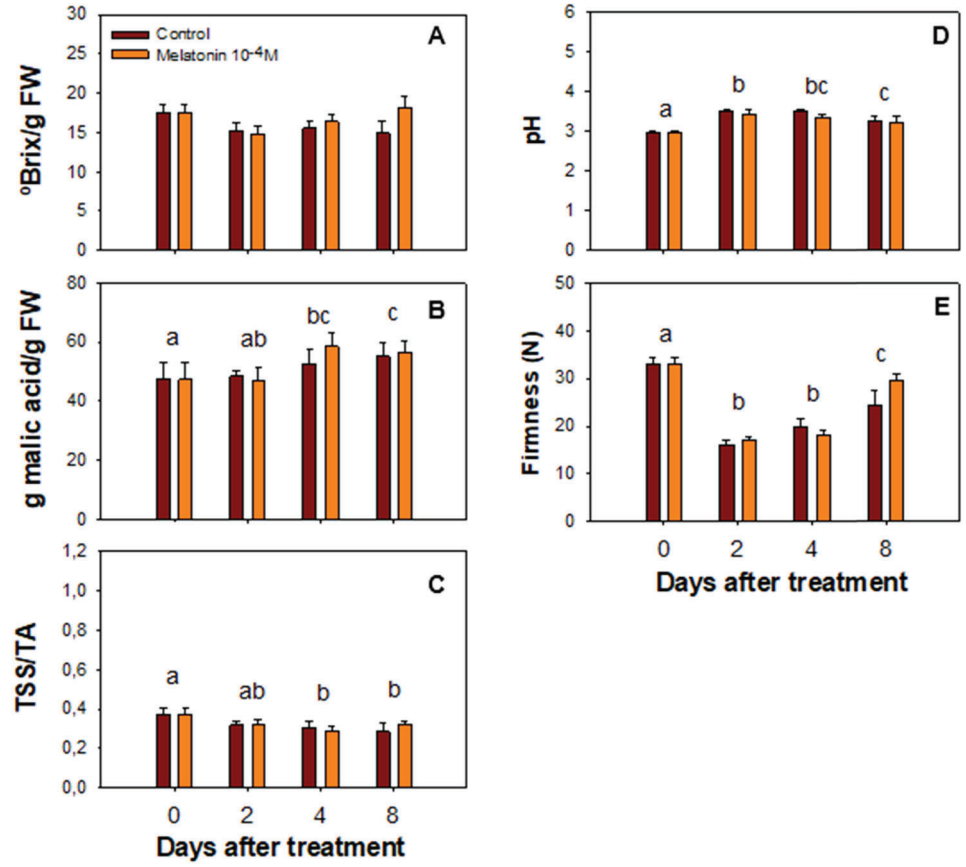
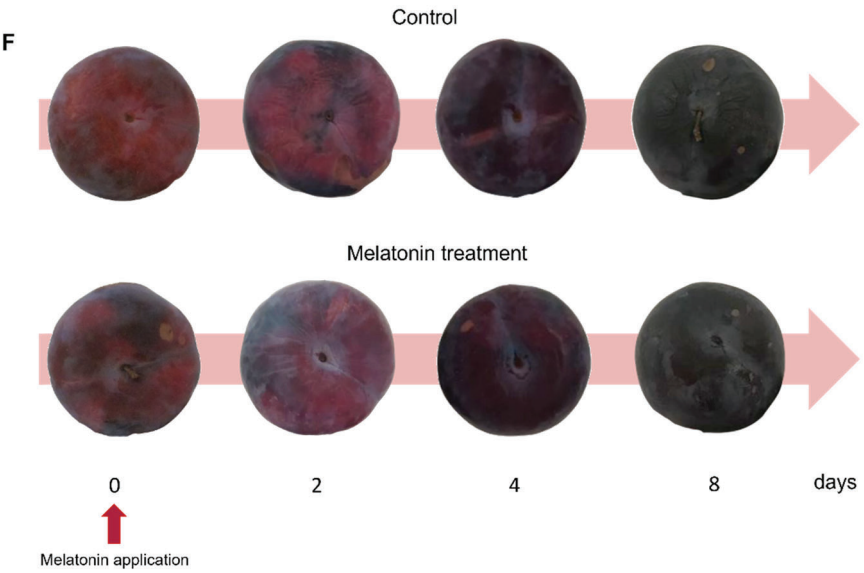


Fig. 1. Evaluation of plum quality parameters. (A) Total Soluble Solids (TSS), (B) Total Acidity (TA), (C) TSS/TA ratio, (D) pH, (E) Firmness and (F) plum phenotype of control and 10^{-4} M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5. Different lower case letters indicate significant differences over time and asterisks indicate significant differences ($p < 0.005$) between control and treated at that point in time. See Table 1 for statistics.



a 0,22 µl PTFE filter (Phenomenex, United States) before UHPLC-ESI MS/MS analysis. High-performance liquid chromatography was coupled to a triple quadrupole mass spectrometer (QTRAP 4000, AB Sciex, Concord, Ontario, Canada) and a LUNA C18 column (Phenomenex Inc., United States (1,6 µm, 100 ×2,1 mm)) was used. Solvent A was water with 0.05% acetic acid and solvent B was acetonitrile with 0.05% acetic acid. The flow rate was set at 0.6 mL/min. Quantification was made considering recovery rates for each sample by using the deuterium-labeled internal standards and calibration curves for each analyte were generated using MultiQuantTM 3.0.1 software.

2.5. Statistical analysis

All data were analyzed by one-way factorial analysis of variance (ANOVA). Multiple comparisons tests were carried out using Tukey's HSD post hoc tests. Differences were considered significant at a probability level of $p \leq 0.05$ and statistical analyses were performed using the IBM SPSS Statistics 19 statistical package. For the correlations, a Pearson correlation was performed between the multiple parameters analyzed using RStudio® software.

3. Results

3.1. Tissue-specific physiological events during plum overripening

To characterize the overripening process and its physiological changes during fruit storage at room temperature, TSS, TA, TSS/TA, pH and firmness were evaluated (Fig. 1). A significant decrease of firmness (Fig. 1E-F) was observed with prolonging storage time (0–8 days) with a reduction between dates of 27%, having an abrupt decrease (50%) at day 2 and a negative impact on plum texture and appearance (Fig. 1F). Furthermore, TA only showed slight increases (around 15%) at day 8 compared to the TA value at the beginning of the experiment (Fig. 1B). In contrast, TSS values were constant (Fig. 1A), which together with the increase of acidity resulted in a 20% decrease in the TSS/TA ratio (Fig. 1C). Additionally, pH measurements also showed variations during postharvest days, having an increase at day 2 and therefore, a slight decrease at day 8 (Fig. 1D).

Analysis of total phenol contents on plums exocarp showed a progressive increase during overripening (Fig. 2A), with maximum contents at day 8, being 28% higher than initial contents. Meanwhile, no changes were observed in the mesocarp, where total phenol contents remained constant and were twice lower than exocarp contents (Fig. 2A). Moreover, anthocyanin contents also increased in the exocarp of Angeleno plums (Fig. 2B), being 58% higher at day 8 than the initial day. Nonetheless, no variations on mesocarp anthocyanin contents were found along the time of evaluation (Fig. 2B). Additionally, anthocyanin contents in the exocarp of plums were 188 and 351-fold higher than in the mesocarp at the beginning and the end of the study, respectively, indicating lower anthocyanin contents in the mesocarp and tissue-specific physiochemical changes during overripening. In this sense, when the values were expressed by 100 g of fruit FW (Fig. 3), total phenol and anthocyanin contents in the mesocarp of plums after 8 days of storage, only represented 1/3 and half of the exocarp amounts, which has important nutritional implications.

Endogenous melatonin contents had a strong decrease during postharvest shelf-life, both in mesocarp and exocarp of Angeleno plum (Fig. 4). Initially, plum tissues showed high values of melatonin contents ($14 \pm 3,6$ and $20 \pm 6,4$ ng·g DW^{-1} in mesocarp and exocarp, respectively). Thereafter, its contents abruptly decreased in both tissues from day 2–8, showing a 90% decrease to almost undetectable contents, less than 2 ng·g DW^{-1} (Fig. 4). Likewise, in plum mesocarp a significant decrease in the content of indole-3-acetic acid (IAA) from day 0–2 were observed and thereafter, its levels remained constant (Fig. S1). Conversely, JA showed a transient increase of 20% at day 2 (Fig. S2), the same as 2iP and IPA (Fig. S3). Instead, jasmonic acid precursor, OPDA, showed an abrupt decrease from day 0–2, just as all other conjugated jasmonates (JA-Ile, JA-Met, JA-Leu, JA-Val), which also showed

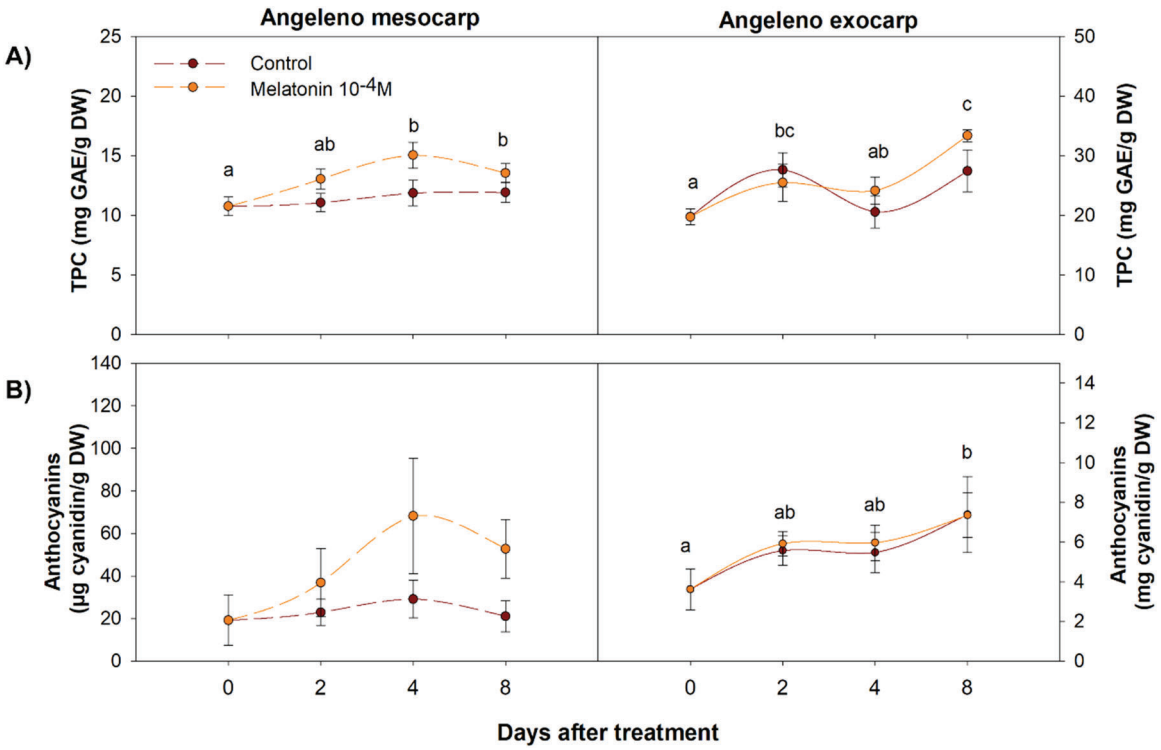


Fig. 2. (A) Total phenol contents (TPC) and (B) anthocyanin contents in the mesocarp and exocarp of control and 10^{-4} M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5. Different lower case letters indicate significant differences over time and asterisks indicate significant differences ($p < 0.005$) between control and treated at that point in time. See Table 1 for statistics.

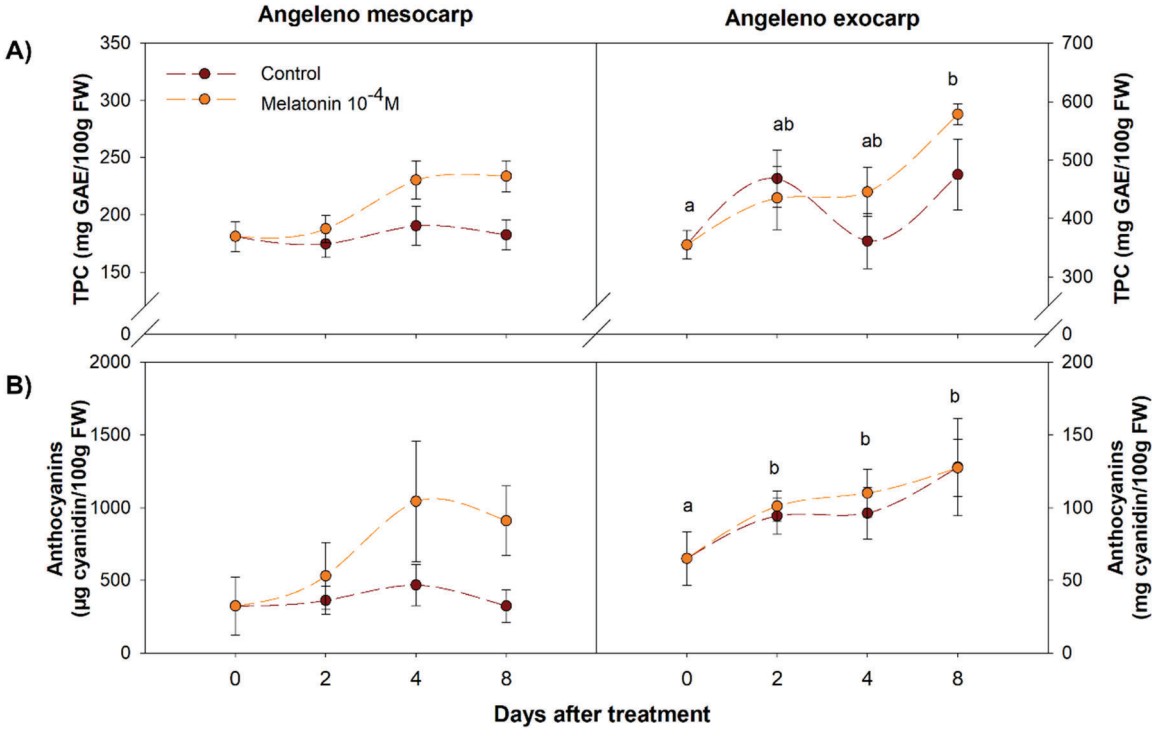


Fig. 3. (A) Total phenol contents (TPC) and (B) anthocyanin contents in the mesocarp and exocarp of control and 10^{-4} M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5. Data represent the means ± standard error of n = 5. Different lower case letters indicate significant differences over time and asterisks indicate significant differences ($p < 0.005$) between control and treated at that point in time. See Table 1 for statistics.

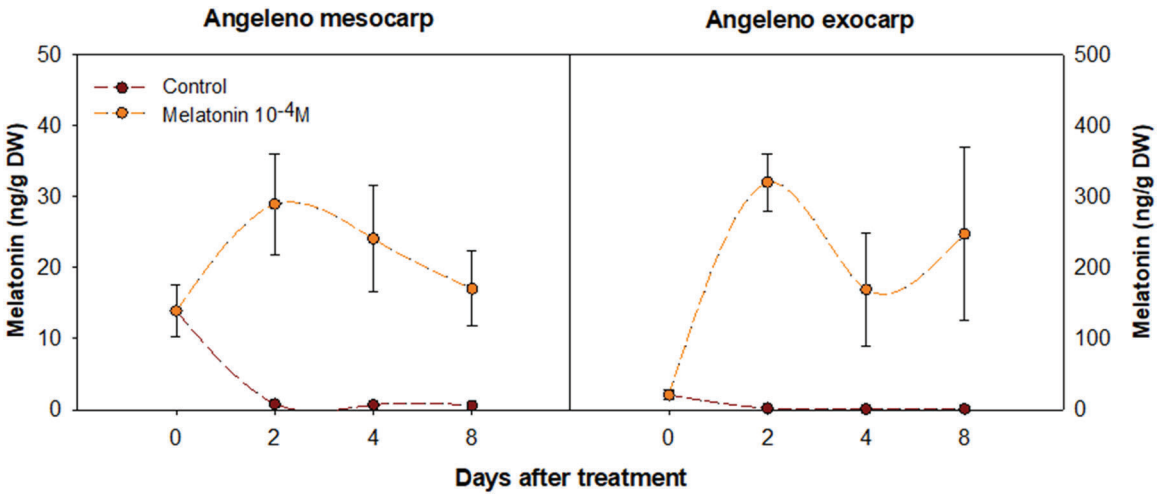


Fig. 4. Endogenous contents of melatonin in the mesocarp and exocarp of control and 10^{-4} M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5. Different lower case letters indicate significant differences over time and asterisks indicate significant differences ($p < 0.005$) between control and treated at that point in time. See Table 1 for statistics.

decreasing contents during plums postharvest (Fig. S2). Additionally, a decrease in *t*-ZR and GA_7 contents to values less than 1 ng·g DW^{-1} was also observed (Fig. S3 and S4), and, in contrast, ABA, SA and ACC levels remained constant (Fig. S1). Therefore, in general, a degradation of a wide variety of phytohormones was occurring during overripening in the mesocarp of Angeleno plums.

Finally, a strong positive correlation was found for firmness with OPDA, JA-Ile and JA-Met but a negative one with JA, *t*-Z, 2iP and IPA (Table S1).

3.2. Exogenous melatonin induces tissue-specific changes in fruit quality

Plum quality was assessed to evaluate the effects of 10^{-4} M exogenous melatonin treatment. No significant differences were observed for TSS, TA, TSS/TA, pH and firmness in treated plums compared to control group (Fig. 1).

In the analysis of nutritional properties, total phenol contents showed significant differences between control and melatonin-treated plums, presenting an increase of 21% in the mesocarp phenol contents of Angeleno-treated fruits after 4 days of application (Fig. 2A). Despite endogenous melatonin contents increased both in mesocarp and exocarp

(97,21% and 99,46% respectively) ($p < 0.001$) after melatonin treatments of Angeleno plums (Fig. 4), no significant differences were found for total phenols in the exocarp of these treated fruits (Fig. 2A). Likewise, anthocyanin contents presented a rise of 58% in the mesocarp of fruits after melatonin treatment mainly from day 4, while the exocarp presented no differences (Fig. 2B). This increase of phenolic compounds was also observed in 100 g of an edible portion (Fig. 3), reaching three times more anthocyanins at the end of the experiment. Moreover, a high correlation with total phenolic compounds (TPC) and melatonin as well as with TPC and anthocyanin were found (Table S1). Therefore, exogenous melatonin showed a differential effect depending on the plum tissue regarding phenolic and anthocyanin composition.

An analysis of regulatory cis-elements (Fig. S5 and S6) on the sequences of CHS and phenylalanine ammonia lyase PAL of *Prunus salicina* showed that both genes had upstream and downstream regions with responsive elements to both melatonin and JA, which could be an additional evidence to elucidate the mechanism behind greater contents on TPC and anthocyanins after melatonin treatments.

3.3. Exogenous melatonin promotes endogenous changes of other hormonal groups in plums

In order to study the possible relationship between melatonin and other phytohormones during postharvest overripening, the endogenous contents of different developmental and ripening-related plant hormones in the exocarp and mesocarp of Angeleno plums were analyzed (Fig. 5). After melatonin treatment, the contents of IAA, SA, ABA and ACC (Fig. S1) did not differ significantly but a transient increase of 28,74% in the contents of OPDA at day 4 were observed ($p = 0.012$), 4-fold more than those registered in control fruits (Fig. S2a). Moreover, JA increased concomitantly with the increase of OPDA at day 4 in melatonin-treated plums, being 14% higher compared to the control fruits, but thereafter, its contents decreased and were 18% lower than non-treated plum fruits (Fig. S2a). Nevertheless, no variations were found in the studied conjugated jasmonates (Fig. S2b). The content of cytokinins, including 2iP, *t*-Z and *t*-ZR, did not show significant differences in treated fruits (Fig. S3). However, IPA contents showed a decrease of 17% on day 8 of the study (Fig. S3). Furthermore, no differences were observed in the content of GAs (Fig. S4) but GA_1 had a sharp increase at day 4 in melatonin treated fruits, and even there were no statistical differences at this point between treatments ($p = 0.065$), the mean values of the contents were 5-fold higher than those in control plums (Fig. S4). Nevertheless, a sturdy correlation between GA_1 and melatonin was found ($p < 0,01$) in Angeleno plums (Table S1).

Overall, quality parameters and hormonal profiling indicated a differential response to postharvest decay for an endogenous condition and exogenous melatonin treatment. While for the first, overripening was mainly characterized by a firmness reduction in the mesocarp as well as an increase of phenolic compounds in the exocarp and a general phytohormones degradation, including melatonin. The second one was characterized by an increase of melatonin in both exocarp and mesocarp in addition to an increase of antioxidant phenolic compounds in the

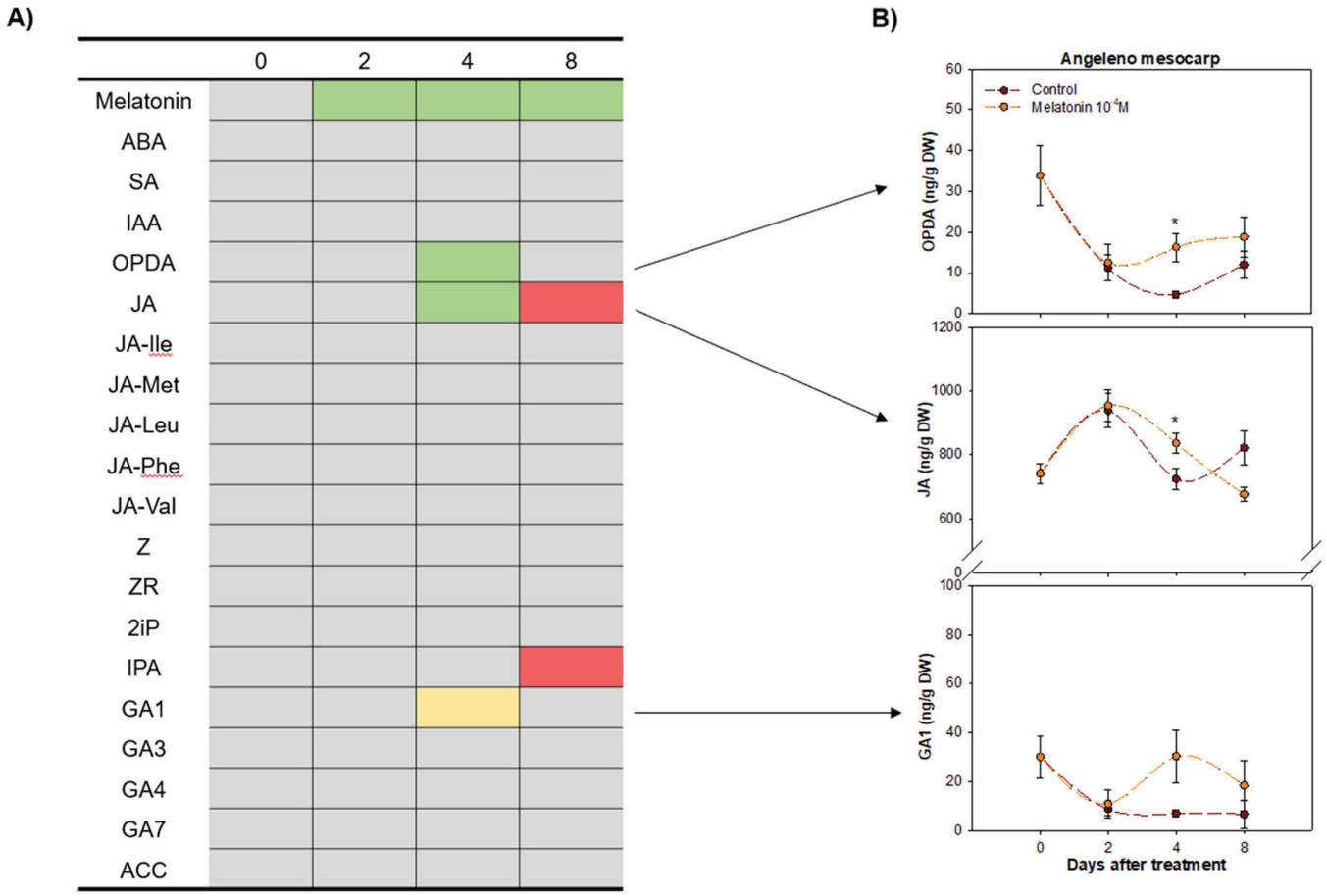


Fig. 5. (A) Variations in the endogenous phytohormones in the mesocarp of Angeleno plum after 10^{-4} M melatonin application during days after treatment. Gray boxes indicate no variations in their content, green boxes indicate an increase, red boxes indicate a decrease and yellow boxes indicate a strong trend to increase. Significant variations were considered when $p < 0,05$. (B) 12-*oxo*-phytodienoic acid (OPDA), jasmonic acid (JA) and gibberellin 1 (GA_1) endogenous content in the mesocarp of control and 10^{-4} M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5 and asterisks indicate significant differences ($p < 0.005$) between control and treated at that point in time. See Table 1 for statistics.

Table 1
P_values of one way ANOVA performed for the time and treatment factors.

	P_{Time}	$P_{Treatment}$
TSS (°Brix·gFW^{-1})	NS	NS
TA (g malic·gFW^{-1})	0.025	NS
TSS/TA	0.046	NS
pH	< 0.001	NS
Firmness (N)	< 0.001	NS
TPC (mg GAE·gDW^{-1}) mesocarp	0.048	0.017
TPC (mg GAE·gDW^{-1}) exocarp	0.016	NS
TPC (mg GAE·100gFW^{-1}) mesocarp	NS	0.019
TPC (mg GAE·100gFW^{-1}) exocarp	0.024	NS
Anthocyanin content (μg cyanidin·gDW^{-1}) mesocarp	NS	0.045
Anthocyanin content (μg cyanidin·gDW^{-1}) exocarp	0.011	NS
Anthocyanin content (μg cyanidin·100gFW^{-1}) mesocarp	NS	0.045
Anthocyanin content (μg cyanidin·100gFW^{-1}) exocarp	0.015	NS
Melatonin (ng·g DW^{-1}) mesocarp	NS	< 0.001
Melatonin (ng·g DW^{-1}) exocarp	NS	< 0.001
IAA (ng·g DW^{-1}) mesocarp	< 0.001	NS
SA (ng·g DW^{-1}) mesocarp	NS	NS
ABA (ng·g DW^{-1}) mesocarp	NS	NS
ACC (ng·g DW^{-1}) mesocarp	NS	NS
OPDA (ng·g DW^{-1}) mesocarp	< 0.001	NS
JA (ng·g DW^{-1}) mesocarp	< 0.001	NS
JA-Ile (ng·g DW^{-1}) mesocarp	< 0.001	NS
JA-Met (ng·g DW^{-1}) Mesocarp	0.004	NS
JA-Leu (ng·g DW^{-1}) mesocarp	< 0.001	NS
JA-Phe (ng·g DW^{-1}) Mesocarp	NS	NS
JA-Val (ng·g DW^{-1}) mesocarp	< 0.001	NS
2-ip (ng·g DW^{-1}) mesocarp	< 0.001	NS
IPA (ng·g DW^{-1}) mesocarp	0.003	NS
Z (ng·g DW^{-1}) Mesocarp	0.001	NS
ZR (ng·g DW^{-1}) mesocarp	< 0.001	NS
GA_1 (ng·g DW^{-1}) mesocarp	NS	NS
GA_3 (ng·g DW^{-1}) mesocarp	0.008	NS
GA_4 (ng·g DW^{-1}) mesocarp	NS	NS
GA_7 (ng·g DW^{-1}) mesocarp	0.013	NS

mesocarp, including anthocyanins leading to a color change in the pulp, while an increase of OPDA and JA was also observed (Fig. 6).

4. Discussion

4.1. Fruit softening and hormonal degradation are events tightly associated with overripening of Angeleno plums

Plums are an important and well-liked stone fruit worldwide valued for their color, palatability and aromatic properties as well as their richness in bioactive compounds, including vitamins, phenols, and anthocyanins (Gil et al., 2002). However, ripe plums are highly perishable and predisposed to a rapid deterioration triggering several physiological and visible changes resulting in a loss of edible quality during their postharvest life, mainly because of overripening (Pott et al., 2020). Fruit softening is a needed and complex regulated process that includes cell wall degradation to become an edible fruit. However, an excessive degree of softening leads to texture decay and has a negative impact on fruit quality (Wang et al., 2018). In our study, plums stored at room temperature for 8 days showed an immediate decline in firmness during storage time and a later recovery, probably due to a typical overripening desiccation process once the fruit is separated from the mother plant, which also adversely affected the appearance and texture of plum fruits. Other quality parameters like TSS, TA, and the ratio TSS/TA are also determining for fruit acceptance and usually, these parameters present several changes after harvested and stored at room temperature (Kappel et al., 2002). Here, postharvest Angeleno plums showed variations in TSS/TA as well as pH alterations through the days of evaluation, which could modify organoleptic properties of plum fruits and compromise their quality, as it has been described for other fruits such as pomegranate, peach, tomato and apple (Hussein et al., 2020; Gohari et al., 2021; Al-Dairi et al., 2021; Nyamende et al., 2021).

Plums are a rich source of antioxidants such as anthocyanins and phenols. In this sense, a high level of phenolic compounds has been reported in plums (Kim et al., 2003b), which have important beneficial

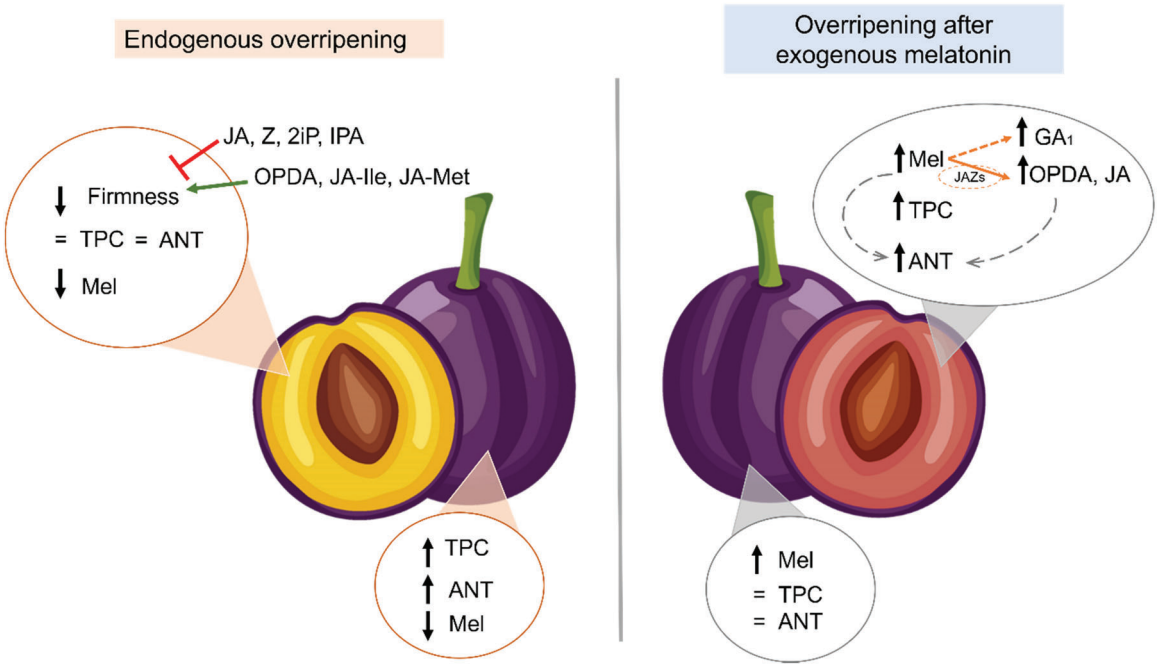


Fig. 6. Proposed model of the different physiological changes during both endogenous and after exogenous melatonin overripening in the mesocarp and exocarp of plum. TPC: total phenol content; ANT: anthocyanin content; Mel: melatonin; JA: jasmonic acid, JAs: jasmonates; OPDA: 12-*oxo*-phytodienoic acid; JA-Met: jasmonoyl-methionine; JA-Ile: jasmonoyl-isoleucine; 2iP: 2-isopentenyl adenine; Z: *trans*-zeatin; ZR; *trans*-zeatin riboside; IAA: indole-3-acetic acid; GA: gibberellin. Discontinuous lines indicate putative relationships. This figure was created with the help of a vector taken from the vecteezy account at https://www.vecteezy.com/free-vector/nature.

health-promoting effects since these bioactive compounds are known to promote free radical scavenging (Di Carlo et al., 1999). Likewise, anthocyanins stand out for being both potent antioxidants and plant pigments responsible for the red, blue and purple colors of many fruits. Angeleno plum is characterized by a high anthocyanin content in the exocarp while very low contents can be found in the mesocarp, acquiring a red-purple coloration in the peel and a yellow one in the pulp. However, from the present study, it was visible that during postharvest storage at room temperature, the content of anthocyanins as well as total phenols in the exocarp was increased, which resulted in a darkening of the exocarp up to almost black color. In plums mesocarp, both anthocyanins and total phenols remained constant until the end of postharvest evaluation. Throughout fruit storage, it is described that there is also accumulation of reactive oxygen species (ROS), being one of the main factors causing fruit decay because their accumulation can lead to cell damage and ultimately cell death (Vicente et al., 2006). Hence, the high content of phenolic compounds in Angeleno exocarp could prevent from oxidative damage given the antioxidant nature of these compounds, especially since fruit exocarp is the most exposed part to abiotic and biotic pressure during fruit postharvest.

Ripening and overripening are complex processes fine-tuned at different physiological levels, including hormonal regulation, which represents a key factor determining fruit quality (Fenn and Giovannoni, 2021). Here, ABA was the most abundant phytohormone in the mesocarp of plums during postharvest and its levels remained constant during postharvest storage. Nevertheless, hormonal content variations such as a decrease of different phytohormones contents also occurred as fruit deterioration progressed, as it was the case for *t*-Z, *t*-ZR and the bioactive gibberellin GA_7. A previous study in the postharvest of sweet cherries showed that there was a high degradation of cytokinins and gibberellins during decay of cherry fruits (Tijero et al., 2019), although a higher decrease was observed for free cytokinin bases more than their ribosides, contrary to what happened in plums of the present study. In another study with strawberries, exogenous cytokinin applications induced a defense response against abiotic and biotic stresses, maintaining postharvest fruit quality (Li et al., 2016b). Likewise, preharvest applications of GA_3in apples improved their quality during storage especially by reducing firmness loss (Souza et al., 2016). These results, together with those found for plums in the present study, strike gibberellins and cytokinin contents as important factors to either determine quality or fruit shelf-life. On the other hand, a decrease in the contents of OPDA, conjugated jasmonates and IAA were also observed during postharvest of plums. In plants, OPDA, which is the precursor of JA, could play a role both by itself and JA-dependent (Böttcher and Pollmann, 2009) in redox signaling (Müller et al., 2017) and plant defense against biotic stress (Gleason et al., 2016; Scalschi et al., 2015; Raacke et al., 2006). Previous studies also reported that exogenous auxin application prevents cell wall degradation in postharvest of strawberries (Chen et al., 2016; Figueroa et al., 2012b). Hence, this wide variety of phytohormones degradation may also contribute to fruit deterioration in plums stored at room temperature although further investigations are required to unravel the possible roles of these plant hormones in plum overripening. Furthermore, although cytokinins and JA have been described to improve quality maintenance and delay softening (Rojas et al., 2021; Roussos et al., 2021; Massolo et al., 2014; Balbontín et al., 2018; Saracoglu et al., 2017), here a negative correlation was found with firmness and JA, Z, 2iP and IPA which showed an increase at day 2 corresponding with the main decrease of firmness. In contrast, a positive correlation with firmness and OPDA, JA-Ile and JA-Met were observed, suggesting that these phytohormones might be involved in the modulation of fruit firmness, although its role is less clear.

4.2. Melatonin acts as a signal molecule in plum postharvest quality inducing tissue-specific phenolic compounds and plant hormones

Melatonin is a multifunctional molecule distributed ubiquitously in diverse plant organs and is responsible for stimulating various physiological processes (Debnath et al., 2019). In fruits, the endogenous melatonin content differs between different species and varieties. In this case, 13 ng·g DW^{-1} of endogenous melatonin have been detected in the mesocarp of Angeleno plums at the start of the experiment. These values are similar to those detected in other species such as 3–12 ng·g DW^{-1} in some varieties of cherry (Kirakosyan et al., 2009) but higher than others as grapes (<1 ng·g DW^{-1}) (Iriti et al., 2006). However, in other fruits like cranberries or tomatoes higher contents have been found (25–96 ng·gDW^{-1} and 7–240 ng·g DW^{-1} respectively) (Brown et al., 2012; Riga et al., 2014). Nevertheless, during postharvest storage, it is common for fruits to experience a general decrease of endogenous melatonin contents to very low values, as it was observed in Angeleno plums, which could contribute to postharvest decay of plums.

Previous studies have described the effects of exogenous melatonin treatments for maintaining or improving quality properties of fruits as well as nutritional compounds during storage delaying postharvest deterioration by regulating redox metabolism (Gao et al., 2016; Liu et al., 2018), maintaining fruit firmness and TSS levels (Zhai et al., 2018; Rastegar et al., 2020) as well as increasing resistance to pathogens (T. Li et al., 2019). Despite this, its role in delaying postharvest decay and its relationship to other plant hormones during postharvest is still unclear. To elucidate the putative role of melatonin during plum fruit overripening, the tissue-specific effects of 10^{-4} M melatonin treatment were evaluated. In the current study, contrary to the studies aforementioned, exogenous melatonin did not influence the main organoleptic properties of plum fruits such as TSS/TA, pH and firmness. The maintenance of the nutritional quality of fruits after exogenous melatonin improving anthocyanin and phenolic compounds accompanied by an increase of biosynthesis-related genes expressed has also been described in litchi (Zhang et al., 2018), strawberry (Aghdam and Fard, 2017), jujube (Wang et al., 2021), grapes (L. Wang et al., 2020) and recently in plums (Bal, 2019; Xu et al., 2022; Yan et al., 2022). In fact, work from Xu et al. (2022) showed that redness index was strongly variable upon melatonin dosage, where melatonin applications of higher concentrations led to a reduction in redness index and anthocyanin accumulation, while lower melatonin applications at μmolar level increased plum postharvest redness index. Similar results have been found in the present experiment, where melatonin applications at 10^{-4} M on plum fruits resulted in increased total phenol and anthocyanin contents in the mesocarp, while the exocarp remained unaffected. In other words, despite melatonin application was exogenously in the exocarp and it increased the content of endogenous melatonin in both mesocarp and exocarp, the increase of phenolic compounds was activated in the mesocarp, not in the exocarp, and most particularly from day 4. Indeed, the concentration of anthocyanins in the mesocarp of treated plums was almost 3-fold higher, leading to higher quality and resulting in a pulp reddish coloration. To our knowledge, this is the first evidence of a differential contribution of melatonin at different tissue levels on these antioxidants during the postharvest shelf-life of plums. Since, phenolic compounds stand out for their antioxidant properties and therefore, their increase could lead to human health benefits and could also participate in the biotic defense response, being able to contribute to the maintenance of the postharvest quality and delaying fruit senescence (Singh et al., 2010; Liu et al., 2014; Meena et al., 2001).

It has been shown that melatonin acts as a general antioxidant, capable of interacting with ROS and RNS (Galano et al., 2011) being a key compound reducing oxidative stress in the biological membranes (García et al., 2014) and a master regulator of redox homeostasis in plants (Arnao and Hernández-Ruiz, 2019). However, the action of melatonin in many physiological processes inducing considerable changes in gene expression and the discovery of its receptor in plants in 2018 indicate that it could be considered as a plant hormone (Arnao and Hernández-Ruiz, 2020). Likewise, whether melatonin is acting as an antioxidant or as a signaling molecule in different physiological processes is still unclear. In this study, the high increase of total phenols in

the mesocarp of plum fruits might be due to both an increase of total phenols resulting from exogenous melatonin and/or an increase of endogenous melatonin, because of its antioxidant properties and the unspecificity limitation of the method. However, the enhanced accumulation of anthocyanins suggest that this increase could be derived by a regulatory role of melatonin acting as a signal molecule by itself. In addition, the possible relationship between melatonin and other plant hormones has been described (Arnao and Hernández-Ruiz, 2018). Mansouri et al. (2021) also showed a higher endogenous accumulation of ABA attributed to exogenous melatonin during strawberry fruit ripening. Despite this, there is a lack of information about the joint contribution of melatonin with other plant hormones in postharvest quality. Thus, in our study, the endogenous content of different phytohormones of Angeleno plums was analysed. The hormonal analysis reveals that an increase of 4-fold OPDA contents occurred in the mesocarp of melatonin-treated plums at day 4, concomitantly with the increase on phenolic compounds in the mesocarp aforementioned. JA also presented higher values at this point of the experiment compared to control plums. It is known that JA is involved in promoting phenylpropanoid compounds biosynthesis such as anthocyanins (Pauwels et al., 2008) by regulating the key WD-repeat/bHLH/MYB complex for its activation (Shan et al., 2009; Qi et al., 2011; Gális et al., 2006). Indeed, Liu et al. (2019a) reported that melatonin treatment increased MeJA contents and JA signaling pathway in tomato fruit disease resistance to *B. cinerea* resulting in a general increase of phenylpropanoid derivates. Since previous work has related increased JA accumulation after melatonin treatments through downregulation of the JASMONATE-ZIM DOMAIN (JAZ) repressor family proteins, which encode for jasmonate co-receptors and transcriptional repressor in JA signaling pathway (Liu et al., 2019b; Tan et al., 2019), the present study might indicate that melatonin treatments could elicit JA signaling to increase endogenous contents of TPC and anthocyanins. However, there is no previous information about the regulatory role of OPDA by itself in the phenylpropanoid pathway beyond being the precursor of JA and even less during postharvest decay. Additionally, analysis of regulatory cis elements in the sequences of CHS and PAL, revealed that melatonin and JA could be interacting with these enzymes, since different responsive elements for these phytohormones were identified upstream and downstream of their sequence, which adds further evidence to the direct regulation of the phenylpropanoid pathway by melatonin or JAs. Moreover, even if not statistically significant, a strong upward trend of 5-fold more GA_1 was noticed at day 4. This result linked to a high significant correlation between GA_1 and melatonin, suggest a close interaction of these phytohormones in plum fruits. In other models such as petunia flowers, gibberellins have been shown to be required for the anthocyanin biosynthesis and accumulation (Weiss et al., 1995), but in other fruits such as strawberries (Martinez et al., 1996; Lin et al., 2021) or Arabidopsis leaves (Loreti et al., 2008) gibberellins inhibited anthocyanin accumulation, indicating contradictory results and an unclear role of GAs on anthocyanin synthesis. Results derived from the present study also suggest that melatonin may be increasing phenolic contents during postharvest storage at room temperature not only through direct regulation but also by signaling with other phytohormones, such as OPDA and JA (Fig. 6). Further studies are however needed to confirm this approach and keep investigating the possible cross-talk between melatonin and other hormones and how these interactions contribute to an improvement of postharvest fruit quality.

