

Melatonin Is Involved in Citrus Response to the Pathogen Huanglongbing via Modulation of Phytohormonal Biosynthesis¹

Yasser Nehela,^{a,b} and Nabil Killiny^{a,2,3}

^aDepartment of Plant Pathology, Citrus Research and Education Center, University of Florida, Lake Alfred, Florida 33850

^bDepartment of Agricultural Botany, Faculty of Agriculture, Tanta University, 31512 Tanta, Egypt

ORCID IDs: 0000-0003-1177-8099 (Y.N.); 0000-0002-3895-6943 (N.K.)

Huanglongbing (HLB) is a devastating citrus disease worldwide that is putatively caused by *Candidatus Liberibacter asiaticus* and transmitted by *Diaphorina citri*. Melatonin is a ubiquitously distributed auxin-like metabolite found in both prokaryotes and eukaryotes. In this study, we used integrative metabolomic and transcriptomic approaches to investigate the potential role of melatonin in citrus response against HLB and to understand the relationships between melatonin and the stress-associated phytohormones at molecular and metabolic levels. Melatonin was detected in the leaves of Valencia sweet orange (*Citrus sinensis*) after derivatization with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide using a targeted gas chromatography-mass spectrometry running in selective ion monitoring mode-based method. *Ca. L. asiaticus* infection and *D. citri* infestation significantly increased endogenous melatonin levels in Valencia sweet orange leaves and upregulated the expression of its biosynthetic genes (*CsTDC*, *CsT5H*, *CsSNAT*, *CsASMT*, and *CsCOMT*). However, infection with *Ca. L. asiaticus* had a greater effect than did infestation with *D. citri*. Melatonin induction was positively correlated with salicylic acid content, but not that of trans-jasmonic acid. Moreover, melatonin supplementation enhanced the endogenous contents of the stress-associated phytohormones (salicylates, auxins, trans-jasmonic acid, and abscisic acid) and the transcript levels of their biosynthetic genes. Furthermore, melatonin supplementation diminished the *Ca. L. asiaticus* titer within the infected leaves, which suggests that melatonin might play an antibacterial role against this bacterium and gram-negative bacteria in general. These findings provide a better understanding of the melatonin-mediated defensive response against HLB via modulation of multiple hormonal pathways. Understanding the role of melatonin in citrus defense to HLB may provide a novel therapeutic strategy to mitigate the disease.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a ubiquitously distributed compound, found in both prokaryotes such as bacteria and eukaryotes including fungi, algae, plants, and animals (Hardeland et al., 2011; Lee et al., 2014; Arnao and Hernández-Ruiz, 2015). Melatonin was discovered first in the kingdom Animalia in 1958 (Lerner et al., 1958) and then in Plantae in 1995 (Dubbels et al., 1995; Hattori et al., 1995). Melatonin plays pleiotropic biological roles in both animals and plants. In mammals, it acts as a neurohormone, secreted by the pineal gland, which regulates numerous

physiological processes (Jan et al., 2009; Carrillo-Vico et al., 2013; Cipolla-Neto et al., 2014). It acts as a sleep regulator (Jan et al., 2009), an immunomodulatory molecule (Galano et al., 2011; Carrillo-Vico et al., 2013), a regulator of the circadian temporal internal order (Cipolla-Neto et al., 2014), and a natural antioxidant (Galano et al., 2011). In Plantae, melatonin (also known as phytomelatonin) modulates many physiological functions. For example, it stimulates plant growth and development in dicots (Hernández-Ruiz et al., 2004) and monocots (Hernández-Ruiz et al., 2005). Melatonin promotes adventitious and lateral root regeneration and elongation (Arnao and Hernández-Ruiz, 2007; Sarropoulou et al., 2012; Zhang et al., 2013). Additionally, it has a key role in seedling growth enhancement (Byeon et al., 2014), flowering delay (Kolar et al., 2003; Byeon and Back, 2014), and fruit ripening and fruit quality improvement (Sun et al., 2015).

As a secondary metabolite, melatonin is implicated in tolerance to abiotic stresses such as drought (Zhang et al., 2013; Wei et al., 2015), salinity (Zhang et al., 2014; Wei et al., 2015), freezing (Uchendu et al., 2013), and high temperature (Tiryaki and Keles, 2012). On the contrary, the protective role of melatonin against biotic

¹This work was supported by the USDA National Institute of Food and Agriculture (grant no. 2016-70016-24844 to N.K.).

²Author for contact: nabilkilliny@ufl.edu.

³Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Nabil Killiny (nabilkilliny@ufl.edu).

N.K. conceptualized the idea; N.K. together with Y.N. designed the experiments; Y.N. carried out the experiments, analyzed the data, finalized the figures, and drafted the article; N.K. and Y.N. revised and finalized the article.