5. Conclusions

Overall, we have seen that differential and tissue-specific responses to plum postharvest occurred for an endogenous condition and an exogenous melatonin treatment. Endogenous plum overripening is mainly characterized by an immediate loss of firmness while there is an active degradation of melatonin and other phytohormones, and increased darkening of plum exocarp by an accumulation of phenolic compounds. In contrast, during overripening after exogenous melatonin, a high increase of endogenous melatonin in both tissues occurred, leading to tissue-specific effects, including increased anthocyanin and phenol contents in the mesocarp. It is suggested that melatonin may be playing a role as a signal molecule enhancing these antioxidant compounds, which contribute to the maintenance of the fruit quality. However, it is also suggested that melatonin could be acting extending the shelf-life of plums fruits rising these phenolic compounds not only by direct regulation but also through signaling with other plant hormones, increasing their contents during overripening. Therefore, the present study provides valuable information to contribute to the knowledge of the regulatory role of melatonin, which seems to be an interesting molecule to delay senescence and extend the postharvest shelf-life of plum fruits. Further researches are however needed to deeply understand the tissue-specific role of melatonin as well as the hormonal cross-talk and its underlying molecular mechanisms occurring during postharvest overripening.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111287.

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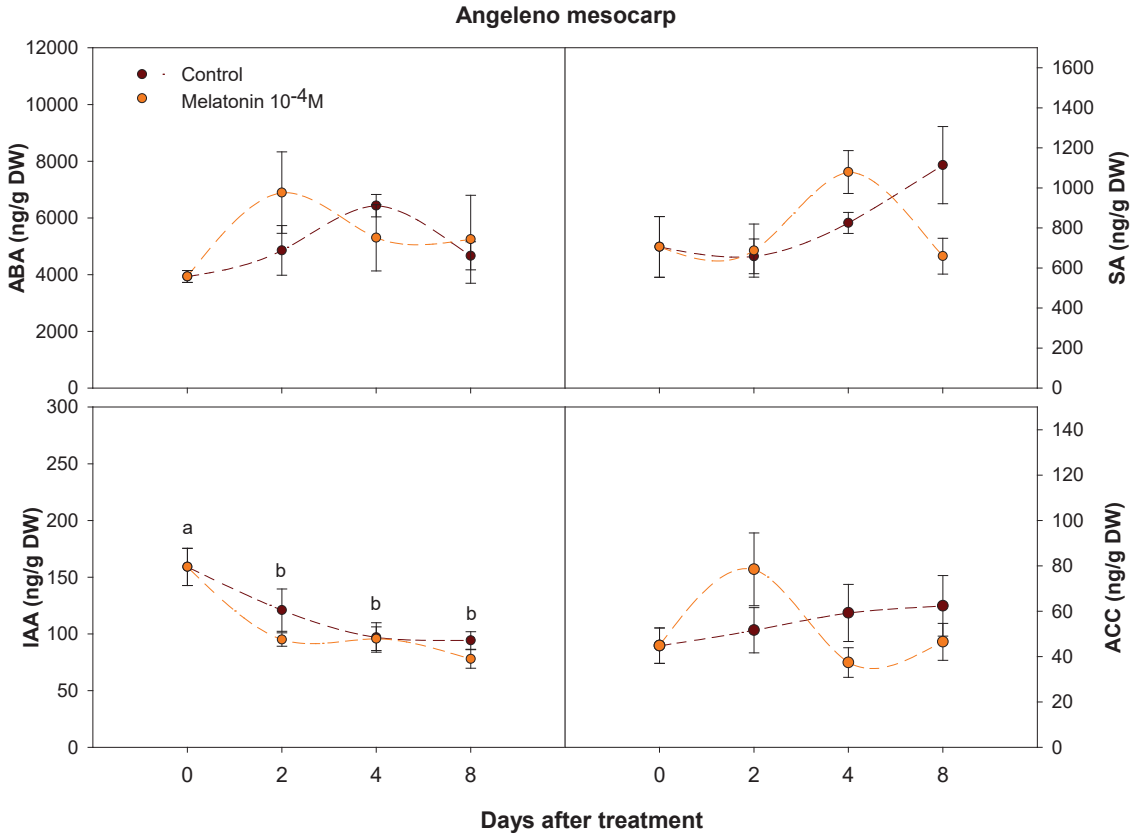


Figure S1. Endogenous content of abscisic acid (ABA), salicylic acid (SA), indole-3-acetic acid (IAA) and 1-amino-cyclopropane-1-carboxylic acid (ACC) in the mesocarp of control and 10^{-4}M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5. Different lower case letters indicate significant differences over time. See table S1 for statistics.

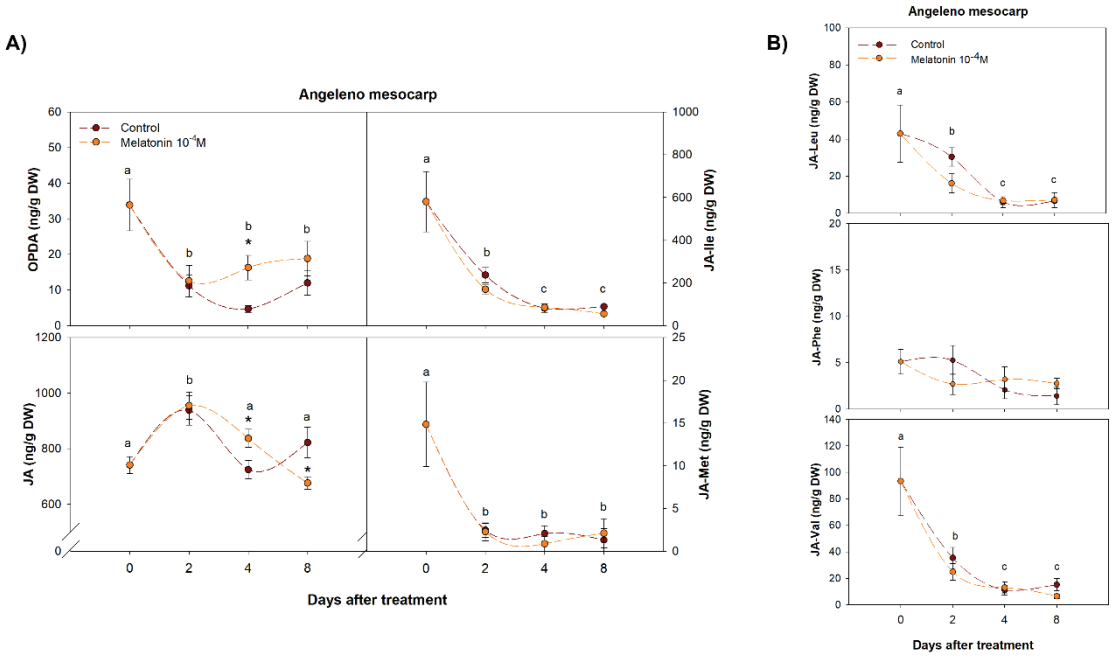


Figure S2. Endogenous content of (A) jasmonic acid (JA), its precursor 12-oxo-phytodienoic acid (OPDA) and its conjugates jasmonoyl-methionine (JA-Met), jasmonoyl-isoleucine (JA-Ile) (B) jasmonoyl-leucine (JA-Leu), jasmonoyl-phenylalanine (JA-Phe) and jasmonoyl-valine (JA-Val) in the mesocarp of control and 10^{-4}M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5. Different lower case letters indicate significant differences over time and asterisks indicate significant differences (p<0,005) between control and treated at that point in time. See table S1 for statistics.

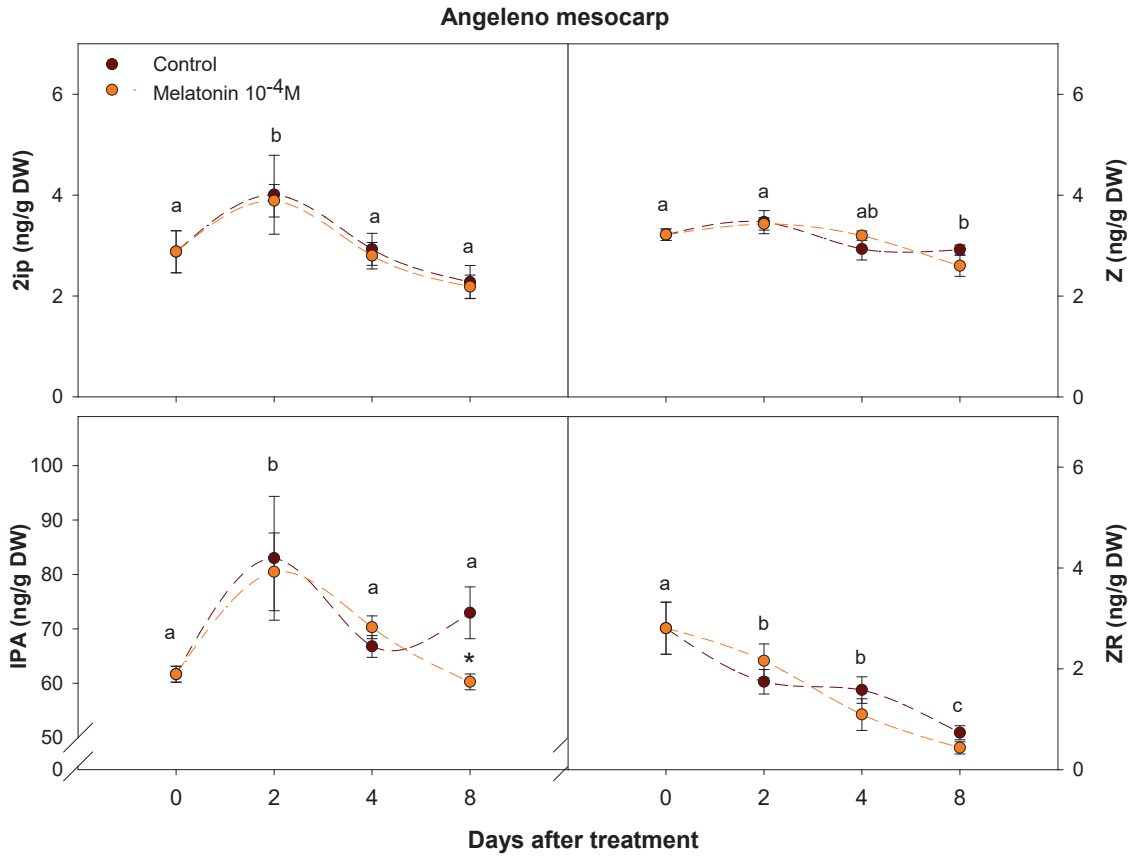


Figure S3. Endogenous content of the cytokinins 2-isopentenyl adenine (2iP), its riboside isopentenyl adenosine (IPA), trans-zeatin (Z) and its riboside trans-zeatin riboside (ZR) in the mesocarp of control and 10^{-4}M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5. Different lower case letters indicate significant differences over time and asterisks indicate significant differences between control and treated plums at that point in time. See table S1 for statistics.

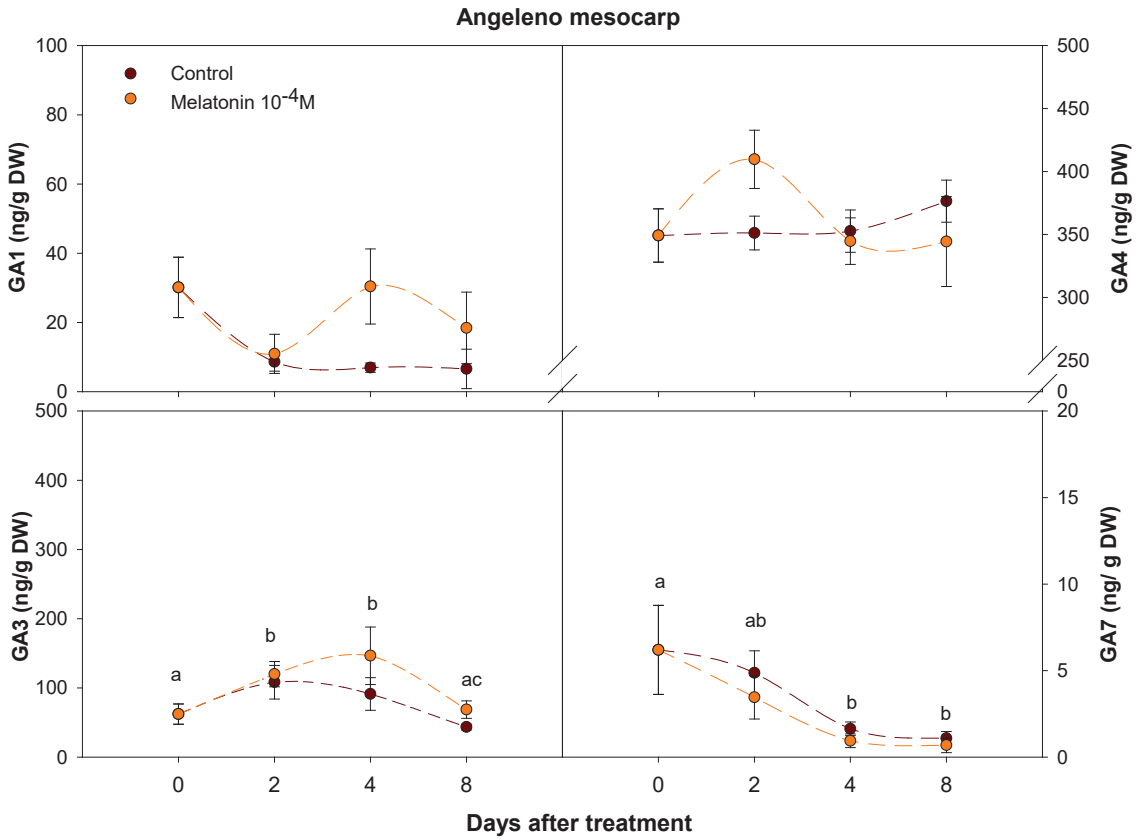


Figure S4. Endogenous content of the gibberellins GA1, GA3, GA4 and GA7 in the mesocarp of control and 10^{-4}M melatonin treated Angeleno plums during days after treatment. Data represent the means ± standard error of n = 5. Different lower case letters indicate significant differences over time. See table S1 for statistics.

>CHS (289590-290885)

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>5' upstream region (288541-289590)

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>Downstream complementary 5’-3’

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Figure S5. Coding sequence for chalcone synthase (CHS) of *Prunus salicina* retrieved from NCBI accession number CM035341.1:289590-290885. Highlighted are ORFs (green), candidate cis elements for melatonin (red) and jasmonic acid (purple). See materials and methods for references.

>PAL Prunus salicina (10256063-10259379)

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>5’ Upstream region (10255061-10256063)

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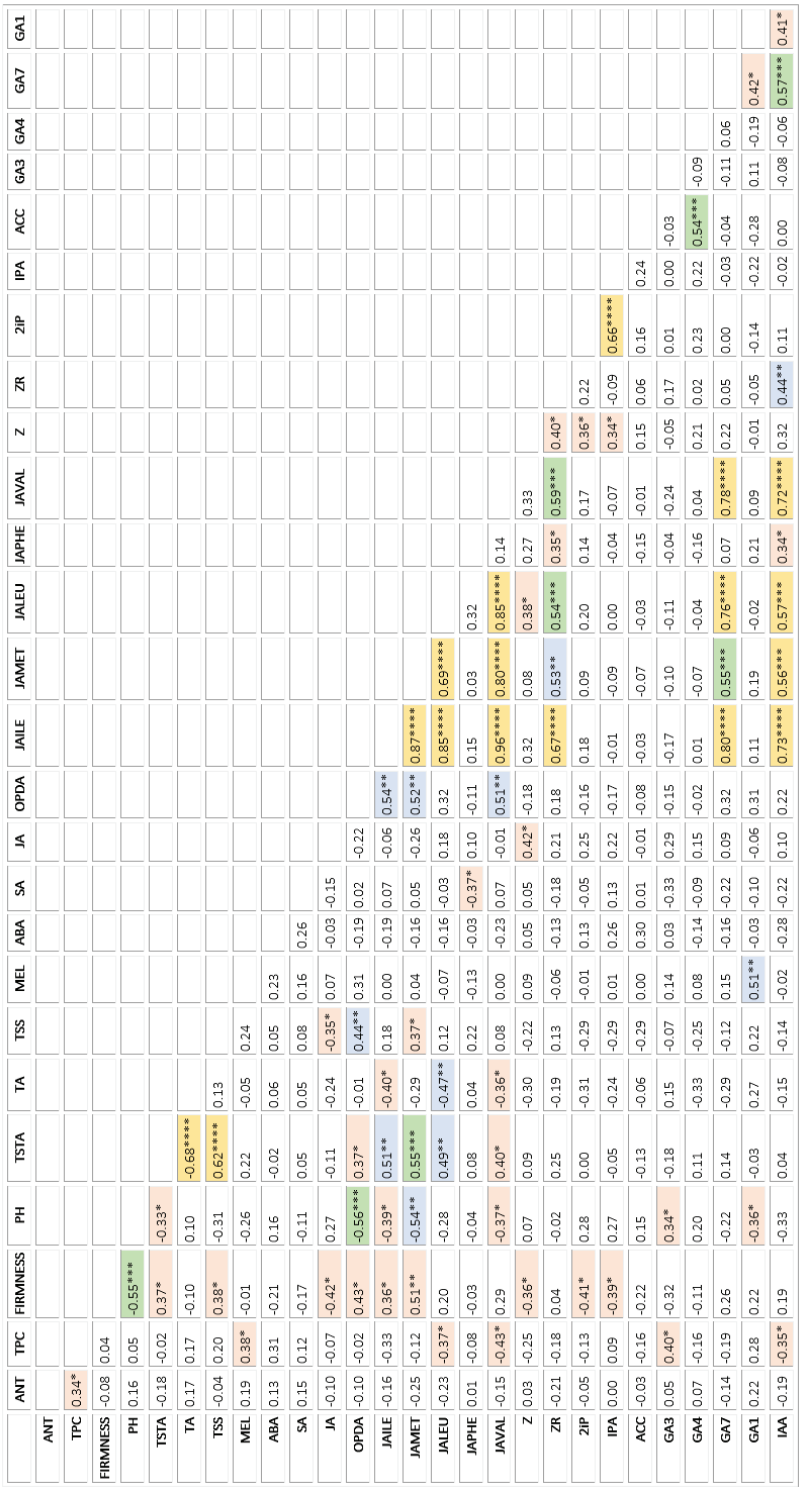
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Figure S6. Coding sequence for phenylalanine ammonia lyase (PAL) of *Prunus salicina* retrieved from NCBI accession number CM035342.1:10256063-10259379. Highlighted are ORFs (green), candidate cis elements for melatonin (red) and jasmonic acid (purple). See materials and methods for references.

Table S1. Linear correlations between the different parameters analyzed in the mesocarp of Angeleno plums. The data are n = 35 and the statistical test used has been Pearson correlation (significant differences p-values≤0.05 with a 95% confidence level). Colored boxes indicate significant differences; four asterisks (****) indicate p <.0001 (yellow), three asterisks p <.001 (green), two asterisks p <.01 (blue) and one asterisk p <.05 (orange).



CAPÍTOL 4

La variabilitat en els túnels de cultiu de maduixot afecta la qualitat del fruit i limita els efectes de la melatonina



Chapter 4. Variability in strawberry tunnels impacts fruit quality and limits melatonin effects

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RESUM

Les fluctuacions en les condicions ambientals dins dels camps i el rendiment dels cultius vegetals poden afectar significativament la producció i els estàndards de qualitat. Aquests factors són especialment rellevants per als productors, que necessiten una producció òptima i sostinguda per obtenir beneficis de petits marges. Aquestes fluctuacions poden veure's exacerbades al final de la temporada de cultiu, quan cap dels factors esmentats es troba en les seves condicions òptimes. En aquest estudi integrat, avaluem la qualitat nutricional dels maduixots i l'impacte del moment de la collita, les condicions del túnel i la variabilitat interindividual en un túnel de producció mediterrani dividit en blocs, on es van realitzar dues collites amb 3 setmanes de diferència. A més, també es van avaluar els efectes de l'aplicació de melatonina al final de la temporada productiva.

La collita al final de la temporada va impactar negativament la hidratació dels fruits, la capacitat antioxidant i les fitohormones relacionades amb la maduració en els maduixots. A més, la distribució dins del túnel va influir en la qualitat nutricional dels fruits, sent la radiació lumínica el principal factor variable que alterava els continguts antioxidants. Els nutrients van mostrar una gran variabilitat interindividual entre les plantes, representant una variació del 20%, i estaven fortament correlacionats amb la hidratació dels fruits i les fitohormones relacionades amb la maduració. Finalment, les aplicacions de melatonina no van afectar ni la producció de fruits ni els paràmetres nutricionals, ja que probablement els efectes es van veure atenuats per la variabilitat intrínseca dels maduixots. En general, els resultats subratllen les limitacions d'aquest tipus d'aplicació per a la seva implementació en el camp.

La variació de la qualitat dels fruits en els camps de maduixots es pot explicar per la variabilitat ambiental i interindividual. De la mateixa manera, la implementació de molècules reguladores com la melatonina en aplicacions de camp depèn de l'homogeneïtat del cultiu i pot tenir una aplicabilitat limitada en sistemes productius heterogenis. Per tant, identificar i reduir la variabilitat del microclima en els camps de producció és fonamental per avançar en les pràctiques agrícoles i mantenir uns estàndards constants en la qualitat dels fruits.

Research Article

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Variability in strawberry tunnels impacts fruit quality and limits melatonin effects

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Abstract

BACKGROUND: Fluctuations in environmental conditions within fields and crop plant performance can greatly affect production and quality standards. These factors are particularly relevant for producers, who require sustained optimal production to profit from small margins. Fluctuations might be exacerbated at the end of the crop season, where neither of the aforementioned factors are optimal. In the present integrated study, we assess strawberries' nutritional quality and the impact of harvest timing, tunnel conditions and inter-individual variability in a Mediterranean production tunnel divided into blocks, where two harvests were performed 3 weeks apart. In addition, the effects of sprayed melatonin at the end of productive season were also evaluated.

RESULTS: End-season harvesting negatively impacted fruit hydration, antioxidant capacity and ripening-related hormones in strawberry fruits. Additionally, tunnel distribution influenced fruit nutritional quality, with light radiation being the main variable factor disturbing antioxidant contents. Nutrients exhibited high inter-individual plant variability, accounting for 20% variation, and were strongly correlated with fruit hydration and ripening-related phytohormones. Finally, melatonin applications affected neither fruit production, nor nutritional parameters, for which the effects were masked by the intrinsic strawberry variability. Overall, the results underline the limitations of this type of application for field implementation.

CONCLUSION: Fruit quality variation in strawberry fields is explained by environmental and inter-individual variability. Likewise, the implementation of regulatory molecules such as melatonin in field applications relies on crop homogeneity and might have limited applicability in heterogeneous productive systems. Consequently, identifying and reducing microclimate variability in productive fields is paramount for advancing agricultural practices to uphold unwavering standards on fruit quality.
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Supporting information may be found in the online version of this article.

Keywords: strawberry quality; off-season; tunnel variability; melatonin; biostimulant; phytohormones

INTRODUCTION

Over recent decades, expanding food production and economic growth have been a priority over fruit quality. Currently, fruits are mostly selected based on their organoleptic characteristics associated with the synthesis of biomolecules, which are strongly influenced by a combination of environmental and genetic factors.[1] Strawberries are a great example of extensive breeding focused on production and development of early varieties, challenging the overall crop performance. These fruits are highly appreciated by consumers and are included in the Mediterranean diet because of their high content of vitamin C and other antioxidant compounds, constituting an important source of minerals.[2] Indeed, strawberries have several health benefits related to their consumption.[3,4] However, such benefits may be hindered by diverse microclimatic conditions such as light and temperature in fields, which may lead to variations in strawberry growth, ripening and, ultimately, quality parameters.[5,6] Therefore, in-depth studies on their effects in productive tunnels are essential to ensure best field practices.

The Maresme area of Catalonia stands as the second-largest producer of strawberries in Spain. Renowned for their exceptional quality, Maresme strawberries owe their excellence to the region's perfect climate and sloping topography. This unique combination of factors positions the area as an ideal hub for cultivating strawberries at their optimal ripeness to ensure maximum quality and

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local consumption. In this region, the strawberry season starts in March and continues until around mid-June.[7] At this time, environmental conditions start being unfavorable because of an increase of humidity and maximum temperatures exceeding 30 °C. These conditions mark the final peak of commercial production because, under elevated temperatures, strawberries start declining their productivity. Indeed, ambient temperatures rising above 30 °C contribute to reductions in flowering, pollination, fruit set and compromise quality.[8] Strawberries are mostly cultivated in open tunnels, which create greater controlled conditions, protecting plants from adverse climate. In this sense, it was reported that, during summer months, air temperature in tunnels can be up to 3.5–6.2 °C lower compared to open field conditions, leading to better microclimate management, increasing fruit quality and yield, and extending the period of strawberry fruit availability.[9]

Fruit ripening and final quality are physiologically regulated by endogenous molecules such as phytohormones.[10] Abscisic acid (ABA), ethylene, auxins, gibberellins, jasmonates and brassinosteroids are some of the most implicated plant hormones in ripening regulation of non-climacteric fruits.[11] At the same time, environmental conditions influence their action mode and induce particular triggering signals. For example, the interplay of temperature with other factors such as relative humidity and light under agricultural systems can significantly influence fruit ripening, with high temperatures dramatically accelerating this process and altering final fruit quality.[12] Interaction between endogenous signals and environmental factors modulate the biosynthetic pathways of biomolecules at the same time as promoting the regulatory network of phytohormones to alleviate negative effects.[13] Therefore, there is an important hormonal response to abiotic stress that can affect the fruit ripening process, thereby modifying final fruit quality.

Strawberries have been reported to be one of the crops with higher requirements with respect to the use of phytosanitary products for their cultivation.[14,15] Currently, within the framework of the European Green Deal[16] to address climate change, the European Union (EU) has rejected its plans to halve pesticide usage by 2030 following a farmers' protest. They asserted that phytosanitary reductions lack support from companies and producers, who have no viable alternative solutions to sustain crop productivity, thereby compromising food security, prices and food production in the EU. However, concerns regarding this resolution should be raised because there is good evidence that the abusive use of phytosanitary substances contributes to the deterioration of agricultural systems, implicating the presence of high chemical residues in fruits that could affect human health.[17-19] In this context, it is essential to address the conflict by focusing on sustainable agriculture, utilizing biosolutions, biotechnology, and digital and precision farming techniques. An agroecological alternative can be the use of biostimulants, which boost plant growth, development, fruit quality and yield, at the same time as improving stress tolerance and nutrient absorption.[20,21]

Melatonin (*N*-acetyl-5-methoxytryptamine) is a natural biomolecule that has aroused great interest the last years with significant functions as both antioxidant and regulatory molecule in several plant physiological processes.[22] Moreover, melatonin's potential as a biostimulant for mitigating abiotic and biotic stresses in diverse crops and environmental conditions has been mainly described after performing exogenous melatonin applications. The results from these studies indicate that melatonin is a versatile regulatory molecule with a great potential to increase plant performance and fruit quality.[23-26] Despite all this knowledge, the limits and conditions where melatonin functions as biostimulant are scarce. To address these questions, the present integrated study investigates how harvest timing, variability within a production tunnel and genotypic variability together with melatonin application impact the nutritional quality of strawberries at the end of the season during a high-temperature period. We hypothesized that both microclimatic and inter-individual variability could negatively affect ripening-related phytohormones altering strawberry quality parameters and tested whether melatonin could alleviate unfavorable productive tunnel conditions.

MATERIALS AND METHODS

Experimental design, melatonin application and samplings

This experiment was conducted in a strawberry production tunnel at Can Marpons estate, located in Arenys de Munt, Catalonia, Spain (41°36′48.7″N, 2°33′01.7″ E). The strawberry plants (*Fragaria* × *ananassa* var. Albion) were fully developed and productive in a hydroponic cultivation system on sloping terrain to facilitate water drainage and solar radiation exposure facing (north to south and east). Within the tunnel, plants were arranged in six rows, with three rows serving as barriers, whereas another three were used for the experiment. Within each row, the plants were divided into eight randomized blocks of eight plants per block (Fig. 1), with four plants assigned for production monitoring and four for samplings. Plants were irrigated as per crop requirement with water and throughout the experiment, the tunnel's production temperature and relative humidity were monitored using a data logger (TinyTag Ultra 2 TGU4500; Gemini Data Loggers, Chichester, UK) situated in the center of the tunnel at plant level (Fig. 2). The photosynthetically active radiation (photosynthetically active radiation, PAR) for each plant was determined at midday between 12.00 pm and 14.00 h using a fluorometer (MINI-PAM-II; Walz, Pfullingen, Germany) with an integrated PAR sensor and expressed in μmol photons m^{-2} s^{-1}.

Distribution and samplings to determine the variability and temporal effects of strawberry late-season harvest in fruit quality

Two samplings were conducted using four plants from each block, with 32 individuals in total. The first sampling (harvest 1) took place at the end of June when the end of the strawberry season begins (28 June 2022), and the second sampling (harvest 2) was performed 3 weeks later when the harvest is off-season (19 July 2022). For each sampling, a single commercially ripe fruit was collected from each plant, immediately frozen in liquid nitrogen, and kept at −80 °C until subjected to analysis. Moreover, these eight blocks were used to determine the variability within blocks of the same production tunnel ($n = 8$), whereas the four plants in each block were used to assess inter-individual variability ($n = 4$). Variability was expressed as a percentage standard deviation using the formula: (SD/mean) × 100.

Melatonin application and samplings

Melatonin application at 10^{-4} M was carried out based on a survey of current literature[23] to assess its potential as a bioactive stimulant. In this case, plants from eight tunnel blocks served as a control group, whereas the treatment application was performed on plants from an additional eight blocks, each containing eight plants (four plants assigned for production monitoring and four for samplings) (Fig. 1). In both experimental conditions, a solution

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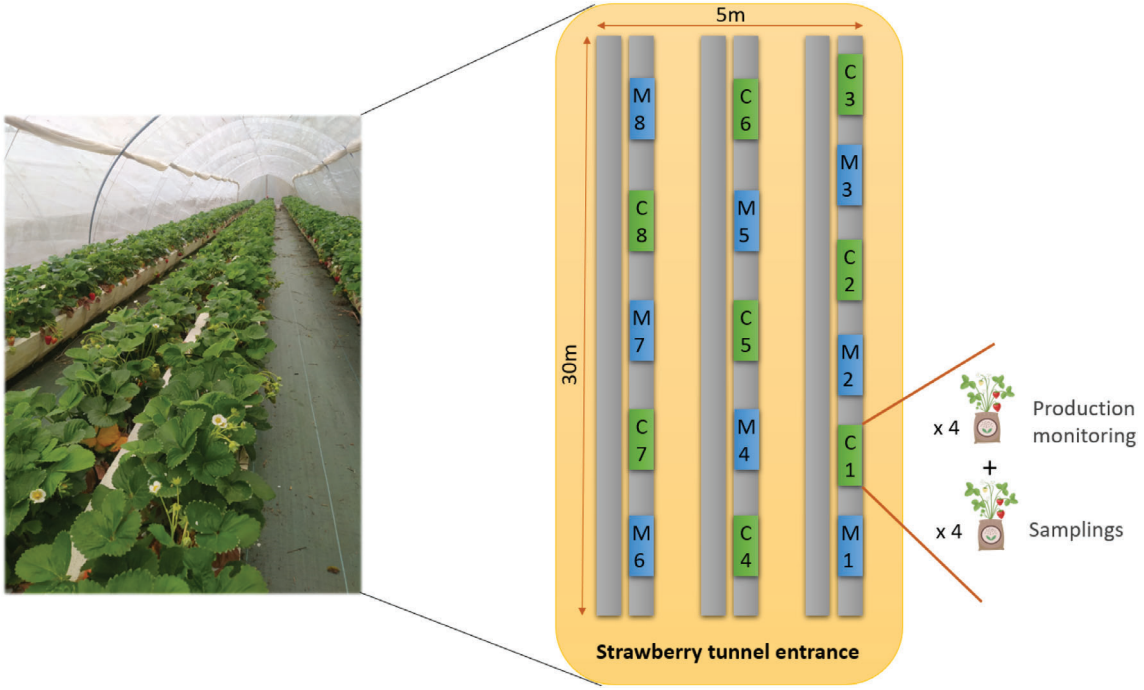


Figure 1. Distribution scheme of the different production blocks within the strawberry cultivation tunnel. Each green and blue box represents a control and melatonin block respectively. Each block contains eight plants, with four dedicated to fruit sampling and four allocated for production monitoring. C, control; M, melatonin.

of H_2O MilliQ (Merck Millipore, Burlington, MA, USA) was employed as a control, or an aqueous solution of melatonin at 10^{-4} M was uniformly sprayed on the entire plant through a nebulizer. To facilitate proper solution absorption, 0.1% Tween 20 was used as a surfactant in control and treatment solutions. These applications were conducted at the start of the experiment on 28 June 2022, with fruit sampling taking place 3 weeks later on 19 July 2022. During sampling, all fruits at the commercial ripening stage of each plant were harvested to first determine fruit biomass. Subsequently, one randomly selected fruit for each plant was chosen to evaluate firmness using an FT 327 penetrometer (QA Supplies, Norfolk, VA, USA) and taking two measurements for each fruit. Finally, fruits were promptly frozen in liquid nitrogen and preserved at −80 °C until quality analysis. Additionally, at intervals of two or three times per week, plant production was systematically monitored throughout the experiment. This entailed the harvesting and counting of all commercially ripe fruits from four plants in each block from both control and melatonin treatment groups.

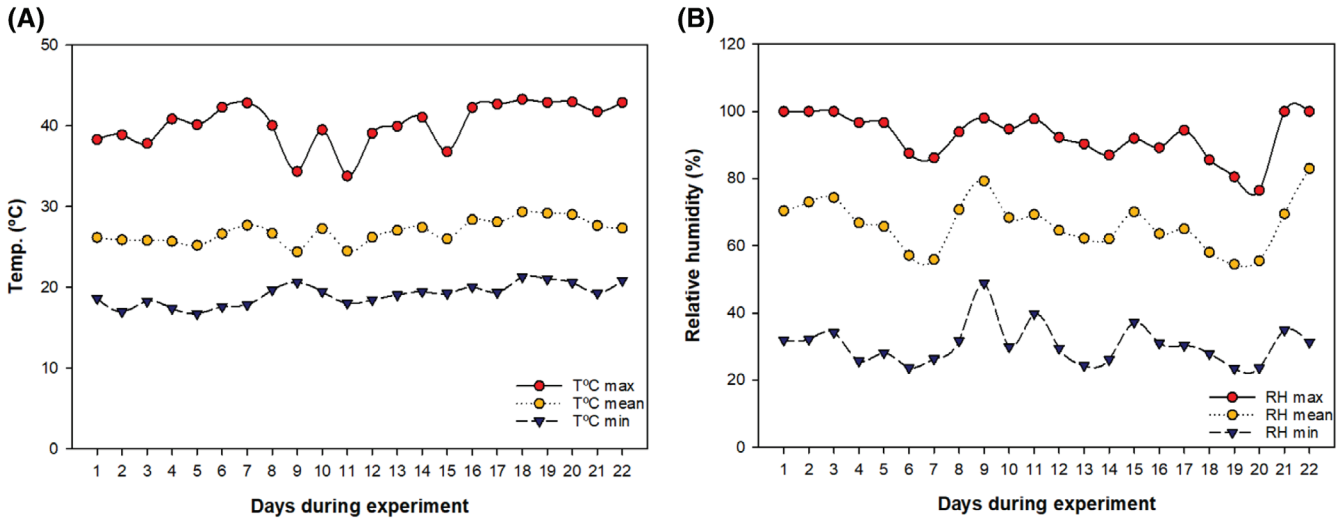


Figure 2. Temperature (A) and relative humidity (B) record of maximum (red), mean (yellow) and minimum (blue) values throughout the entire experiment. Day 1 corresponds to the first day of the experiment (28 June 2022) and day 22 corresponds to the last day (19 July 2022).

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Basic quality parameters

For estimating fruit quality, total soluble solids (TSS), titratable acidity (TA) and pH were assessed. For the analysis of TSS, 2 g of powdered samples was homogenized with 10 mL of H_2O MilliQ and the obtained juice was utilized to determine °Brix in 1 mL using a refractometer (Hannah Instruments, Villafranca Padovana, Italy), as described by Boulton *et al.*[27] Then, the method described by Latimer[28] was followed to estimate the quantity of major acid in the strawberry (g citric acid kg^{-1} fresh weight strawberry). Furthermore, pH was evaluated using a MicropH 2002 pH meter (Crison Instruments, S.A., Barcelona, Spain). Finally, the hydration of the fruits (*H*) was analyzed using the formula: (fresh weight − dry weight)/dry weight.

Vitamin E

For vitamin E analysis, 100 mg of each frozen strawberry sample was extracted with 1.9 mL of cold methanol. High-performance liquid chromatography (HPLC) was performed, using a Waters 600 controller pump and Waters 717 plus autosampler (Waters Corp., Milford, MA, USA) and a FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The different vitamin E compounds (α-, β-, δ- and γ-tocopherols, and tocotrienols) were separated on a normal phase column (Inertsil 100A, 5 µm, 30 × 250 mm; GL Sciences, Tokyo, Japan) using a mobile phase of *n*-hexane and 1,4-dioxane (95.5:4.5, v/v). Fluorescence detection was performed with an excitation of 295 nm and emission at 330 nm. Vitamin E compounds were quantified using a calibration curve with authentic standards (Sigma-Aldrich, Steinheim, Germany).

Vitamin C

For the analysis of vitamin C, both the reduced form (ascorbic acid, AA) and the oxidized form (dehydroascorbate, DHA) were determined following the procedure described by Queval & Noctor[29] with some modifications. The extractions were performed using cold extraction buffer (6%, w/v, meta-phosphoric acid and 0.2 mM diethylenetriaminepentaacetic acid) and vitamin C determination was carried out in triplicate using a 96-well quartz plate measuring absorbance at 265 nm by spectrophotometry (xMarkTM Microplate Spectrophotometer; Bio-Rad, Hercules, CA, USA). The total vitamin C content was calculated as the sum of AA + DHA, and the redox state was calculated as: AA/(AA + DHA) × 100.

Anthocyanins, total phenols and total antioxidant capacity

To determine total anthocyanin, phenol content and antioxidant capacity, the methanolic extracts prepared for vitamin E analysis were utilized. For anthocyanins, an acidification was carried out with 10 N HCl (approximately 37%) and absorbance was measured at 530 nm using a spectrophotometer (CE Aquarius UV/Visible; Cecil Instruments Ltd, Cambridge, UK). The anthocyanin content was calculated as described by Siegelman & Hendricks.[30] Total phenolic content (TPC) was estimated using Folin–Ciocalteu reagent and a gallic acid standard curve as described by Singleton & Rossi[31] and Li *et al.*,[32] expressed in g gallic acid equivalents (GAE) kg^{-1} fresh weight. The total antioxidant capacity (radical scavenging activity, RSA) was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as was described in Barros *et al.*[33] and Poojary *et al.*[34]

Mineral composition

For minerals analysis, 100 mg of the lyophilized sample was used. The analysis of calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn) and phosphorus (P) was conducted using inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 8300; PerkinElmer, Waltham, MA, USA). Copper (Cu), sodium (Na) and zinc (Zn) analysis was performed using inductively coupled plasma mass spectrometry (ICP-MS) (Nexion 350d; PerkinElmer). Both ICP-OES and ICP-MS techniques were carried out by the metal analysis unit at the Scientific and Technological Centers of the University of Barcelona (CCiTUB).

Hormonal content

Ripening-related phytohormones content, including ABA, jasmonic acid (JA), ethylene precursor 1-amynocyclopropane-1-carboxylic acid (ACC) and melatonin, was analyzed by ultra HPLC coupled with electrospray ionization tandem-MS as described by Müller and Munné-Bosch[35] using methanolic extracts. HPLC was coupled to a triple quadrupole mass spectrometer (QTRAP 4000; AB Sciex, Concord, ON, Canada) and a LUNA C18 column (1,6 µm, 100 × 2,1 mm) (Phenomenex Inc., Torrance, CA, USA) was used. Quantification was made considering recovery rates for each sample by using the deuterium-labeled internal standards and calibration curves for each analyte were generated using MultiQuant, version 3.0.1 (Sciex, Framingham, MA, USA).

Statistical analysis

All data were analyzed using Rstudio (https://posit.co/products/open-source/rstudio). For all analyzed parameters, two-way analysis of variance was performed considering harvest time or treatment and block as factors. Multiple comparisons were conducted using Tukey's post-hoc test. Spearman's correlation tests were also conducted for all analyzed parameters. $P < 0.05$ was considered statistically significant.

RESULTS

Impact of harvest time on strawberry nutritional quality and ripening-related phytohormones content

To assess the impact of harvest timing on the quality of Albion strawberries, an initial harvest was conducted followed by a second harvest after 3 weeks of elevated temperatures (Fig. 2A). During off-peak production period, a significant decrease in fruit hydration was observed, below 20% initial contents at harvest 1 (Fig. 3). Additionally, a 17% reduction in total antioxidant activity was shown in fruits from harvest 2 ($P < 0.001$), resulting in values below 50% (Fig. 3). However, no decrease in the content of specific antioxidants, such as vitamin C, E or total anthocyanins, was observed (see Supporting information, Fig. S1). By contrast, TPC increased by 33% ($P < 0.001$) in strawberries at harvest 2 (see Supporting information, Fig. S1). Furthermore, harvest time neither influenced any essential parameters for both producers and consumers, such as TSS and TA, which remained constant. Moreover, there were no significant differences in micronutrient content based on harvest time (see Supporting information, Fig. S2).

To explore the relationship between quality parameters and phytohormones related to stress and fruit ripening during off-peak production, their endogenous contents were analyzed. Results showed that hormonal levels of ACC, JA and melatonin were affected by the harvest time at the end of the productive

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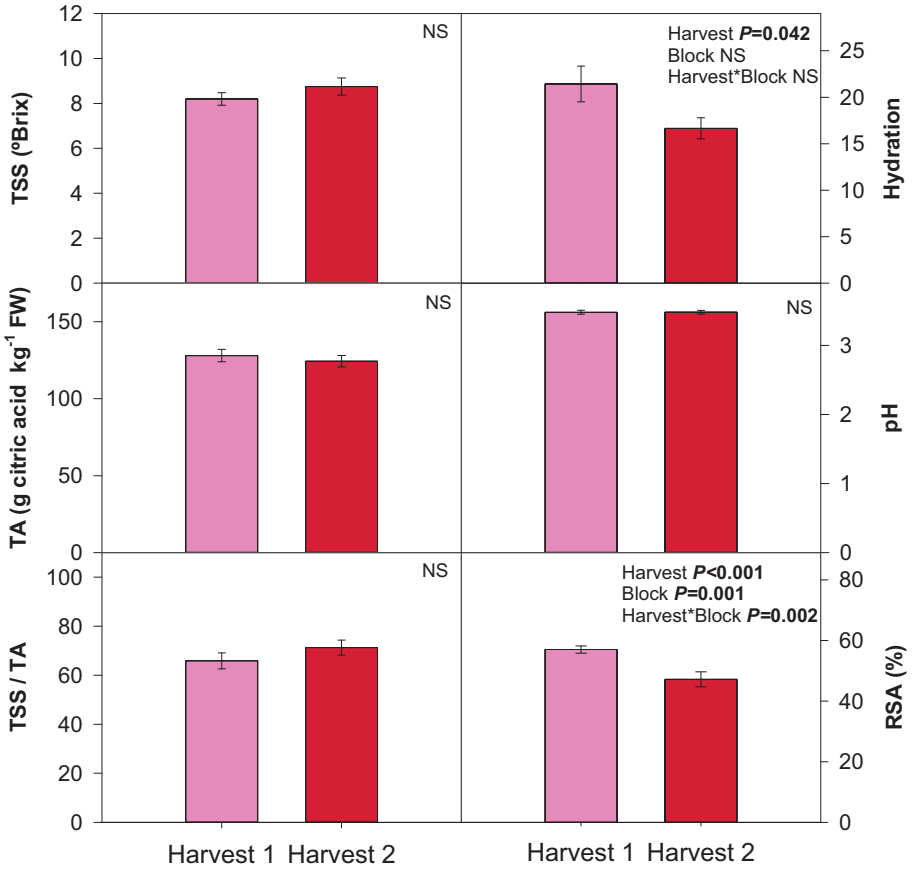


Figure 3. Basic quality parameters of strawberries from the two harvests carried out 3 weeks apart, in the off-season between June and July. TSS, total soluble solids; TA, total acidity; RSA, radical scavenging activity. Data represent the mean ± SE of $n = 32$. NS, no significant differences over harvest ($P > 0.05$).

season, but no statistical differences were found for ABA (Fig. 4). During harvest 2, the content of JA and ACC presented a sharp decrease by 50% and 26%, respectively (Fig. 4). Likewise, melatonin content had a remarkable decrease, being 3.5-fold lower than in harvest 1 ($P < 0.001$), reflecting a 72% reduction and attaining contents below 1 ng g^{-1} dry weight (Fig. 4).

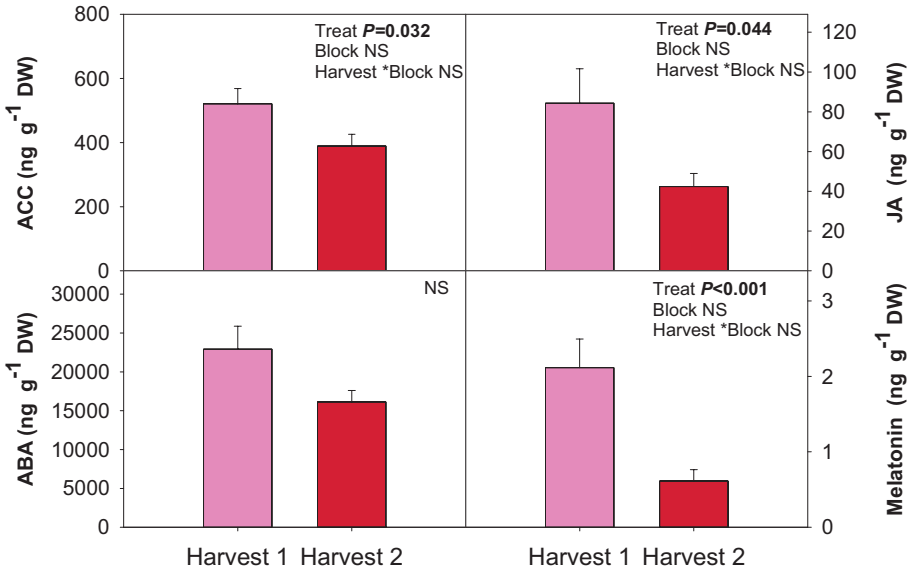


Figure 4. 1-amynocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), jasmonic acid (JA) and melatonin content of strawberries from the two harvests carried out 3 weeks apart, in the off-season between June and July. Data represent the mean ± SE of $n = 32$. NS, no significant differences over harvest ($P > 0.05$).

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Determination of field block variability in antioxidant compounds and light incidence

In both harvests, subdivision of the productive tunnel in eight blocks allowed the identification of substantial variability in the main antioxidant compounds. This was particularly relevant in γ-tocopherol and DHA, where the coefficient of variation (CV) between bocks surpassed 30% for γ-tocopherol and even 50% in DHA (Fig. 5A,D). In harvest 1, other antioxidant compounds such as TPC and AA showed a CV higher than 20%, and only RSA displayed less than 10% (Fig. 5A). By contrast, in harvest 2, TCP and AA had a CV lower than RSA, which increased to more than 20% (Fig. 5D). Likewise, the CV between blocks for PAR was higher in the first harvest exceeding 20%, whereas, for harvest 2, it only represented 10% of variation (Fig. 5A,D).

Furthermore, in harvest 1, the antioxidant compounds TPC, DHA, redox state of vitamin C and γ-tocopherol showed significant differences among blocks (Fig. 5B). Regarding TPC, block 1 presented values of 0.529 ± 0.138 g GAE kg^{-1} fresh weight, whereas block 8 showed 1.183 ± 0.73 g GAE kg^{-1} fresh weight, being two-fold higher. In the case of DHA, block 5 was five-fold lower than block 7. Moreover, PAR also had significant differences among the different blocks, where blocks 1, 2 and 3 showed values that were 35% higher than those for the remaining blocks (Fig. 5B). In harvest 2, RSA and PAR had significant differences among blocks. RSA showed a 48% decrease between block 1 and block 5. As in harvest 1, the PAR values for blocks 1, 2 and 3 in harvest 2 were also more elevated, being two-fold higher than those for blocks 6, 7 and 8 (Fig. 5E). Furthermore, the different antioxidant compounds had strong positive correlations with each other, especially between RSA, TPC and vitamin E in both harvests (Fig. 5E,F). By contrast, negative correlations were observed between PAR and TPC ($P < 0.01$) in harvest 1 (Fig. 5C) and PAR and anthocyanins ($P < 0.05$) in harvest 2 (Fig. 5F).

Evaluation of inter-individual and inter-block variability of nutrient content and its association with fruit hydration and plant hormones

In harvest 1, nutrient levels presented a high CV among blocks, all of them exceeding 20%, with Fe standing out at almost 40% and Na reaching almost 50% (Fig. 6A). By contrast to nutrient levels, organoleptic quality parameters demonstrated lower CV, except for fruit hydration, which exceeded 25% (Fig. 6A). Additionally, nutrients showed higher inter-individual variability compared to organoleptic quality parameters, especially when CV exceeded 100%, such as Fe in block 1, Ca in block 8 and Na showing the highest inter-individual variability, surpassing 100% in blocks 2, 7 and 8 (Fig. 6B). However, fruit hydration also exhibited high inter-individual CV, being higher than 50% in blocks 1, 6 and 7. Furthermore, fruit hydration showed a strong negative correlation with the content of all nutrients ($P < 0.001$), whereas all nutrients were positively correlated with each other ($P < 0.001$) (Fig. 6C).

In the case of harvest 2, the CV among blocks for nutrient levels was not as high as in harvest 1. In comparison to harvest 1, Na exhibited the highest variability with 35% and Cu with 25%, whereas the rest remained below 20% (Fig. 7A). Nevertheless, similar to harvest 1, nutrient content also showed increased inter-

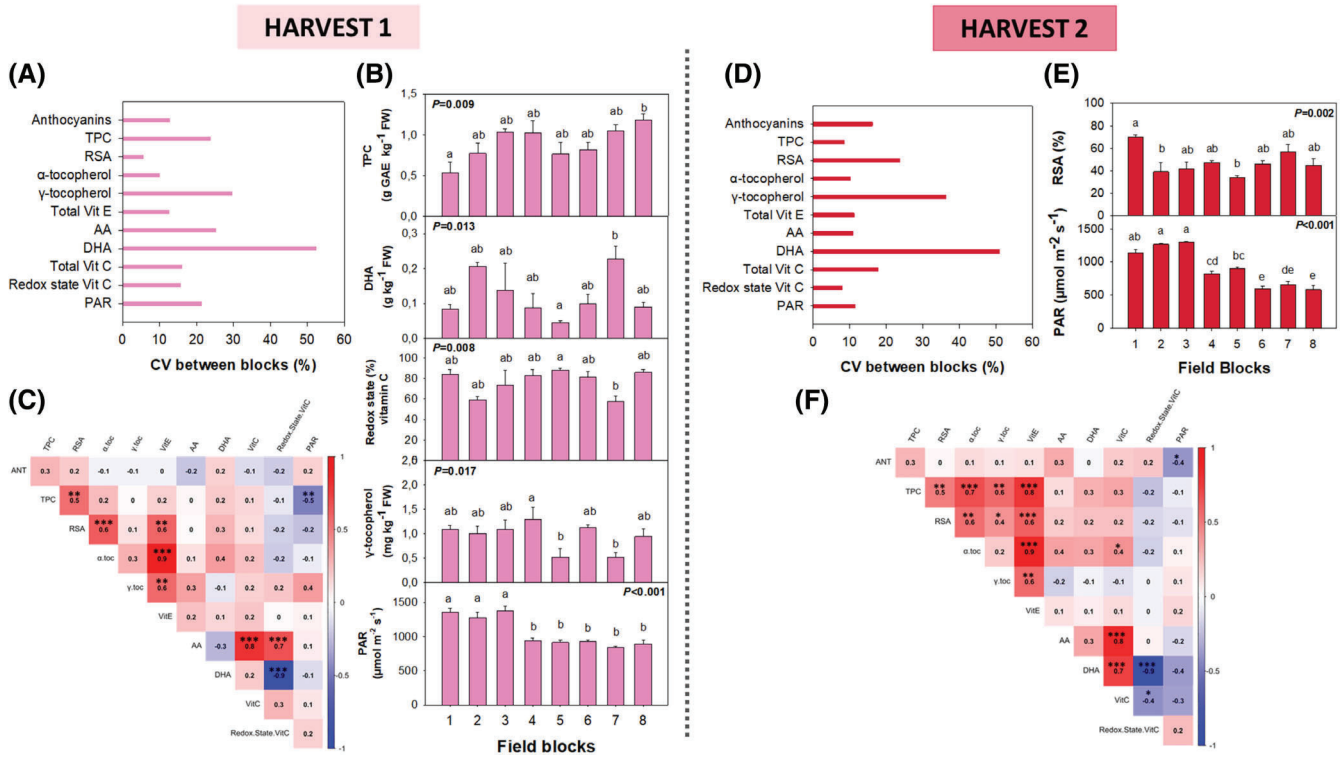


Figure 5. (A, D) Coefficient of variation (CV) of antioxidant compounds in the fruit and PAR among different blocks of the field ($n = 8$) in harvests 1 and 2. (B, E) Parameters that have shown significant differences between blocks in harvest 1 and harvest 2. Data represent mean $\pm$ SE of $n = 4$. (C, F) Spearman correlation matrix between antioxidant parameters and PAR in harvest 1 and harvest 2. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. The color gradient represents correlation intervals from blue (negative) to red (positive). TPC, total phenol content; RSA, radical scavenger activity; AA, ascorbic acid; DHA, dehydroascorbate; PAR, photosynthetically active radiation.

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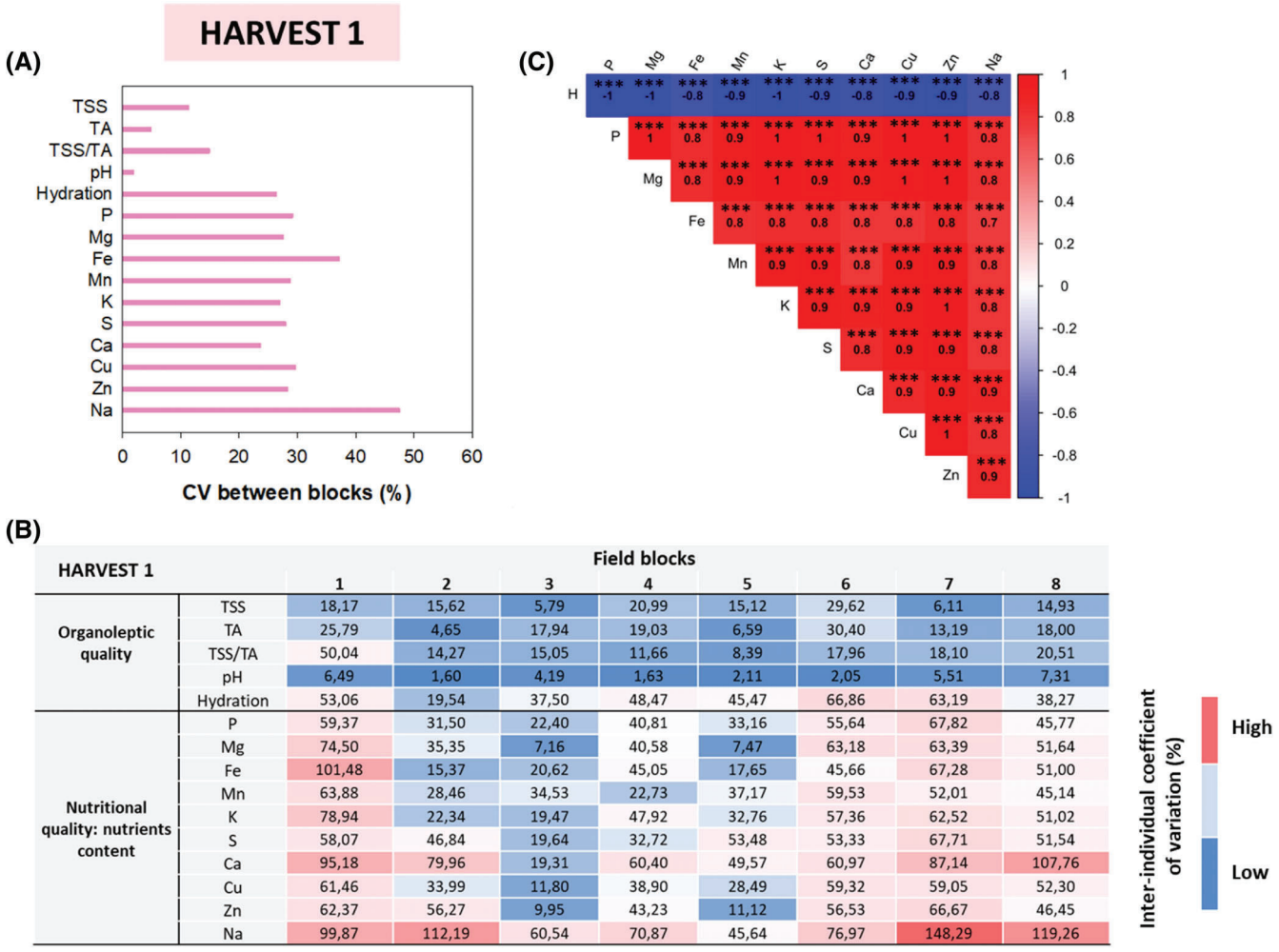


Figure 6. (A) Coefficient of variation of basic quality parameters and nutrients content among different blocks of the field ($n = 8$) in harvest 1. (B) Coefficient of variation of basic quality parameters and nutrients content among individuals (n = 4) within each block in harvest 1. Red and blue colorations indicate high and low values, respectively. (C) Spearman correlation matrix between nutrients content and hydration in harvest 1. ***$P < 0.001$. The color gradient represents correlation intervals from blue (negative) to red (positive). TSS, total soluble solids; TA, total acidity; H, hydration; Ca, calcium; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; Na, sodium; P, phosphorus; Zn, zinc, Zn.

individual CV, particularly in blocks 4, 6, 7 and 8. Especially, Ca and Na surpassed 100% CV in block 7 and Cu in block 6 (Fig. 7B). Among the organoleptic quality parameters, fruit hydration also presented the highest inter-individual variability (Fig. 7B) and, consistent with harvest 1, a strong negative correlation was observed between hydration and nutrient levels ($P < 0.001$) (Fig. 7C).

Furthermore, in both harvests, a positive correlation was observed between fruit hydration and plant hormones, particularly exhibiting a strong correlation in the case of ACC and ABA ($P < 0.001$), followed by melatonin ($P < 0.01$) and JA ($P < 0.05$) (Fig. 8). ACC and ABA also displayed a robust negative correlation with different nutrients, significantly stronger in harvest 1 ($P < 0.01$ for all nutrients). Melatonin also demonstrated a negative correlation with P, Mg, K, S and Na in both harvests, along with a negative correlation with Mn, Ca, Cu and Zn in harvest 1 (Fig. 8).

Validation of the regulatory role of melatonin as a natural biostimulant to enhance strawberry crops

The endogenous melatonin content of ripe fruits treated with melatonin 21 days ago was seven-fold higher than control fruits, which represents an increase of 93% rising from 0.6 ± 0.15 ng g^{-1} dry weight to 4.3 ± 1.22 ng g^{-1} dry weight (Fig. 9B). Despite this considerable increase in fruit melatonin content, no differences were observed in yield compared to control plants (Fig. 9A). Likewise, the principal organoleptic quality parameters of the fruit remained unchanged (Fig. 10) as well as no changes were observed in the total vitamin C content or the total antioxidant capacity of the fruit (Fig. 10; see also Supporting information, Fig. S3), nor in other quality parameters analyzed such as firmness, vitamin E, phenolic compounds and nutrients (see Supporting information, Figs S3–S5).

DISCUSSION

Harvest timing negatively impacts fruit quality decreasing the antioxidant activity and strawberry hydration

Environmental factors such as temperature are one of the main quality-impacting factors in fruit production.[36-38] In strawberries, an upper critical threshold has been reported at 33 °C, where there may be induction of oxidative stress in the plant.[39] Throughout the present study, temperatures around 40 °C were recorded,

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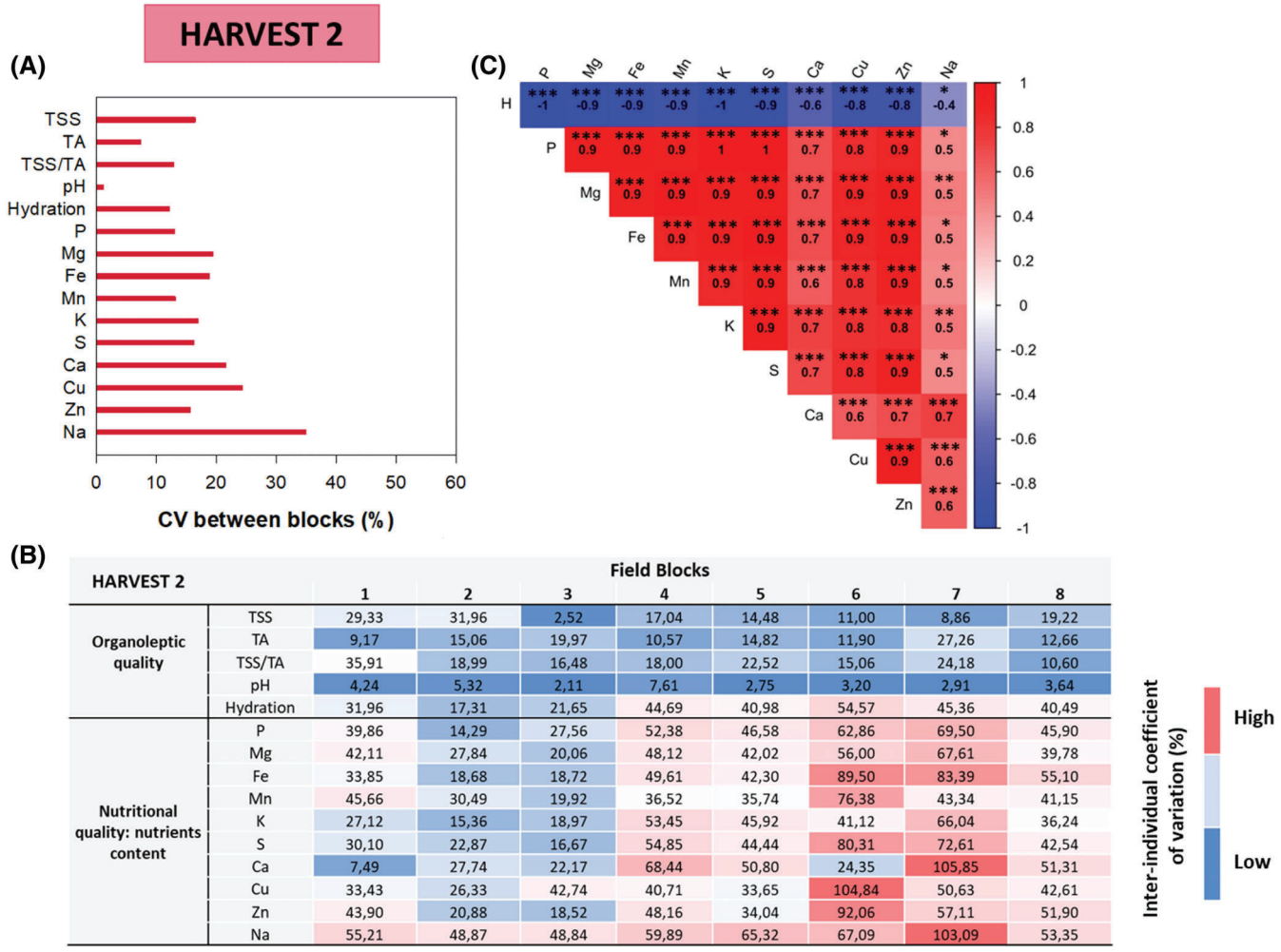


Figure 7. (A) Coefficient of variation of basic quality parameters and nutrients content among different blocks of the field ($n = 8$) in harvest 2. (B) Coefficient of variation of basic quality parameters and nutrients content among individuals (n = 4) within each block in harvest 2. Red and blue colorations indicate high and low values, respectively. (C) Spearman correlation matrix between nutrients content and hydration in harvest 2. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. The color gradient represents correlation intervals from blue (negative) to red (positive). TSS, total soluble solids; TA, total acidity; H, hydration; Ca, calcium; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; Na, sodium; P, phosphorus; Zn, zinc.

which is well above the optimal range for cultivation. This increase in temperature at end-season harvest was supported by increased TPC in Albion strawberries. This pattern was also observed in other studies such that by Balasooriya *et al.*,[40] who described increases in total polyphenols, flavonoids, anthocyanin and antioxidants in Albion cultivars under higher temperature conditions (30 °C) compared to ambient conditions (25 °C). Likewise, Wang & Zeng[41] reported that strawberry growth under high-temperature conditions (30 °C/22 °C) significantly enhanced flavonoids content and the antioxidant capacities in Earliglow and Kent cultivars. Increases in TPC may be induced by activity stimulation of the two main enzymes in the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS).[42] However, in contrast to that described, here, an increase in the anthocyanin content was not observed and a reduction in the RSA occurred. Thus, the increased TPC may be attributed to other phenolic or hydrophilic compounds present among the main strawberry antioxidants.[43] The environmental conditions experienced by the strawberry plants in the present study were quite extreme, surpassing those of prior studies by more than[40,41] 5 °C. Therefore, extreme temperatures can lead to an increase in TPC but not in other antioxidant compounds, and even result in a reduction of some bioactive compounds, thereby decreasing the overall antioxidant capacity of the fruit. Unfortunately, decreases in RSA could not be related to any of the analyzed antioxidants but it may be relevant because the fruit antioxidant capacity at the time of harvest is a key parameter for minimizing oxidative stress and maintaining quality during post-harvest. Hence, a reduced RSA in commercial ripe fruits could lead to a shorter post-harvest shelf-life and an increase in food waste.[44,45]

Consumers purchase strawberries on the criteria of visual quality, freshness and taste.[46] Water loss in strawberry leads to commercial and physiological deterioration of fresh fruit, in the form of wilting and shriveling, as well as a decrease in stiffness, turgidity and succulence.[47] In this regard, besides the reduction in RSA, a decrease in fruit hydration was observed during the off-season strawberry production. Transpiration is one of the main physiological processes contributing to water loss. Elevated temperatures, coupled with lower relative humidity, constitute two critical environmental factors influencing the transpiration process in strawberries. As temperature rises, transpiration will increase accompanied by reductions in relative humidity.

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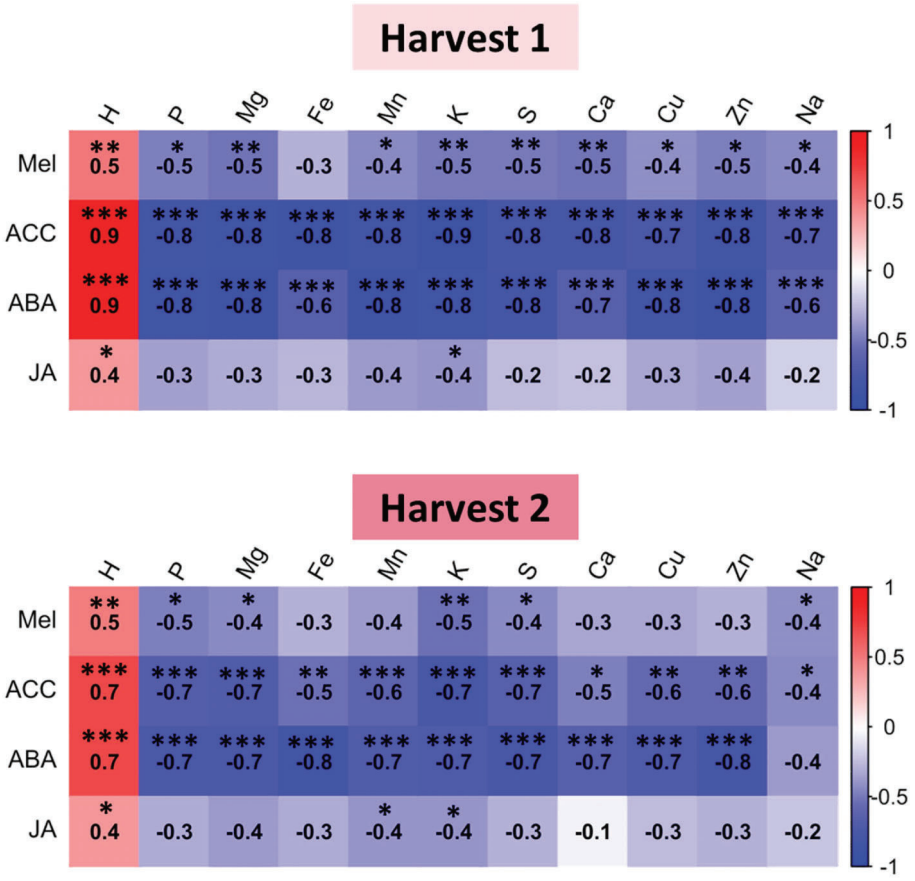


Figure 8. Spearman correlation matrix between hormone content and hydration in harvests 1 and 2. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. The color gradient represents correlation intervals from blue (negative) to red (positive). H, hydration; Mel, melatonin; ACC, 1-amynocyclopropane-1-carboxylic acid; ABA, abscisic acid; JA, jasmonic acid.

Moreover, aerobic respiration also generates some heat, with most of it being released from the produced fruit surface by evaporating water vapor from upper tissular layers.[48] Consequently, respiration and transpiration directly affect fruit quality and shelf life both of them affecting water loss.[49] Thus, even though, in the present study, increased temperatures did not affect TSS, TA or anthocyanins in strawberries out of the productive period, a reduction in fruit hydration and antioxidant capacity was observed and this can directly impact the appreciation of these fruits by consumers and accelerate their deterioration during post-harvest period.[45,50]

Furthermore, the decline in quality towards the off-peak production was accompanied by a reduction in phytohormones and regulatory molecules associated with stress and ripening, such as JA, ACC and melatonin. Melatonin is involved in increasing the biosynthesis of antioxidant compounds[23,51,52] and therefore it is closely associated with the antioxidant capacity of the fruit. Moreover, previous studies demonstrated that melatonin could improve the skin strength properties of fruit, reducing the gradient of water vapor pressure between the fruit and the surrounding atmosphere as a result of respiration and transpiration.[45,53] Hence, a reduction in its levels may have a direct relationship with a decrease in both antioxidant capacity and fruit hydration. Likewise, both ethylene and JA are phytohormones that play a critical signaling role in abiotic stress conditions and fruit ripening. Ethylene can increase antioxidant enzymes and regulate osmolytes, nutrients and other plant hormones for stress tolerance. JA also has an essential role under abiotic stresses improving plant stress tolerance affecting fruit ripening as well as increasing antioxidant capacity enhancing anthocyanins and polyphenols.[54,55] Moreover, JAs are key signals triggering the closure of stomata, synthesis of cuticles and formation of different osmotic compounds.[56] Hence, the role of JAs in maintaining crop water content and preventing water stress is critical for plant survival, crop productivity and the prevention of postharvest losses. Beyond their protective function, ethylene and JA also play an important role in fruit ripening by enhancing antioxidants and phenolic compounds boosting fruit nutritional value.[57-61] Besides, a close cros-stalk exists between ethylene and JAs in different plants,[62,63] which is consistent with results obtained in the present study where a decrease in ACC, the precursor of ethylene, could lead to a reduction in ethylene and could thereby be related to JA decrease, hence affecting both the antioxidant capacity and hydration of the fruit. Therefore, considering the protective role of melatonin, JA and ethylene against adverse environmental conditions and their role in fruit antioxidant accumulation, the reduction in their contents at the end of the season could be one of the main causes compromising the quality of strawberries.

High variability in PAR incidence between field blocks influences fruit antioxidant content

Growing conditions or environmental factors play a significant role in determining the nutritional value of strawberries.[64,65] In the present study, the existence of distinct microclimatic regions

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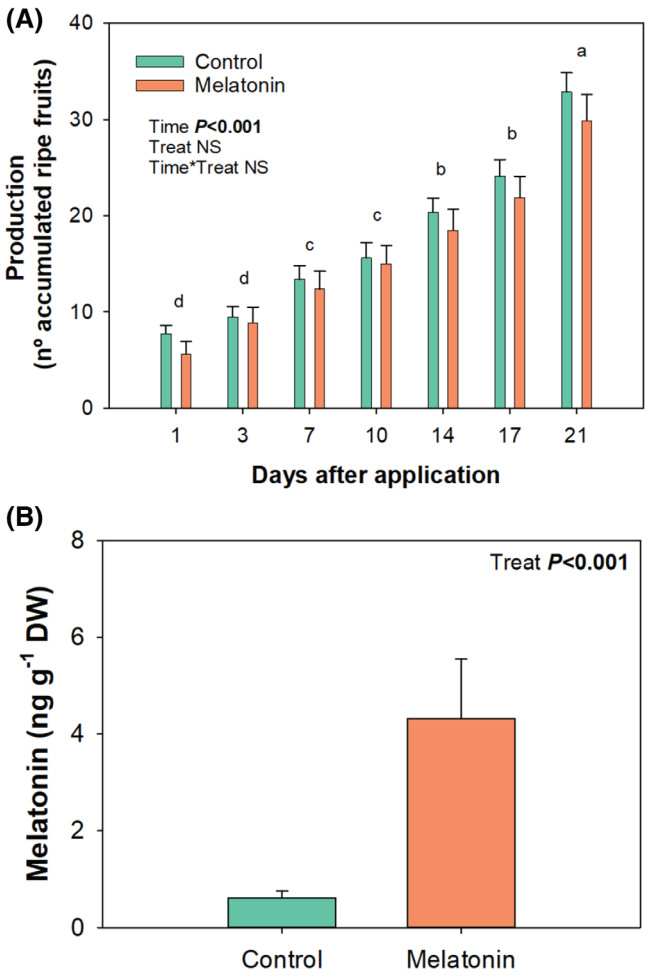


Figure 9. (A) Production of strawberry plants (number of accumulated ripe fruits) and (B) melatonin content of control and melatonin-treated strawberries. Data represent the mean ± SE of $n = 32$. NS, no significant differences ($P > 0.05$). Different letters indicate significant differences over time ($P \leq 0.05$).

within the same production tunnel was observed. PAR values were extensively variable regardless of productive time and were negatively correlated with the content of antioxidant compounds in the fruit, including TPC in harvest 1 and anthocyanins in harvest 2. Light intensity directly impacts strawberry quality because it is a crucial factor for the proper development of leaves and fruits. It is well-known that light influences the synthesis of phenolic compounds including anthocyanins.[66-68] Sharma *et al.*[69] demonstrated that plants growing under low light intensity conditions (336 µmol m^{-1} s^{-1}) tend to produce a higher proportion of albino fruits compared to conditions of higher light intensity (720 µmol m^{-1} s^{-1}). Nevertheless, light conditions exceeding photosynthetic saturation levels may lead to an increase in fruit temperature and result in damage and quality loss.[70] Moreover, although light has been recognized as an important regulator of flavonoid accumulation in species of the Rosaceae family, the induction of flavonoid biosynthesis by light can differ among cultivars depending on their sensitivity.[5,71] Here, higher light intensities around 1300 µmol m^{-1} s^{-1} were negatively correlated with anthocyanin contents and thereby with fruit quality loss. These results indicate that the light received by plants is influenced by their position within the tunnel and affects their antioxidant content. In addition, there is significant variability in different antioxidant compounds among various zones within the production tunnel, which generates heterogeneity in the nutritional quality of the fruits depending on the specific zone in which they are located. Furthermore, variability differs between harvest times because there is a higher number of antioxidants that have shown significant differences in harvest 1 but not in harvest 2. However, significant differences were present in PAR values among blocks within the tunnel in both harvests. Blocks that experienced higher light intensity during the first harvest are identical to those that received more light intensity during the second harvest, coinciding with the ones situated on the right side of the tunnel. Therefore, monitoring positions within the tunnel that receive more PAR and correlates with lower RSA, can be used to implement specific measures in different zones of a production tunnel or field. For example, it would be possible to mitigate excess light by implementing covering material to further shield the crop areas where nutritional quality of the fruit may be compromised. In this sense, innovative and accurate measurements through the use of a large number of sensors distributed within a tunnel or alternatively, mobile sensors can be proposed to estimate the microclimate of the tunnel.[72] Similarly, our results promote the idea that the development of tools such as wireless sensors, active machine learning or remote sensing techniques to deliver detailed data about the structure and thermal composition of environments is essential for optimizing the nutritional quality of fruits.[73,74] Maps of thermal and light regimes below different conditions can help managers to optimize planting in accordance to variety-specific temperature and light adaptations as well as obtain more realistic predictions.[75] This will contribute to managing agricultural systems where microclimates affect yield and fruit quality. Hence, in conjunction with other precision agriculture approaches, such as non-destructive methods for detecting sugars and antioxidant compounds,[76,77] there is the potential to significantly improve the detection of crop variability. This, in turn, can facilitate the adoption of more precise agricultural practices aimed at enhancing crop quality.

Nutrient content presents a high inter-block and inter-individual variability and is conditioned by fruit hydration and phytohormone content

Minerals in strawberries, such as Mg, K, Mn, Cu, Fe and P are crucial for brain function, blood pressure regulation, muscle health and immune system support.[78] Genetics is a paramount factor in determining the nutritional quality of strawberries because the accumulation of micronutrients can substantially differ depending on the variety.[79,80] The significant variability observed not only within the tunnel, but also within plants of the same block at the end of the season indicates high inter-block and inter-individual variability. Nutrients such as Cu, Na, Fe and Ca exhibited the most variability. Considering that this significant variation in nutrient levels could be even more pronounced in depleted soils, it is important to note that the use of cultivation techniques such as hydroponic sack production could help increase nutrient homogeneity to prevent a greater impact of variability. Furthermore, the strong negative correlation of hydration with all nutrients suggests that nutrient content and availability could be influenced by fruit hydration. In this sense, transpiration represents a key driver for the accumulation of xylem-born minerals. Morandi *et al.*[81] reported that water loss

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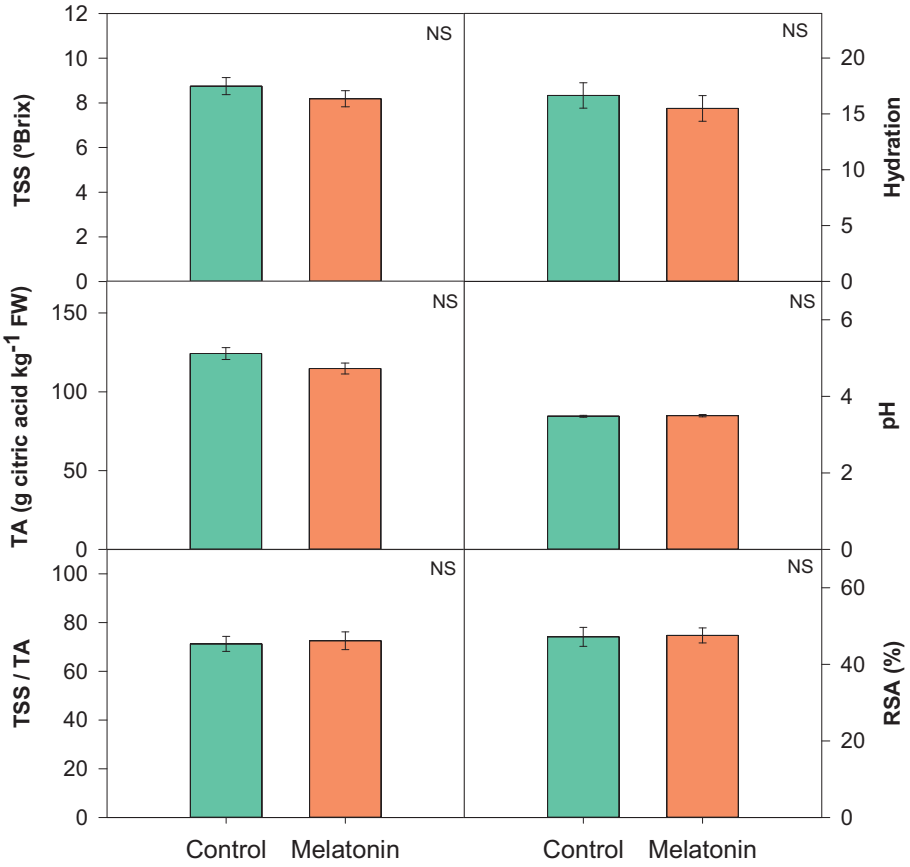


Figure 10. Basic quality parameters of control and melatonin-treated strawberries. TSS, total soluble solids; TA, total acidity; RSA, radical scavenging activity. Data represent the mean ± SE of $n = 32$. NS, no significant differences ($P > 0.05$).

induces a decrease in fruit water potential via an increase in the osmotic concentration and a decrease in the turgor pressure. Accordingly, as more water is lost by fruits, more water can be drawn from the phloem to xylem streams into the fruit itself. Hence, an increase in transpiration will increase water loss and would increase accumulation of dry matter. Because the correlation between hydration and nutrient content is negative, it may be that a decrease in off-season hydration is favorable for better nutrient availability at the end of the season. In addition, the high positive correlation between melatonin, ABA, ACC, JA and fruit hydration and the negative one with nutrient content could indicate that the decrease in fruit hydration may be linked to the decrease in essential regulatory molecules and both condition the nutrient content. The relationship between plant hormones and micro and macronutrients is fundamental for the development and metabolism of plants. Hormones appear to establish links between nutrient levels and their effects on overall growth, development and stress protection. ABA can affect water and nutrient absorption by modulating stomatal opening, limiting nutrient uptake and influencing nutrient redistribution in plants.[82,83] The nutrient content and its ratio can change with the intensity of stress, and hormones may be involved in this process. On the other hand, it has also been observed that melatonin can modulate the acquisition, assimilation and distribution of nutrients in plants, ultimately influencing aspects such as growth and crop yield under stress conditions.[84,85] Nevertheless, the relationship between plant hormones and nutrient content in fruits is still complex and multifaceted.

The effect of melatonin as a biostimulant can be affected by the significant variability within the tunnel

In agriculture, the frequent use of pesticides and fertilizers is common to achieve increased plant yield and productivity, as well as better quality fruits. However, in recent years and particularly in the EU region, there has been a proposal to reduce the application of these synthetic chemical products and promote the use of biostimulants based on natural products that may also have a positive impact on plants. Specifically, in the present study, an application of melatonin at a concentration of 10^{-4} M was performed to assess whether this molecule could alleviate the potential effects of unfavorable production conditions on the quality of Albion strawberries. However, in contrast to several prior publications applying melatonin in productive crops,[86-89] the foliar application of melatonin in strawberry plants bearing fruits did not have any impact on their production and nutritional quality of the fruit. Nevertheless, melatonin applications lead to an increase in endogenous melatonin in the fruits up to 21 days after application, coinciding with the decreased melatonin levels in the control fruits. Manafi *et al.*[90] reported that foliar treatments of melatonin at 10^{-4} M can enhance antioxidant defense in strawberry leaves during high temperatures (35–40 °C). The same was observed in the study of Khan *et al.*,[91] where the same melatonin concentration mitigated the effects of drought stress in strawberries, increasing both enzymatic and non-enzymatic antioxidants. Likewise, melatonin at 10^{-4} M enhanced the content of anthocyanins, flavonoids and ascorbic acid in strawberry leaves under cadmium stress.[92] Similarly, Zahedi *et al.*[93] reported that 10^{-4} M exogenous

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melatonin leads to an increase in strawberry fruit yield and quality parameters such as TSS, TA and TPC in fruits under salinity stress. However, the majority of studies have been conducted under controlled greenhouse conditions. In this context, Okatan *et al.*[94] reported that melatonin application improved both the yield and quality of strawberries, although these effects were more pronounced in controlled environments compared to open fields. Furthermore, Mukherjee *et al.*[95] recently reported that the prolonged stability of biostimulants such as melatonin within a plant tissue is vital to enable efficient management of growth regulation and stress tolerance under normal and challenging environments. This is aligned with the fact that, 3 weeks after application, the treated fruits still had elevated melatonin levels, whereas the control fruits experienced a substantial decrease in their content. Nonetheless, the complexities associated with its implementation within a production tunnel must be considered. As demonstrated in the present study, the plethora of environmental and genetic factors that interact in fruit production makes biostimulants success difficult. Therefore, even though scientific evidence suggests that melatonin 10^{-4} M could have potential applications in strawberry fields, the intrinsic aspects of the environment and the plant itself interfere with the putative biostimulant effect. These factors should be taken into account in future crop enhancement projects to optimize the use of melatonin as a biostimulant.