www.plantphysiol.org/cgi/doi/10.1104/pp.20.00393

stresses in plants is still unclear and not well studied. A few reports discussed the mechanisms of melatonin-dependent pathways in the plant response to phytopathogen infection (Yin et al., 2013; Lee et al., 2014, 2015; Shi et al., 2015; Lee and Back, 2016). For example, in apple trees (*Malus prunifolia*), exogenous melatonin treatment improved resistance to *Diplocarpon mali*, the fungal pathogen of Marssonina apple blotch (Yin et al., 2013). Additionally, melatonin and its derivatives, *N*-acetylserotonin and 2-hydroxymelatonin, improved plant resistance against the bacterial pathogen *Pseudomonas syringae* pv *tomato* strain DC3000 (*Pst*-DC3000) in *Arabidopsis* (*Arabidopsis thaliana*; Lee et al., 2014, 2015; Shi et al., 2015; Lee and Back, 2016) and *Nicotiana benthamiana* (Lee et al., 2014; Lee and Back, 2016). The protective role of melatonin is associated with the pathogenesis-related proteins (PRs; Yin et al., 2013; Lee et al., 2014; Shi et al., 2015), mitogen-activated protein kinases (MAPKs; Lee and Back, 2016), *C-REPEAT-BINDING FACTORS/DROUGHT RESPONSE ELEMENT-BINDING1 FACTORS* (Shi et al., 2015, 2016), nitric oxide (NO; Shi et al., 2015), and phytohormones such as salicylic acid (SA; Lee et al., 2014, 2015).

Phytohormones play key roles in host responses to a wide range of pathogen infections, insect attacks, and abiotic stresses (Bari and Jones, 2009). SA is associated with plant defense responses to biotrophic and hemibiotrophic pathogens and plays a key role in the establishment of systemic acquired resistance (Hatcher et al., 2004; Glazebrook, 2005; Bari and Jones, 2009). NPR1, a key signaling molecule in the SA-mediated pathway, acts as a receptor for SA (Wu et al., 2012). On the other hand, the jasmonic acid/ethylene (JA/ET)-mediated pathway is involved in defense responses against necrotrophic pathogens and insect herbivory (Mur et al., 2006; Bari and Jones, 2009). Many signaling molecules are implicated in the JA/ET-mediated pathways, such as MAPKs (Turner et al., 2002; Meldau et al., 2012) and *ETHYLENE INSENSITIVE2* (which interacts with *ETHYLENE RECEPTOR1*; Bisson et al., 2009).

Huanglongbing (HLB) is a devastating disease causing great losses in citrus industries worldwide (Bové, 2006). HLB is associated with *Candidatus Liberibacter* species, a fastidious, gram-negative, phloem-limited, and nonculturable α -proteobacterium (Bové, 2006; Gottwald, 2010). Taxonomically, three bacterial species have been identified as causal pathogens of HLB based on the characteristic 16S rDNA sequences and their geographic distribution (Bové and Ayres, 2007; Tatineni et al., 2008; Gottwald, 2010; Wang and Trivedi, 2013). *Candidatus Liberibacter asiaticus* is a heat-tolerant species spreading in the Arabian Peninsula, Africa, Asia, Brazil, and North America; *Candidatus Liberibacter americanus* is a heat-tolerant species spreading only in Brazil; and *Candidatus Liberibacter africanus* is a heat-sensitive species spreading in numerous countries in Africa (Bové and Ayres, 2007; Tatineni et al., 2008; Gottwald, 2010; Wang and Trivedi, 2013). The three *Candidatus Liberibacter* species are

transmitted by two species of citrus psyllids. Both *Ca. L. asiaticus* and *Ca. L. americanus* are typically transmitted by the Asian citrus psyllid *Diaphorina citri*, while *Ca. L. africanus* is transmitted by *Trioza erytreae* (Bové and Ayres, 2007; Gottwald, 2010; Wang and Trivedi, 2013).

Citrus plants have developed an innate immune system with multilayered complex defense responses to mediate the harmful effects of *Ca. L. asiaticus* infection and its vector, *D. citri*. These defense mechanisms are not only mechanical but also include the accumulation of defense molecules, alteration of the plant signaling system, changes in primary and secondary metabolites, and other biochemical and physiological modifications. Our previous studies showed that several leaf metabolites are involved directly or indirectly in plant defense response against HLB. These metabolites include volatile organic compounds (Hijaz et al., 2013), proteinogenic amino acids (Killiny and Hijaz, 2016; Killiny and Nehela, 2017a), nonproteinogenic amino acids and polyamines (Nehela and Killiny, 2019), organic and fatty acids (Killiny and Nehela, 2017a), tricarboxylic acid-associated compounds and γ -aminobutyrate shunt (Nehela and Killiny, 2019), phytohormones (Nehela et al., 2018), leaf pigments (Killiny and Nehela, 2017b), and other secondary metabolites. However, the interaction between these metabolites is poorly understood. We believe that the identification of these compounds and understanding the relationships between them will further help us to clarify the underlying molecular mechanisms of the citrus immunity system. Although the role of phytohormones, such as SA and JA, in citrus response to HLB is well reported, the potential role of melatonin in citrus response against HLB is poorly studied, and its role in citrus immunity/response remains unclear. Furthermore, the mechanisms behind the melatonin-mediated defensive response are inadequately explored.

Although some previous studies reported that exogenous melatonin application may trigger the plant defense responses against phytopathogenic bacteria (Lee et al., 2014, 2015; Qian et al., 2015; Shi et al., 2015; Zhao et al., 2015; Lee and Back, 2016, 2017a; Wei et al., 2018a; Chen et al., 2019b; Xian et al., 2020), none of these studies have investigated its relationships with the major groups of stress-associated phytohormones, particularly SA and trans-jasmonic acid (*t*JA). In this study, we used integrative metabolomic and transcriptomic approaches to (1) investigate the potential role(s) of melatonin in citrus response to different biotic stressors, including the phytopathogenic bacterium *Ca. L. asiaticus* and its insect vector *D. citri*; (2) provide a better understanding of the melatonin-mediated defensive response and its relationships with the major groups of stress-associated phytohormones, including salicylates (SAs), auxins, *t*JA, and abscisic acid (ABA), and the gene expression patterns of their biosynthetic genes; and (3) elucidate the antibacterial role of exogenous melatonin against *Ca. L. asiaticus* within HLB-infected Valencia sweet orange (*Citrus sinensis*) plants. We believe that melatonin is not

only involved in the induction of both the SA-mediated pathway against *Ca. L. asiaticus* and the JA-mediated pathway against *D. citri* but also might be involved with other hormonal pathways, such as auxins and ABA, to enhance the disease resistance for HLB. Understanding the role of melatonin in citrus defense to HLB may provide a novel therapeutic strategy to mitigate the disease.