In summary, the present integrated study demonstrated that substantial variability exists in the antioxidant content attributed to microclimatic fluctuations in the tunnel and in the nutrient content of strawberry fruit as a result of inter-individual differences linked to fruit hydration and hormonal content. Harvesting at the end of the production period predominantly compromises antioxidant capacity and fruit hydration, affecting final quality. In consideration of the use of melatonin as a biostimulant, it demonstrates inefficacy in ameliorating the loss of quality or enhancing productivity, suggesting that variability within a tunnel may influence its potential benefits. Hence, it is essential for agricultural improvement programs, including the incorporation of biostimulants, to direct their investigation toward the factors that have shown to be crucial, such as light intensity and hormonal content. This knowledge is critical for implementing environmentally friendly techniques that enhance both the uniformity and nutritional quality of the crop.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

AA, SM-B and PM were responsible for reviewing and editing. AA was responsible for writing the original draft. AA and SM-B were responsible for funding acquisition. AA and PM were responsible for conceptualization. NP and SM-B were responsible for formal analysis. NP was responsible for methodology. SM-B and PM were responsible for project administration. PM was responsible for writing the original draft.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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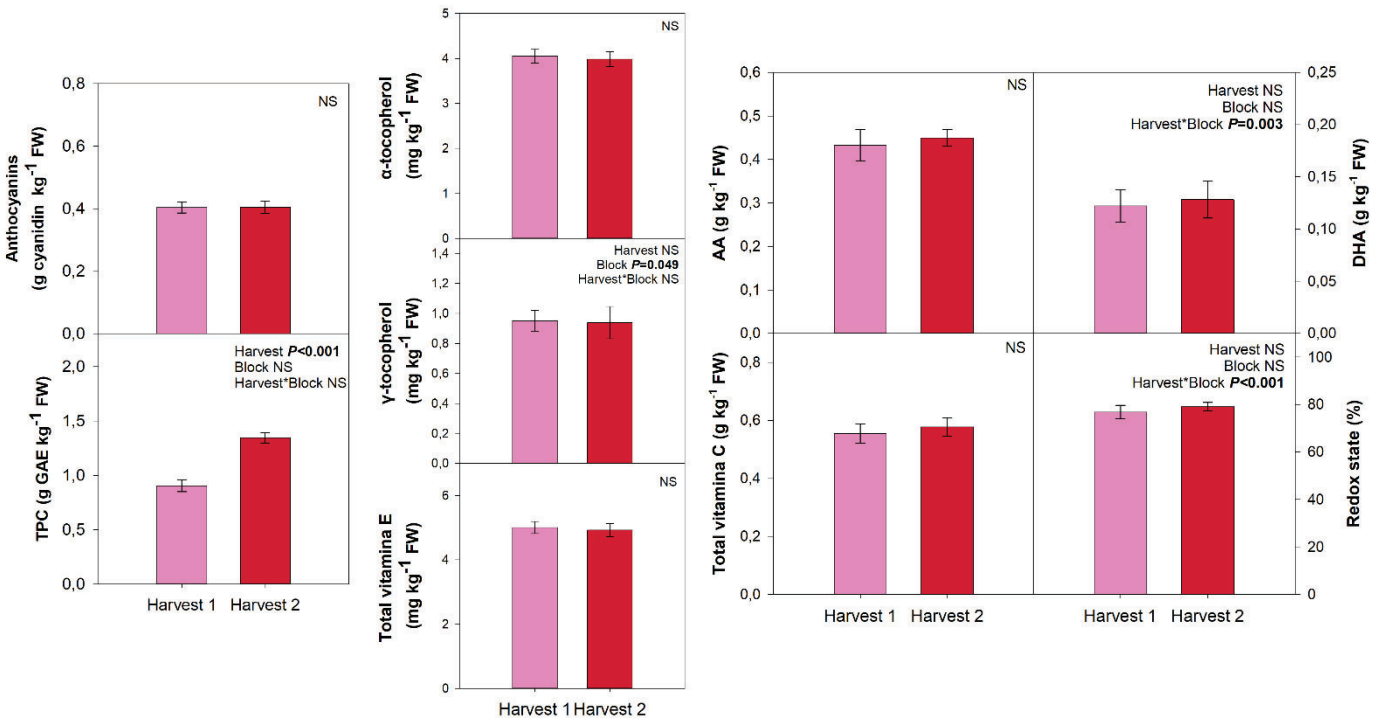


Figure S1. Antioxidant compounds of strawberries from the two harvests carried out 3 weeks apart, in the off-season between June and July. Data represent the mean ± standard error of n = 32. NS indicates that there are no significant differences over harvest ($P > 0.05$). Total Phenol content, TPC; Ascorbic acid, AA; Dehydroascorbate, DHA.

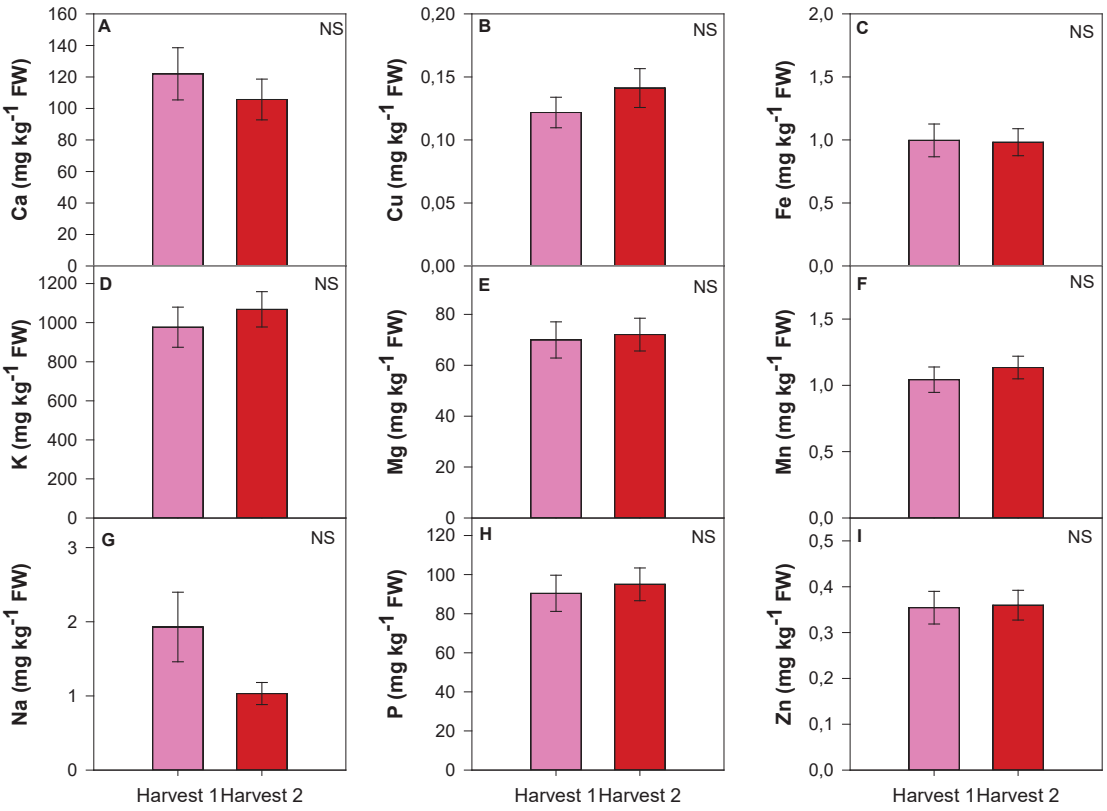


Figure S2. Mineral content of strawberries from the two harvests carried out 3 weeks apart, in the off-season between June and July. (A) Calcium, Ca; (B) Copper, Cu; (C) Iron, Fe; (D) Potassium, K; (E) Magnesium, Mg; (F) Manganese, Mn; (G) Sodium, Na; (H) Phosphorus, P; and (I) Zinc, Zn. Data represent the mean ± standard error of n = 32. NS indicates that there are no significant differences over harvest ($P > 0.05$).

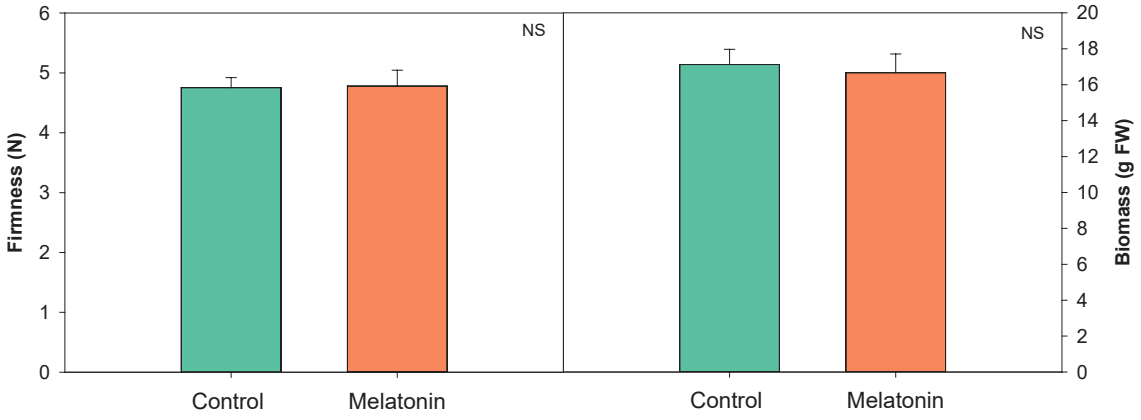


Figure S3. Firmness and fruit biomass of control and melatonin treated strawberries. Data represent the mean ± standard error of n = 32. NS indicates no significant differences (P > 0.05).

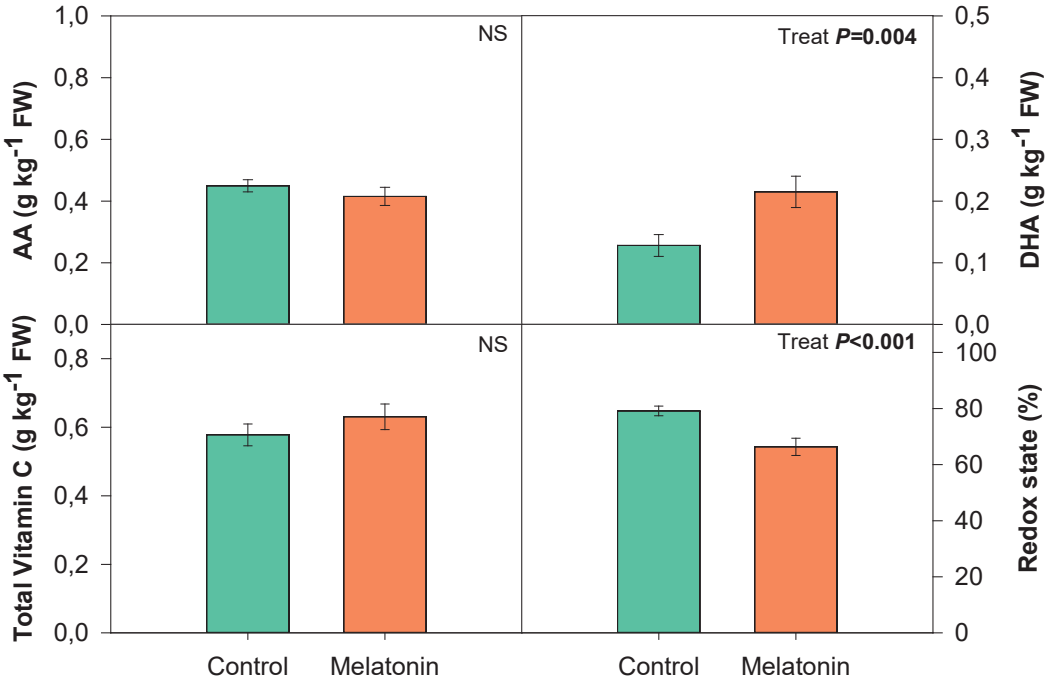


Figure S4. Vitamin C content of control and melatonin treated strawberries. Data represent the mean ± standard error of n = 32. NS indicates no significant differences (P > 0.05). Ascorbic Acid, AA; Dehydroascorbate, DHA.

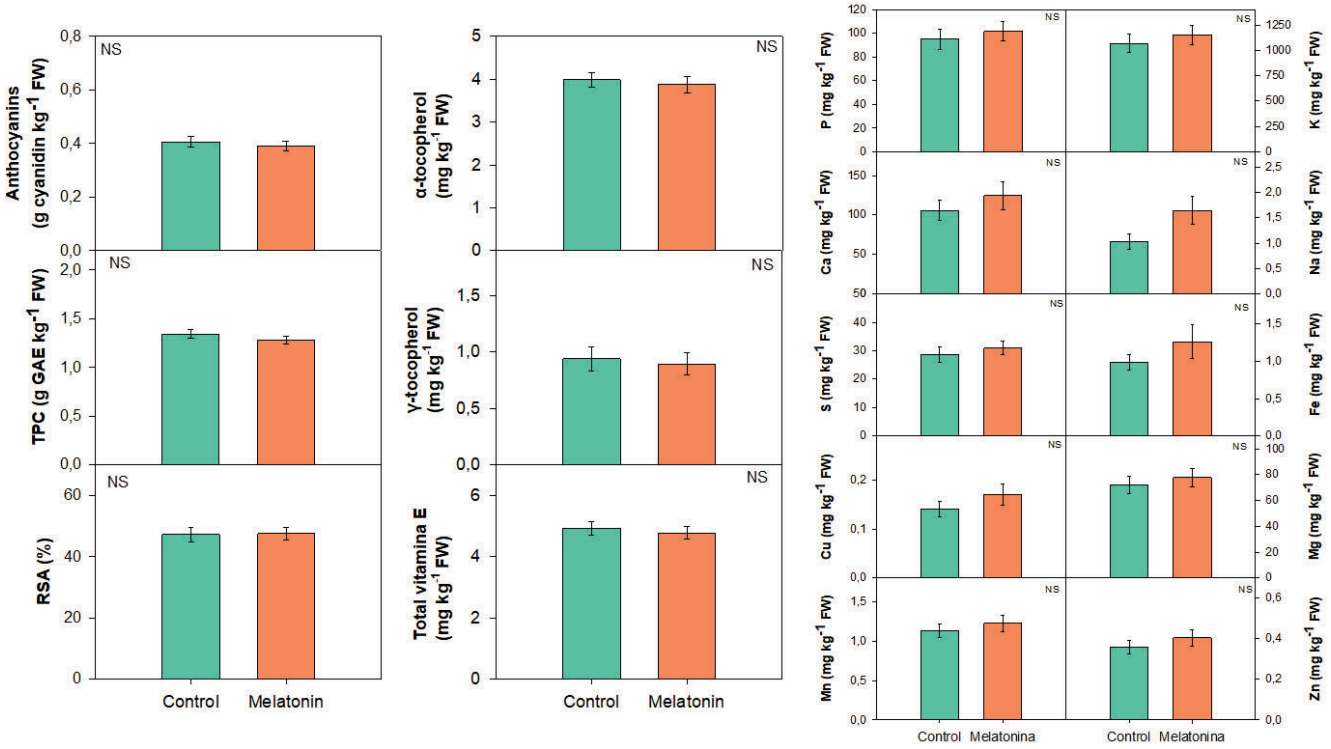


Figure S5. Antioxidants and mineral content of control and melatonin treated strawberries. Data represent the mean ± standard error of n = 32. NS indicates no significant differences ($P > 0.05$). Radical Scavenging Activity, RSA; Total Phenol Content, TPC; Calcium, Ca; Copper, Cu; Iron, Fe; Potassium, K; Magnesium, Mg; Manganese, Mn; Sodium, Na; Phosphorus, P; Sulfur, S; Zinc, Zn.

DISCUSSIÓ



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Fa tres dècades, amb el descobriment de la presència de melatonina en plantes, es van obrir les portes a tot un nou camp de recerca. Des de llavors, la comunitat científica està dedicant un esforç significatiu per respondre preguntes fonamentals com per exemple: "com es duu a terme la seva biosíntesi en plantes?, "quina és la seva localització i en quins nivells es troba?", o "quin paper fisiològic exerceix?". De fet, una cerca en bases de dades com Scopus, utilitzant les paraules clau "Melatonin" i "Plant" juntes en el títol, resum o paraules clau, revela un total de 3.520 articles publicats entre 1995 i 2025. D'aquests, però, 2.236 corresponen només als últims cinc anys, evidenciant el creixent interès científic en aquest camp durant el darrer lustre. A partir dels descobriments realitzats per diversos autors que han estudiat la melatonina en diferents espècies, condicions ambientals i etapes de desenvolupament, aquesta molècula s'ha anat consolidant com un potent antioxidant i alhora, com una molècula multifuncional capaç de regular una gran diversitat de processos fisiològics, destacant-se especialment en el creixement, desenvolupament i resposta a estressos (a)biòtics. No obstant això, la seva implicació en la maduració i la senescència dels fruits també ha estat suggerida, fet que ha generat un creixent interès en el sector agroalimentari i posiciona la melatonina com una molècula de gran rellevància en el context actual per al seu possible ús en la millora de la qualitat dels fruits. En aquest marc, aquesta tesi doctoral ha buscat aprofundir en el paper fisiològic de la melatonina en la maduració i sobremaduració dels fruits, per millorar la comprensió de la seva funció en aquests processos i obrir noves perspectives per a la seva aplicació en la conservació i qualitat dels fruits.

1. Continguts de melatonina en fruits

La melatonina és una molècula activa present de manera ubiqua en els vegetals, amb nivells endògens que poden variar significativament no només entre espècies, sinó també entre varietats, depenent en gran manera de la localització, l'estat de creixement i l'òrgan específic de la planta (Murch et al., 2009; Arnao & Hernández-Ruiz, 2013; Okazaki & Ezura, 2009; Hernández-Ruiz & Arnao, 2008). De forma general, s'ha acceptat que els nivells més elevats de melatonina es troben en les llavors, fulles i flors (Nawaz et al., 2016; Mannino et al., 2021; Arnao, 2014), mentre que els fruits són els òrgans on s'ha detectat un contingut

més baix d'aquesta molècula. En aquesta tesi, la concentració de melatonina ha estat quantificada en diversos fruits incloent maduixots, cireres, nabius, gerds i prunes, sota diferents condicions experimentals i mitjançant UHPLC-MS/MS (**Taula 1**). La tècnica analítica utilitzada té un impacte significatiu en els resultats quantitatius, ja que és un dels factors que contribueixen a la gran variabilitat de concentracions reportades a la literatura. D'ençà que es va notificar per primera vegada la presència de melatonina en plantes, la seva extracció i quantificació per avaluar-ne la funció ha representat un gran repte. L'adaptació de tècniques utilitzades per la seva detecció en animals, com els assaigs immunològics (radioimmunoassaig, RIA, o assaigs immunoabsorbents lligats a enzims, ELISA) ha generat resultats poc fiables en matrius vegetals, degut a la seva complexitat química. Això pot provocar interferències que deriven en falsos positius o negatius, o bé en la sobreestimació o subestimació dels nivells de melatonina (Gomez et al., 2013). Altres tècniques com la cromatografia líquida d'alta resolució (HPLC) acoblada a detectors electroquímics (ECD), de fluorescència (FD) o de llum ultraviolada (UV), també s'han utilitzat àmpliament per a la quantificació de melatonina (Arnao, 2014). Tot i demostrar una sensibilitat elevada, només mètodes com la cromatografia de gasos (GC) o la HPLC acoblada a espectrometria de masses (MS) han mostrat una fiabilitat suficient per a la quantificació precisa d'aquesta molècula. Van Tassel et al. (2001) van evidenciar que les concentracions de melatonina en tomàquet obtingudes per RIA sobreestimaven els continguts respecte quan s'analitzaven amb GC-MS, tot i que aquesta presenta el desavantatge de requerir una derivació prèvia. En aquest context, el mètode HPLC-MS amb ionització per electroesprai positiva (ESI+) i monitorització de reaccions múltiples (MRM) s'ha consolidat com una tècnica altament sensible i específica, amb límits de detecció més baixos que les altres metodologies (Huang & Mazza, 2011; Gomez et al., 2013). Per tant, la quantificació de melatonina en fruits al llarg dels anys ha mostrat grans variacions, en part, a causa de les tècniques analítiques utilitzades. Per exemple, a la dècada dels 90, Hattori et al. (1995) van detectar 0,01 ng/g de melatonina en maduixots mitjançant RIA validat amb HPLC-FD, mentre que el 2011, Stürtz et al. van identificar valors d'entre 1 i 11 ng/g en diferents varietats de maduixot utilitzant LC-MS, resultats més similars als obtinguts en el **capítol 1**, **2** i **4** per a la varietat Albion de maduixot, on els nivells de melatonina es situen en el rang de 0,5 a 17 ng/g de pes sec (PS) (**Taula 1**).

En general, la quantificació mitjançant UHPLC-MS/MS revela que la melatonina es troba en concentracions relativament baixes en els fruits estudiats, sense superar els 20 ng/g PS. Aquestes concentracions, però, varien significativament segons l'espècie, destacant la pruna com el fruit amb els nivells més elevats entre els analitzats (**Taula 1**). Tot i aquesta variabilitat entre espècies, també s'han identificat variacions dins d'una mateixa en funció de les condicions ambientals. En el **capítol 4** es mostra que, en els maduixots cultivats en condicions de camp, la concentració de melatonina varia segons el moment de la collita. Els fruits procedents de la collita realitzada al juliol, durant una intensa onada de calor amb temperatures superant els 35 °C, presenten una concentració de melatonina inferior a la meitat de la registrada en la collita prèvia al mes de juny. Aquesta disminució podria atribuir-se a l'ús de la melatonina com a mecanisme de defensa antioxidant davant l'estrès oxidatiu induït per les temperatures elevades (Ahammed et al., 2019). Els **capítols 1** i **4** permeten comparar diferents condicions de cultiu i demostren que aquestes influeixen de manera significativa en els nivells de melatonina del maduixot Albion. Els fruits madurs cultivats en hivernacle presenten concentracions de melatonina més elevades en comparació amb els cultivats en camp. A l'estudi d'Arnao & Hernández-Ruiz (2013) també es va observar que les concentracions de melatonina en diferents òrgans de la tomaquera (fulla, tija i arrel) variaven segons el sistema de cultiu (in vitro, cambra o camp), però en el seu cas les concentracions més altes es van detectar en tots els òrgans sota condicions de camp, tot i que malauradament no reporten dades sobre el fruit. No obstant això, en aquesta tesi els nivells més baixos de melatonina s'han registrat, en general, en condicions de postcollita, quan el fruit ja no està unit a la planta mare. De fet, durant la postcollita, excepte en el cas de les prunes, els fruits vermells incloent maduixots, gerds, nabius i cireres presenten nivells de melatonina generalment inferiors a 1 ng/g PS. Aquests valors són similars als reportats en la postcollita d'altres fruits com plàtans, taronges, dàtil vermell o raïm (Hu et al., 2017; Johns et al., 2013; Wang et al., 2021a; Mercolini et al., 2012), i poden estar associats a la degradació d'antioxidants i hormones durant aquest període, tal com s'observa als **capítols 2 i 3**, on els nivells de melatonina juntament amb el d'hormones vegetals com les Cks, GAs, IAA i JAs disminueixen a mesura que avança la sobremaduració. A més, s'ha reportat que l'enzim SNAT presenta una baixa eficiència catalítica en condicions de senescència i pot actuar com a coll d'ampolla (Byeon et al., 2015a), per la qual cosa això també podria contribuir als baixos nivells de melatonina en la postcollita.

L'acumulació de la melatonina dins d'un mateix fruit també varia segons el tipus de teixit. En el **capítol 1** es mostra com el maduixot presenta concentracions més elevades al receptacle carnós que als aquenis (els veritables fruits), amb variacions associades a l'estadi de maduració, el qual es discuteix en més profunditat a la **secció 2**. En el **capítol 3**, en la pruna, un model que ha permès estudiar de forma diferencial els diferents teixits del fruit carnós, s'observa que l'exocarpi (pell) presenta nivells més elevats de melatonina que el mesocarpi (polpa). Aquest patró també s'ha documentat en altres fruits, com la poma o el raïm, on la capa més externa mostra concentracions més altes que la part interna (Verde et al., 2022; Vitalini et al., 2011). Aquesta distribució diferencial, caracteritzada per un increment del contingut en el teixit en contacte directe amb l'ambient, es podria relacionar amb la funció protectora de la melatonina, atès que s'ha descrit com una molècula capaç de conferir resistència enfront estressos abiòtics i biòtics, gràcies a la seva dualitat antioxidant i reguladora (Cao et al., 2016; Li et al., 2022b; Li et al., 2015). Per altra banda, a més dels factors esmentats anteriorment, els nivells generalment baixos de melatonina detectats en els fruits, en comparació amb els descrits en altres òrgans (Nawaz et al., 2016), podrien estar relacionats amb la tendència de les plantes a concentrar aquesta molècula en òrgans més exposats a condicions adverses, com les fulles, per protegir-se de l'estrès oxidatiu.

En resum, a través dels diferents models i condicions experimentals utilitzats en aquesta tesi, es demostra que la concentració de melatonina en el maduixot està condicionada per factors com les condicions ambientals i de cultiu, el moment de la collita i l'estadi de maduració del fruit, observant-se una disminució dels nivells sota condicions de camp i en situacions ambientals adverses. En general, les condicions de postcollita redueixen la concentració de melatonina en els fruits, tot i que també es manifesta l'efecte del genotip, com es pot observar en el cas de la pruna, que presenta concentracions notablement més altes en comparació amb els fruits vermells analitzats després de la collita. La combinació d'aquests factors, juntament amb l'ús de processos d'extracció poc eficients i de tècniques analítiques amb limitacions al llarg dels anys, probablement contribueix a l'elevada variabilitat en els nivells de melatonina reportats, fins i tot dins d'una mateixa varietat.

Taula 1. Quantificació del contingut de melatonina per UHPLC-MS/MS en diversos fruits sota diferents condicions experimentals, estadis i teixits al llarg dels diferents capítols de la tesi doctoral. PS: pes sec.

Fruit	Condició experimental	Estadi	Òrgan/teixit	ng/g PS
Maduixot (*Fragaria* x *ananassa* cv. Albion)	Precollita camp (juny)	Madur	Fruit sencer	2,12
	Precollita camp (juliol)	Madur	Fruit sencer	0,61
	Precollita hivernacle	Verd	Aqueni	4,7
	Precollita hivernacle	Verd	Receptacle	2,6
	Precollita hivernacle	Inici maduració	Aqueni	1,68
	Precollita hivernacle	Inici maduració	Receptacle	8,7
	Precollita hivernacle	Madur	Aqueni	1,69
	Precollita hivernacle	Madur	Receptacle	17,44
	Postcollita	Madur	Fruit sencer	0,555
Gerd (*Rubus idaeus* cv. Driscoll's)	Postcollita	Madur	Fruit sencer	0,24
Nabiu (*Vaccinium corymbosum* cv. Duke)	Postcollita	Madur	Fruit sencer	0,34
Cirera (*Prunus avium* cv. Lapins)	Postcollita	Madur	Fruit sencer	0,45
Pruna (*Prunus salicina* cv. Angeleno)	Postcollita	Madur	Exocarpi	20,66
	Postcollita	Madur	Mesocarpi	13,91

Nota**:** Per a una major claredat, l'error estàndard no s'ha inclòs en aquesta taula. No obstant això, totes les dades detallades es poden consultar als capítols corresponents de la tesi.

2. Paper fisiològic de la melatonina en la maduració i sobremaduració dels fruits

La maduració dels fruits carnosos és un procés de desenvolupament altament complex, en què es produeixen transformacions estructurals, bioquímiques i metabòliques, que impliquen l'activació de centenars de milers de gens que acabaran definint el seu color, sabor, textura i aroma. Per tant, aquest procés requereix una estricta coordinació i regulació a nivell genètic, principalment per l'acció fonamental de diverses hormones vegetals, amb una dinàmica espacial i temporal que resulta clau pel correcte desenvolupament del fruit (Klee & Giovannoni, 2011). Tot i això, altres biomolècules actives també estan involucrades en la regulació de la maduració i, per tant, en la qualitat final dels fruits (Corpas et al., 2022). En aquest sentit, la melatonina ha emergit com una molècula multifuncional en plantes, però el seu paper en la maduració dels fruits encara no és clar, fet que genera interrogants sobre si actua com a promotora o inhibidora del procés. Per desxifrar i comprendre millor la funció de la melatonina, es poden aplicar diverses aproximacions experimentals, una de les quals és l'avaluació de la seva concentració endògena en diferents òrgans i etapes del desenvolupament per establir una possible causalitat (**Annex I**). Les molècules reguladores de la maduració modulen vies metabòliques mitjançant l'expressió gènica, fet que provoca variacions significatives en els seus nivells endògens al llarg del creixement i la maduració del fruit. Entendre aquestes variacions és fonamental per determinar el seu paper biològic i mode d'acció. Fins ara, els estudis sobre la melatonina en la maduració dels fruits s'han centrat principalment en l'anàlisi del fruit sencer després d'aplicacions exògenes (Hu et al., 2017; Wang et al., 2020b; Mansouri et al., 2023), mentre que la informació sobre la seva distribució endògena és escassa, fet que ens impedeix determinar la seva funció fisiològica en la regulació dels canvis fisiològics associats al procés de maduració, depenent del teixit. Per abordar aquesta qüestió, en el **capítol 1** s'ha caracteritzat la distribució de la melatonina en els estadis del desenvolupament del maduixot en els dos òrgans que conformen el fals fruit, l'aqueni i el receptacle, tenint en compte que el maduixot és un dels sistemes més utilitzats per estudiar la maduració de fruits no climatèrics (Giovannoni, 2001). Els resultats obtinguts en aquest capítol mostren que la melatonina segueix una distribució espaciotemporal, depenent no només de l'òrgan del fals fruit, sinó també de l'estadi de desenvolupament (**Capítol 1**). Específicament, la concentració màxima de melatonina en els

aquenis es detecta durant el quallat del fruit (FS, Fruit Set en anglès) i el primer estadi verd de creixement (SG, Small Green en anglès), i disminueix progressivament arribant als nivells més baixos abans de l'inici de la maduració (IR, Initial Red en anglès). A més, els seus precursors principals, el triptòfan i la serotonina, mostren un patró d'acumulació molt similar, indicant també una limitació de substrat (**Capítol 1, Figura 8**).

L'aqueni i el receptacle són dos òrgans connectats per feixos vasculars, però molt diferents en termes de morfologia, xarxes metabòliques i rol fisiològic dins del fals fruit. Cal tenir en compte que la contribució de cada un al fruit complet depèn en gran mesura de l'estadi de desenvolupament. En els estadis primerencs, els aquenis presenten un metabolisme altament coordinat, centrat en l'acumulació de reserves, compostos protectors i precursors de fitohormones, essencials tant pel desenvolupament i la maduració de la llavor com pel manteniment de la seva quiescència (Fait et al., 2008). El desenvolupament del maduixot s'ajusta al creixement dels aquenis, i el desenvolupament de la part carnosa es reprimeix fins que els aquenis fecundats alliberen senyals d'auxina a la paret de l'ovari i al receptacle (Tian et al., 2022), contribuint així a la divisió cel·lular i afavorint l'expansió del receptacle en longitud i diàmetre a través de vies dependents i independents de GAs (Liao et al., 2018). Per tant, que els nivells més elevats de melatonina en els aquenis es trobin en els estadis inicials del desenvolupament suggereix que té una funció fisiològica relacionada amb el procés de creixement. A més, el patró d'acumulació de la melatonina observat en els aquenis segueix una dinàmica molt similar a la descrita per l'IAA (**Figura 8**), indicant que, com ocorre amb aquesta fitohormona promotora del creixement, la disminució dels nivells de melatonina en l'aqueni és necessària per al progrés adequat de la maduració. En concordança, la melatonina presenta un patró d'acumulació oposat al de l'ABA, la principal hormona promotora de la maduració en fruits no climatèrics (**Figura 8**). Els aquenis, com a veritables fruits, són els centres reguladors no només del desenvolupament de la llavor i el creixement del receptacle, sinó també de la maduració comercial. L'eliminació dels aquenis altera l'inici del procés de maduració, demostrant el seu paper fonamental en la regulació d'aquest procés (Li et al., 2024a). A més, també està demostrat que l'acumulació d'ABA en els aquenis té un rol més rellevant que en el receptacle sobre el ritme de maduració i el seu paper en la maduració del receptacle es pot atribuir, en part, a la seva regulació en els aquenis (Li et al., 2022c). De fet, s'ha descrit que l'ABA es pot sintetitzar de forma

autocatalítica en els aquenis per promoure la maduració, però no en el receptacle, el qual també fa destacar les diferències entre ambdós òrgans (Li et al., 2022c). Per tant, tot i que la maduració comercial es manifesta en el receptacle, els senyals provinents dels aquenis són un factor determinant en aquest procés. Així, tenint en compte que tant la melatonina com l'IAA deriven del triptòfan, la seva similitud estructural i el paral·lelisme en la seva acumulació en els aquenis, els resultats obtinguts en el **capítol 1** demostren que la melatonina endògena té un paper clau en el creixement i desenvolupament del maduixot, i, en conseqüència, en la regulació negativa de la maduració. La implicació de la melatonina en les primeres fases del creixement, on es promou la divisió i expansió cel·lular, també s'ha reportat en estudis previs amb altres fruits no climatèrics. En el cas de les cireres, Tijero et al. (2019) i Zhao et al. (2013) han detectat concentracions més elevades de melatonina durant les primeres etapes de creixement, les quals disminueixen abans de l'inici de la maduració. De manera similar, Xia et al. (2020) han observat també els màxims nivells de melatonina durant el quallat del fruit, alhora que Wang et al. (2016a) han registrat una reducció del seu contingut abans del canvi de color en mores.

En el receptacle, la melatonina segueix un patró oposat al trobat als aquenis, amb els nivells més baixos durant el quallat del fruit i un increment gradual a mesura que avança la maduració, assolint el pic màxim a l'estadi completament madur, quan el fruit ha adquirit la coloració vermella (FR, Full Red en anglès, **Figura 8**). De fet, en aquest punt, presenta uns nivells sis vegades superiors als observats en el quallat (**Capítol 1**). En el receptacle, l'etapa final de la maduració es caracteritza per un augment notable de la respiració i l'estovament del fruit (Kumar et al., 2016; Lombardo et al., 2011), fenòmens que estan associats a la desintegració de la paret cel·lular i sobretot, a una major producció de ROS. Així doncs, que l'augment més pronunciat de melatonina coincideixi amb el de H_2O_2 en el receptacle (**Figura 8**), suggereix que la seva funció en aquest òrgan està vinculada amb la creixent demanda d'antioxidants i a l'increment de l'activitat metabòlica, en resposta a les necessitats del fruit. S'ha acceptat que el cloroplast i el mitocondri són els principals llocs biosintètics de la melatonina (Hardeland, 2019), el qual va estretament vinculat a la seva naturalesa com a antioxidant, ja que aquests són fonts importants de producció de ROS a nivell cel·lular (Muñoz & Munné-Bosch, 2018). Sabent això, és probable que el seu increment a la darrera etapa de desenvolupament del fruit es pugui atribuir a la seva naturalesa antioxidant. De fet,

els nivells de melatonina 4 vegades més alts al receptacle a l'estadi FR que en els aquenis al quallat del fruit indiquen una especialització fisiològica, possiblement relacionada amb les necessitats funcionals específiques de cada òrgan al llarg del desenvolupament. Mentre que als aquenis la melatonina està involucrada en els processos de regulació del creixement, al receptacle, on l'augment de ROS es produeix principalment a nivell mitocondrial a mesura que avança la maduració, l'increment de melatonina en l'estadi madur podria constituir una resposta adaptativa al punt màxim d'estrès oxidatiu. De fet, en aquest estadi, els nivells de H_2O_2 al receptacle tripliquen els màxims observats als aquenis, reforçant la idea que la funció principal de la melatonina en aquest òrgan madur pot estar relacionada amb la protecció antioxidant. Per tal de reforçar aquesta idea, serien necessaris estudis complementaris que combinin l'anàlisi espaciotemporal de la distribució de la melatonina amb l'avaluació funcional de la seva activitat, com per exemple la localització tissular i subcel·lular a través de tècniques de microscòpia de fluorescència o immunolocalització. La manipulació genètica com línies mutants en gens clau de la seva biosíntesi també serien clau per tal d'establir correlacions funcionals més robustes entre els nivells de melatonina i les necessitats específiques de cada òrgan en funció de l'estadi de desenvolupament. No obstant això, en els aquenis, els nivells més alts de H_2O_2, tot i ser inferiors als del receptacle, es registren durant el quallat, coincidint amb el moment de màxima presència de melatonina, cosa que suggereix també un possible efecte protector d'aquesta sobre les llavors immadures (**Figura 8**). Aquests fets indiquen que la demanda de melatonina per part del fruit està directament relacionada amb la seva doble funció. D'una banda, com a antioxidant, especialment al receptacle, ja que s'ha demostrat que l'acumulació de melatonina és paral·lela a la de ROS com el H_2O_2 a mesura que avança la maduració (**Figura 8**), i que la melatonina és eficaç en la reducció de l'estrès oxidatiu, fins i tot a concentracions baixes (Galano & Reiter, 2018; Khan et al., 2020). De l'altra, com a regulador de vies metabòliques i molècules clau implicades en el desenvolupament del fruit i en el manteniment de l'equilibri redox, funció predominant en els aquenis durant les primeres etapes de desenvolupament.

En aquest context, l'estudi de Mansouri et al. (2021) també va analitzar el contingut endogen de melatonina en quatre estadis del desenvolupament del maduixot. A diferència dels resultats obtinguts en el **capítol 1**, Mansouri et al. detecten el pic màxim de melatonina en el canvi de color, suggerint que la melatonina està involucrada en l'inici de la maduració,

amb una posterior disminució en l'estadi FR. Aquesta discrepància podria ser deguda a diversos factors. D'una banda, el seu estudi no inclou dades sobre el contingut de melatonina durant el quallat del fruit i els resultats s'expressen en pes fresc en lloc de pes sec, fet que genera estimacions inexactes dels nivells de melatonina a causa de les variacions en el contingut d'aigua entre òrgans i estadis de desenvolupament. D'altra banda, el fet que l'anàlisi es realitzés sobre el fruit sencer, sense distingir entre aquenis i receptacle, influeix en els resultats, ja que ambdós òrgans presenten diferències en l'acumulació de compostos bioactius i en els mecanismes reguladors durant el desenvolupament i la maduració (Li et al., 2022c; Enomoto et al., 2018). Concretament, aquestes diferències respecte als resultats del **capítol 1** mostren que l'heterogeneïtat entre òrgans pot generar un biaix en la interpretació del paper fisiològic de la melatonina si no es tenen en compte les particularitats de cadascun. Tot i la gran quantitat d'estudis sobre la melatonina els últims anys, són escassos els que analitzen les seves variacions segons el tipus de teixit. Un dels pocs exemples reportats a la literatura, que destaca com la separació per teixits influeix en la quantificació i determinació de la melatonina, és el cas del raïm. L'estudi de Xu et al. (2018) demostra que el pic de melatonina durant el desenvolupament del fruit es produeix en el moment de transició de color, del verd al vermell fosc, i disminueix un cop iniciada la maduració. En canvi, l'estudi de Vitalini et al. (2011) revela que el moment en què s'assoleix el màxim nivell de melatonina en raïm varia segons el teixit analitzat, amb el pic màxim abans del canvi de color en la pell, mentre que en la polpa i les llavors aquest pic es produeix durant la transició del color, tal com es descriu també en l'anàlisi del fruit sencer (Xu et al., 2018). En altres fruits com la poma, també s'ha descrit que la dinàmica de la melatonina varia segons el teixit, ja que no es va detectar cap patró clar a la polpa, però si a la pell, on la melatonina incrementa al final de la fase d'expansió i assoleix el màxim en el canvi de color (Verde et al., 2022). En conjunt, aquests estudis i els resultats del **capítol 1**, subratllen la importància de diferenciar els teixits o òrgans del fruit per entendre millor les dinàmiques i els mecanismes subjacents a les funcions fisiològiques de les molècules reguladores, com és el cas de la melatonina.

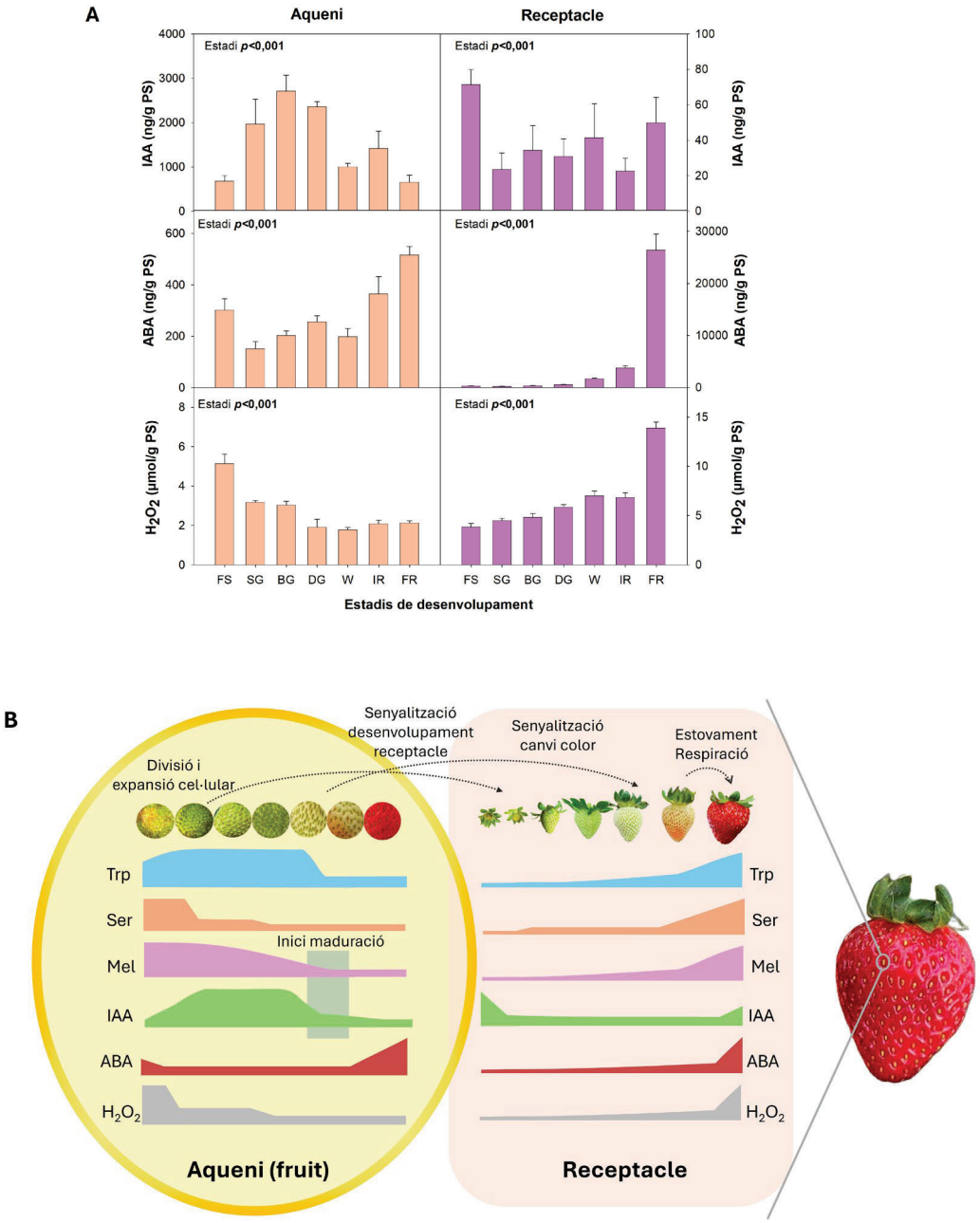


Figura 8. **A)** Concentracions endògenes d'àcid indolacètic (IAA), àcid abscísic (ABA) i peròxid d'hidrogen (H_2O_2) en els aquenis i receptacle del maduixot al llarg dels diferents estadis de desenvolupament. Les anàlisis hormonals s'han realitzat tal com es descriu a Müller & Munné-Bosch (2011) i a la secció de materials i mètodes del **capítol 1**, i el H_2O_2 tal com es descriu a Mesa et al. (2024). Les dades són la mitjana ± error estàndard de *n* = 5 i per l'anàlisi estadística es va dur a terme una ANOVA d'un factor. **B)** Model de l'acumulació de la melatonina, els seus precursors, les principals fitohormones involucrades en la regulació del desenvolupament del maduixot i el H_2O_2 com a senyal redox en l'aqueni i el receptacle, els dos òrgans que componen el maduixot. Trp: triptòfan; Ser: serotonina; PS: pes sec.