RESULTS

Detection of Melatonin in Valencia Sweet Orange Leaves Using GC-MS-SIM

Melatonin was detected in the leaves of Valencia sweet orange after derivatization with 50 μ L of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) containing 1% (v/v) chlorotrimethylsilane at 85°C using targeted gas chromatography-mass spectrometry running in selective ion monitoring mode (GC-MS-SIM). The typical total ion current chromatograms obtained for a prepared melatonin standard of known concentration (20 ppm) and endogenous melatonin from Valencia sweet orange leaves (healthy, *Ca. L. asiaticus* infected, *D. citri* infested, and double attacked) are presented in Figure 1, A and B, respectively. The GC results for the melatonin standard in scan mode showed that the derivatization procedure gives the bis-trimethyl silyl (bis-TMS) derivative of melatonin as a final product. The mass spectrum of the bis-TMS-melatonin standard is presented in Figure 1C (in full scan mode) and Figure 1D (in SIM mode). The constituent mass-to-charge ratio (m/z) ions for melatonin are as follows: m/z M^+ = 376, 304, 246, 245, 232, 202, and 160. In SIM mode, the MS spectrum profile of melatonin in Valencia sweet orange leaves (Fig. 1E) matches the MS spectrum profile of the melatonin standard (Fig. 1D). Seven ions were used for the identification of melatonin in Valencia sweet orange leaves, while the base ion 232 was used for quantification purposes. Although m/z 73 was the most abundant ion in the scan mode, it was not used for the identification or quantification of melatonin because it is a signature ion fragment for TMS derivatives and does not represent melatonin.

Ca. L. asiaticus Infection Altered the Melatonin, SA, and tJA Content of Valencia Sweet Orange Leaves

Our GC-MS results showed that both *Ca. L. asiaticus* infection and/or *D. citri* infestation significantly increased the endogenous melatonin content of Valencia sweet orange leaves, with a greater effect of *Ca. L. asiaticus* alone (Fig. 2A). Both *Ca. L. asiaticus*-infected and double-attacked trees had high concentrations of melatonin ($1,922.69 \pm 797.21$ and $1,591.68 \pm 612$ μ g g^{-1} fresh weight, respectively) without significant differences between them, followed by *D. citri*-infested trees (777.67 ± 404.41 μ g g^{-1} fresh weight), which were lower in melatonin content than *Ca. L. asiaticus*-infected