Entre els diversos metabòlits primaris derivats de l'oxidació de la melatonina, la 2-HM és el més prevalent en plantes, amb nivells que poden superar els de la melatonina (Byeon et al., 2015b; Back, 2021). Aquest fet s'ha demostrat per primera vegada en maduixot al **capítol 1**, on els nivells de 2-HM durant el desenvolupament del fruit són fins a tres vegades més alts que els de la melatonina, tant en aquenis com en receptacle, superant els 50 ng/g PS en aquest últim. Aquesta major presència de 2-HM, també observada en la postcollita dels fruits vermells del **capítol 2**, probablement es deu a una activitat catalítica més elevada de l'enzim M2H, que converteix la melatonina en 2-HM, en comparació amb els enzims implicats en la seva biosíntesi (Byeon & Back, 2015). En plantes, s'ha atribuït a la 2-HM un paper en la defensa contra l'estrès biòtic i abiòtic (Back, 2021; Lee & Back, 2019; Shah et al., 2020), amb funcions similars a les de la melatonina (Korkmaz et al., 2023). Tot i això, les evidències sobre la seva funció exacta encara són limitades i s'han observat diferències importants respecte a la melatonina. Per exemple, mentre que l'aplicació de 2-HM en arròs incrementa la resistència a l'estrès per fred i sequera, aquest efecte no es presenta amb la melatonina (Lee & Back, 2016). A més, s'ha descrit que la 2-HM, però no la melatonina, té propietats prooxidants, induint la producció de ROS en *Arabidopsis* i afavorint la senescència foliar (Lee & Back, 2021). Pel que fa als fruits, la informació respecte a la 2-HM segueix sent pràcticament inexistent. Tot i això, en el **capítol 1** es presenta el primer estudi sobre el patró de 2-HM durant el desenvolupament del maduixot, on segueix la mateixa dinàmica espaciotemporal que la melatonina, amb nivells màxims en els aquenis durant el quallat i al receptacle en l'estadi madur. En aquest sentit, només un altre estudi (Xu et al., 2018) ha analitzat el contingut de 2-HM al llarg de la maduració, en raïm, on la producció de 2-HM es desencadena posterior al pic de melatonina, arribant al màxim al final de la maduració. Aquest patró coincideix amb l'increment de H_2O_2 (Xu et al., 2018) i amb els nostres resultats en el receptacle del maduixot, tot i que també s'ha determinat que la 2-HM presenta una eficiència com a antioxidant molt menor respecte la melatonina (Galano & Reiter, 2018). Per tant, tot i els seus alts nivells en els teixits vegetals, la seva recerca encara es troba en una fase molt primerenca, sense evidència clara sobre si exerceix una funció fisiològica específica en els fruits, ja sigui com a part de l'efecte de la melatonina o de manera independent. Tanmateix, cal considerar que la 2-HM és un producte de degradació de la melatonina, i no es pot descartar que el seu augment sigui simplement un reflex de la seva pèrdua. En aquest sentit, l'anàlisi de la ràtio entre melatonina i 2-HM al llarg del desenvolupament podria

aportar informació rellevant per discernir si l'acumulació de 2-HM respon exclusivament a la degradació de melatonina o si existeixen mecanismes de regulació diferencials.

A més de l'anàlisi de la concentració endògena de melatonina en els diferents punts de desenvolupament del fruit, la seva aplicació exògena és una altra aproximació experimental a aplicar per obtenir informació addicional i determinar si la melatonina exerceix un paper promotor o inhibidor en la maduració (**Annex I**). En els darrers anys, diversos estudis han explorat l'efecte d'aquestes aplicacions tant en la fase precollita com en la postcollita, revelant que la melatonina contribueix a la regulació de canvis fisiològics i bioquímics (**Annex I**). Generalment, les dosis de melatonina exògena aplicades en fruits solen oscil·lar entre 10^{-3}M i 10^{-5}M, sent 10^{-4}M (100 µM) la concentració més emprada a la literatura (Aghdam & Arnao, 2024). Després de testar un ampli rang de dosis (10 µM - 10 mM) en maduixot, s'ha descrit que 100 µM és la concentració que ofereix els millors resultats en termes de qualitat de fruit (Liu et al., 2018a). A més, s'ha reportat un comportament hormètic de la melatonina en plantes (Zhang et al., 2019; Agathokleous et al., 2019), i aplicacions a dosis molt elevades, de l'ordre de 1000 µM, han induït respostes associades a un estrès oxidatiu cel·lular en lloc d'un efecte hormonal (Mansouri et al., 2021). Tenint en compte els nivells endògens de melatonina (ng/g) i les evidències prèvies, s'utilitza una dosi de 100 µM de melatonina als **capítols 1, 2, 3 i 4** per estudiar el seu rol en la maduració i sobremaduració, induint respostes fisiològiques sense comprometre l'estat del fruit.

Seguint aquesta aproximació, en el **capítol 1** s'ha investigat l'efecte de l'aplicació de 100 µM de melatonina mitjançant immersió en diferents etapes clau del desenvolupament del fruit, demostrant que el moment d'aplicació condiciona el fenotip resultant (**Figura 9a**). Concretament, l'aplicació just després de la fecundació, en el quallat, va accelerar l'adquisició del color, fent que l'angle hue dels fruits s'apropés més ràpidament als angles associats al vermell. En canvi, els tractaments en etapes més tardanes, com a l'estadi de desverdiment abans del canvi de color (DG, Degreening en anglès), o al IR, a l'inici de la maduració, van tenir l'efecte contrari, retardant l'aparició de la vermellor aproximadament dos dies, com es reflecteix en la menor progressió de la component a* (color vermell) i el desplaçament més lent del hue cap a tons vermellosos. L'adquisició del color és un dels canvis més rellevants en el fruit durant la maduració, ja que condiciona el moment en què el fruit arriba al mercat comercial. En el cas del maduixot, aquest canvi de color es produeix per la degradació de

clorofil·les i l'acumulació d'antocianines, els pigments responsables de la tonalitat vermella. S'ha descrit que la melatonina pot regular el contingut d'antocianines mitjançant la modificació de l'expressió de gens relacionats amb la seva síntesi com la fenilalanina amoni liasa (PAL), la calcona sintasa (CHS), l'antocianidina sintasa (ANS), la dihidroflavonol 4-reductasa (DFR) o factors de transcripció MYB (Altaf et al., 2024; Marak et al., 2024; Sun et al., 2021b; Xia et al., 2021; Mansouri et al., 2023). En relació amb aquests resultats, altres autors també han observat un endarreriment en el ritme de maduració mitjançant l'aplicació exògena en fruits no climatèrics. Per exemple, el tractament amb 10 µM de melatonina en maduixot i cireres en l'estadi "verd gran" (BG, Big Green en anglès) va estar associat a variacions en els valors de hue i a*, així com a una disminució del contingut d'antocianines (Mansouri et al., 2021; 2023; Tijero et al., 2019; Ruiz-Aracil, 2022). No obstant això, en el mateix estudi de Mansouri et al. (2021) no es detecta cap efecte amb l'aplicació de 100 µM. És probable que les diferències en el mètode d'aplicació (injecció en el pedicel respecte a la immersió, mètode utilitzat en el nostre estudi) i en el moment de l'aplicació (en el seu estudi es va realitzar en l'estadi BG, que se situa entre el quallat i el DG) siguin la causa d'aquestes discrepàncies respecte als nostres resultats. Pel que fa a l'aplicació de 100 µM de melatonina mitjançant esprai en cireres, aquesta va causar variacions en l'acidesa total (Tijero et al., 2019). En canvi, en el **capítol 1**, no s'observen variacions en les principals propietats organolèptiques dels fruits collits en el punt òptim de maduresa comercial després dels tractaments, indicant que la melatonina exògena en maduixots modifica la velocitat de maduració sense perjudicar la qualitat final del fruit. D'altra banda, en altres fruits no climatèrics com el raïm, l'aplicació de melatonina mitjançant immersió en estadis molt inicials de creixement va accelerar la maduració (Xu et al., 2018), uns resultats similars als observats amb l'aplicació de melatonina en el quallat del maduixot (**Capítol 1**). Per tant, aquestes diferències fenotípiques segons l'aplicació exògena suggereixen que l'efecte de la melatonina està fortament associat a l'estadi en què s'aplica així com probablement també al mètode utilitzat.

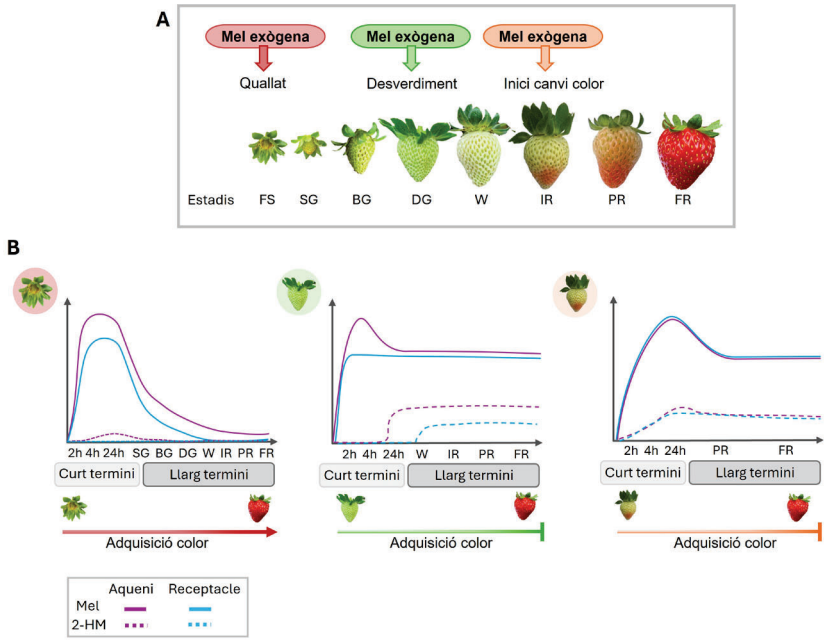
A nivell bioquímic, les diferents aplicacions de melatonina provoquen un increment significatiu de la seva concentració durant les primeres 24 h post-aplicació, amb augments de fins a 200, 100 i 60 cops en els aquenis i de 70, 20 i 15 cops en el receptacle, segons l'aplicació a l'estadi de quallat, DG o IR, respectivament (**Capítol 1**). La melatonina, per la

seva naturalesa amfipàtica, té una gran capacitat per travessar les membranes cel·lulars (Tan et al., 2012a) facilitant així la seva absorció i acumulació, tal com es pot observar amb aquests increments. Tot i això, la mida del fruit i la proporció d'aquenis respecte a la superfície del fruit en el moment de l'aplicació podrien influir en l'absorció i acumulació de la melatonina en funció de l'òrgan. Aquesta alta capacitat d'absorció també s'ha observat en altres estudis, on l'aplicació de melatonina a 100 µM va augmentar la seva concentració fins a 400 vegades en cireres (Tijero et al., 2019). No obstant això, dosis més baixes de melatonina generen un augment molt menor, com per exemple un increment de només 1,2 vegades en maduixots tractats amb 10 µM (Mansouri et al., 2023).

Al cap de 24 hores de l'aplicació, hi ha una resposta diferencial en l'acumulació de melatonina i la seva conversió a 2-HM depenent del moment de l'aplicació (**Figura 9b**). Quan la melatonina s'aplica al quallat del fruit, es produeix un augment inicial en els seus nivells, però aquests disminueixen abans que el fruit comenci a canviar de color, continuant a la baixa fins al final de la maduració, especialment al receptacle, on els nivells s'aproximen als dels fruits control. Aquesta caiguda dels nivells de melatonina abans de la maduració coincideix amb el moment de l'aparició de l'efecte fenotípic, accelerant el canvi de color. A més, aquesta resposta a llarg termini també es reflecteix en l'anàlisi de transcriptòmica, on s'observen molts més canvis significatius en l'expressió diferencial de gens quan el fruit és gairebé madur, 16 dies després de l'aplicació, que no pas a les 24 hores post-aplicació (**Figura 9c, Figura S6 del Capítol 1**). Tanmateix, la disminució de melatonina no va acompanyada d'un augment en el contingut de 2-HM. En canvi, quan la melatonina s'aplica en els estadis avançats DG o IR, els nivells de melatonina es mantenen molt elevats durant tot el procés de maduració respecte als fruits control, i la resposta transcriptòmica és immediata, amb un major nombre de gens expressats diferencialment 24 hores després de l'aplicació (**Figura 9b-c**). Aquest comportament podria explicar el retard en l'adquisició del color observat en aquests estadis, en contrast amb les aplicacions al quallat, reflectint així el paper endogen de la melatonina com a inhibidor de la maduració. A més, 24 hores després de l'aplicació en DG, s'observa un lleuger descens en els nivells de melatonina, que, a diferència de l'aplicació al quallat, aquí sí que va acompanyada d'un increment en l'acumulació de 2-HM, especialment en els aquenis. Una acumulació progressiva de 2-HM també es detecta després de l'aplicació en IR. Per tant, el moment d'aplicació de la melatonina modula l'activitat

metabòlica associada a la seva hidroxilació, afavorint una conversió més gran a 2-HM en estadis posteriors del desenvolupament del fruit. Fins al moment, no s'han registrat altres estudis que avaluïn la conversió de melatonina a 2-HM durant la maduració, per la qual cosa aquests resultats obren noves perspectives per investigar aquest complex equilibri, específic de cada etapa, en la regulació del desenvolupament del fruit.

En resum, es demostra que la melatonina té una funció inhibidora de la maduració òrgan-específica, jugant un paper clau durant les primeres etapes del desenvolupament en els aquenis, els veritables fruits que actuen com a centres reguladors del creixement i la maduració. En canvi, en el receptacle, sembla tenir una funció més associada a la protecció davant l'estrès oxidatiu durant les etapes finals de la maduració, de forma directa i/o indirecta. A més, la funció inhibidora de la maduració de la melatonina es confirma mitjançant la seva aplicació exògena en estadis avançats del creixement o un cop iniciada la maduració, associada a un ajustament en l'acumulació i hidroxilació de la melatonina, sobretot en aquenis. Els resultats de l'aplicació exògena indiquen que l'estat fisiològic del fruit en el moment de l'aplicació és fonamental, probablement relacionat amb els nivells i mecanismes endògens per integrar i metabolitzar la melatonina exògena en aquell moment (generant una resposta a llarg o curt termini). Finalment, aquests resultats obren la porta a noves preguntes sobre la coordinació entre l'aqueni i el receptacle en la regulació de l'acumulació i acció de la melatonina, el qual encara és desconegut.



C

	Curt termini	Llarg termini
Quallat	24h	PR
	DEGs	DEGs
Aqueni	13	36
Receptacle	0	5
Desverdiment	24h	PR
	DEGs	DEGs
Aqueni	90	1
Receptacle	139	8
Inici color	24h	PR
	DEGs	DEGs
Aqueni	4	4
Receptacle	58	5

Figura 9. **A)** Esquema que mostra els estadis en què es va aplicar la melatonina exògena (100µM) en cada tractament, indicant l'aplicació en el quallat del fruit (FS), en el desverdiment (DG) o a l'inici de la maduració (IR). **B)** Dinàmica de l'acumulació de melatonina (línia continua) o 2-hidroximelatonina (línia discontínua) en els aquenis (color lila) o receptacle (color blau) després de l'aplicació en l'estadi FS, DG o IR durant les primeres 24 h (curt termini) o en els estadis de desenvolupament posteriors (llarg termini) fins a arribar a la maduració comercial. **C)** Nombre total de gens expressats diferencialment (DEGs) en l'anàlisi de transcriptòmica, tan regulats a l'alta com a la baixa, a l'aqueni i receptacle després de 24 h de cada tractament (al FS, DG i IR, de dalt a baix) i en l'estadi parcialment vermell (llarg termini). FS: Fruit Set (quallat), SG: Small Green (verd petit), BG: Big Green (verd gran), DG: Degreening (desverdiment), W: White (blanc), IR: Initial Red (Inici color vermell), PR: Partial Red (parcialment vermell), FR: Full Red (completament vermell), Mel: melatonina, 2-HM: 2-hidroximelatonina.

El consum de productes hortícoles com els fruits, altament valorats pel seu sabor i atractiu visual, contribueix a la salut humana prevenint malalties cròniques (Arias et al., 2022). No obstant això, la seva elevada peribilitat durant el període postcollita provoca un deteriorament qualitatiu i quantitatiu que, a més, genera grans pèrdues econòmiques i un fort impacte ambiental. En aquest context, comprendre els mecanismes fisiològics i bioquímics associats a la sobremaduració és clau per desenvolupar estratègies que permetin prolongar la seva vida útil i millorar-ne la comercialització. Després de demostrar al **capítol 1** el paper inhibidor de la melatonina en la maduració del maduixot, als **capítols 2 i 3** es confirma que també actua com a molècula inhibidora de la senescència en maduixots i en altres fruits altament peribles (Kader, 1993), com els gerds, nabius, cireres i prunes. Tal com s'ha comentat a la **secció 1**, els nivells de melatonina en el maduixot són més baixos en el període postcollita, situant-se per sota dels 2 ng/g, igual que en la resta de fruits vermells analitzats al **capítol 2** (**Taula 1**). A més a més, però, aquests nivells continuen disminuint a mesura que avança la postcollita, amb reduccions del 25% en maduixots entre el dia 1 i 2 d'emmagatzematge, i encara més pronunciades en cireres, on es redueixen a la meitat 4 dies després de la collita arribant a valors inferiors a 0,1 ng/g PS (**Capítol 2**). Atès el rellevant patró d'acumulació de la melatonina endògena en funció de l'òrgan durant la maduració del maduixot (**Capítol 1**), en el **capítol 3** es va analitzar també la seva distribució en diferents teixits durant la postcollita, utilitzant la pruna Angeleno com a model. A l'inici de la postcollita, els nivells de melatonina són entre 20 i 40 vegades superiors als dels fruits vermells del **capítol 2**, amb una concentració especialment més elevada a l'exocarpi. No obstant això, 2 dies després de la collita, aquests nivells es redueixen dràsticament, 11 vegades a l'exocarpi i 16 vegades al mesocarpi, amb una disminució progressiva en ambdós teixits fins al final de la vida útil (**Capítol 3**), adquirint valors similars als dels fruits vermells (**Capítol 2**). Aquesta disminució dels nivells tant en l'exocarpi com en el mesocarpi durant l'emmagatzematge també ocorre en el cas d'algunes fitohormones inhibidores de la maduració i la senescència com l'IAA, els JAs o la GA_7 (**Figures S1, S2, S4 del Capítol 3**). Així doncs, en general, es confirma que la pèrdua de melatonina està estretament relacionada amb la deterioració del fruit, disminuint de manera simultània amb la pèrdua de paràmetres de qualitat com el color, la fermesa, els sucres o la hidratació, a mesura que avança la sobremaduració (**Figura 10**). Aquests resultats són coherents amb els observats per altres

autors en raïm, on els nivells de melatonina també disminueixen a mesura que avança la sobremaduració durant l'emmagatzematge (Wang et al., 2020a).

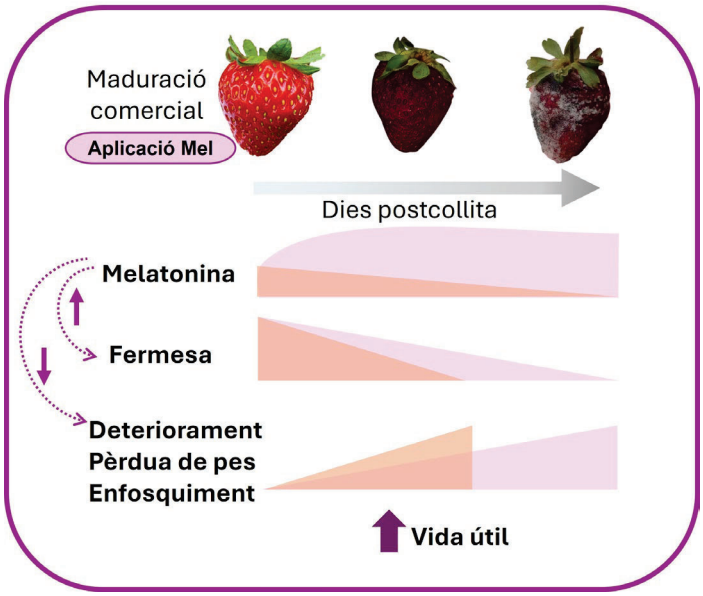


Figura 10. Representació dels canvis en el contingut de melatonina i paràmetres relacionats amb la qualitat postcollita dels fruits com la fermesa, el deteriorament, la pèrdua de pes i l'enfosquiment de forma natural (color taronja) i després de l'aplicació de melatonina (color lila).

La reducció en el percentatge de deteriorament dels maduixots, gerds, nabius i cireres, després de l'aplicació de melatonina a 100 µM reflecteix el seu efecte com a inhibidor de la sobremaduració i senescència en fruits vermells (**Capítol 2**). Aquest efecte inhibidor està relacionat, per una banda, amb els nivells elevats de melatonina després de l'aplicació exògena, que poden ser fins a 1000 vegades més alts en la majoria dels fruits avaluats (**Capítol 2**). Per altra banda, aquesta promoció de melatonina contribueix a mantenir paràmetres essencials per a la qualitat del fruit, com la fermesa, la pèrdua de pes o el color (**Figura 10**). A la literatura ens troben exemples de com la melatonina pot modular els enzims relacionats amb la degradació de la paret cel·lular, com la pectina metilesterasa, poligalacturonasa, la cel·lulasa o la β-galactosidasa, evitant així la seva desestructuració i reduint l'estovament del fruit, fet que també està vinculat amb una menor pèrdua de pes (Qu et al., 2022; Pang et al., 2023). A més, la capacitat de la melatonina exògena per modular paràmetres associats al color, com la component a* i l'angle hue durant la maduració (**Capítol 1**), també ocorre durant la sobremaduració. En aquest cas, ajuda a conservar la coloració

vermella del maduixot i prevenir el deteriorament del color, que es un factor de rebuig visual pels consumidors. Aquesta resposta també s'observa en els gerds i els nabius (**Capítol 2**). Aquestes millores en els paràmetres de qualitat es poden atribuir, per una banda, a la major presència de melatonina i a la seva capacitat de controlar les ROS durant l'emmagatzematge, les quals són responsables de la degradació dels components cel·lulars, i, d'altra banda, a la seva acció en la promoció d'altres antioxidants, com els fenols totals en maduixots i nabius (**Capítol 2**). La melatonina exògena també millora la qualitat en la pruna Angeleno, augmentant els nivells de fenols totals i antocianines al mesocarpi, compostos fenòlics antioxidants associats tant amb beneficis per a la salut com amb una resposta de defensa del fruit (**Capítol 3**). Tanmateix, el fet que aquest increment només es produeix al mesocarpi i no a l'exocarpi, on contribuiria a un major enfosquiment del fruit indicant un avanç de la sobremaduració, demostra que la melatonina actua de forma específica en funció del teixit durant aquest període, tal com també s'observa en la maduració. Tot i que es coneix l'activació de la via fenilpropanoide per l'aplicació de melatonina en altres varietats de pruna, conferint resistència al fruit durant l'emmagatzematge (Yan et al., 2022), la majoria de les investigacions en postcollita analitzen el fruit sencer, la qual cosa limita la comprensió detallada dels mecanismes implicats en cada part del fruit. En aquest sentit, estudis com el del **capítol 3**, on s'avalua el seu efecte específic de teixit, són fonamentals per aprofundir en la comprensió dels mecanismes que la melatonina activa dins del fruit per endarrerir la sobremaduració, proporcionant informació clau per a futures aplicacions. De manera similar, un dels pocs estudis que ha analitzat l'efecte de la melatonina en l'exocarpi i el mesocarpi és el de Cortés-Montaña et al. (2023), també en prunes. Aquest estudi determina que l'aplicació de melatonina a 300 µM tres vegades durant la precollita indueix canvis en la coloració de l'exocarpi, però no en el mesocarpi, reduint així l'enfosquiment del fruit durant l'emmagatzematge a 1 °C. Tot i que les condicions experimentals (moment d'aplicació, temperatura d'emmagatzematge, varietat, etc.) difereixen de les del **capítol 3**, i en conseqüència també els resultats, recolza l'acció diferencial de la melatonina segons el teixit.

L'aplicació de triptòfan en els fruits vermells, tal com es descriu al **capítol 2**, reforça encara més l'efecte inhibidor de la senescència de la melatonina, ja que només ha aconseguit allargar la vida útil dels fruits quan s'ha metabolitzat a melatonina, com en el cas del maduixot i el nabiu. A més, la resposta bioquímica d'aquests fruits tractats amb triptòfan és

molt similar a la dels tractats directament amb melatonina (**Capítol 2**). El triptòfan és un aminoàcid aromàtic que, a més de ser essencial per a la síntesi de proteïnes, actua com a precursor d'indolamines com l'IAA, així com de fitoalexines, glucosinolats, alcaloides, serotonina i melatonina. A més, el seu baix cost l'ha convertit en una eina àmpliament utilitzada en plantes per estimular el creixement i augmentar la productivitat dels cultius (Mustafa et al., 2018). L'aplicació de triptòfan també inhibeix la senescència en alguns vegetals com el bròquil (Sohail et al., 2021), tot i que el mecanisme d'acció encara es desconeix i no hi ha evidències a la literatura sobre l'efecte del triptòfan en la postcollita. Contràriament als resultats obtinguts en el **capítol 2**, a la literatura l'efecte de senyalització del triptòfan en el creixement vegetal i en la resposta de defensa s'ha atribuït habitualment a la seva conversió en IAA (Naveed et al., 2015; Jiang et al., 2022; Wójcik et al., 2019). No obstant això, en el cas de la postcollita de maduixots i nabius, el seu efecte antisenescent no està relacionat amb un increment d'aquesta fitohormona, ni tampoc amb un augment de serotonina, com s'ha suggerit en altres estudis (Ishihara et al., 2008), sinó de melatonina. Per altra banda, l'aplicació de melatonina en els fruits vermells incrementa el contingut de 2-HM durant la postcollita, tal com també es produeix després del tractament amb melatonina exògena durant la maduració (**Capítol 1**), evidenciant una metabolització generalitzada de la melatonina a 2-HM. No obstant això, aquest patró no s'observa després de l'aplicació de triptòfan, ja que, malgrat l'augment de melatonina en maduixots i nabius, no es detecta un increment paral·lel de 2-HM (**Capítol 2**). Això reforça la idea que l'efecte antisenescent de la melatonina és el factor clau en la inhibició de la senescència, per sobre del possible paper de la 2-HM en fruits. En altres contextos fisiològics, com en plantes de maduixot sotmeses a estrès per baixes temperatures, sí que s'ha observat que l'aplicació de triptòfan pot promoure tant el desenvolupament vegetatiu com el quallat de fruits, coincidint amb un augment de serotonina i 2-HM. No obstant això, els nostres resultats en postcollita de fruits s'alineen més amb els de Xiang et al. (2024), on el tractament de triptòfan en condicions precollita de raïm va incrementar el contingut de melatonina en el moment de la collita.

En conjunt, els resultats del **capítol 2** i **3** confirmen que la melatonina exerceix un paper fonamental en la regulació de la sobremaduració i senescència en fruits altament peribles en què la seva qualitat es veu ràpidament compromesa durant la postcollita. La seva disminució endògena està associada amb la pèrdua de qualitat i el progrés de la

sobremaduració, reforçant la seva rellevància en aquest procés. A més, l'aplicació exògena de melatonina no només redueix el deteriorament i prolonga la vida útil del maduixot, el gerd, el nabiu, la cirera i la pruna Angeleno, sinó que també contribueix a mantenir propietats essencials com la fermesa, el color i el contingut de compostos antioxidants. D'altra banda, els experiments amb triptòfan han reforçat aquesta evidència, ja que els seus efectes beneficiosos reduint el deteriorament només es manifesten quan es metabolitza en melatonina i no en altres indolamines. Finalment, l'anàlisi de la seva acció específicament en diferents teixits de la pruna ha revelat que la seva influència no és uniforme, sinó que varia segons el teixit del fruit, proporcionant informació clau per optimitzar les estratègies d'aplicació en postcollita.

3. Interacció de la melatonina amb les fitohormones en la regulació de la maduració i la sobremaduració dels fruits

Entre els diferents mecanismes que s'han descrit en l'acció reguladora de la melatonina, s'ha plantejat que interactua amb altres molècules senyalitzadores de les plantes, especialment les fitohormones com auxines, ABA, SA, JA, etilè i CKs, per ajustar els processos fisiològics i metabòlics de les plantes. Les seves interaccions amb les hormones vegetals han estat objecte d'interès per comprendre millor el seu paper, sobretot en la promoció del creixement i la resposta als estressos (a)biòtics, proposant-se que pot actuar tant de manera independent com modulant rutes hormonals (Arnao & Hernández-Ruiz, 2021). No obstant això, el coneixement sobre aquestes relacions encara es troba en una fase molt inicial, especialment pel que fa al desenvolupament dels fruits. En aquest context, les investigacions sobre la melatonina i la seva interacció amb fitohormones en la maduració i processos postcollita s'han centrat principalment en la seva influència sobre factors relacionats amb l'etilè. La relació entre la melatonina i l'IAA és una de les que també ha generat més interès per la seva similitud estructural, però la recerca s'ha enfocat en processos de creixement, arrelament i gravitropisme (Liang et al., 2017; Zhang et al., 2022b), mentre que la seva interacció en la maduració de fruits continua sent pràcticament desconeguda. Fins ara, s'ha establert que la relació entre la melatonina i l'IAA és complexa i dosi-dependent: a nivells alts, la melatonina té una relació antagònica amb l'IAA, reduint el creixement, mentre que a nivells baixos actua sinèrgicament, promovent la síntesi d'IAA i el

creixement de les arrels, a més de regular els elements de senyalització d'auxina (Yang et al., 2021; Chen et al., 2009; Wang et al., 2016b). En el **capítol 1**, descobrim que l'endarreriment en la maduració després de l'aplicació de melatonina en l'estadi DG s'associa, en part, amb una regulació a la baixa de gens relacionats amb les auxines, com *FaGH3.9*, tant en l'aqueni com en el receptacle al cap de 24 h de l'aplicació exògena. Aquest gen codifica per una IAA amido sintetasa, que catalitza la conjugació de l'IAA lliure i actiu amb aminoàcids com l'aspartat (Asp) i el glutamat (Glu), convertint-lo així en una forma no activa (Ludwig-Müller, 2011). Aquest resultat es reflecteix bioquímicament amb una disminució del contingut d'IAA-Asp i un increment de la relació IAA/IAA-Asp, també 24 h després de l'aplicació (**Capítol 1**). En la regulació hormonal, els gradients creats per la integració de la biosíntesi, el catabolisme, la conjugació i la translocació de l'auxina són indispensables per a les diverses respostes a aquesta fitohormona. En aquest context, la família de proteïnes GH3 és clau per mantenir l'homeòstasi de l'IAA durant la maduració. Tot i que alguns estudis han suggerit una possible funció biològica de l'IAA-Asp en l'aparició de malalties (González-Lamothe, 2012; Grover et al., 2024), no s'ha establert una funció biològica clara i directe, i actualment es considera com un dels primers intermediaris en la degradació de l'IAA. De fet, s'han reportat nivells més alts d'IAA-Asp i IAA-Glu en aquenis en estadis previs al canvi de color i en fruit vermell (Gu et al., 2019), el que demostra que de forma natural es produeix una inactivació de l'IAA a través de la seva conjugació perquè procedeixi la maduració. La capacitat de la melatonina de modular l'expressió de GH3 s'ha reportat en *Arabidopsis* (Weeda et al., 2014) i s'ha descrit també sota condicions d'estrès abiòtic (Duan et al., 2024). Els nostres resultats mostren per primera vegada que també modula la seva expressió durant la maduració de fruits, similar a com ho fan altres hormones com l'etilè i l'ABA (Liu et al., 2005; Böttcher et al., 2010). Per tant, l'endarreriment de la maduració per l'aplicació exògena de melatonina, a més de relacionar-se amb l'estadi de desenvolupament del fruit i amb una dinàmica diferencial de melatonina i 2-HM específica de l'òrgan, també depèn de l'equilibri entre la forma activa i inactiva de l'IAA, mantenint una major proporció d'IAA respecte a l'IAA-Asp (**Figura 11**). La conjugació de l'IAA pot reduir el senyal d'auxina (Woodward & Bartel, 2005) i, per tant, una menor destinació d'IAA cap al catabolisme i un increment de la seva disponibilitat relativa podria estar modulant la seva percepció a nivell cel·lular. No obstant això, són necessaris més estudis per confirmar i aprofundir en aquest mecanisme d'acció. En particular, seria necessari analitzar línies mutants en gens clau de la biosíntesi de

melatonina i de conjugació de l'IAA, com *GH3*, per establir una relació funcional directa. Aquestes anàlisis no s'han pogut dur a terme en aquesta tesi per les dificultats tècniques associades a la generació de mutants en maduixera octoploide, com s'especifica en més profunditat a la **secció 4**. En relació amb aquests resultats, en altres fruits com les cireres, l'endarreriment de la maduració induït per la melatonina s'ha associat també amb la regulació del contingut d'hormones inhibidores d'aquest procés fisiològic, com les CKs (Tijero et al., 2019).

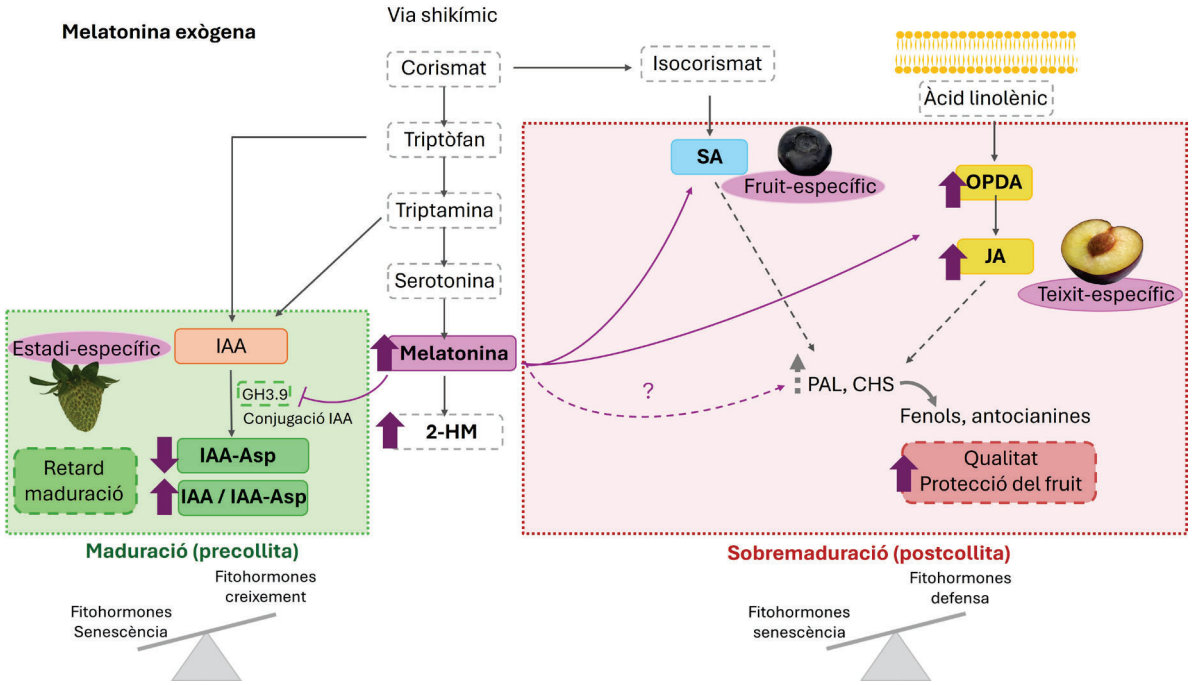


Figura 11. Model de la interacció de la melatonina amb les fitohormones durant la maduració i la sobremaduració dels fruits. IAA: àcid indolacètic, Asp: aspartat, GH3.9: Gretchen Hagen 3.9, 2-HM: 2-hidroximelatonina, SA: àcid salicílic, OPDA: àcid 12-*oxo*-fitodienoic, JA: àcid jasmònic, PAL: fenilalanina amoni liasa, CHS: calcona sintasa.

Tot i que l'ABA s'ha establert com una de les principals hormones reguladores de la maduració en fruits no climatèrics com el maduixot, en aquesta tesi no s'ha trobat cap evidència clara que la melatonina estigui regulant la maduració a través de l'ABA. En el **capítol 1**, les aplicacions de melatonina en diferents moments del desenvolupament que han accelerat o retardat la maduració, no comporten canvis bioquímics en els nivells d'ABA, ni en aquenis ni en receptacle, així com tampoc s'han detectat gens expressats diferencialment

relacionats amb la síntesi o senyalització de l'ABA en l'anàlisi de transcriptòmica realitzat. Tot i que els nostres resultats no mostren aquesta relació, altres estudis han trobat que l'efecte de la melatonina en l'acceleració o endarreriment de la maduració es relaciona amb canvis en els nivells d'ABA, induint-los o disminuint-los, respectivament, mitjançant la modulació de gens implicats en la seva biosíntesi, com la 9-cis-epoxicarotenoide dioxigenasa (NCED) (Mansouri et al., 2021; 2023; Xu et al., 2018). Un possible motiu d'aquesta diferència podria ser l'enfocament metodològic, ja que en el **capítol 1** les mostres es van seleccionar per estadis de desenvolupament, evitant així la comparació de fruits control i tractats en estadis diferents, en lloc de per dies post-aplicació, com en els estudis mencionats. Això indica que, quan els fruits tractats arriben a cadascun dels estadis de desenvolupament, el seu contingut d'ABA és similar al dels controls. Tanmateix, en alguns estudis com els de Tijero et al. (2019), l'endarreriment de la maduració en cireres per melatonina exògena tampoc comporta canvis significatius en el contingut d'ABA, per la qual cosa la interacció melatonina-ABA durant la maduració dels fruits requereix encara més evidències. Generalment, també s'ha descrit una relació antagònica entre la melatonina i l'ABA durant la senescència de les plantes, principalment sota condicions d'estrès, reduint l'expressió de NCED i d'activadors transcripcionals de la família ABF i induint gens del seu catabolisme, com la monooxigenasa CYP (Tan et al., 2019; Zhang et al., 2017; Li et al., 2015). En la postcollita de fruits com el mango i el litxi, la melatonina inhibeix la síntesi d'aquesta hormona i desencadena la transcripció de reguladors negatius de la senyalització d'ABA (com la fosfatasa 2C o F-box), prolongant així la vida útil del fruit (Liu et al., 2020; Zhang et al., 2021b). En els **capítols 2 i 3**, amb l'aplicació de melatonina tampoc s'han detectat canvis en els continguts d'ABA en cap dels fruits vermells ni en la pruna durant la sobremaduració, indicant que en aquests casos l'efecte de la melatonina no està associat a una degradació d'hormones promotores de la senescència. En canvi, l'aplicació de triptòfan al maduixot sí que disminueix un 45% els nivells d'ABA (**Capítol 2**). Considerant que l'efecte beneficiós del triptòfan s'atribueix a l'increment de melatonina, com s'explica a la **secció 2**, i que no s'ha descrit una relació directa entre el triptòfan i l'ABA, s'hipotetitza que aquest canvi també es deu a l'augment de melatonina. Es requereixen, però, més evidències per entendre per què aquesta disminució no s'observa en l'aplicació de melatonina i confirmar el possible mecanisme subjacent.

L'efecte de la melatonina en la postcollita està vinculat a un increment en les hormones implicades en la defensa i la resposta a l'estrès, com el SA i el JA (**Figura 11**). Per exemple, en nabius, el contingut de SA augmenta a l'inici del període postcollita (**Capítol 2**). L'aplicació de melatonina i triptòfan acceleren l'augment dels nivells de SA, elevant-los un 125% i un 46%, respectivament, ja al cap de dos dies d'emmagatzematge (on els fruits control ja assolien el 25% de deteriorament), oferint una protecció precoç als fruits tractats (**Capítol 2**). A nivell molecular, en models com *Arabidopsis* o tabac, s'ha descrit que la resposta defensiva induïda per la melatonina depèn de SA, promovent l'expressió dels gens implicats en aquesta via (Lee et al., 2014; Weeda et al., 2014). Els nostres resultats en nabius coincideixen amb els obtinguts en altres models com el tomàquet o el préssec, on la melatonina millora la resistència a malalties en postcollita, augmentant tant el contingut com la via de senyalització de SA (NPR1, el factor de transcripció TGA o la proteïna PR), així com el metabolisme fenòlic (Gao et al., 2018; Li et al., 2022a). Considerant que en nabius la melatonina també incrementa el contingut de fenols i que se sap que el SA regula positivament els metabòlits secundaris, com els compostos fenòlics, que reforcen la resistència del fruit (Wu et al., 2025), és probable que l'increment de SA observat estigui estretament vinculat amb l'augment de fenols, contribuint així a una major resistència. En l'estudi de Qu et al. (2022) també en nabius, la melatonina va millorar la seva resistència postcollita activant la via del JA i la fenilpropanoide. Aquesta diferència respecte als nostres resultats podria deure's, d'una banda, al tipus de patogen, ja que el SA s'associa a la tolerància a patògens biotròfics i el JA a necrotròfics, i de l'altra, a les condicions postcollita, perquè en l'estudi de Qu et al. els nabius es van conservar a 0 °C fent possible que l'augment de JA respongui també a un mecanisme de tolerància al fred (Li et al., 2024b). En el **capítol 3**, la melatonina sí que incrementa els continguts de JA i també fins a 4 vegades els del seu precursor, OPDA, en el mesocarpi de la pruna al quart dia d'emmagatzematge, coincidint amb l'increment de fenols i antocianines (**Capítol 3**). El JA està involucrat en la biosíntesi de compostos fenilpropanoides, com les antocianines, regulant el complex WD-repeat/bHLH/MYB per activar-los (Qi et al., 2011). Per altra banda, també s'ha demostrat que l'OPDA pot tenir un rol específic en la regulació de vies de senyalització independents de COI1, activant l'expressió de diferents gens relacionats amb la defensa contra l'estrès biòtic (Scalschi et al., 2015). Tanmateix, encara no es coneix la seva implicació directa en la via fenilpropanoide, i per això és necessari realitzar més investigacions per aclarir els

mecanismes específics d'aquesta senyalització. En tomàquet s'han reportat resultats similars als nostres, on la melatonina incrementa la via de senyalització de JA millorant la resistència a patògens i associats també a un increment dels compostos fenilpropanoides (Liu et al., 2019a). Per tant, és probable que la melatonina activi la senyalització de JA per incrementar els continguts endògens de fenols totals i antocianines en la polpa de la pruna. A més, la identificació d'elements de resposta a JA i melatonina en la zona promotora dels gens CHS i PAL (**Figures S5 i S6 del capítol 3**), claus en la biosíntesi de compostos fenòlics, revela que ambdues molècules podrien estar modulant aquests enzims. Així, no es pot descartar que la melatonina moduli la síntesi d'aquests compostos tant a través d'una regulació directa de la via com mitjançant la senyalització de fitohormones com l'OPDA i el JA.

El perfil hormonal avaluat en el **capítol 2** inclou les principals fitohormones actives implicades en la regulació dels processos fisiològics de les plantes, però en fruits com els gerds, les cireres o els maduixots no s'observen canvis significatius en cap de les més de deu hormones analitzades. Per altra banda, el tractament amb melatonina en la postcollita de fruits vermells no altera el balanç entre les hormones promotores de la senescència (com ABA i ACC) i les inhibidores (com les CKs i l'IAA) però sí que disminueix la relació ABA/Mel i ACC/Mel (**Capítol 2**), la qual cosa suggereix que l'efecte antisenescent ve donat més per un increment directe de la melatonina que no pas per canvis en el balanç hormonal. De forma similar, al **capítol 1** tampoc es pot associar l'efecte promotor de la maduració de la melatonina aplicada en el quallat del fruit amb canvis en el contingut de cap de les fitohormones analitzades. Per tant, en aquests casos, l'acció reguladora de la melatonina sembla estar relacionada amb mecanismes no hormonals o respostes antioxidants, activant altres vies de regulació. No obstant això, calen estudis específics i més profunds per entendre millor els mecanismes subjacents a l'efecte de la melatonina en aquestes situacions i poder determinar el seu mecanisme.

En resum, segons els nostres resultats, la resposta inhibidora de la melatonina sobre la maduració precollita dels maduixots està vinculada a la regulació de l'homeòstasi d'hormones que actuen com a inhibidores d'aquest procés, com l'IAA. Els canvis observats en la relació entre l'IAA i la seva forma conjugada subratllen la importància de considerar no només la biosíntesi, sinó també altres mecanismes com la conjugació i el catabolisme, estretament relacionats amb el balanç hormonal, per comprendre millor la interacció entre

la melatonina i les fitohormones. En canvi, en la postcollita, la resposta hormonal induïda per la melatonina està més associada a hormones implicades en mecanismes de defensa, com el SA i el JA, que no pas a la inhibició de fitohormones promotores de la senescència, com l'ABA. Cal destacar, però, que l'activació hormonal per part de la melatonina és específica tant a nivell de fruit com de teixit, ja que la resposta no és consistent en tots els fruits sota condicions experimentals similars, tenint un fort impacte la variabilitat interespecífica.

4. Es pot considerar la melatonina una nova hormona vegetal en fruits?

Donat que la melatonina és una biomolècula present des dels inicis de l'evolució, la seva funció en les plantes resulta complexa, comprenent des d'una simple acció química fins a un procés de senyalització. La melatonina té una forta activitat antioxidant que protegeix les plantes a concentracions adequades, independentment de la senyalització cel·lular. Durant l'evolució, les plantes han optimitzat el seu ús també com a senyal, millorant la regulació i la diversificació de les respostes a través de les vies de transducció de senyals (Khan et al., 2023). Tot i que aquesta diversitat de funcions és un benefici per les plantes, genera també una notable confusió entre els investigadors d'aquest àmbit, i fa que en diverses situacions fisiològiques ens preguntem si actua com a antioxidant, com a molècula reguladora o si s'ha de considerar una hormona vegetal. Les bases per considerar un compost com una hormona vegetal s'han determinat i són conegudes per la comunitat científica (Ludwig-Müller & Lüthen, 2015). Per definició, les fitohormones són petites molècules orgàniques que estan presents en baixes concentracions (nano o micromolar) en les plantes i juguen un paper clau en la senyalització i acció hormonal. El coneixement de la seva biosíntesi, degradació, conjugació, transport, receptor(s), cadena de transducció de senyal i efecte(s) fisiològic(s) és fonamental perquè una molècula sigui considerada com una possible hormona.

El fet que un nombre limitat de fitohormones pugui regular una àmplia diversitat de respostes fisiològiques es deu, en part, a la seva acció pleiotròpica. Aquesta característica també s'observa en la melatonina, a la qual se li han atribuït nombrosos efectes en la regulació de diversos aspectes relacionats amb l'estrès abiòtic, el creixement i el

desenvolupament (Wei et al., 2015; Hassan et al., 2022). En molts casos, aquests efectes es produeixen a través d'una xarxa d'interaccions amb altres fitohormones. Concretament en els fruits, la melatonina també presenta pleiotropisme, ja que modula diversos processos fisiològics d'aquest òrgan. Com s'ha demostrat al **capítol 1**, regula el procés de maduració a través de la seva distribució espaciotemporal, i també, en part, modulant gens relacionats amb les auxines. A més, com es detalla als **capítols 2** i **3**, també actua en la senescència, augmentant la resistència dels fruits mitjançant una acció directa, promovent les principals hormones de defensa i contribuint a millorar la seva aparença i qualitat nutricional. L'efecte de l'aplicació exògena d'hormones en fruits depèn de com i quan és aplicada, sent l'estadi de desenvolupament en el moment de l'aplicació un factor determinant. Per exemple, el tractament amb ABA en maduixots en estadi verd gran o durant l'acumulació de color promou la coloració vermella mitjançant l'increment de les antocianines (Li et al., 2014; Chen et al., 2016b), mentre que quan l'aplicació es realitza quan el fruit té ¾ de la superfície vermella, no es produeix aquesta acceleració (El-Mogy et al., 2019). En altres hormones com el MeJA, també s'han descrit efectes diferenciats en fruits, incrementant o reduint la fermesa depenent de si el fruit es troba en un estadi madur o immadur en el moment del tractament, respectivament (El-Mogy et al., 2019; Concha et al., 2013). Aquestes diferències van lligades al seu efecte pleiotròpic i als requeriments bioquímics de cada estadi. En aquest context, al **capítol 1** també es demostra que l'estadi del fruit en el moment de l'aplicació és un factor determinant en l'efecte de la melatonina, tal com ocorre amb altres fitohormones.

El metabolisme de la melatonina en plantes és més complex que en animals, però en els darrers anys s'han fet grans avanços en la seva caracterització (Back, 2021). En els fruits, la melatonina s'acumula en concentracions similars a les de les fitohormones, de l'ordre de 10^{-9} grams. Per exemple, els seus nivells en maduixot són relativament baixos en comparació amb l'IAA, però es troben en valors semblants als d'altres hormones com les CKs o alguns JAs (**Capítols 1** i **2**). A més, les fitohormones presenten una distribució diferencial en funció de l'estadi de maduració, el teixit o les condicions ambientals (Kim et al., 2019; Ribalta-Pizarro et al., 2021), una dinàmica espaciotemporal que també segueix la melatonina, com es mostra als **capítols 1, 3** i **4**. No obstant això, els llocs específics de biosíntesi de la melatonina encara no estan completament identificats. Tot i que la presència d'enzims en cloroplasts i mitocondris suggereix que aquests són els principals llocs de síntesi, encara manca evidència

clara sobre la localització tissular dels gens implicats i la seva organització subcel·lular precisa. En fruits, tampoc se sap amb certesa si la seva síntesi es redistribueix entre orgànuls segons l'estadi de maduració, produint-se, per exemple, als cloroplasts en estadis verds i als mitocondris en fruits madurs. A més, continua sense estar definit si les diferents vies de síntesi, que impliquen intermediaris diferents, funcionen en paral·lel o si una d'elles predomina segons l'estadi de desenvolupament del fruit o les condicions ambientals.

Pel que fa a la regulació de la melatonina, aquesta es biosintetitza i es catabolitza com altres fitohormones, i s'integra dins una xarxa reguladora interactiva que permet a les plantes optimitzar els nivells hormonals i satisfer les seves demandes fisiològiques. Hormones com les CKs, les GAs i el MeJA, poden modular els nivells de melatonina en plantes regulant l'expressió de gens relacionats amb la seva biosíntesi (Bychkov et al., 2023; Hwang & Back, 2022; Yin et al., 2023). Recentment, Gao et al. (2025) han identificat que l'element de resposta a etilè ERF5 es pot unir a la zona promotora del gen ASMT promovent així la síntesi de melatonina en llavors de raïm. Tot i aquest estudi, en fruits és encara un terreny molt inexplorat i els mecanismes específics que governen aquesta regulació continuen sense estar definits. Al mateix temps, la melatonina també té un paper en la regulació de l'homeòstasi hormonal. Al **capítol 1** es demostra com la melatonina infereix en la conjugació de l'IAA en maduixot, mentre que la bibliografia també recull evidències del seu paper en la síntesi de l'etilè, modulant l'expressió dels gens implicats en la seva biosíntesi (Verde et al., 2023). Encara es desconeix, però, com es percep la melatonina i com regula l'expressió gènica. En raïm, s'ha descrit que la melatonina indueix l'expressió d'un factor de transcripció tipus MYB, que s'uneix a la regió promotora del gen ACS, implicat en la síntesi d'etilè, afavorint així la síntesi de metabòlits secundaris (Ma et al., 2021). Recentment, en poma, s'ha descobert que la melatonina modula l'expressió de factors de transcripció REM, que interactuen amb factors de resposta a l'etilè com ERF3 i en regulen la producció modulant la transcripció de l'ACS (Li et al., 2025). A més, Gao et al. (2025) han identificat que ERF104, prèviament induït per melatonina, s'uneix en un element de resposta a melatonina al promotor del gen MYB14 en raïm, implicat en l'activació de la síntesi de polifenols. Per tant, les evidències sobre la senyalització de la melatonina en fruits, especialment vinculades a l'etilè, comencen a emergir i suposen un gran avenç en la comprensió de la seva senyalització, tot i que calen més estudis per conèixer el seu mecanisme a nivell genètic en la regulació de l'homeòstasi

hormonal i les seves interaccions. Els futurs estudis haurien d'enfocar-se en (1) els models de senyalització de la melatonina i (2) els gens que codifiquen els components clau de les vies de senyalització de fitohormones, per determinar si aquests regulen la biosíntesi de la melatonina o, al contrari, són regulats per ella, així com (3) caracteritzar factors de transcripció que estimulen la seva biosíntesi. S'ha detectat que, per exemple, en tomàquet i poma, els seus gens de síntesi estan regulats positivament per llum vermella i el factor de transcripció WRKY, respectivament (Zhang et al., 2023b; Song et al., 2023) però la senyalització i la regulació de la seva via de síntesi és encara molt desconeguda.

En la planta model *Arabidopsis*, s'ha caracteritzat un receptor de melatonina, el CAND2/PMTR1, per la transducció de senyals (Wei et al., 2018). CAND2 és una proteïna localitzada a la membrana plasmàtica i interactua amb la subunitat α de la proteïna G (GPA1). La unió de la melatonina amb aquest possible receptor, activa la producció de H_2O_2 dependent de NADPH oxidasa (RBOH), promovent l'entrada de Ca^{2+} i la sortida de K^+, ocasionant el tancament d'estomes. Tanmateix, també s'ha proposat que la senyalització de la melatonina a través de PMTR1 prevé no només la pèrdua d'aigua sinó també la invasió de bacteris (Li et al., 2022d). La seva integritat com a receptor, però, requereix més evidència, ja que Lee & Back (2020) van informar que CAND2 es localitza al citoplasma i no a la membrana a més que el mutant *cand2* d'*Arabidopsis* també indueix gens de defensa mitjançant l'activació de cascades MAPK en resposta a la melatonina, igual que el control. Tanmateix, altres investigacions han caracteritzat que homòlegs de PMTR1 en espècies com el tabac, l'alfals o el blat de moro, actuen com a proteïna de membrana i són necessaris per percebre la senyalització de la melatonina (Kong et al., 2021; Yu et al., 2021; Wang et al., 2022). No obstant, cap receptor de melatonina s'ha descrit en fruits. El fet que encara no es coneixen bé els mecanismes que vinculen els receptors PMTR1 i les cascades MAPK, ni com PMTR1 reconeix i transmet els senyals de la melatonina, fa que encara sigui un tema obert a debat i es consideri una proteïna d'unió a melatonina més que un receptor. A més, la naturalesa amfipàtica de la melatonina i la seva capacitat de difondre a través de la membrana plasmàtica també posa en dubte la necessitat de la melatonina d'unir-se a una proteïna de la membrana plasmàtica, ja que té la capacitat d'interactuar directament amb components de la cascada de senyalització com H_2O_2 o NO en la regulació de processos fisiològics.

El transport és un altre element clau en l'acció hormonal i en el cas de la melatonina, és encara molt poc entès. Tot i que s'ha descrit que per exemple la melatonina exògena al sistema radicular s'absorbeix i es transporta fins a les fulles seguint el flux transpiratori en blat de moro (Yoon et al., 2019), el transport a llarga distància de la melatonina relacionat amb la regulació del desenvolupament del fruit no s'ha identificat.

Per determinar la funció d'una molècula reguladora en un procés fisiològic, les aproximacions transgèniques són una eina fonamental. En aquest context, tecnologies d'edició genètica com la sobreexpressió, el silenciament gènic mitjançant ARN d'interferència (ARNi) o mutacions dirigides amb el sistema CRISPR/Cas9 (repeticions palindròmiques curtes agrupades i regularment interespaiades associades a la proteïna 9) permeten aprofundir en la biosíntesi de la melatonina i aclarir-ne el paper hormonal. Aquests enfocaments faciliten la modulació de l'expressió de gens implicats en la seva biosíntesi o senyalització i analitzar-ne el fenotip resultant (**Annex I**). En general, els estudis que han emprat l'edició genètica per investigar la funció de la melatonina s'han centrat principalment en el seu paper en la tolerància a l'estrès, i revelen que el mecanisme està associat en l'alteració de l'acumulació i senyalització d'altres hormones com l'ABA o l'IAA (Gao et al., 2024; Miao et al., 2024). En fruits, la informació mitjançant línies mutants o sobreexpressades és encara molt limitada. Els pocs treballs que han sobreexpressat gens de la seva biosíntesi en plantes amb fruit s'han dut a terme en tomaquera, però no han analitzat l'impacte d'aquesta alteració en el desenvolupament, la maduració o la qualitat del fruit (Yan et al., 2019; Liu et al., 2019b). L'estudi de He et al. (2023) és el primer que demostra que el silenciament de l'enzim COMT en tomàquet impacta significativament en el creixement i desenvolupament del fruit, reduint la biomassa, la producció de llavors i el quallat. A més, el seguiment dels diferents estadis del desenvolupament va revelar que la disminució de melatonina alterava la relació sucres/acidesa, afectava la síntesi de carotenoides i, per tant, l'inici de l'acumulació del color. Així, aquest estudi posa de manifest que uns nivells adequats de melatonina són essencials per al correcte desenvolupament del fruit i la seva qualitat, tot i que no mostren informació de l'efecte sobre les fitohormones i cal esclarir els mecanismes reguladors subjacents. Ara bé, cal considerar que l'obtenció de fruits modificats genèticament per estudiar el paper regulador de la melatonina en la maduració presenta grans reptes. D'una banda, generar mutants *knock-out* per a la melatonina és especialment

complex, ja que en cada etapa enzimàtica de la seva biosíntesi intervenen múltiples isògens (Ikram et al., 2024) i en la majoria de fruits no es coneix específicament la via completa de biosíntesi. D'altra banda, models d'estudi com el maduixot cultivat, amb un genoma octaploide i altament heterozigot, dificulten la identificació de gens diana i en compliquen l'eficiència. A més, els quatre subgenomes homòlegs del maduixot no estan clarament separats, ja que han experimentat nombrosos intercanvis genètics, cosa que afegeix una capa addicional de complexitat genòmica (Whitaker et al., 2020). En canvi, la maduixa diploide representa un model més senzill, ja que el seu genoma està completament seqüenciat (a diferència del maduixot, que encara no compta amb una seqüenciació i anotació completes) i la seva transformació és més accessible. Tot i això, els seus fruits són fràgils i difereixen de les varietats comercials en mida, fermesa, qualitat postcollita i metabolisme (Carvalho et al., 2016), fet que en limita la transferibilitat dels resultats al fruit consumit comercialment.

En resum, els resultats dels **capítols 1, 2** i **3**, juntament amb els diversos estudis citats, recolzen el paper regulador de la melatonina en la maduració dels fruits, independentment de la presència d'un receptor específic. Tot i compartir característiques amb les fitohormones, com la concentració i patró d'acumulació, la biosíntesi, el catabolisme i un efecte pleiotròpic, encara és imprescindible identificar un receptor específic de melatonina en fruits per poder afirmar la seva acció hormonal. A més, és fonamental caracteritzar el seu mecanisme de senyalització, incloent-hi la identificació dels components de la cascada de senyalització, l'activació de factors de transcripció i el mode d'acció en la regulació de fitohormones. També és crucial caracteritzar específicament la biosíntesi de la melatonina en fruits, així com els factors que la regulen i la seva regulació transcripcional associada. Aquest coneixement facilitarà l'obtenció de fruits, com el maduixot, amb una síntesi de melatonina enriquida o inhibida, fet que serà determinant per desxifrar els mecanismes moleculars implicats i considerar-la, o no, una nova hormona vegetal.

5. La melatonina com a estratègia eficaç i sostenible per prolongar la vida útil dels fruits

El gran valor comercial dels fruits, tant pel seu sabor com pel seu alt contingut en compostos fitoquímics beneficiosos per a la salut, ha impulsat la recerca de diverses estratègies per prolongar-ne la conservació. Aquesta necessitat és encara més evident en aquells fruits de textura tova i especialment vulnerables a la deshidratació i la descomposició. S'han provat i implementat diverses tècniques postcollita per preservar la qualitat i reduir les pèrdues, però molts dels tractaments utilitzats no són viables a causa del seu alt cost o de la baixa acceptació per part dels consumidors. Així mateix, l'ús de productes químics sovint està restringit per motius mediambientals o de seguretat alimentària, fet que fa necessari investigar alternatives sostenibles per reduir la senescència dels fruits (El-Mogy et al., 2019). En aquest sentit, els **capítols 2** i **3** han establert l'impacte positiu de l'aplicació de melatonina en la postcollita de fruits altament peribles com els fruits vermells o els de pinyol com la pruna, una alternativa respectuosa amb el medi ambient i la salut humana. Com a molècula amfipàtica, presenta una excel·lent capacitat d'absorció i penetració al fruit, fet que es demostra amb l'increment de melatonina després de l'aplicació en els fruits. A més, exerceix una funció inhibidora de la senescència, que s'ha analitzat en profunditat a la **secció 2**. Actualment, tot i que hi ha un gran interès per l'ús de la melatonina en la conservació i millora de qualitat dels productes hortícoles, les investigacions sobre la seva aplicació encara es troben en una fase inicial, amb diverses limitacions que cal superar per consolidar-ne l'eficàcia. Un dels principals desafiaments que cal aclarir és la falta d'estandardització en els tractaments amb melatonina, la qual cosa provoca que els resultats de les recerques siguin sovint contradictoris, depenent del tipus de fruit o varietat estudiada, així com per les àmplies variacions en les concentracions aplicades en cada estudi (Ze et al., 2021; Aghdam & Arnao, 2024). A més, la melatonina en postcollita s'acostuma a analitzar de manera aïllada en diferents models, fet que en dificulta l'aplicació generalitzada en la producció industrial i la postcollita de cultius hortícoles. Aquest aspecte clau s'aborda al **capítol 2** amb l'objectiu d'estandarditzar i oferir una visió integradora de l'aplicació de la melatonina. Per a això, es van tractar exògenament quatre fruits vermells botànicament diferents, amb una vida útil variable però una elevada i similar susceptibilitat al deteriorament, utilitzant la mateixa dosi, mètode d'aplicació i condicions experimentals

(temperatura ambient). El retard en la sobremaduració de tots quatre fruits (maduixot, gerd, nabiu i cirera), a través de la disminució del deteriorament, la reducció de la seva severitat, la pèrdua de pes i el manteniment de la fermesa durant l'emmagatzematge, demostra que l'aplicació exògena de melatonina per esprai a una dosi de 100 µM té una efectivitat consistent i uniforme per aplicar-se en la postcollita dels fruits vermells (**Figura 12**). L'efecte de la melatonina reduint el percentatge de deteriorament en fruits vermells té una gran efectivitat a l'inici de l'emmagatzematge, on el minimitza fins a un 50%, i en alguns casos, com el maduixot i el gerd, fins i tot un 60% (**Capítol 2**). La collita en el punt òptim de maduració comercial és crucial per assegurar la qualitat dels fruits vermells, però està estretament relacionada amb l'acceleració del seu deteriorament per la seva alta susceptibilitat. Aquest deteriorament inclou lesions, símptomes de sobremaduració i creixement fúngic visible. Per tant, retardar aquesta immediata disminució de qualitat és fonamental per evitar pèrdues ràpides dels fruits, facilitar la seva comercialització i allargar la finestra de consum. Tot i aquest potent efecte inicial en la reducció del percentatge de deteriorament, és cert que la seva eficàcia es debilita a mesura que avancen els dies d'emmagatzematge en la majoria dels fruits estudiats, excepte en el cas dels nabius on al final del període encara presenten una reducció del 30%. No obstant això, aquesta pèrdua d'eficàcia no sembla estar relacionada amb una disminució dels nivells de melatonina durant l'emmagatzematge post-aplicació. Tanmateix, tot i que la diferència en el percentatge de deteriorament és major a l'inici, el manteniment d'alguns paràmetres específics, com la fermesa, és inclús més notable cap al final de l'emmagatzematge en gerds, maduixots i cireres. El mateix passa amb la qualitat nutricional dels fruits, ja que l'increment de fenols es fa evident a l'etapa més avançada de la sobremaduració en els nabius (**Capítol 2**), mentre que en les prunes es detecta des de la meitat del període postcollita i fins al final de la seva vida útil (**Capítol 3**).

Un aspecte que ha estat poc explorat en els últims anys de recerca sobre la melatonina en postcollita és el seu mecanisme fisiològic. Es desconeix específicament com interactua amb altres reguladors de la senescència dels fruits. Tot i observar-se una resposta bastant uniforme de la melatonina en la reducció del deteriorament en els fruits vermells (**Capítol 2**), el mecanisme d'acció relacionat amb la regulació hormonal, com s'ha mencionat a la **secció 3**, és específic de cada fruit i no presenta una resposta homogènia. Per tant,

comprendre millor el mecanisme regulador de la melatonina en la postcollita podria ser fonamental per optimitzar la seva aplicació i mantenir els seus efectes en el temps (**Figura 12**). A més, investigar els receptors i les vies de senyalització de la melatonina permetria aprofundir en com aquesta molècula interactua amb els processos fisiològics dels fruits i les variacions en funció del fruit, la qual cosa contribuiria a millorar i prolongar l'eficàcia de la melatonina i a dirigir la seva acció de manera més precisa. Tanmateix, altres alternatives a contemplar inclouen l'ús de nanotransportadors, ja que tot i que la melatonina és una molècula amfipàtica, s'ha demostrat que l'ús de nanopartícules de quitosà pot accelerar la seva captació i millorar la seva estabilitat, prevenint l'oxidació prematura (Mukherjee et al., 2024). Tot i això, aquest àmbit encara està en etapes molt primerenques en el maneig postcollita i els possibles impactes ambientals i els riscos per a la salut humana s'haurien de determinar. Atès que la melatonina està implicada en diverses vies de senyalització, el tractament combinat amb altres molècules de senyalització com l'òxid nítric també es podria considerar per millorar-ne l'eficiència, tot i que encara cal aprofundir en aquestes possibles sinergies perquè estudis recents com el de Cheng et al. (2025) demostren que la combinació de melatonina i SA no presenta efectes additius.

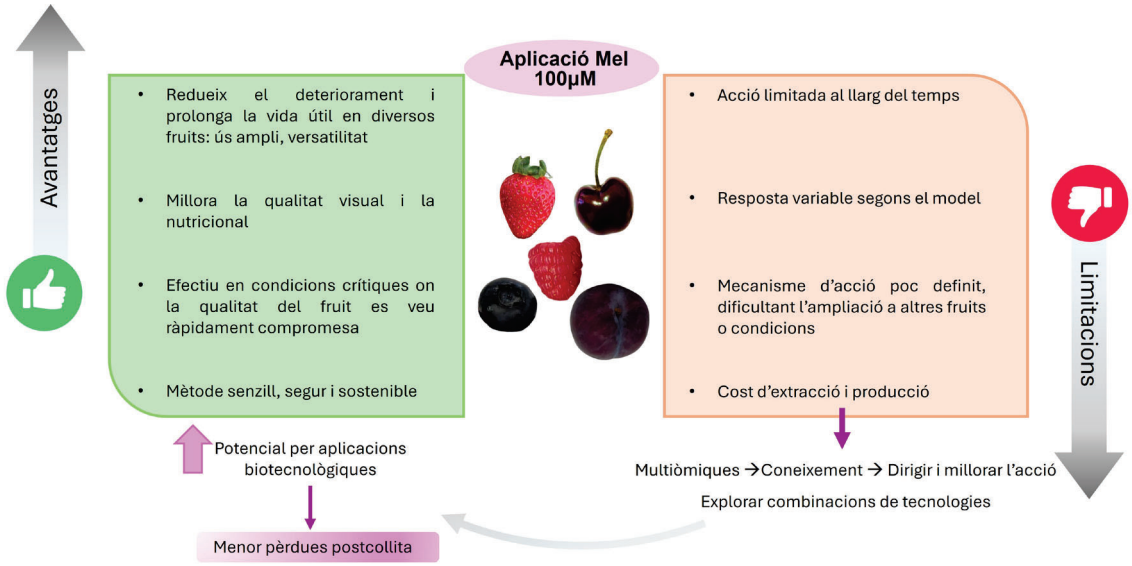


Figura 12. Avantatges i limitacions de l'ús de melatonina com a principi actiu en solucions postcollita per prolongar la vida útil i mantenir la qualitat dels fruits.

Per altra banda, en poques ocasions els estudis reporten com els tractaments afecten els nivells endògens de melatonina i la seva persistència en la pell o polpa dels fruits. Les anàlisis de melatonina dels **capítols 2** i **3** demostren que els alts continguts de melatonina al fruit després del tractament exogen es prolonguen al llarg de la postcollita, tot i ser una molècula sensible a la llum. En relació amb això, un dels avantatges més importants de la melatonina com a tractament postcollita, en comparació amb altres reguladors de creixement (Le et al., 2020), és que s'ha demostrat que no té efectes nocius pel medi ambient i que la seva toxicitat és molt baixa per a la salut humana, fins i tot a dosis extremes d'1 g/dia (Galano & Reiter, 2018; Andersen et al., 2016). Actualment, no existeix una regulació específica per a l'ús de la melatonina en solucions postcollita, però està regulada com a complement alimentari, i l'AESAN (Agència Espanyola de Seguretat Alimentària i Nutrició) ha informat que, igual que en altres països de la Unió Europea, a Espanya la melatonina es pot comercialitzar com a complement alimentari en dosis de fins a 2 mg (AESAN, 2013). En aquest context, el contingut de melatonina en els fruits és molt inferior a aquest límit. Per exemple, en el cas del maduixot, després de l'aplicació exògena de melatonina a 100 μM, el fruit adquireix una concentració de 2469 ng/g en pes sec (**Capítol 2**), la qual cosa es tradueix aproximadament en 200 ng/g en pes fresc. Tenint en compte el pes mitjà del maduixot (≈ 15 g), els nivells de melatonina després de l'aplicació són 667 vegades inferiors als 2 mg establerts com a límit per als complements alimentaris, de manera que per assolir aquesta dosi seria necessari consumir 10 kg de maduixots, una quantitat difícil d'aconseguir en condicions normals de consum. Així doncs, els baixos nivells endògens de melatonina presents als fruits, molt per sota dels establerts per als complements alimentaris, juntament amb els beneficis observats en la conservació de la qualitat dels fruits, fan que la seva aplicació en el sector hortícola sigui una opció molt atractiva i segura, sense implicar efectes en la salut humana associats a un contingut elevat de melatonina.

Per avaluar l'impacte de la melatonina en la conservació postcollita també és fonamental considerar situacions d'emmagatzematge que reflecteixin les condicions reals de la cadena de distribució. Tot i que l'emmagatzematge en fred és la condició generalment utilitzada pels estudis de melatonina (Miranda et al., 2020; Qu et al., 2022), cal considerar que no sempre és viable mantenir els fruits a baixes temperatures, especialment pel cost i

pels danys que el fred pot provocar en molts d'ells. En situacions com el transport des dels camps fins a les plantes de processament, en els punts de distribució als magatzems, en diverses etapes del processament tecnològic, o fins i tot un cop en el punt de venda, els fruits es mantenen sovint a temperatura ambient (Shoji et al., 2022). De fet, l'emmagatzematge en condicions no òptimes és una de les causes més comunes de pèrdues postcollita, tant en qualitat com en quantitat. Per tant, mantenir el valor comercial i prevenir el deteriorament de la qualitat del fruit és un dels principals desafiaments en la cadena de subministrament sota aquestes condicions crítiques que comprometen la seva integritat. Per aquest motiu, els efectes i mecanismes de la melatonina descrits als **capítols 2** i **3**, centrats en fruits vermells i pruna a temperatura ambient, proporcionen un enfocament diferencial respecte als descrits a la bibliografia, ja que la seva regulació pot diferir segons les condicions d'emmagatzematge.

D'altra banda, en el **capítol 2** es presenta el triptòfan com una solució econòmica per al tractament postcollita, però els resultats obtinguts són inconsistents. El seu efecte depèn de la capacitat del fruit per metabolitzar la melatonina, un procés que no es produeix de manera homogènia, sinó que varia en funció de les necessitats fisiològiques i metabòliques específiques de cada fruit. Així, tot i que el tractament amb triptòfan no comporta riscos associats a residus ni toxicitat, ja que no es produeix un augment dels seus nivells en el fruit després de l'aplicació (probablement perquè és precursor de diverses molècules), la seva eficàcia com a tractament postcollita és inferior a la de la melatonina i està limitada al tipus de fruit i els seus requeriments metabòlics.

Per tant, el fet que la melatonina sigui un compost amb propietats inhibidores de la sobremaduració que es troba de manera natural en els fruits, juntament amb la seva baixa toxicitat, l'absència d'acumulació de residus perjudicials i el seu caràcter respectuós amb el medi ambient, la converteixen en una alternativa viable per a l'aplicació en postcollita i la millora de la qualitat dels fruits. La seva aplicació a través d'esprai a 100 µM ha demostrat un efecte ampli i consistent en el manteniment de la qualitat i la prolongació de la vida útil del maduixot, gerd, nabiu i cirera, especialment en els primers dies després de la collita, així com en la millora nutricional de fruita de pinyol com la pruna, destacant el seu potencial pel sector postcollita. No obstant això, cal aprofundir en el seu mecanisme fisiològic i explorar possibles combinacions amb altres tecnologies o molècules per optimitzar i dirigir millor la seva acció, amb l'objectiu d'estendre la seva eficàcia al llarg del temps (**Figura 12**).

6. Limitacions de l'aplicació de la melatonina com a bioestimulant en l'agricultura

El maduixot Albion és una varietat de dia neutre reconeguda per la seva excel·lent qualitat destacant especialment per la fermesa i el color brillant dels seus fruits (Taghavi et al., 2016). Tot i això, el maduixot és un cultiu molt sensible a la presència constant de patògens, així com a l'estrès causat per les altes temperatures i humitat (Otero et al., 2023). En condicions de temperatures superiors als 30 °C, el rendiment del cultiu disminueix i la qualitat dels fruits es veu compromesa (Muneer et al., 2017; Taghavi et al., 2016). En aquest sentit, les condicions ambientals en el moment de la collita són un factor determinant en la qualitat i el valor comercial dels fruits. Estudis anteriors han descrit que paràmetres de qualitat com la relació sucres/àcids o compostos antioxidants varien segons el mes de l'any en què es fa la collita i s'observa, per exemple, una qualitat inferior en les collites extra primerenques (Winardiantika et al., 2015; Akhatou & Fernández-Recamales, 2014). De forma similar, els maduixots Albion collits a un camp de producció del Maresme (Catalunya, Espanya), durant la tercera setmana de juliol (final de la temporada productiva) en condicions ambientals desfavorables per les fortes onades de calor (amb temperatures al voltant dels 40 °C), tenen una reducció del 20% en la hidratació i la capacitat antioxidant total del fruit, en comparació a la collita feta al juny (**Capítol 4** i **Figura 13**). Aquests paràmetres estan directament implicats en l'apreciació dels fruits per part dels consumidors i en la determinació de la vida útil durant la postcollita. A més, es van relacionar també amb una disminució del contingut de molècules clau en la defensa enfront estressos abiòtics i l'acumulació d'antioxidants, incloent-hi el JA i la melatonina (**Capítol 4**).

Generalment, en el cultiu del maduixot s'ha prioritzat la productivitat sovint en detriment de la qualitat organolèptica i nutricional del fruit. En aquest context, els maduixots han estat assenyalats com un dels cultius amb majors requeriments en l'ús de productes fitosanitaris per satisfer les demandes de producció, a causa de la seva sensibilitat ambiental (Otero et al., 2023). Es coneix bé que un ús abusiu d'aquestes substàncies implica una presència de residus químics en els fruits que pot afectar a la salut humana (Shao et al., 2021). En aquest context, l'aparició dels bioestimulants ofereix solucions innovadores per la seguretat agrícola. Per definició, la nova regulació de la Unió Europea (EU) 2019/1009 (EU, 2019b) defineix els bioestimulants com substàncies, microorganismes o extractes que

estimulen els processos de nutrició de les plantes independentment del seu contingut de nutrients per millorar l'absorció o l'eficiència de nutrients, la tolerància a l'estrès abiòtic o la qualitat i rendiment dels cultius, sense imposar cap risc ecològic. La creixent acumulació d'evidències sobre el rol regulador de la melatonina ha confirmat el seu potencial com a bioestimulant per combatre els estressos abiòtics i biòtics en diverses condicions agroclimàtiques, especialment a través de les seves aplicacions exògenes (Jindal et al., 2024). Les evidències del **capítol 1**, que mostren el paper regulador de la melatonina en la maduració dels fruits, juntament amb els resultats dels **capítols 2 i 3** sobre la seva influència en la qualitat, suggereixen que l'ús de la melatonina com a bioestimulant podria ser una estratègia efectiva per millorar la qualitat organolèptica i nutricional del maduixot Albion cultivat al Maresme al final de la temporada productiva. Estudis previs també han demostrat el paper bioestimulant de la melatonina en el cultiu del maduixot a una dosi de 100 µM. Per exemple, aplicacions foliars en condicions de temperatures elevades han millorat la defensa antioxidant de les fulles (Manafi et al., 2022). A més, aquesta mateixa dosi ha incrementat el rendiment del cultiu i la qualitat dels fruits, augmentant el contingut de sucres, acidesa i fenols totals en situacions d'estrès salí (Zahedi et al., 2020). En el **capítol 4**, l'aplicació exògena de melatonina a 100 µM en maduixeres al final de la temporada provoca un increment de fins a 7 vegades en el contingut de melatonina dels fruits el dia de la collita, 21 dies després del tractament, cosa que indica una bona absorció i estabilitat de la melatonina. No obstant això, contrari al que s'esperava, aquest augment no es tradueix en canvis en la producció ni en diferències en les propietats organolèptiques o en el perfil nutricional complet (**Figura 13**).

La majoria d'estudis sobre la funció bioestimulant de la melatonina descrits a la literatura s'han dut a terme en entorns controlats, com ara hivernacles. En canvi, la nostra aplicació s'ha realitzat en condicions reals de camp, on la variabilitat és inherent al sistema de producció. En aquest context, el **capítol 4** posa de manifest la gran heterogeneïtat present en un túnel de producció. Factors microclimàtics, com les fluctuacions en la irradiació lumínica segons la posició de les plantes dins del túnel, influeixen en el contingut d'antioxidants. A més, la variabilitat interindividual de les maduixeres, condicionada per aspectes com la hidratació del fruit i els nivells de fitohormones, afecta la concentració de nutrients en els fruits. En alguns casos, nutrients com el sodi, el ferro o el calci presenten

coeficients de variació interindividual fins i tot superiors al 100% (**Capítol 4**). La limitació de la gran variabilitat associada al sistema de cultiu en l'eficàcia de la melatonina també es reflecteix en estudis com el d'Okatan et al. (2022), on s'identifica que la millora del rendiment i la qualitat del maduixot pel tractament de melatonina és molt més pronunciada en el cultiu d'hivernacle en comparació amb el cultiu en camp obert. Recentment, Ruiz-Aracil et al. (2024) també han descrit que l'aplicació de melatonina en camps de cireres millora paràmetres de qualitat com la fermesa, sucres o acidesa, però depenent de la temporada. Per tant, els aspectes intrínsecs del medi ambient i de la mateixa planta interfereixen en l'efecte bioestimulant de la melatonina (**Figura 13**).

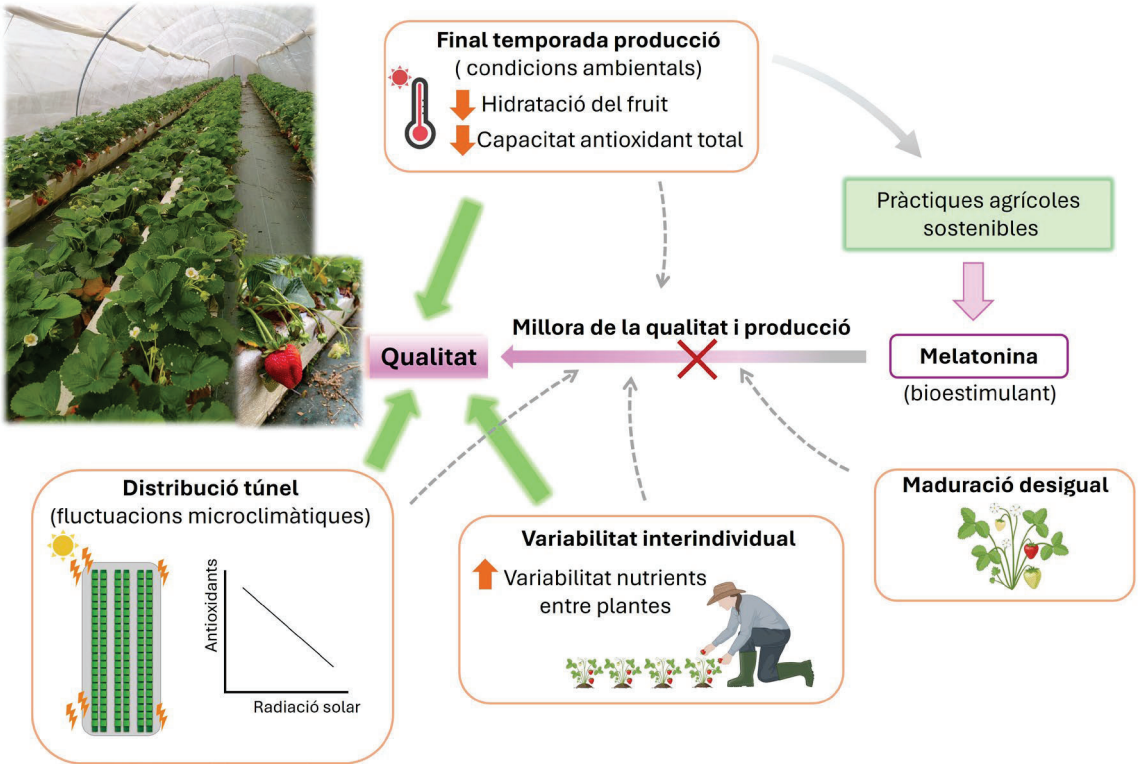


Figura 13. Factors, tant ambientals com intrínsecs del fruit, que determinen la qualitat final dels maduixots en el moment de la collita cultivats en un túnel de producció i que se suggereix que interfereixen també en el potencial efecte positiu de la melatonina en la millora de la qualitat.

Per altra banda, el cultiu de maduixot es caracteritza per una maduració no homogènia, amb fruits en diferents estadis de desenvolupament dins de la mateixa planta. Aquesta variabilitat en l'estadi de maduració pot influir significativament en la resposta a la melatonina, ja que al **capítol 1** es demostra que l'efecte de la melatonina depèn en gran

mesura de l'estadi de desenvolupament en què es troben els fruits en el moment de l'aplicació. Per tant, aquest factor pot ser limitant a l'hora de garantir una resposta homogènia en condicions de camp a causa de la inevitable maduració no uniforme del cultiu. En altres fruits com la pruna, l'aplicació de melatonina com a bioestimulant en la precollita millorava la qualitat del fruit si s'aplicava en tres estadis clau de desenvolupament, però no si es feia en només dos dels estadis, concloent també que el nombre de vegades i en quin estadi es fa l'aplicació és clau per garantir la seva efectivitat (Cortés-Montaña et al., 2023).

En resum, tot i que la bibliografia recull nombrosos estudis que evidencien l'ús de la melatonina com a bioestimulant per a la millora de cultius hortícoles, la seva aplicació en condicions de camp en cultius heterogenis com el maduixot presenta diverses limitacions. La melatonina estimula i regula processos fisiològics clau en la maduració i qualitat dels fruits, tal com s'ha demostrat al llarg d'aquesta tesi. No obstant això, potenciar aquest efecte a través d'aplicacions exògenes en condicions no controlades pot ser ineficaç degut a la variabilitat inherent al sistema de producció. Factors com el moment de la collita, la variabilitat genètica, la variabilitat en les condicions microclimàtiques i ambientals de cada temporada, així com l'estadi de desenvolupament dels fruits en el moment de l'aplicació o el nombre d'aplicacions, poden condicionar l'efectivitat del bioestimulant (**Figura 13**). Aquestes consideracions esdevenen encara més rellevants en cultius amb una elevada heterogeneïtat intrínseca, com és el cas del maduixot. Per tant, l'ús de la melatonina com a bioestimulant podria ser més efectiu en cultius amb una maduració més homogènia o en aquells que es desenvolupen en condicions més controlades, com els cultius en hivernacle. A més, el desconeixement actual sobre els mecanismes de regulació de la melatonina en plantes i el seu putatiu receptor també dificulta la seva aplicació en sistemes de cultiu a gran escala. El coneixement de la seva acció a través d'un receptor ben caracteritzat permetria millorar la seva resposta en aplicacions exògenes. En el futur, el desenvolupament de varietats de cultius amb una major producció de melatonina o una senyalització modificada de melatonina sens dubte donarà lloc a nous paradigmes per a l'aplicació de la melatonina en cultius hortícoles.

CONCLUSIONS



CONCLUSIONS

i. Durant el creixement i la maduració del maduixot, la melatonina s'acumula de manera diferencial segons l'òrgan i l'estadi de desenvolupament, exercint un rol inhibidor de la maduració. Així, en l'aqueni, el veritable fruit, els nivells més elevats de melatonina es produeixen durant el quallat i disminueixen abans del canvi de color. En canvi, al receptacle, l'increment més significatiu es produeix en l'estadi madur, on s'associa a una funció protectora contra l'estrès oxidatiu.

ii. L'efecte de l'aplicació de melatonina exògena està condicionat per l'estadi del desenvolupament del fruit en el moment de l'aplicació, accelerant la coloració en el quallat i endarrerint-la en estadis avançats com el desverdiment o just a l'inici de la maduració.

iii. L'efecte inhibidor de la melatonina després de l'aplicació en l'estadi de desverdiment es deu, en part, a la modulació del gens relacionats amb les auxines, incrementant la disponibilitat de la forma lliure de l'IAA respecte a la conjugada.

iv. La melatonina endògena té una funció inhibidora de la sobremaduració. Durant la postcollita, es produeix una disminució general dels nivells de melatonina en els fruits, a la vegada que es veu limitada la seva qualitat.

v. La qualitat dels fruits vermells es veu compromesa ràpidament just després de la collita, i el tractament amb melatonina per esprai a 100 µM té un efecte d'ampli espectre en la prolongació de la vida útil i el manteniment de la seva qualitat a temperatura ambient.

vi. L'aplicació de triptòfan, tot i ser una opció molt econòmica, és menys eficaç que la melatonina en la reducció del deteriorament dels fruits vermells i el seu efecte positiu només ocorre quan és metabolitzat a melatonina, un procés que varia depenent del fruit.

vii. En la pruna, la melatonina exògena actua de manera diferencial segons el teixit del fruit per endarrerir la sobremaduració. Incrementa el contingut d'antioxidants a la polpa, però no a la pell, millorant la protecció i la qualitat nutricional del fruit alhora que evita un enfosquiment no desitjat a la capa externa.

viii. Durant la postcollita, l'aplicació exògena de melatonina desencadena una regulació hormonal per endarrerir la sobremaduració, incrementant el contingut d'hormones de defensa, com el SA en el nabiu i l'OPDA i el JA en la pruna, els quals semblen ser responsables dels canvis associats a l'augment de compostos fenòlics en aquests fruits. Aquesta resposta hormonal, però, és específica de cada fruit i també del teixit.

ix. L'eficàcia de la melatonina com a bioestimulant per millorar la qualitat dels fruits es veu limitada en cultius heterogenis com el maduixot en condicions de camp. Se suggereix que la gran variabilitat en el contingut d'antioxidants i nutrients dels fruits, derivada de les fluctuacions microclimàtiques i de la variabilitat interindividual, emmascara el seu potencial efecte. Cal considerar aquests elements en el desenvolupament de nous bioestimulants que incloguin la melatonina com a ingredient actiu.