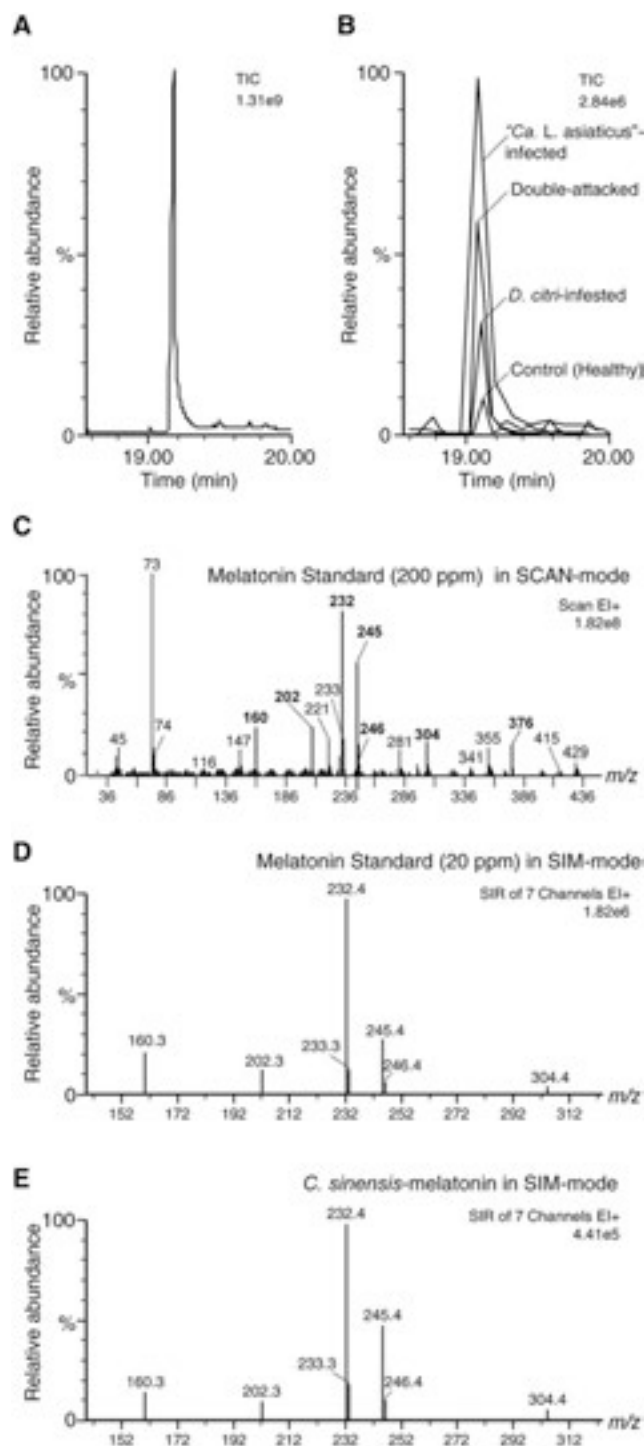


Figure 1. Chromatographic characterizations of phytomelatonin detected in Valencia sweet orange leaves after infection with *Ca. L. asiaticus* and/or infestation with *D. citri* using targeted GC-MS-SIM. A, Chromatogram of melatonin authentic standard (20 ppm). B, Phytomelatonin from healthy (control), *Ca. L. asiaticus*-infected, *D. citri*-infested, or double-attacked (*Ca. L. asiaticus* infected and *D. citri* infested at the same time) Valencia sweet orange leaves. C to E, Mass spectra of melatonin authentic standard (20 ppm) in full scan mode, melatonin authentic standard (20 ppm) in SIM mode, and phytomelatonin from Valencia sweet orange leaves in SIM mode, respectively.

trees but higher than the control ($164.50 \pm 30.25 \text{ pg g}^{-1}$ fresh weight; Fig. 2A).

Likewise, SA has almost the same profile of melatonin, and it has been associated with citrus defense response to *Ca. L. asiaticus* infection (Fig. 2B). *Ca. L. asiaticus*-infected trees had the highest concentration of SA ($2,601.22 \pm 609.36 \text{ ng g}^{-1}$ fresh weight), followed by double-attacked and *D. citri*-infested trees ($1,896.37 \pm 370.08$ and $1,106.22 \pm 227.44 \text{ ng g}^{-1}$ fresh weight, respectively), which were also higher in SA content than the control ($554.28 \pm 88.91 \text{ ng g}^{-1}$ fresh weight; Fig. 2B).

On the other hand, *t*JA has been associated with citrus defense response against *D. citri*. Infestation with *D. citri* significantly increased the *t*JA content ($395.56 \pm 39.38 \text{ ng g}^{-1}$ fresh weight), followed by double-attacked trees ($265.29 \pm 28.08 \text{ ng g}^{-1}$ fresh weight), while it did not affect the *t*JA levels in control or *Ca. L. asiaticus*-infected trees (138.67 ± 12.46 and $166.22 \pm 16.62 \text{ ng g}^{-1}$ fresh weight, respectively; Fig. 2C).

Furthermore, to understand the relationship between the endogenous melatonin content and the endogenous levels of SA and *t*JA, data were fitted using a simple linear regression (SLR) model and a second-degree polynomial regression model. Interestingly, the SLR showed that the endogenous melatonin content was positively correlated with the level of SA ($y = 0.69x + 774.79$; $R^2 = 0.476$, $R^{2\text{adj}} = 0.462$, and $P < 0.0001$; Fig. 2D), while no significant correlation was observed between endogenous melatonin content and *t*JA levels ($y = -0.0011x + 242.67$; $R^2 = 0.0001$, $R^{2\text{adj}} = 0.026$, and $P = 0.9551$; Fig. 2E).

Furthermore, to study the nonlinear phenomena between melatonin content and the levels of SA and *t*JA, data were fitted with a second-degree polynomial regression model. Data presented in Figure 2, D, and E, show the polynomial fit models and their 95% confidence curves for the estimated regression for both SA and *t*JA, respectively. Generally, the relationship between endogenous melatonin content and SA levels followed a positive and quadratic model, which were described by the equation $y = -0.00039x^2 + 1.78x + 331.43$ ($R^2 = 0.602$, $R^{2\text{adj}} = 0.581$, and $P < 0.0001$; Fig. 2D). On the other hand, although the relationship between endogenous melatonin content and *t*JA levels followed a positive and quadratic model ($y = -0.000058x^2 + 0.16x + 177.08$), this relationship was very weak ($R^2 = 0.188$, $R^{2\text{adj}} = 0.144$, and $P < 0.0212$; Fig. 2D).

***Ca. L. asiaticus* Infection Altered the Gene Expression of Melatonin Biosynthetic Genes in Valencia Sweet Orange Leaves**

We investigated the transcript levels of 15 melatonin biosynthetic genes of citrus (Fig. 3A). Four reference/housekeeping genes, *CsEF1*, *CsF-box*, *CsGAPDH*, and *CsSAND* (Supplemental Table S1), were used for gene expression data normalization, which previously showed high stability for transcript normalization in citrus under biotic stress (Mafra et al., 2012; Wei et al., 2014a, 2014b). There were no significant differences between

the normalized transcript levels using the four housekeeping genes. The differential relative expression levels of the 15 melatonin biosynthetic genes are visualized as a heat map combined with a nonstandardized two-way HCA and presented in Figure 3A. Generally, the presence of *Ca. L. asiaticus* induced the expression levels of all investigated genes (Fig. 3A).

The total HCA dendrogram among treatments (presented at the bottom of Fig. 3A) showed that the transcript levels of *Ca. L. asiaticus*-infected and double-attacked trees were closer to each other and clustered together (the half-square Euclidean distance was less than 3; Fig. 3A). In contrast, *D. citri*-infested treatment was clustered with the control (healthy) trees (the half-square Euclidean distance was approximately 6).

Furthermore, the total HCA dendrogram among the tested melatonin biosynthetic genes showed that all 15 genes separated into two distinct clusters. Cluster I included the majority of studied genes (nine genes), which were expressed at higher levels in both *Ca. L. asiaticus*-infected and double-attacked trees, with a relative fold change greater than 4 (Fig. 3A). These genes included *CsTDC1* (or *CsAADC-like*), *CsSNAT-2*, and seven *CsASMT* genes. Cluster II included only six genes, which were expressed at higher levels in both *Ca. L. asiaticus*-infected and double-attacked trees, but with a relative fold change less than 4 (Fig. 3A). Genes in cluster II included *CsSNA-1*, *CsT5H* (or *CsCYP71P1*), *CsTDC2*, and three *CsCOMT* genes. According to these findings, the gene expression results supported our findings from the GC-MS work.

Melatonin Supplementation Altered the Gene Expression of Melatonin Biosynthesis Genes in Valencia Sweet Orange Trees

We investigated the impact of exogenous melatonin supplementation on the transcript levels of melatonin biosynthesis genes of citrus. In general, the 15 genes studied were upregulated (up to 5.2-fold) at 72 h post-treatment (hpt) in all melatonin treatments compared with control trees (Fig. 3B). The transcript levels of melatonin biosynthesis genes were elevated by the low melatonin concentrations (0.05 and 0.1 mM), which clustered together and separately from other treatments (the half-square Euclidean distance was less than 6; Fig. 3B). Furthermore, the total HCA dendrogram among the melatonin biosynthesis genes showed that the different melatonin concentrations separated the 15 genes into four distinct clusters. Cluster I includes five genes (*CsTDC1* [or *CsAADC-like*], *CsCOMT-1*, and three *CsASMT1* genes), which were expressed at higher levels after the treatment of 0.1 mM melatonin (Fig. 3B). Likewise, Cluster II includes five genes (*CsTDC2*, *CsT5H* [or *CsCYP71P1*], *CsASMT2*, and two *CsSNAT* genes), which were expressed similarly in all treatments, but higher than the control (Fig. 3B). Cluster III includes three *CsASMT* genes, which were expressed similarly when the trees were treated with 0.05 or