CONCLUSIONS

i. During strawberry growth and ripening, melatonin differentially accumulates subject to the organ and developmental stage, playing an inhibitory role in ripening. In the achenes, the true fruit, the highest levels of melatonin are produced during fruit set and decrease before color change. In contrast, in the receptacle, the most significant increase occurs at the ripe stage, where melatonin is associated with a protective role against oxidative stress.

ii. The effect of exogenous melatonin application is conditioned by the developmental stage of the fruit at the time of application, accelerating color acquisition at fruit set and delaying it in advanced stages such as degreening or initial red.

iii. The inhibitory effect of melatonin after application at degreening stage is partly due to modulation of auxin-related genes, increasing the availability of the IAA free form in relation to the conjugated form.

iv. Endogenous melatonin plays an inhibitory role in overripening. During postharvest, melatonin levels decrease, coinciding with a reduction in their quality.

v. The quality of red fruits is quickly compromised after harvest, and treatment with 100 µM melatonin spray shows a broad-spectrum effect in prolonging shelf life and maintaining fruit quality at room temperature.

vi. The application of tryptophan, although being an economic option, it is less effective than melatonin in reducing red fruit deterioration, and its positive effects only occur when it is metabolized into melatonin, a process that varies depending on the fruit.

vii. In plum, exogenous melatonin differentially acts, subject to the fruit tissue to delay overripening. It increases antioxidant contents in the pulp but not in the skin, improving protection and nutritional quality while preventing undesirable darkening of the outer layer.

viii. In postharvest conditions, exogenous melatonin triggers hormonal regulation to delay overripening, increasing the levels of defense hormones, such as SA in blueberries and OPDA and JA in plums. These phytohormones appear to be responsible for the associated increase in phenolic compounds in these fruits. However, this hormonal response is fruit- and tissue-specific.

ix. The effectiveness of melatonin as a biostimulant to improve fruit quality is limited in heterogeneous crops like strawberries under field conditions. The significant variability in antioxidant and nutrient content, as a result from microclimatic fluctuations and interindividual variability masks its potential effect. These factors should be considered when developing new biostimulants that include melatonin as an active ingredient.

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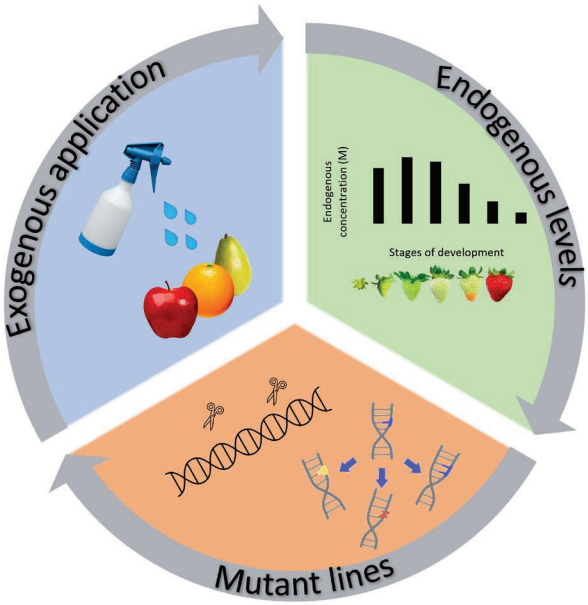
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ANNEXOS



ANNEX I:

Aproximacions experimentals en l'estudi de biomolècules actives que modulen la maduració del fruit: La melatonina com a cas d'estudi



Annex I. Experimental approaches in studying active biomolecules modulating fruit ripening: Melatonin as a case study

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RESUM

Les fitohormones són petites molècules orgàniques naturals que es troben en baixes concentracions en les plantes i realitzen funcions essencials en els processos de creixement i desenvolupament, des de la iniciació d'òrgans fins a la senescència, inclosa la maduració dels fruits. Aquestes molècules reguladores s'estudien mitjançant diferents aproximacions experimentals, com les aplicacions exògenes, l'avaluació de nivells endògens i/o l'obtenció de línies genèticament modificades. En aquest treball es discuteixen els avantatges i les limitacions de les aproximacions experimentals utilitzades actualment per l'estudi de biomolècules actives que modulen la maduració dels fruits, centrant-se en la melatonina. Tot i que la melatonina està involucrada en la maduració dels fruits en diversos models de cultius fruiters, els coneixements actuals estan afectats per les diferents aproximacions experimentals utilitzades, que han donat resultats diferents i, a vegades, fins i tot contradictoris. Els mètodes d'aplicació i les dosis utilitzades han produït resultats diferents en estudis basats en aplicacions exògenes, mentre que diferents mètodes de mesura i formes d'expressar els resultats expliquen la major part de la variabilitat en els estudis que utilitzen anàlisis correlatives. A més, els estudis sobre cultius genèticament modificats s'han centrat únicament en plantes de tomàquet (*Solanum lycopersicum* L.). No obstant això, les metodologies TILLING i CRISPR s'estan convertint en eines essencials per complementar els resultats de les aproximacions experimentals descrites anteriorment. Això no només ajudarà a la comunitat científica a entendre millor el paper de la melatonina en la modulació de la maduració dels fruits, sinó que també ajudarà a desenvolupar avenços tecnològics per millorar el rendiment i la qualitat dels fruits en els principals cultius. La combinació de diverses aproximacions experimentals conduirà, sens dubte, a una comprensió completa de la funció de la melatonina en la maduració dels fruits en un futur pròxim, de manera que aquest coneixement es pugui transferir de manera efectiva al camp.

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Update

Experimental approaches in studying active biomolecules modulating fruit ripening: Melatonin as a case study

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Abstract

Phytohormones are naturally occurring small organic molecules found at low concentrations in plants. They perform essential functions in growth and developmental processes, from organ initiation to senescence, including fruit ripening. These regulatory molecules are studied using different experimental approaches, such as performing exogenous applications, evaluating endogenous levels, and/or obtaining genetically modified lines. Here, we discuss the advantages and limitations of current experimental approaches used to study active biomolecules modulating fruit ripening, focusing on melatonin. Although melatonin has been implicated in fruit ripening in several model fruit crops, current knowledge is affected by the different experimental approaches used, which have given different and sometimes even contradictory results. The methods of application and the doses used have produced different results in studies based on exogenous applications, while different measurement methods and ways of expressing results explain most of the variability in studies using correlative analyses. Furthermore, studies on genetically modified crops have focused on tomato (*Solanum lycopersicum* L.) plants only. However, TILLING and CRISPR methodologies are becoming essential tools to complement the results from the experimental approaches described above. This will not only help the scientific community better understand the role of melatonin in modulating fruit ripening, but it will also help develop technological advances to improve fruit yield and quality in major crops. The combination of various experimental approaches will undoubtedly lead to a complete understanding of the function of melatonin in fruit ripening in the near future, so that this knowledge can be effectively transferred to the field.

Introduction

Fruit ripening entails multiple coordinated physiological, biochemical, and organoleptic modifications that affect the color, flavor, aroma, and texture of ripe fruits (Giovannoni et al. 2017). Metabolic changes such as starch and cell-wall degradation, chlorophyll loss, as well as the accumulation of free sugars, carotenoids, anthocyanins, and aromas provide the main desirable attributes of fruits (Prasanna et al. 2007). Ripening constitutes a complex physiological process that is highly regulated by both environmental and endogenous signals. Although different biomolecules are involved in modulating ripening, **phytohormones** (Box 1) and their interactive network represent one of the main endogenous regulators determining the final fruit quality. Phytohormones are small organic molecules that are present at low concentrations in plants and play a key role in signaling. Therefore, the presence of a receptor triggering a cellular signaling cascade and **hormonal action** are required, together with the short- and/or long-distance transport of the hormones (Davies 2004; Santner et al. 2009). Furthermore, it is not the action

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Annexos

of a single phytohormone, but the **crosstalk** between multiple hormones that regulates a physiological process, making this event even more complex (Fenn and Giovanoni 2021).

Melatonin (N-acetyl-5-methoxytryptamine) is a multifunctional and pleiotropic indolamine synthesized from tryptophan, with serotonin an essential metabolic intermediary (Back et al. 2016). Since the first report of the presence of melatonin in plants almost three decades ago (Dubbels et al. 1995), much progress has been made in improving our knowledge on its physiological functions in plants. Its presence has been reported in more than 50 plant species (Nawaz et al. 2016), and its involvement in fruit development and ripening has been proposed in several plant models (Arnao and Hernández-Ruiz 2019; Wang et al. 2020b; Ze et al. 2021). However, there is still apparently inconclusive information about its function in the regulation of fruit ripening due to several different and/or controversial results varying according to species, tissue, and method of study.

To unravel and gain a deeper insight into the function of various regulatory biomolecules (Box 2), including melatonin, studies have been performed using different experimental approaches (Fig. 1). First, exogenous applications of a biomolecule can determine if it has a promoting or inhibitory role in a given physiological process. However, this response is dose-dependent and may differ depending on the method of application and the developmental stage at which the biomolecule is applied, resulting in different phenotypes and, consequently, different interpretations of its role. Second, an evaluation of the endogenous concentration of the molecule under study is highly needed to quantify organ and tissue levels, even at different points of time throughout the process, to establish possible **causation**. Finally, generating specific transgenic lines by genetic engineering based on the silencing or overexpression of genes related to the biosynthesis or signal transduction of the molecule under study is required to determine the degree of relevance of the molecule in the process. The use of only one approach could lead to inconclusive results, making it difficult to draw critical conclusions and transfer them to the field.

Here, we discuss different experimental methods determining the function of active molecules in modulating fruit ripening, considering their advantages and limitations, and focusing on the example of melatonin. In addition to describing the experimental approaches, we will discuss the need to include, or at least take into consideration, all the information from the three experimental approaches to achieve a complete and better understanding of the physiological importance of melatonin. An integration of all this knowledge will be essential in boosting technological applications in agrifood biotechnology and agronomy to improve fruit yield and quality.

Exogenous applications: spray and pray

Plant growth regulators have been classically used for a plethora of biotechnological applications since their discovery. More recently, they have been replaced by plant **biostimulants** in modern biotechnology (Rouphael and Colla 2020; Giordano et al. 2022). Melatonin is a good example of the worldwide popularity of biostimulants. However, since its use, there has been a focus on "what melatonin does" instead of "how it does it", overlooking its mechanisms of action. Nowadays, important advances are being made in discovering the physiological role of melatonin in plants, which will undoubtedly be used to achieve a more suitable use of this biostimulant (Yakhin et al. 2017; Sudiro et al. 2022).

Over the last few years, several studies based on exogenous applications have been performed to study the biostimulatory effect of melatonin, revealing that it is a signal molecule

Box 1. Glossary

Antioxidant: enzyme or low-molecular-weight compound that prevents the oxidation of essential cellular components

Biostimulant: a substance or microorganism that stimulates natural processes in plants to improve either water or nutrient use efficiency, tolerance to abiotic stress, or crop quality and yield

Causation: the relationship between cause and effect; causality

Correlation: statistical measure that expresses the extent to which two variables are linearly related

Derivatization: process of chemically altering an analyte or analytes

Hormonal action: physiological function exerted by a hormone as a result of recognition of a given hormone by its receptor and the subsequent activation of a signaling pathway leading to the activation or repression of gene expression

Hormonal crosstalk: interaction between two or more hormones or hormone pathways in cell signaling at the molecular level

Matrix: components of a sample other than the analyte of interest

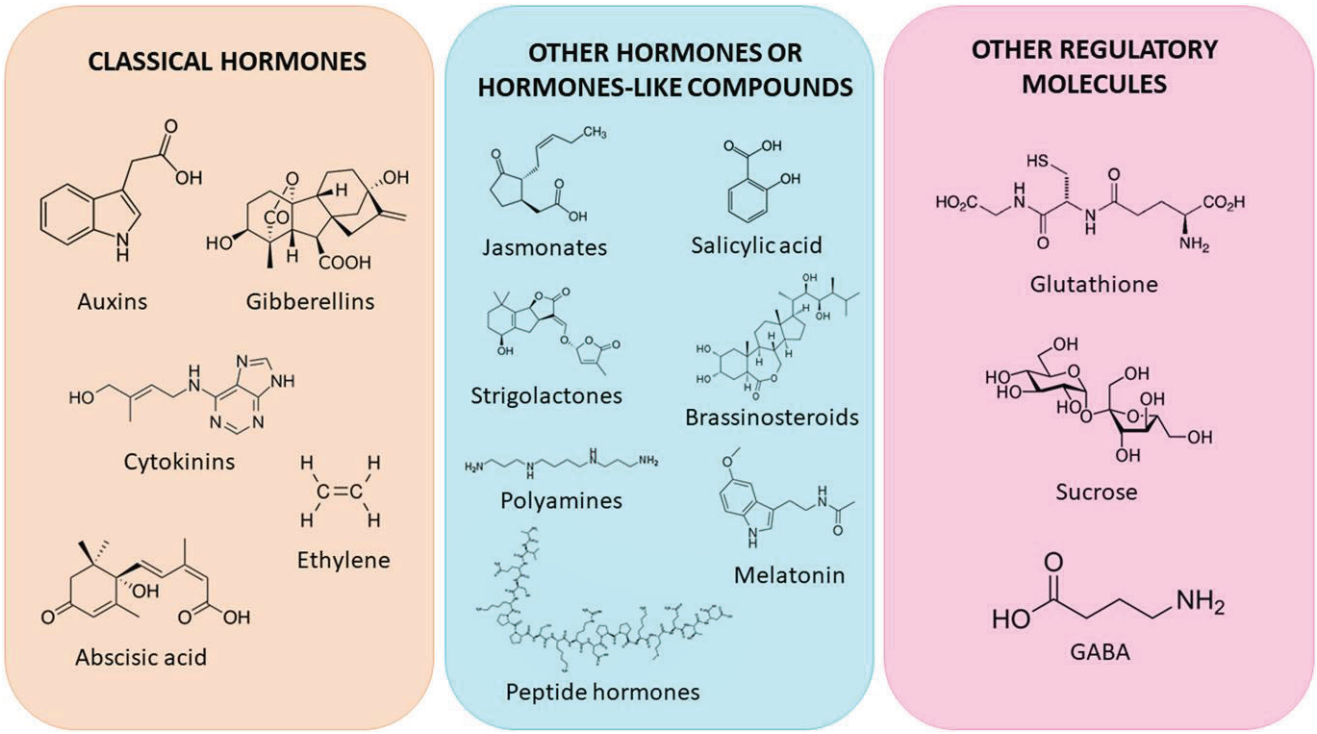
Phytohormone: naturally occurring organic compound found at nano- or micromolar concentrations that has a hormonal action at short and/or long distances in plants

Plant growth regulator: chemical used to modify any plant growth or developmental process, such as altering fruit ripening

Veraison: change of color of grape berries that characterizes the onset of ripening

Box 2. The regulatory role of melatonin in fruit ripening is still not fully established.

Phytohormones are small organic molecules synthesized de novo by plants. They have the ability to regulate physiological processes at much lower concentrations than nutrients or vitamins (<1 μM). Phytohormones act as chemical messengers, coordinating multiple cellular processes (Davies 2004). They can be transported to their site of action by transporters to regulate their distribution. Likewise, the effects of the hormones are determined by the sensitivity or responsiveness of plant tissues to hormones. Thus, to be considered a plant hormone, the presence of receptors and signal-transduction components are needed. These receptors must show a specific and reversible binding of the hormone, consequently inducing a biological response. Melatonin is a natural indoleamine molecule that was identified in plants in 1995 (Dubbels et al. 1995). Since then, much progress has been made in determining the melatonin biosynthetic routes as well as the products of its catabolism (Back et al. 2016; Lee and Back 2021). Its presence has been detected in a wide variety of plant species, organs, and tissues, thus making it ubiquitous (Nawaz et al. 2016). Furthermore, melatonin is involved in the regulation of several physiological plant processes (Lee et al. 2014; Sun et al. 2015; Zhang et al. 2018; Ye et al. 2020; Gholami et al. 2022). Recently, a melatonin putative receptor, *Cand2/PMTR1*, was identified in *A. thaliana*. It localizes to the plasma membrane, interacting with the alpha subunit of the G protein (GPA1) (Wei et al. 2018). Despite this, the signal transduction mechanism of melatonin has not been identified yet. Furthermore, there is still a lack of knowledge about fruit melatonin receptors, as well as the melatonin signaling process and the mechanisms for melatonin transport. Melatonin can be classified as a hormone-like compound, but we still do not know enough about it to consider it a hormone in fruit ripening regulation (see Box 2 Figure).



Box 2 Figure. Regulatory plant molecules, their chemical structure, and classification as hormones, hormone-like compounds, and other regulatory molecules.

involved in the regulation of numerous physiological processes (Murch et al. 2009; Lee et al. 2014; Sun et al. 2015; Arnao and Hernández-Ruiz 2020; Ye et al. 2020). Multiple experiments have demonstrated that the exogenous application of melatonin enhances several traits of interest in plants such as productivity (Liu et al. 2016), resistance to biotic and abiotic factors (Aghdam and Fard 2017; Gholami et al. 2022), tolerance to heavy metals (Nawaz et al. 2018), as well as improvements in fruit quality and shelf-life (Zhang et al. 2018). Several studies have carried out exogenous applications of melatonin both in pre- and postharvest fruits. In this regard, melatonin application has been described to contribute to the regulation of biochemical and physiological changes that preserve many quality attributes,

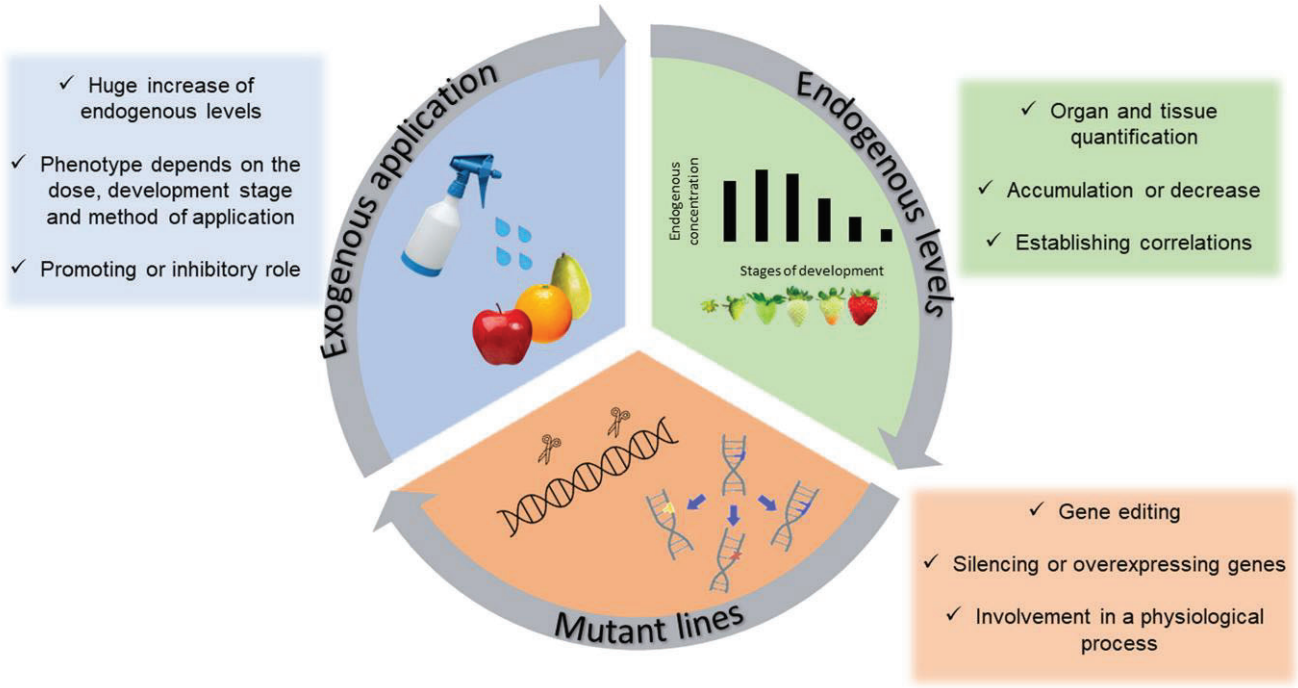


Figure 1. Diagrammatic representation of the three experimental approaches used to study active biomolecules in plants.

enhancing postharvest shelf-life, and inducing changes in the ripening rate (Table 1).

Generally, the dose of exogenous melatonin applied to fruits has been 10^{-3} to 10^{-5} M in both pre- and postharvest studies. However, varying effects of melatonin have been reported. During preharvest, in climacteric fruits such as apples (*Malus domestica* Borkh.), the exogenous application of 10^{-4} M melatonin at the green stage just before the onset of ripening has been reported to cause an acceleration in ripening parameters such as ethylene levels, sugar concentrations, weight, color, and firmness (Verde et al. 2022). Likewise, the application of 10^{-4} M melatonin in Zaosu pears (*Pyrus bretschneideri* Rehd.) has been demonstrated to cause a higher accumulation of sugars at the ripening stage (Liu et al. 2019c). On the contrary, melatonin applied at 10^{-5} M on nonclimacteric fruits such as sweet cherries (*Prunus avium* L.) at the green stage in a tree orchard inhibited ripening by delaying anthocyanin accumulation and increasing the cytokinin content (Tijero et al. 2019). The same effect was observed when melatonin was exogenously applied at 10^{-5} M to strawberries (*Fragaria* × *ananassa* Duch.) at the green stage, since it delayed their ripening (Mansouri et al. 2021). However, when melatonin was applied at 10^{-3} M, this accelerated the ripening of the strawberries by increasing the levels of ABA, hydrogen peroxide (H_2O_2), and anthocyanins. Moreover, in contrast to the effects of melatonin on cherries and strawberries, melatonin applied at 10^{-5} M and at 10^{-4} M promoted grape (*Vitis vinifera* L.) berry ripening by increasing the levels of ABA, ethylene, and H_2O_2 (Xu et al. 2018).

Changes in ripening induced by melatonin treatment during postharvest life have also been studied, particularly in climacteric fruits. In tomatoes, the exogenous application of melatonin at 5×10^{-5} M at the green mature stage was shown to induce ethylene and carotenoid biosynthesis as well as cell-wall modifications, demonstrating accelerated ripening (Sun et al. 2015, 2020). By contrast, when plums (*Prunus salicina* L.) were harvested at the green mature stage and treated with melatonin at 5×10^{-5} M and 10^{-4} M, their ripening slowed, as indicated by the firmness, respiration rate, and ethylene production (Yan et al. 2022). Similarly, it has been described that the application of melatonin at a concentration of 2×10^{-4} M and 5×10^{-4} M to mature unripe bananas (*Musa acuminata* L.) represses the expression of *1-AMINO CYCLOPROPANE-1-CARBOXYLIC ACID OXYDASE 1* (*MaACO1*) and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 1* (*MaACS1*), thereby inhibiting ethylene biosynthesis and delaying fruit ripening (Hu et al. 2017). In addition, 10^{-4} M and 5×10^{-4} M of melatonin were observed to delay the process of ripening and softening in mango (*Mangifera indica* L.) fruits by inhibiting ethylene production as well as suppressing ABA accumulation and pectin depolymerization (Liu et al. 2020; Dong et al. 2021). Hence, there is an antagonistic relationship between melatonin and ethylene in most climacteric fruits during postharvest that involves changes in the expression of ethylene biosynthesis-related genes (Table 1).

Several factors determine the outcome of the exogenous application of melatonin. First, the method of application, for instance, by spraying, dipping or infiltration, influences

Table 1. Exogenous applications of melatonin carried out during pre- and postharvest of fruits

Fruit	Developmental stage	Method	Applied concentration (M)	Main effects	References
Preharvest					
Apple	Green	Spray	5×10^{-4}	Accelerated ripening (ethylene) and improved quality (↑ fruit size, weight, sugars, and firmness)	Verde et al. (2022)
Cherry	Green large size	Spray	10^{-5}	Delayed ripening (↓ anthocyanins)	Tijero et al. (2019)
	During fruit growth cycle	Spray	10^{-4}	Maintained postharvest quality properties	Carrión-Antolí et al. (2022)
Grape	Pre-veraison	Spray	4×10^{-4}	Increased fruit weight (mechanism unclear)	Meng et al. (2015)
	Onset of veraison	Dipping, 10 s	10^{-4}	Enhanced antioxidant capacity (↑ anthocyanins, phenols, flavonoids, and proanthocyanidins)	Xu et al. (2017)
	60 d after full bloom	Dipping, 10 s	10^{-4}	Accelerated ripening (↑ ABA, H_2O_2 and ethylene)	Xu et al. (2018)
	70 d after full bloom	Dipping, 5 s	5×10^{-5}	Accelerated ripening (↑ ethylene), improved quality (phenylpropanoids and lipids)	Ma et al. (2021)
	During veraison	Spray	10^{-4}	Improved quality (↑ sugars, N, K, Cu, Fe, Zn)	Xia et al. (2021)
Pear	Anthesis	Spray	10^{-4}	↑ Parthenocarpy	Liu et al. (2018b)
	20 d after pollination	Spray	10^{-4}	Increased fruit weight and quality (↑ photosynthetic efficiency and ↑ sugars)	Liu et al. (2019c)
	100 d after blooming	Spray	5×10^{-5}; 2×10^{-4}	Increased anthocyanins and flavonols concentrations	Sun et al. (2021b)
Strawberry	Light green	Injection	10^{-5}	Limited effect on ripening	Mansouri et al. (2021)
(*Fragaria* × *ananassa*)	Light green	Injection	10^{-3}	Accelerated ripening (↑ ABA and H_2O_2)	
Apricot	During fruit growth cycle	Spray	10^{-4}	Delayed postharvest ripening (↓ethylene) Maintained quality properties during storage	Medina-Santamarina et al. (2021a, b)
Pomegranate	During fruit growth cycle	Spray	10^{-4}	Increased fruit crop yield and quality Maintained postharvest quality properties	Medina-Santamarina et al. (2021a, b)
Tomato	Plants at fifth true leaf stage up to fruit set	Spray	10^{-4}	Improved defenses against abiotic stress (simulated acid rain)	Debnath et al. (2018)
Postharvest					
Apple	Commercial maturity	Spray	10^{-3}	Delayed ripening (↓ ethylene) and maintained quality	Onik et al. (2021)
	Commercial maturity	Dipping, 10 min	5×10^{-5}	Delayed ripening (↓ ethylene and respiratory intensity) and maintained quality (↑ firmness, ascorbic acid, TSS, and TA)	Fan et al. (2022b)
	Immature green stage	Dipping, 2 h	5×10^{-5}	Improved quality and stimulate ethylene production	Verde et al. (2023)
Banana	Mature unripe	Dipping, 2 h	5×10^{-5}; 2×10^{-5}	Delayed ripening (↓ ethylene)	Hu et al. (2017)
	Commercial maturity	Dipping, 3 min	10^{-5}	Improved defenses against biotic stress (*Colletotrichum musae*)	Li et al. (2019)
Cherry	Commercial maturity	Dipping, 5 min	10^{-4}	Senescence inhibition and maintained quality (↓ weight loss and decay incidence, ↑ firmness, TSS, and TA)	Wang et al. (2019a)
Kiwi fruit	Physiological maturity	Dipping, 30 min	10^{-4}	Senescence inhibition (↑ antioxidants and ↓ MDA)	Wang et al. (2019b)
	Physiological maturity	Dipping, 10 min	10^{-4}	Delayed ripening (↓ ethylene, respiration rate) and maintained quality (reduced ethanol production)	Cheng et al. (2022)
	Physiological maturity	Dipping, 10 min	5×10^{-4}	Delayed ripening (↓ ethylene, respiration rate) and maintained quality (↑firmness, ascorbic acid, and TA) but reduced TSS	Luo et al. (2022)

(continued)

Table 1. (continued)

Fruit	Developmental stage	Method	Applied concentration (M)	Main effects	References
Lychee fruit	Commercial maturity	Dipping, 5 min	4×10^{-4}	Senescence inhibition (↓ H_2O_2, O_2^-, and MDA)	Zhang et al. (2018)
Mango	Mature green	Dipping, 1 h	5×10^{-4}	Delayed ripening (↓ ethylene and ABA)	Liu et al. (2020)
	70% matured	Dipping, 30 min	$2\cdot10^{-4}$	Delayed ripening and maintained membrane integrity (↓ H_2O_2 and MDA)	Dong et al. (2021)
Papaya	Breaker	Dipping, 2 h	4×10^{-4}	Improved defenses against biotic stress (*Colletotrichum* spp.)	Fan et al. (2022a)
Peach	Commercial maturity	Dipping, 10 min	10^{-4}	Senescence inhibition (↓ ROS)	Gao et al. (2016)
Pear	Commercial maturity	Dipping, 12 h	10^{-4}	Senescence inhibition (↓ ethylene)	Liu et al. (2019b)
	Before breaker	Dipping, 1 h	$2\cdot10^{-4}$	↑anthocyanins and anthocyanin biosynthesis genes ↑ H_2O_2	Sun et al. (2021a)
	Commercial maturity	Dipping, 6 h	10^{-4}	Improved defenses against biotic stress (*Botryosphaeria dothidea*)	Wang et al. (2022)
Plum	Commercial maturity	Dipping	10^{-4}; 10^{-3}	Improved cold tolerance (h ascorbic acid, phenols, and antioxidant activity)	Bal (2019)
	Commercial maturity	Spray	10^{-4}	Improved post-harvest life (↑ anthocyanin and phenol content)	Arabia et al. (2022)
	Green mature	Dipping, 1 h	5×10^{-5}; 10^{-4}	Delayed ripening (↑ firmness, ↓ respiration rate, and ethylene)	Yan et al. (2022)
Pomegranate	Commercial maturity	Dipping, 15 min	10^{-4}	Improved cold tolerance (↓ MDA and H_2O_2)	Jannatizadeh (2019)
Strawberry (*Fragaria* × *ananassa*)	Commercial maturity	Dipping, 5 min	10^{-4}	Improved cold tolerance (↑ GABA-T enzyme activity)	Aghdam and Fard (2017)
	Commercial maturity	Dipping, 5 min	10^{-4}; 10^{-3}	Senescence inhibition and improved cold tolerance (↑ phenols and flavonoids, ↓ MDA and H_2O_2)	Liu et al. (2018a, b)
	Commercial maturity	Dipping, 5 min	10^{-4}	Senescence inhibition (↓ weight loss and decay incidence)	El-Mogy et al. (2019)
Tomato	Green	Dipping, 2 h	5×10^{-5}	Ripening acceleration and improved quality (↑ ethylene and lycopene)	Sun et al. (2015)
	Mature green	Dipping, 5 min	10^{-4}	Improved cold tolerance (↑ polyamines, proline and NO)	Aghdam et al. (2019)
	Mature green	Dipping, 5 min	10^{-4}	Improved cold tolerance (↑ H_2O_2 and GABA shunt pathway)	Sharafi et al. (2019)
	Breaker	Dipping, 2 h	5×10^{-5}	Improved quality (↑ αβ-carotene and lycopene)	Sun et al. (2020)
	Mature green	Dipping, 10 min	5×10^{-5}	improved quality (↑ lycopene) and increased demethylase activity	Shan et al. (2022)

The developmental stage during application, the method used, the concentration applied, and the main effects are shown.
ABA, abscisic acid; GABA, γ-aminobutyric acid; MDA, malondialdehyde; NO, nitric oxide; ROS, reactive oxygen species; TA, titratable acid; TSS, total soluble solids.

the absorption of the molecule into the fruit, while the use of surfactants can also play a role, thereby determining treatment effectiveness. Indeed, all examples found in literature for melatonin involvement on fruit ripening and quality (Table 1) have overlooked root application, even if this method would be the easiest to implement in fields for biostimulant purposes. While some examples can be found for melatonin irrigation to evaluate plant performance in horticultural crops (Supplemental Table S1), to our knowledge, only one study evaluated the effects of melatonin by root application versus leaf spraying in tomato seedlings (Yang et al. 2021), but tomato quality or its implications in fruit formation were not investigated. A second factor affecting melatonin impact would be whether applications were performed during fruit pre- or postharvest, which can have a differential influence, since the sink strength exerted by cytokinin in attached fruits can substantially alter the effects of exogenous melatonin. Third, the differences in physiology between climacteric and nonclimacteric fruits, simple and aggregated fruits, or even true and false fruits according to their origin and constitution might explain some observed disparities. The melatonin dose applied is also an essential factor, since different doses can produce opposite phenotypes, as seen in strawberries (Mansouri et al. 2021). It must be considered that exogenous melatonin applications may lead to a huge increase in its endogenous concentration, generating nonphysiological endogenous levels (Sun et al. 2015; Liu et al. 2018a; Wang et al. 2021b). In that manner, high doses may contribute to creating unnatural physiological conditions, resulting in unexpected outcomes. Moreover, another key point in the exogenous application of melatonin is the moment of application, in other words, at which fruit developmental stage the application is performed. The physiological and biochemical events taking place in fruits differ depending on the stage of development (Kapoor et al. 2022) and elicit different responses. Since the transfer of knowledge to the field of biotechnology is one of the main objectives of research on biostimulants, the spray mode of application in preharvest crops and/or dipping in postharvest fruits are the most effective and easiest options to implement in the field. Nevertheless, several factors influence the outcome and much more research is needed to better understand the factors influencing the final effect of melatonin applied exogenously.

Hence, studies performing exogenous applications of biomolecules can improve knowledge on the role of regulatory molecules and their capability to stimulate or inhibit metabolic pathways. Comprehending the biostimulant function of a molecule is of great interest for its application in agrifood biotechnology. Moreover, assessing the effects of exogenous applications is a simple, economical, and fast technique to gain a better understanding of the physiological involvement of the molecule under study. Nevertheless, some limitations must be contemplated and multiple studies are required to define the optimal dose, mode, and moment of application for each fruit variety. Finally, it is also essential to unravel the mechanisms and modes of action of these regulatory molecules because the knowledge of their role will undoubtedly make exogenous applications more efficient and provide a better prediction of the desired effects.

Correlative analyses and causation

Fruits show different physiological characteristics between the beginning of development, when growth occurs through cell expansion and division, and the beginning of ripening, when seed maturation is complete and ripening occurs, arresting growth and triggering different processes to generate the final quality attributes. Fruit ripening is tightly controlled by multiple regulatory biomolecules that are involved in the up- or downregulation of metabolic pathways, inducing or repressing the transcription of different genes (Mariotti et al. 2011; Kim et al. 2019). For that reason, the endogenous contents of regulatory molecules differ throughout the different stages of fruit development. Thus, analyzing their endogenous concentrations at different points of growth, ripening, and postharvest periods is of great relevance in establishing causation about their biological role.

Melatonin concentration has been quantified in several species and fruit varieties by different analytical techniques (Table 2). In fruits, the presence of melatonin has been described in a wide variety of plant families, indicating that it is ubiquitous at a concentration of ng/g (Nawaz et al. 2016). However, its endogenous concentration differs across species and varieties, even between particular tissues of the same fruit. Therefore, specific tissue analysis is necessary to deepen our understanding of its role, which is conditioned by its localization and the specific metabolic processes taking place there. For example, in climacteric fruits such as Golden Delicious apples, the contents of endogenous melatonin differ between the skin and the pulp, being up to 60 times higher in the former than in the latter. In fact, marked spatiotemporal variations in melatonin contents occur in these fruits; melatonin levels in the skin are very low during the first stages of apple growth, but increase abruptly before the onset of ripening before decreasing constantly until the end of the process (Verde et al. 2022). In suppressed climacteric fruits such as Angeleno plums, higher melatonin contents in the skin than in the pulp have also been reported (Arabia et al. 2022). This higher content in the outer layer of the fruit, which is in contact with the environment, could be related to a protective role. Indeed, melatonin has been described to be involved in stomatal closure to cope with abiotic stress (Li et al. 2015, 2022; Wei et al. 2018). Therefore, its higher levels in the exocarp might be a protective mechanism against environmental stress and might be related not only to its role as a regulatory molecule but also to an additional putative **antioxidant** role.

Variations in endogenous melatonin contents during fruit development have been also reported in various nonclimacteric fruits. In Moldova grapes, melatonin levels increase rapidly during **veraison**, reaching a concentration of 8 ng/g DW,

Table 2. Endogenous concentrations of melatonin in different fruits

Fruit	Developmental stage	Endogenous concentration (ng/g)	Analysis method	References
Apple	Full maturity	0.04 FW	HPLC–FD	Hattori et al. (1995)
	Full maturity	0.16 FW	GC–MS	Badria (2002)
	30 d postanthesis	0.25 (skin); 0.02 (pulp) FW	HPLC–FD	Verde et al. (2022)
	100 d postanthesis	1 (skin); 0.006 (pulp) FW		
	Onset of ripening	2 (skin); 0.012 (pulp) FW		
	Full maturity	0.5 (skin) 0.012 (pulp) FW		
Banana	Full maturity	0.47 FW	GC–MS	Dubbels et al. (1995)
	Full maturity	0.66 FW	GC–MS	Badria (2002)
	Full maturity	0.01 FW	HPLC–FD	Johns et al. (2013)
	Full maturity	0.02 to 0.07[a] FW	ELISA	Hu et al. (2017)
	6 d postharvest	0.035 to 0.34[a] FW		
Cherry	Full maturity	0.00 to 0.224[a] FW	HPLC–ESI–MS	González-Gómez et al. (2009)
	Initial green	10 FW	HPLC–FD	Zhao et al. (2013)
	Green	35 FW		
	Full maturity	10 FW		
	Initial green	25 DW	UHPLC–ESI–MS/MS	Tijero et al. (2019)
	Full maturity	10 DW		
	Full maturity	1.43 FW		
Grape	Green	2 DW	UHPLC–MS	Xu et al. (2018)
	Onset of ripening	8 DW		
	Full maturity	1 DW		
	Full maturity	1.2 to 1.5[a] FW	HPLC–FD	Mercolini et al. (2012)
	Full maturity	0.005 to 0.965[a] (skin, FW)	ELISA	Iriti et al. (2006)
Kiwi fruit	Full maturity	0.24 FW	HPLC–FD	Hattori et al. (1995)
Mango	Full maturity	0.7 FW	HPLC–FD	Johns et al. (2013)
Orange	Full maturity	0.15 FW	HPLC–FD	Johns et al. (2013)
Papaya	Full maturity	0.24 FW	HPLC–FD	Johns et al. (2013)
Pineapple	Full maturity	0.04 FW	HPLC–FD	Hattori et al. (1995)
	Full maturity	0.28 FW	GC–MS	Badria (2002)
	Full maturity	0.3 FW	HPLC–FD	Johns et al. (2013)
Plum	Full maturity	20 (skin); 14 (pulp) DW	UHPLC–ESI–MS/MS	Arabia et al. (2022)
Pomegranate	Full maturity	0.17 FW	GC-MS	Badria (2002)
Strawberry (*Fragaria magna*)	Full maturity	0.01 FW	HPLC–FD	Hattori et al. (1995)
	Full maturity	0.14 FW	GC–MS	Badria (2002)
Strawberry (*Fragaria* × *ananassa*)	Full maturity	1.4 to 11.26[a] FW	LC–MS	Stürtz et al. (2011)
	Light green	7 FW	HPLC–FD	Mansouri et al. (2021)
	Initial red	27 FW		
	Full maturity	20 FW		
Mulberry	Fruit set	5.76 to 10.34[a]	HPLC–ESI–MS/MS	Wang et al. (2016a)
	Full maturity	0.58 to 1.41[a]		
Tomato	Full maturity	0.11 to 0.51[a] FW	GC–MS	Dubbels et al. (1995)
	Full maturity	0.3 FW	GC–MS	Badria (2002)
	Immature green	1.5 FW	ELISA	Okazaki and Ezura (2009)
	Full maturity	2.7 FW	ELISA	Okazaki and Ezura (2009)
	Full maturity	4.1 to 114.5[a] FW	LC–MS	Stürtz et al. (2011)

Data are shown as the mean concentration expressed in ng/g. FW, fresh weight; DW, dry weight; HPLC, high-performance liquid chromatography; ESI, electrospray; MS, mass spectrometry; FD, fluorescence detector; UHPLC, ultra-high-resolution liquid chromatography; MS/MS, tandem mass spectrometry; GC, gas chromatography; LC, liquid chromatography; ELISA, enzyme-linked immunosorbent assay.
[a]Measures in different varieties.

before decreasing abruptly to the lowest level of around 1 ng/g DW at the ripe stage (Xu et al. 2018). Indeed, a drastic decrease occurs in the endogenous melatonin content from 25 ng/g DW at the first green stage of sweet cherry development to 10 ng/g DW at the fully ripe stage (Tijero et al. 2019). Likewise, endogenous melatonin levels peak at the green developmental stages of Hongdeng and Rainier cherries, decreasing once ripening starts (Zhao et al. 2013). Moreover, in Sabrina strawberries, melatonin contents sharply increase at the onset of ripening, reaching 27 ng/g FW, before

gradually decreasing as the ripening progresses (Mansouri et al. 2021). This pattern of melatonin levels decreasing with fruit ripening has been shown also in black and white mulberries (*Morus nigra* L. and *Morus alba* L., respectively, Wang et al. 2016a).

There are several methods to quantify the endogenous levels of molecules in fruits that differ in their sensitivity and specificity. Some of the most widely used methods, from the least to the most sensitive, are enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), high-performance liquid chromatography (HPLC) coupled to an electrochemical detector (ECD) or a fluorescence detector (FD), gas chromatography (GC), and HPLC coupled to mass spectrometry (MS) (Feng et al. 2014). On the one hand, the detection of endogenous levels of molecules depends on intrinsic factors of the sample such as the mass/water/oil ratio of the tissue under study, as well as the factors depending on the chemical properties of the **matrix**, such as its extraction capacity by organic solvents. Thus, an appropriate choice of the extraction solvent is essential. On the other hand, extrinsic factors such as the sensitivity of the instrument determine the capacity of detection. Immunological techniques such as RIAs and ELISAs are based on the principle of competition. However, several molecules present in fruits may cross-react with the antibodies and enzymes used, causing an overestimation of melatonin levels (Johns et al. 2013). In fact, van Tassel et al. (2001) showed that the melatonin concentration obtained with RIAs was overestimated when compared to that obtained with GC–MS due to the relatively high levels of compounds that cross-reacted with the antibody used in the RIAs. Although useful for its relatively low cost, ELISA techniques with low specificity or low sensitivity can lead to incorrect results that over- or underestimate the levels of the molecules of interest. Consequently, chromatographic methods have been the most widely used for melatonin detection in recent years. Although GC–MS stands out for its high sensitivity, specificity, and reproducibility, HPLC does not require previous **derivatization** or high temperatures and is also a powerful and precise technique (Chafi and Ballesteros 2022). However, it is important to consider its limitations such as interferences due to matrix complexity as well as the huge amount of organic solvent needed. Huang and Mazza (2011) reported that HPLC–MS presented the lowest limits of melatonin detection, being more sensitive than HPLC–FD, HPLC–ECD, and HPLC–UV. Moreover, the multiple reaction monitoring mode allowed the production of multiple specific ion fragments from their specific precursors, providing more sensitivity and specificity (Huang and Mazza 2011). Thus, HPLC–MS is one of the most reliable techniques to quantify melatonin, showing high sensitivity and even more specificity than other techniques.

The study of endogenous levels of melatonin in a spatio-temporal manner, both at different stages and in different tissues, will determine its accumulation pattern, providing essential information for unraveling its regulatory role. However, studies on endogenous melatonin present differences in the analyzed melatonin levels as well as in the units used to express the results. Several studies report the endogenous levels as ng/g of fresh weight, whereas others use g of dry weight or g of tissue. During the analysis of the levels of regulatory molecules at different developmental stages or in tissues, differences between them must be taken into account. For instance, the water content is higher in the flesh tissues or at the fully ripe stage than in the skin of the fruit or at the initial greener stages. Consequently, in physiological terms, by not considering this water status depending on the stage or tissue analyzed, expressing the results as fresh weight may cause an over- or underestimation of the endogenous levels of the regulatory molecules. Therefore, it may be possible that one of the principal factors influencing the high variability in the melatonin levels reported in the literature and even the contradictory results between fruits in various studies is the way the results have been expressed, making comparisons extremely difficult (if not impossible).

A growing number of studies are describing and quantifying the endogenous levels of not only melatonin, but also of other molecules such as the phytohormones and antioxidants involved in the ripening process, as well as the expression of the genes related to their biosynthesis, by performing statistical **correlation** analyses (Zhao et al. 2013; Tijero et al. 2019; Verde et al. 2019). Establishing a significant positive or negative correlation between melatonin and other compounds or genes using the Pearson or Spearman coefficient could help better comprehend the main metabolic pathways involved. However, it must be considered that a correlation does not imply causation. There may be other factors involved. To confirm this possible interaction, it is necessary to perform in-depth genetic studies that will provide advanced and more specific knowledge from a mechanistic point of view. Indeed, a complex aspect to be additionally considered in the interpretation of variations in the endogenous contents of melatonin is that this compound may both act as a signal molecule and as an antioxidant at the same time. Therefore, assigning specific physiological roles and discussion of possible functions for melatonin and their mode of action based on changes of melatonin contents should be made very carefully, especially if one aims to establish causation.

Overall, despite differences in the analytical methodology used and the units applied to express these results, data from most of the studies suggest that melatonin could have a different regulatory role depending on the stage of development, and there might be differences between climacteric and nonclimacteric fruits (Fig. 2). Melatonin is a natural indoleamine and its biosynthesis involves the chorismate pathway. Chorismate gives rise to tryptophan and after several reactions, it can generate melatonin, but also phytohormones such as the auxin indole-3-acetic acid (IAA). Melatonin and IAA share a common precursor and a very similar chemical structure (Pérez-Llorca et al. 2019). In addition, it has been found that melatonin has auxin-like

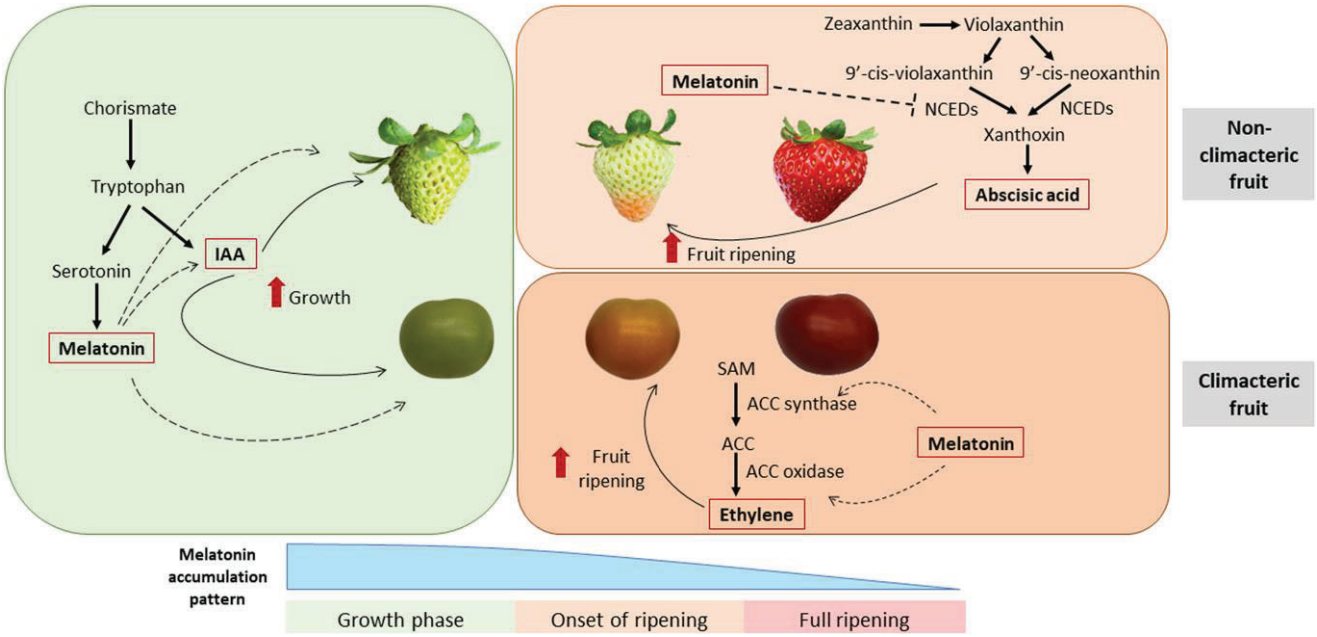


Figure 2. Proposed model of melatonin accumulation and its regulatory role in fruit ripening during different developmental stages in climacteric and nonclimacteric fruits. It is suggested that melatonin promotes fruit growth by itself or by a possible crosstalk with IAA in both climacteric and nonclimacteric fruits. In nonclimacteric fruits, melatonin may act in the advanced developmental stages, interacting antagonistically with ABA through the downregulation of its biosynthesis-related genes such as *NCED*. By contrast, melatonin might have a positive crosstalk with ethylene, positively influencing its biosynthesis and signaling in climacteric fruits. ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indole-3-acetic acid; NCED, 9-*cis*-epoxycarotenoid dioxygenase; SAM, S-adenosyl-methionine.

functions, such as in root growth (Chen et al. 2009; Wen et al. 2016). During fruit development, IAA is principally involved in fruit set and growth, reaching its highest levels during the initial stages of fruit development before decreasing prior to the onset of ripening (Hu et al. 2018). Therefore, we suggest that melatonin may have an auxin-like role during fruit development, promoting growth at the green stages by itself and/or through interactions with auxins, possibly regulating their accumulation in fruits (Fig. 2). Moreover, although auxins have been described to repress ripening, crosstalk between auxins and ethylene has been reported in the ripening of climacteric fruits, with some genes in the ethylene domain regulated by auxins (Trainotti et al. 2007; Zhang et al. 2020; Wang et al. 2021c; Gambhir et al. 2022). Likewise, melatonin can upregulate the expression of genes encoding ethylene biosynthesis enzymes, such as 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO), as well as influence ethylene signaling (Sun et al. 2015; Verde et al. 2023). In this sense, Shan et al. (2022) showed that melatonin postharvest treatments in green tomato fruits could induce ethylene production by demethylation of CpG islands (DNA regions that contain a high frequency of CpG dinucleotides located around the gene transcription initiation sites) of ethylene biosynthesis (*SlACS1*) and signal transduction, such as *ETHYLENE RESPONSE FACTOR A1* (*SlERF-A1*) genes, thereby promoting fruit ripening. Likewise, it has been reported that increased production of lycopene and other carotenoids after melatonin treatments in green tomato fruits could be partially explained by increases in the ethylene production mediated by melatonin (Sun et al. 2020). This hypothesis is based on the melatonin effects in both green tomato fruits and *never ripe* (*nr*) mutants, insensitive to ethylene signaling (ETHYLENE RECEPTOR 3), where melatonin treatments on *nr* fail to promote carotenoid biogenesis that can be seen in nonmutagenic tomatoes. Although this work highlights the relationship between melatonin and ethylene, it fails to describe a direct interaction between melatonin and ethylene-related genes. Similarly, Ma et al. (2021) found that exogenous melatonin promoted ethylene biosynthesis in grape berries during on-tree development when treated at green stages and demonstrated that this interaction could be related to *VvMYB14* transcription, encoding a transcription factor that binds to the promoter region of *VvACS1*. Although this study showed that *VvACS1* expression was downregulated in *VvMYB14* suppressed lines when melatonin was applied, the experiment did not demonstrate a direct interaction between VvMYB14 and melatonin, therefore leaving a gap in the melatonin signal transduction. However, these experiments evidence that in climacteric fruits, melatonin could also act by promoting ethylene biosynthesis through synergistic crosstalk during ripening (Fig. 2). On the contrary, an antagonistic relationship between IAA and ABA has been described during nonclimacteric fruit ripening, with IAA inhibiting the expression of *NCED* (which encodes 9-*cis*-epoxycarotenoid dioxygenase that is required for ABA

biosynthesis) and *PYRABACTIN RESISTANCE 1* (which encodes the receptor for ABA) (Jia et al. 2016). Likewise, melatonin and ABA generally have an antagonistic relationship, with melatonin decreasing ABA levels via the downregulation of ABA biosynthetic genes, the upregulation of its catabolic genes, and the inhibition of its signaling components during stress conditions (Li et al. 2015, 2021; Zhang et al. 2017b; Tan et al. 2019; Wang et al. 2021a). Therefore, in nonclimacteric fruits, it is possible that melatonin might interact with ABA, suppressing its biosynthesis by downregulating the genes associated with ABA synthesis and inhibiting ABA signaling at the onset of ripening.

In summary, measuring endogenous melatonin concentrations can potentially establish, in a spatiotemporal manner, its stimulatory or inhibitory role (reflected by an increase or reduction in its levels, respectively) in the regulation of fruit ripening. To date, some scientific evidence has been obtained not only on the role of melatonin in the modulation of ripening, but also on the metabolic pathways and other molecules involved in the regulation of this process by melatonin. However, as discussed before, some critical factors must be considered to correctly establish causation and avoid quantification mistakes that may sometimes lead to misleading conclusions. Furthermore, it is still not fully clear whether melatonin acts as an antioxidant or signal molecule, more specifically in those cases where endogenous melatonin tissue concentrations are high. It is essential that changing patterns of melatonin are consistent with that of ripening-related indexes to establish causation effects, and this is not clearly observed in all cases, thus suggesting that melatonin might not be a key regulator in fruit ripening, at least not in all fruits.

Evidence in mutant and transgenic lines

Genetics and genomics have revolutionized our knowledge about fruit ripening, being used in studies that have provided important information about the function of genes and metabolites involved in fruit ripening (Giovanoni et al. 2017). There is a plethora of techniques used to obtain transgenic organisms for research purposes in order to identify key regulatory signals or enzymes involved in a given process. One example is RNA interference (RNAi), which consists of downregulating the expression of the gene of interest to study its function and regulation (Baulcombe 2004). There are many methods for transferring dsRNA into plant cells to trigger RNAi pathways, for instance, by developing specific vectors for *Agrobacterium tumefaciens* transformation and infiltration into plant cells or by infecting plants with autonomous viral vectors (Lin et al. 2013). To better understand the function of a target gene and possible competition with other pathways, it is important not only to study the effects of its silencing, but also those of its overexpression. Overexpression can be achieved through the amplification and cloning of the coding region of the gene of interest into a binary vector with restriction enzymes and using this vector to transform *A. tumefaciens* (Zhou et al. 2022). Virus-induced gene silencing is also a fast and efficient method for fruit transformation, most specifically for fruit crops that are recalcitrant to *Agrobacterium*-mediated transformation or for fruit functional studies when gene silencing could induce abnormal plant development (Senthil-Kumar et al. 2011a). Indeed, transgenic lines overexpressing or silencing the gene of interest should be compared to unravel and verify its molecular functions. However, there are some limitations to these methods such as incomplete and temporary knockdowns or nonspecific insertions, leading to ambiguous results and unpredictable effects (Senthil-Kumar et al. 2011b; Casacuberta et al. 2015).

By contrast, specific target mutagenesis is a more efficient method for gene disruption, using engineered nucleases to create a double-strand break that is repaired by homologous or nonhomologous recombination (Wang et al. 2018). Precise genome editing systems using zinc-finger nucleases, transcription activator-like effector nucleases or clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) are promising tools that reduce long cycles to obtain transgenic offspring with homozygosity and avoid nonspecific mutagenesis. However, there are some limitations when applied to fruit studies. Many fruit crops have heterozygous or even polyploid genomes, hybrid incompatibility, and long growth cycles, particularly fruit trees, where initial fruit production can take many years, thus complicating directed mutagenesis (Wang et al. 2018). The CRISPR/Cas9 system uses a guiding RNA molecule to target and cleave specific DNA regions in complex genomes. This system stands out as the most efficient for fruit mutagenesis studies as it is simple and efficient and is not time-consuming (Zhang et al. 2017a), even though its use in fruits is still far from being optimized. For instance, stable transformations in strawberries (*Fragaria* × *ananassa*) are complex due to their octoploid genome and their high level of heterozygosity. Indeed, transforming methods must have a higher percentage of effectiveness to obtain satisfactory results in polyploid crops. In these cases, transient expression studies using the agroinfiltration of a CRISPR/cas9 cassette are satisfactory alternatives (Martín-Pizarro et al. 2019). Therefore, the different advantages and limitations of different powerful techniques as well as the added complexity of fruit genomes must be considered in each case when elucidating the function of a particular gene (Fig. 3).

In plants, melatonin is synthesized by six enzymes: tryptophan decarboxylase (TDC), tryptophan hydroxylase, tryptamine 5-hydroxylase, serotonin N-acetyltransferase (SNAT), N-acetylserotonin O-methyltransferase (ASMT), and caffeic acid O-methyltransferase (COMT). This synthesis includes four reactions that start with tryptophan, with serotonin an essential intermediary (Back et al. 2016). Thus, downregulation or overexpression of some of these genes in fruits, especially the most specific ones involved in the last steps of melatonin biosynthesis (*SNAT*, *ASMT*, and *COMT*), can be used to clarify melatonin function. Studies of the genes

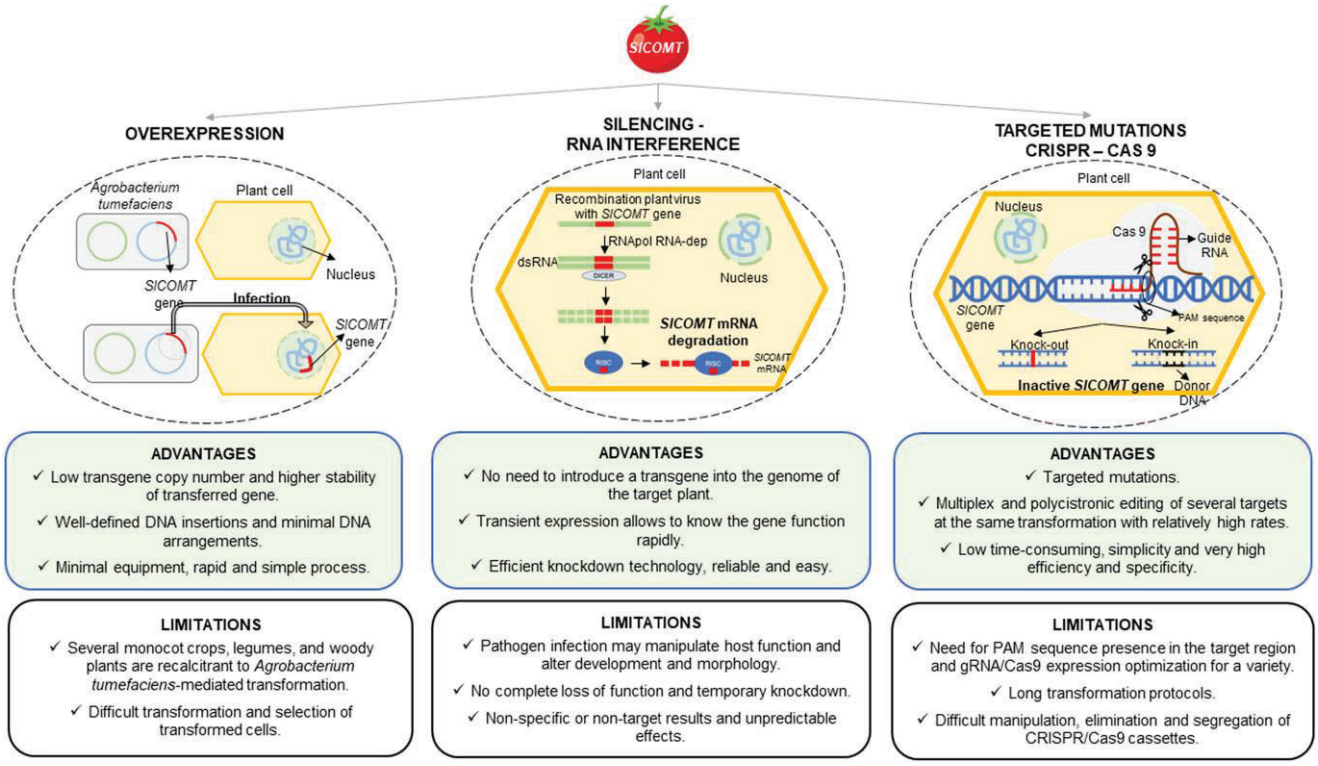


Figure 3. Schematic diagram showing the three techniques used to obtain a mutant tomato line, using the *SlCOMT* gene (caffeic acid O-methyltransferase) as an example. Gene overexpression using *A. tumefaciens* transformation (left), gene silencing with RNA interference (center), and targeted mutations with the CRISPR/Cas9 cassette (right) are shown. The advantages and limitations are discussed for each technique.

associated with melatonin biosynthesis using genetic transformations have mainly focused on their role in abiotic stress resistance, such as mitigating toxicity (Li et al. 2016; Yan et al. 2019), cold-induced oxidative stress (Wang et al. 2020a), and heat stress in tomato plants (Ahammed et al. 2019), by silencing *COMT* or *TDC* with *Agrobacterium* transformation and infiltration. However, none of these studies have evaluated fruit ripening or the regulation of quality attributes to identify the role of melatonin in fruit development. In fact, most gene editing studies in fruit crops have analyzed the expression and regulation of the genes associated with melatonin biosynthesis in vegetative organs such as leaves. Thus, there is a great lack of studies using gene-editing techniques to study melatonin in fruits (Table 3). It was previously shown that the ectopic expression of the ovine homologs of *SNAT* (*oAANAT*, from *arylalkylamine N-acetyltransferase* that is equivalent to the *SNAT* gene) and *ASMT* (*oHIOMT*, from *hydroxyindole-O-methyltransferase*) in Micro-Tom tomato plants, involving *Agrobacterium*-mediated transformation, caused a higher accumulation of melatonin in fruits and reduced total plant IAA levels, possibly due to competition for the precursor substrates (Wang et al. 2014). That study did not report reductions in fruit set, deformities in the fruit morphology or parthenocarpy, which would have been promoted by reductions in IAA levels as IAA plays a role in fruit architecture and seed formation (Wang et al. 2005; Batista-Silva et al. 2022). However, it did report that the ectopic expression of *oAANAT* and *oHIOMT* induced a severe loss of apical dominance in plant growth by reducing IAA levels in the stems and leaves. Thus, the increased melatonin levels in these plants could not compensate for the IAA deficiency and throws into question the auxin-like function

Table 3. Tomato mutant lines, technique used to generate the line, and the main effects of melatonin are shown

Fruit	Technique	Main effects	References
Tomato	Transgenic tomato plants overexpressing *oAANAT* and *oHIOMT* using *A. tumefaciens*	↑Endogenous melatonin ↓Indoleacetic acid Highest expression in leaves than in ripened fruits	Wang et al. (2014)
	Transgenic tomato plants overexpressing *SlCOMT1* using *A. tumefaciens*	Improved defenses against salinity stress Highest expression in fruits	Liu et al. (2019a)
	Transgenic rootstock: overexpression of *COMT1* using *A. tumefaciens*	↑Melatonin in fruit ↓Carbendazim residues (↓ROS and MDA)	Yan et al. (2019)

COMT1, caffeic acid *O*-methyltransferase 1; MDA, malondialdehyde; ROS, reactive oxygen species; *oAANAT*, arylalkylamine *N*-acetyltransferase from the pineal gland of *Ovis aries* (sheep); *oHIOMT*, hydroxyindole-*O*-methyltransferase from the pineal gland of *Ovis aries* (sheep).

attributed to melatonin, at least for growth. Liu et al. (2019a) also examined the overexpression of *SlCOMT1*, which is associated with melatonin biosynthesis, in tomato plants to evaluate its role in salt stress resistance. They showed that this gene was highly expressed in fruits at the ripe stages, suggesting its involvement in fruit development. However, the study did not measure fruit production, fruit size or biomass, the ripening dynamics related to color acquisition, starch content, or any basic fruit quality parameter such as sugar content and fruit firmness. In another study, rootstock transformation of tomato plants overexpressing *SlCOMT1* increased the total melatonin content in tomato fruits without increasing the expression of *SlCOMT1* in fruits (Yan et al. 2019). Yet again, no analyses were performed to evaluate fruit growth and ripening, and the only conclusions made were related to pesticide metabolism through GSH conjugation.

Overall, current experimental approaches using gene editing to determine melatonin function have provided very little information about the actual role of melatonin in fruits and whether this molecule is essential enough for fruit growth and ripening to be considered a hormonal regulator. Directed mutagenesis to suppress melatonin expression involving knockout mutants or gene silencing has been mostly performed in Arabidopsis (*Arabidopsis thaliana* [L.] Heynh.) or rice (*Oryza sativa* L.) models to determine the effects on stress tolerance [see recent reviews by Back (2021) and Liu et al. (2022a, b)]. These studies indicate that melatonin deficiency strongly influences pathogen sensitivity (Lee et al. 2015) and the **hormonal crosstalk** with auxins and gibberellins (GAs) in root architecture and flowering, respectively (Lee et al. 2015; Yang et al. 2021). These effects of melatonin could be of particular interest for understanding the regulation of both fruit development and fruit preservation during postharvest. To our knowledge, the only study referring to melatonin deficiency in fruit crops was based on the silencing of *COMT1* in tomato, demonstrating that the silencing aggravated heat stress-related symptoms, but not analyzing the implications of this deficiency for fruit development (Ahammed et al. 2019).

Another important aspect is identifying the signaling pathways elicited by both endogenous melatonin and melatonin applied exogenously as a biostimulant in fruits. In this sense, it is known that melatonin can activate the transcription of ripening-related genes in different fruits, as has been observed after the application of exogenous melatonin. Transcriptomic analysis after melatonin application in fruits has demonstrated that it specifically activates the genes related to the antioxidant system (Zhang et al. 2018; Ze et al. 2021). Modulation of gene transcription by melatonin could be exerted through the direct activation of specific promoter regions, such as the ones described for other phytohormones (Leng et al. 2014; Liu et al. 2015), or by specific interactions with other molecules, such as the ones described for ROS or NO signaling (Corpas et al. 2018; Muñoz and Munné-Bosch 2018). For the former, melatonin would need a specific receptor for its signal transduction, while for the latter, melatonin would need to modify the chemical or structural properties of different molecules to elicit a signaling cascade. Although a lot of effort has been made to identify melatonin-specific transmembrane receptors and signal transduction, there is still a lot of missing gaps, particularly in fruit-related studies. Several recent reviews have proposed potential melatonin-signaling pathways (Back 2021; Liu et al. 2022a, 2022b), such as the one where melatonin interacts with the only melatonin transmembrane receptor identified in plants, the putative phytomelatonin receptor (PMTR1/CAND2) leading to downstream signaling via the activation of the Gα subunit of the receptor or via the mitogen-activated protein kinase (MAPK) signaling cascades. The lack of fruit mutants with impaired melatonin biosynthesis as well as the dearth of melatonin-specific receptors in fruits make it very difficult to classify melatonin as an essential hormonal regulator of fruit development (Box 3). It is important to complement fruit studies, both using exogenous applications and studies on variations in endogenous contents, with transgenic approaches to consider melatonin as an essential molecule in the regulation of fruit development. In this sense, new genome editing tools such as CRISPR/Cas9 and the willingness to develop strategic integrative studies on fruit development are essential in answering outstanding questions related to the role of melatonin in fruit development and ripening.

Complementary studies integrating knowledge

Experimental design is the basis of producing rigorous data that can elucidate and validate our hypotheses. Studies integrating different experimental approaches can provide relevant information to better understand relationships governing fruit growth, ripening, and postharvest physiological events, which can then improve knowledge for biotechnological and environmentally friendly applications in fruit production. Here, three experimental approaches have been described to illustrate the complexity of characterizing regulatory molecules in fruit ripening, but all are essential and complementary to establish such a role.

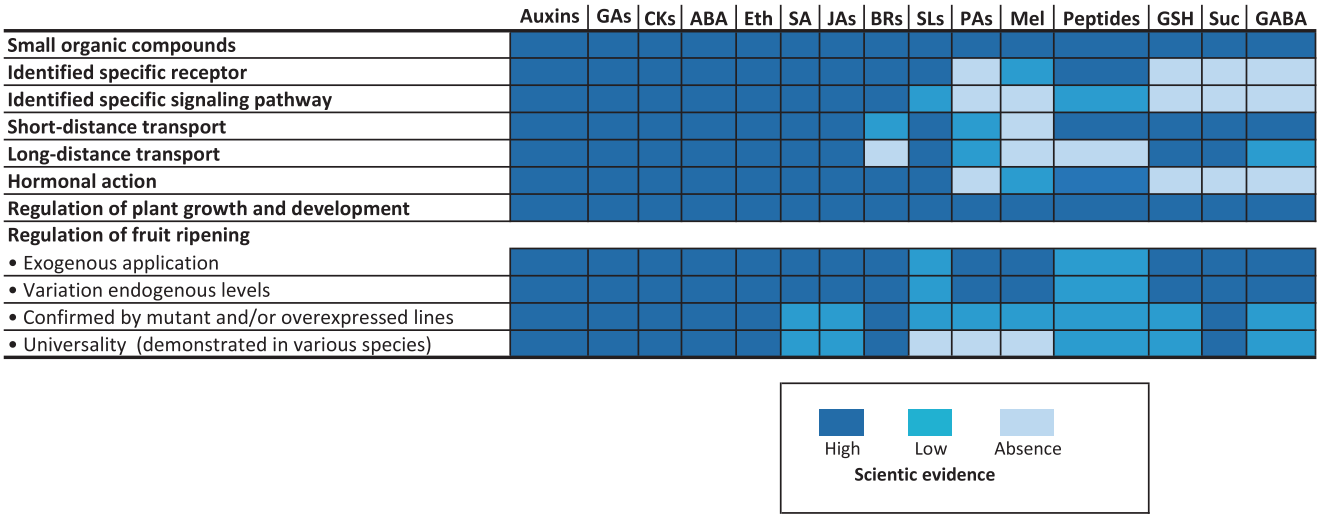
Melatonin has been described to be a putative hormone that can regulate fruit quality attributes and fine-tune the ripening process by crosstalk with other hormones. After a thorough analysis of all the studies related to melatonin and fruit ripening using different experimental approaches, it appears that melatonin promotes the initial phases of fruit development (Table 2). This is mainly because endogenous levels in most fruits show a pattern of higher contents at the beginning of fruit development and reductions prior to the onset of ripening, either with a progressive decline during fruit growth or through an accumulative peak before maturation (Fig. 2). This function of melatonin is further validated by the exogenous applications of melatonin. Although there is an important effect of melatonin dosage and the fruit

developmental stage, doses of around 10^{-4} M applied at the beginning of fruit development have been shown to delay the onset of ripening and influence the expression of growth-promoting phytohormones (such as IAA and GAs), while partially repressing the elicitors of fruit ripening (such as ABA and ethylene) (Table 1; Fig. 2). However, it should be noted that molecular analyses in fruits harvested at the early stages of development and detached from plants do not accurately capture the actual functions of the molecule, particularly if the subject of discussion is whether this molecule has a hormonal-like function and, therefore, is transported over short or long distances.

Integrating the findings from the experimental approaches of assessing the effects of exogenous applications and measuring endogenous levels as well as considering the structural nature of melatonin, it appears that melatonin might act as a classical hormone regulating fruit ripening. However, its functions are not different from those of other low-molecular-weight organic molecules that are not considered phytohormones such as GSH and GABA. To truly ascertain whether melatonin acts as a hormonal signal in fruit growth and ripening, it is important to include genetic studies. However, to date, genetic studies on fruit crops have evaluated the effects of melatonin from a stress-related perspective, without considering fruit development and quality parameters. We can speculate from other mutagenesis studies, which have evaluated melatonin function from a mechanistic point of view with growth-promoting phytohormones such as IAA and GAs, that melatonin has a positive relationship with these phytohormones. However, it cannot compensate for their physiological functions when they are deficient, as shown in examples of IAA precursor competition (Wang et al. 2014), IAA mutants (Wang et al. 2016b; Yang et al. 2021), and gibberellin inhibition (Liu et al. 2018b). Melatonin may trigger its function in fruits through IAA-dependent signaling, interacting with other phytohormones, including GAs, ethylene, and ABA. In this model (a), melatonin fine-tunes IAA levels at the initial stages of fruit growth development, integrating circadian or environmental signals that can alter melatonin contents, induce metabolic changes in IAA accumulation, and activate IAA-dependent signaling, while IAA activates nuclear transcriptomic changes to regulate fruit growth architecture and the ripening onset through its interactions with ethylene and ABA (Fig. 4). In a second model (b), melatonin activities are independent of IAA and even if melatonin affects the

Box 3. Requirements that organic compounds must meet to be considered a phytohormone in the regulation of fruit ripening.

Not all regulatory biomolecules meet the requirements to be considered a phytohormone involved in the regulation of fruit ripening (see Box 3 Figure). Aside from the classic five (auxins, GAs, cytokinins, abscisic acid, and ethylene), salicylic acid and jasmonates fulfil all requirements (despite some additional evidence using mutant and overexpressed lines as well as in different models is required). Furthermore, brassinosteroids and strigolactones also meet all requirements, except transport and universality, respectively. Other regulatory biomolecules, such as melatonin, meet a variable number of requirements, but not all. These gaps underline future lines of research.



Box 3 Figure. Requirements met by various regulatory biomolecules. ABA, abscisic acid; BRs, brassinosteroids; CKs, cytokinins; Eth, ethylene; GABA, γ-aminobutyric acid; GAs, gibberellins; GSH, glutathione; JAs, jasmonates; Mel, melatonin; PAs, polyamines; Peptides, peptide hormones; SA, salicylic acid; SLs, strigolactones; Suc, sucrose.

endogenous contents of this growth-promoting phytohormone, melatonin can directly activate specific promoter regions of genes associated with promoting growth and the ripening-related phytohormones in fruits (Fig. 4), as evidenced by the direct increases in GAs (Zhang et al. 2014) and in particular with ethylene (Sun et al. 2020), either promoting demethylation activity of CpG regions in ethylene-related genes (Shan et al. 2022) or promoting the transcription of specific transcription factors such as those related to the MYB family (Ma et al. 2021). Likewise, because melatonin regulates the redox balance by activating antioxidant systems, a third model (c) considers melatonin an endogenous regulator of ROS levels and, therefore, an activator of signaling cascades, primarily to activate the antioxidant defense system or to elicit controlled ROS-dependent signaling (Fig. 4). This model is supported by work showing that exogenous melatonin triggers transient H_2O_2 accumulation at the early developmental stages by activating NADPH oxidase (RBOH), which further induces anthocyanin accumulation in pears (Sun et al. 2021a), and also in postharvest models, where melatonin enhances total antioxidant activity to regulate total ROS production and ameliorate fruit decay (Bal 2019; Wang et al. 2019b; Arabia et al. 2022). Furthermore, this model would explain one of the putative melatonin-signaling pathways involving the increased activation of MAPK, which has been long related to the redox status and dependent on H_2O_2 production control in plants (Kovtun et al. 2000).

At the moment, models (a) and (c), which are not mutually exclusive, could be considered the most plausible for the regulation of fruit development, but further research is needed because knockout mutants have not been developed, the short- or long-distance transport of melatonin has not been validated, and no promoter regions recognizing melatonin have been identified. Major breakthroughs in understanding the function of melatonin in fruit development would be the identification of a specific receptor of

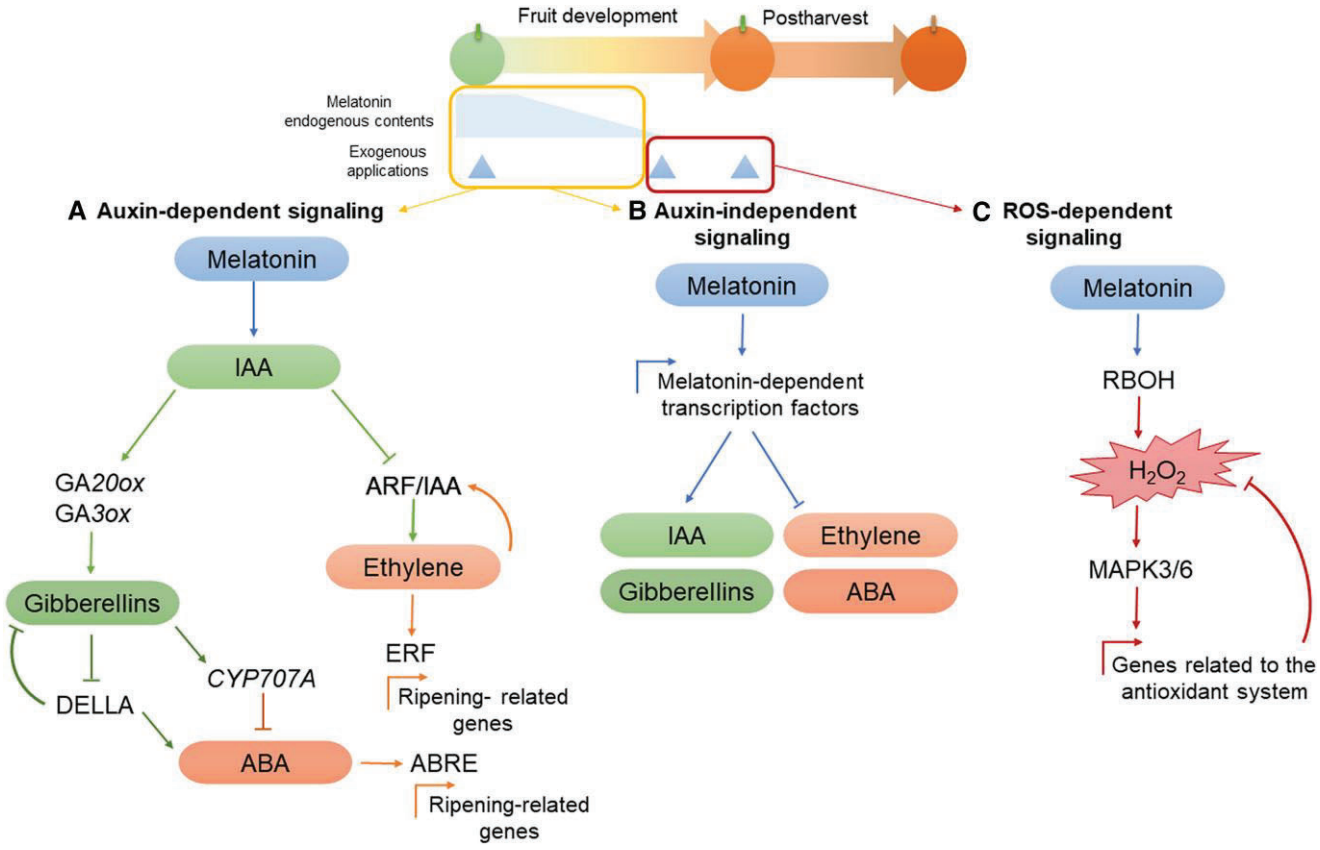


Figure 4. Proposed models for the role of melatonin in fruits. In general, higher melatonin contents are found at the early stages of fruit development. Endogenously, at the early stages, melatonin may interact with other phytohormones to regulate fruit growth and ripening. In the first model **A)** melatonin, at low concentrations, interacts with auxins (IAA) and elicits IAA-dependent signaling, where high IAA contents promote the activity of gibberellins (GAs), but inhibit both ethylene and ABA. In the second model **B)**, melatonin acts independently of IAA and activates the expression of specific transcription factors that then interact with other promoter regions of important phytohormones in fruit development, promoting IAA and GA accumulation, while inhibiting ethylene and ABA. Lastly, in the model **C)**, exogenous melatonin, especially when applied at the advanced stages of fruit development or during postharvest, elicits ROS-dependent signaling and induces a moderate production of hydrogen peroxide (H_2O_2) by the activation of NADPH oxidase (RBOH), which in turn triggers the MAPK signaling cascade to enhance the fruit antioxidant machinery. ABRE, ABA-responsive element-binding factors; ARF, auxin response factors; DELLA, negative protein regulators of GAs; ERF, ethylene response factors.

melatonin in fruits and the effects of its downregulation, determining the mechanism of melatonin transport and signal transduction, and identifying specific promoter motifs that recognize melatonin. Undoubtedly, integrative studies that use all experimental approaches would give more information about the role of melatonin, but the limitations of the techniques and budget must be considered. In this sense, cumulative efforts from the scientific community working towards standardized and focused fruit melatonin research would give a complete picture on this topic.

Conclusions and future perspectives

We show here that a combination of the three complementary experimental approaches (exogenous applications, analysis of endogenous contents, and mutagenesis studies) must be considered in elucidating the actual function of active biomolecules, including melatonin in fruit development and ripening. Exogenous applications of melatonin indicate that melatonin could act as a growth promoter and ripening retardant. Due to its high similarity with IAA and given that mutagenesis studies show an interaction between melatonin and IAA, it can be concluded that melatonin acts through auxin-dependent signaling at the early stages of growth. However, the involvement of melatonin in ROS regulation cannot be excluded, especially at the later stages of fruit development or during post-harvest, where it has a biostimulant-like function. At the same time, melatonin cannot be classified as a hormonal regulator of fruit development and ripening until its transport and signaling have been completely elucidated. Altogether, the analysis of this molecule shows the need for integrative studies in fruit development and ripening to discern between phytohormones and other regulatory molecules. Melatonin studies aimed at elucidating the hormonal role of melatonin in fruit development and ripening should include a complete spatiotemporal evaluation of melatonin biosynthesis and signaling, whether the molecule is required at specific phases of fruit development or whether tissular gradients determine its mode of action. Functional studies with mutagenic lines will also validate whether this molecule is essential for fruit development and ripening, including examples in nonmodel species with different patterns of hormonal regulation, where sensitiveness to a lack of melatonin could have particular outcomes. At the same time, in silico studies for the discovery of melatonin ligands at upstream regions of melatonin-differentially expressed genes should be a priority taking advantage of the appropriate bioinformatic analyses and the plethora of RNAseq already available from studies already made that are based on melatonin applications. Ultimately, integrative studies considering melatonin involvement in fruit ripening from a whole plant perspective will undoubtedly help elucidating key open questions (see "Outstanding Questions") related to the possible hormonal role of this molecule, including root-to-shoot and shoot-to-root translocation and evaluation of plant performance, so that new innovative solutions can be implemented for improving ongoing technological agricultural practices.

ADVANCES BOX

- Integrative studies including various experimental approaches (based on exogenous applications, analysis of endogenous contents, and mutagenesis) can give us a complete understanding of the function of phytohormones and other regulatory biomolecules in plants.
- Melatonin is a hormone-like compound with growth-promoting effects in fruit development.
- Melatonin promotes auxin signaling and this model may explain several of the functions of melatonin observed in fruits.
- ROS-melatonin interactions may explain its biostimulant function, particularly at the later stages of fruit development and during postharvest.

OUTSTANDING QUESTIONS BOX

- Will additional studies using fruits with altered melatonin contents, either by TILLING or CRISPR/Cas9 methods, help us clarify the putative hormonal role of melatonin in fruit development and ripening?
- Will a melatonin receptor and the underlying signaling components be identified in fruits?
- Will long-distance transport of melatonin related to the regulation of fruit development and ripening be identified in the near future?
- Does melatonin applied as a biostimulant have contrasting dose–response effects in climacteric and nonclimacteric fruits?

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Supplemental data

The following materials are available in the online version of this article.

Supplemental Table S1. Exogenous treatments of melatonin applied by irrigation in fruity crops.

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Conflict of interest statement. None declared.

Data availability

No new datasets were generated or analysed in this study.

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Supplemental Information

Table S1. Exogenous treatments of melatonin applied by irrigation in fruity crops. The developmental stage during application, the method used, the concentration applied, and the main effects are shown. As shown here, several studies have described the biostimulant function of melatonin by root application in fruit plants. However, most of them have been focused on its protective role against abiotic stresses, and how these treatments could affect fruit development or fruit ripening and quality are still unexplored.

Fruit	Developmental stage	Method	Applied concentration (M)	Main effects	Reference
Kiwifruit	10-leaf seedling	Irrigation, 2-day interval for 8 days	10^{-4}	↑ Root system architecture, ↓ Lipid peroxidation and pigment degradation ↑ Photosynthesis ↑ Adaptability to drought stress	Liang et al., 2019
	6-leaf seedling	Irrigation, twice at 3-day intervals	10^{-4}	Promoted shoot biomass and antioxidant enzyme activities Improved abiotic resistance	Xia et al., 2022
Cucumber	3-leaf seedling	Irrigation	10^{-4}	↑ Photosynthesis rate ↑ Antioxidant levels Promoted water-stress tolerance	Zhang et al., 2012
	2-leaf seedling	Irrigation, once daily, 6 days	$2 \cdot 10^{-4}$	↓ Photoinhibition Promoted tolerance to chilling conditions	Zhao et al., 2022
	Seedling	irrigation	10^{-5}	Tolerance to cinnamic acid stress	Li et al., 2017
	Seedling	Irrigation, 3 days	10^{-4}	Enhanced nitrate stress tolerance	Zhang et al., 2017
Tomato	4-leaf seedling	Irrigation, hydroponic culture	10^{-4}	Mitigated cadmium stress (↑ root architecture and mineral nutrient uptake)	Altaf et al., 2022
	Seedling	Irrigation, once every third day	10^{-4}	Enhanced salt stress tolerance (↑ growth, biomass production, antioxidant enzymes activity, root growth	Altaf et al., 2020

				and photosynthetic pigments content)	
	Seedling	Irrigation, every 2 days for 25 days	$2 \cdot 10^{-5}$ -$5 \cdot 10^{-5}$	Improved salt stress tolerance (↑ antioxidant system, proline and carbohydrates) Improved plant growth	Siddiqui et al., 2019
	6-leaf seedling	Irrigation, 3 consecutive days	10^{-4}	Mitigated low-temperature stress (↓ photosynthetic apparatus damage)	Yang et al., 2018
	Seedlings	Irrigation, twice at 3-day intervals	10^{-4}	Improved drought tolerance	Liu et al., 2015
Apple	After 3 months of growth	Irrigation	10^{-4}	Delayed drought-induced leaf senescence	Wang et al., 2013
	Seedling	Irrigation	10^{-6}	Mitigated UV-B stress	Wei et al., 2019
	Seedlings	Irrigation, hydroponic culture	10^{-7}	Enhanced tolerance and adaptability to future salt and nutrition deficiency stresses	Li et al., 2016
	Seedling	Irrigation, hydroponic culture	10^{-4}	Alleviated cadmium toxicity (↑ antioxidant defense)	He et al., 2020
Bitter orange	Seedlings	Irrigation, once a week for 30 days	10^{-6}	Improved salt adaptation	Kostopoulou et al., 2015

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