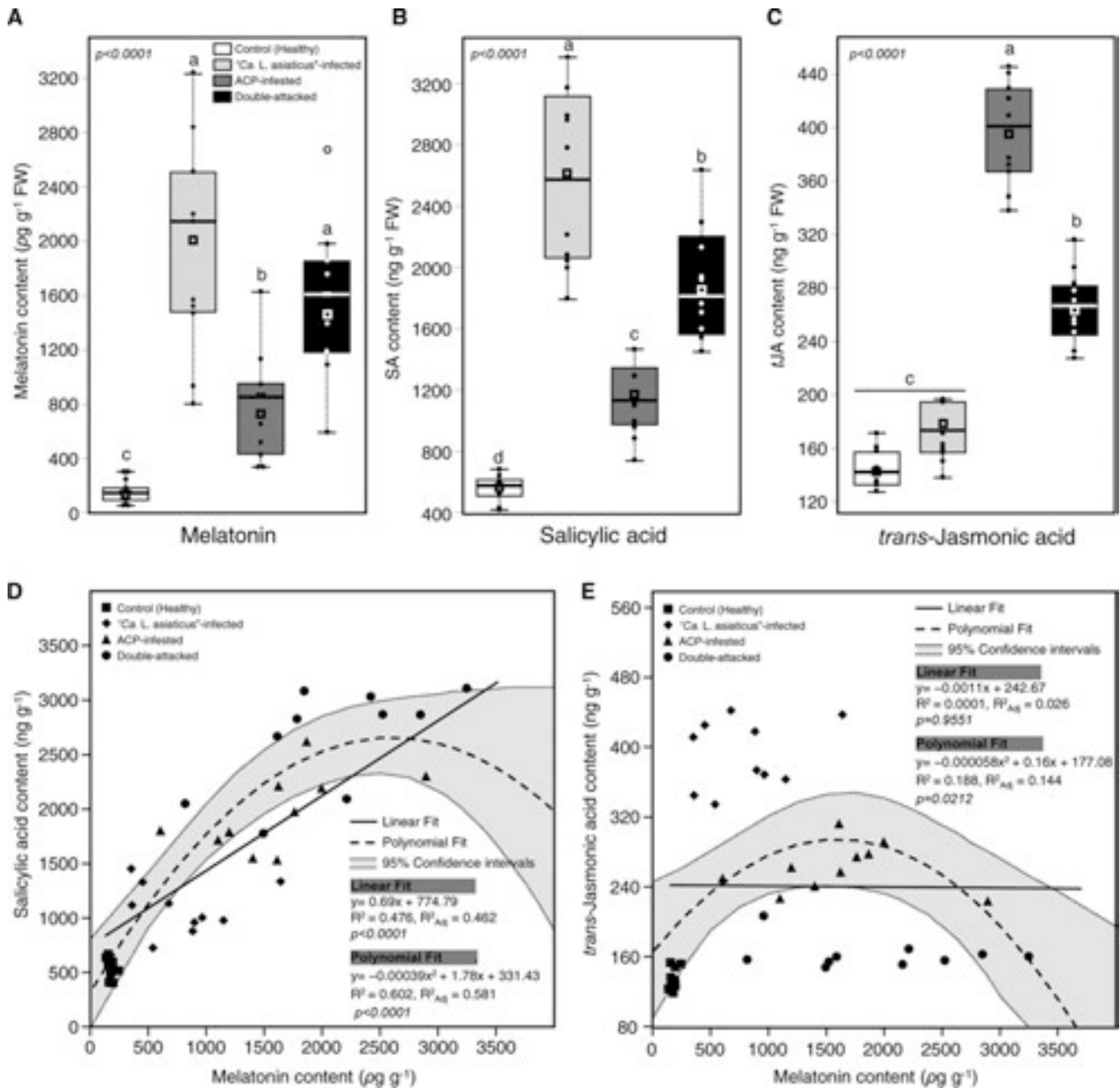


Figure 2. Endogenous contents of phytochemicals detected in Valencia sweet orange leaves after infection with *Ca. L. asiaticus* and/or infestation with *D. citri* using GC-MS-SIM. A to C, Endogenous contents of phytochemicals (pg g⁻¹ fresh weight [FW]; n = 10), SA (ng g⁻¹ fresh weight; n = 10), and tJA (ng g⁻¹ fresh weight; n = 10), respectively, from healthy (control), *Ca. L. asiaticus*-infested, *D. citri*-infested, or double-attacked Valencia sweet orange leaves. Whiskers indicate the minimum and maximum values of the data, horizontal thick lines show the medians, black or white open squares signify the means, black or white dots indicate the raw data, and boxes show the interquartile ranges (25th to 75th percentiles of the data). Different letters indicate statistically significant differences among treatments, while the same letter signifies no significant differences between them using the Tukey-Kramer honestly significant difference test (Tukey's HSD; P < 0.05). D and E, Simple linear regression and quadratic polynomial regression analyses between endogenous phytochemicals and SA (D) and tJA (E). Black shapes represent the raw data (n = 10; see the key at the top left corner of the graph). The fitted regression line is presented as a solid line, while polynomial regression models are presented as dashed lines. The 95% confidence intervals for the estimated regression are gray shaded and edged by dotted lines. Regression equations, R², R^{2adj}, and P value based on the F test (P ≤ 0.05) were also obtained and are presented within the graph.

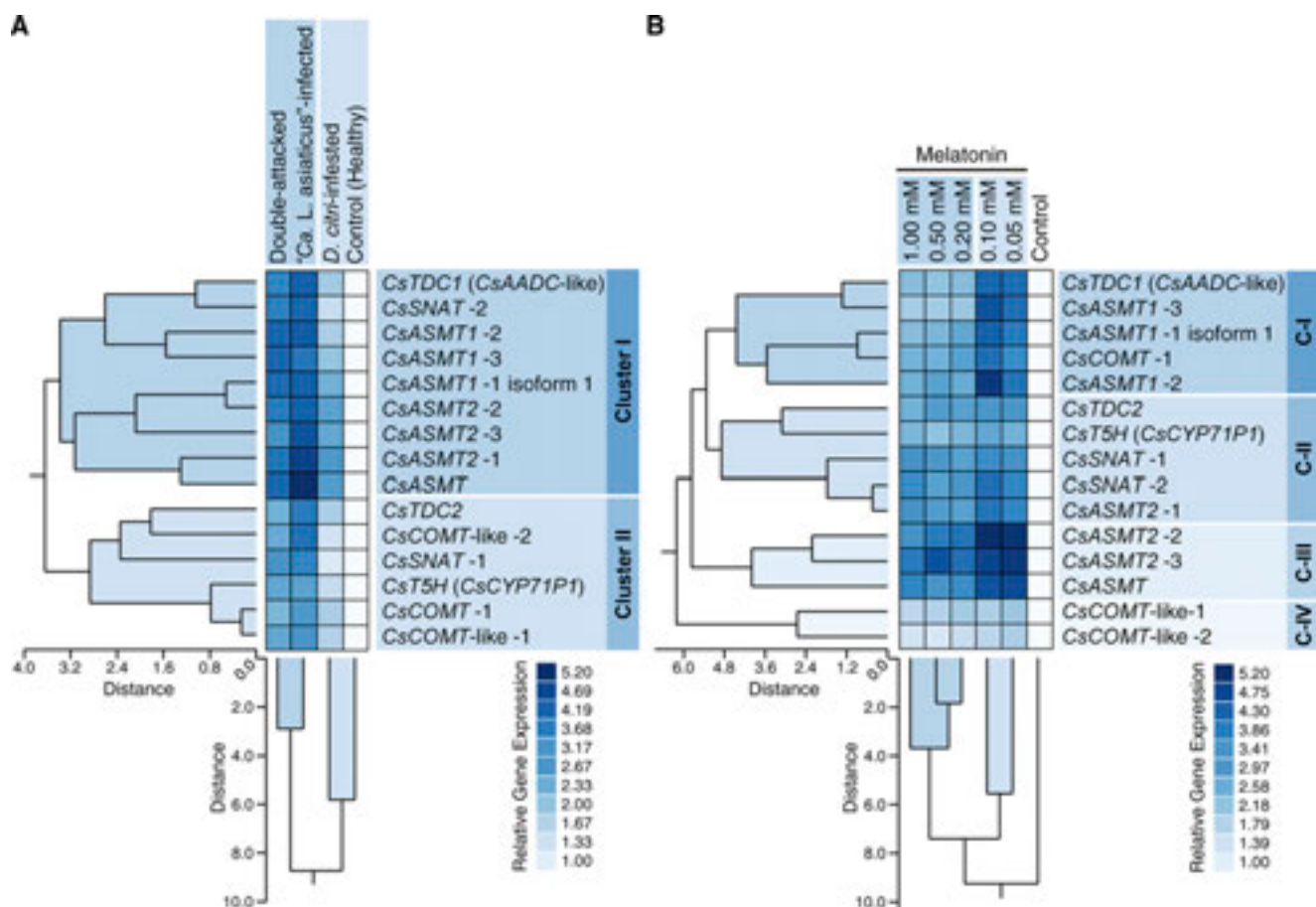


Figure 3. Relative expression of genes involved in the melatonin biosynthesis pathway in Valencia sweet orange. Two-way hierarchical cluster analysis (HCA) and its associated heat map diagram of differential nonstandardized gene expression patterns of the predicted melatonin biosynthetic genes in Valencia sweet orange leaves after infection with *Ca. L. asiaticus* and/or infestation with *D. citri* (A) or after melatonin supplementation (B) are shown. Rows represent the genes, whereas columns represent different treatments. Low expression levels are colored light blue and high expression levels are colored dark blue (see the scale at the right bottom corner of the heat map). Treatments and genes in the HCA were organized using Ward's minimum variance method (Ward, 1963). The full list of expressed genes, names, accession numbers, and primers is available in Supplemental Table S1.

0.1 mM melatonin, but much higher than other treatments. Cluster IV includes two *CsCOMT-like* genes, which were slightly induced (approximately twofold increases) after melatonin treatment (Fig. 3B). Generally, the expression levels of most, if not all, of the melatonin biosynthetic genes were increased after melatonin supplementation, particularly after the treatment with a low concentration (0.05 or 0.1 mM melatonin), which supported our GC-MS findings.

Melatonin Supplementation Enhanced the SAs Content of Valencia Sweet Orange Leaves

The impact of treatment with melatonin on SAs, including benzoic acid (BA), trans-cinnamic acid (tCA), and SA, was investigated. In general, at 12 hpt, the application of 0.1 mM melatonin induced the accumulation of both BA (Fig. 4A) and tCA (Fig. 4B) but not SA (Fig. 4C). In all melatonin treatments, BA, tCA, and SA

began to rise markedly at 48 hpt until they reached their highest peak at 72 hpt, then plummeted when measured at 96 and 120 hpt. The trees treated with 0.1 mM melatonin had the highest BA, tCA, and SA levels throughout the experiment, particularly at 72 hpt (3.23-, 2.33-, and 2.26-fold higher, respectively; Fig. 4).

To understand the relationship between the additive melatonin and the endogenous content of different SAs, data were fitted with a second-degree polynomial regression model, because our data follow nonlinear phenomena. Data presented in Figure 4, D to F, show the fit means models, polynomial regression models, and the 95% confidence curves for the estimated regression for BA, tCA, and SA, respectively. Generally, the relationship between supplementary melatonin rates and different SAs followed a positive and quadratic model. The relationship between exogenous melatonin rates and BA is described by the equation $BA = -2,908.6x^2 + 1,206.5x + 4,857.3$ ($R^2 = 0.074$, $R^2_{adj} = 0.018$, and $P = 0.2826$), while for tCA it is described by

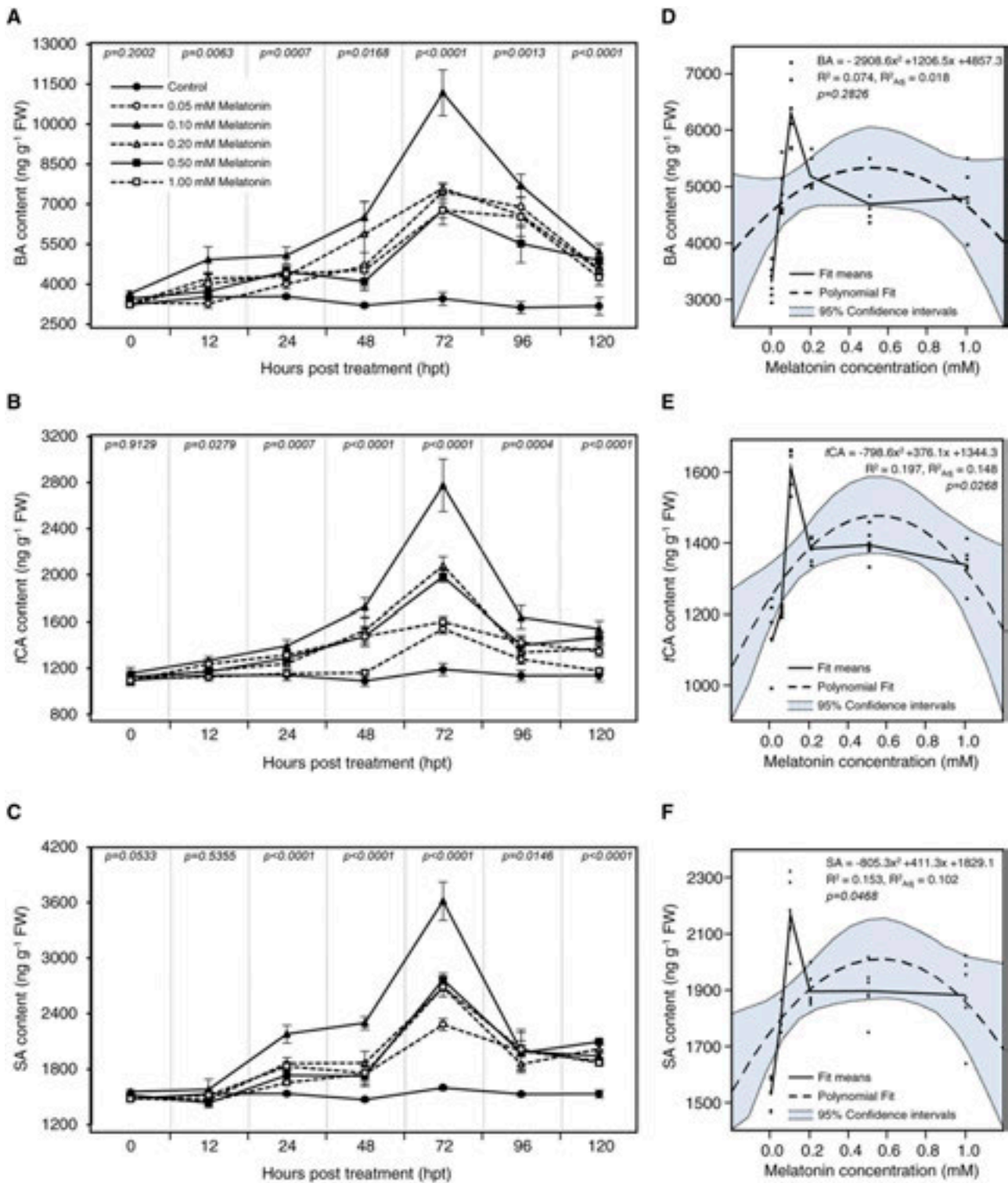


Figure 4. Effects of exogenous melatonin supplementation on the endogenous SAs content of Valencia sweet orange. A to C, Endogenous levels of BA (A), tCA (B), and SA (C) after the exogenous melatonin treatments. Valencia sweet orange trees were treated with 150 mL of Milli-Q water (control; 0 mM melatonin) or 0.05, 0.1, 0.2, 0.5, or 1 mM melatonin. Data shown are means \pm SD ($n = 5$). $P < 0.05$ indicates statistically significant differences among treatments, while $P > 0.05$ indicates no significant differences among them using Tukey's HSD. D to F, Fit means models and quadratic polynomial regression analysis between exogenous melatonin concentrations and endogenous contents of BA (D), tCA (E), and SA (F). Black dots represent the raw data ($n = 6$). The fit means line is presented as a solid line, while polynomial regression models are presented as dashed lines. The 95%

tCA = $-798.6x^2 + 376.1x + 1,344.3$ ($R^2 = 0.197$, $R^{2\text{adj}} = 0.148$, and $P = 0.0268$) and for SA it is described by SA = $-805.3x^2 + 411.3x + 1,829.1$ ($R^2 = 0.153$, $R^{2\text{adj}} = 0.102$, and $P = 0.0468$). In addition, the fit means model for each value showed that the treatment with 0.1 mM melatonin induced the accumulation of BA (Fig. 4D), tCA (Fig. 4E), and SA (Fig. 4F) to higher levels compared with nontreated trees and other treatments, regardless of the sampling time.

Melatonin Supplementation Increased the Auxin Content of Valencia Sweet Orange Leaves

We investigated the effect of supplementary melatonin on three auxins, indole-3-acetic acid (IAA; Fig. 5A), indole-3-propionic acid (IPA; Fig. 5B), and indole-3-butyric acid (IBA; Fig. 5C). Like SA, the application of exogenous melatonin did not significantly affect any of the auxin contents at 12 hpt, while the auxin concentrations began to rise markedly at 24 hpt until the end of the experiment. Interestingly, the trees treated with 0.1 or 0.2 mM melatonin had the highest IAA and IBA levels during the experiment (Fig. 5, A and C, respectively), without significant differences between either treatment in most sampling points. On the other hand, treatment with 0.05 or 0.1 mM melatonin led to the highest IPA levels (Fig. 5B), particularly at the end of the experiment (72, 96, and 120 hpt), without significant differences between the two treatments. No noticeable reduction was recorded in the contents of the three auxins during the experiment until after 96 hpt, although some treatments showed a slight reduction after 120 hpt.

Because of the nonlinear relationship between exogenous melatonin concentrations and the endogenous content of different auxins, data were fitted using a second-degree polynomial regression model and are presented in Figure 5, D to F. Generally, the relationship between supplementary melatonin rates and different auxins followed a positive and quadratic model. The relationship between exogenous melatonin rates and IAA is described by the equation $IAA = -166.6x^2 + 76.4x + 283.5$ ($R^2 = 0.139$, $R^{2\text{adj}} = 0.086$, and $P = 0.0358$), while for IPA it is described by $IPA = 6x^2 + 2.6x + 340.4$ ($R^2 = 0.022$, $R^{2\text{adj}} = 0.037$, and $P = 0.6911$) and for IBA it is described by $IBA = -28.3x^2 + 10.9x + 346.7$ ($R^2 = 0.173$, $R^{2\text{adj}} = 0.123$, and $P = 0.0432$). In addition, the fit means model for each value showed that the accumulation of IAA was induced by treatment with 0.1 or 0.2 mM melatonin, without significant differences between them (Fig. 5D). Similarly, the accumulation of IPA was induced by treatment with 0.05 or 0.1 mM melatonin, without significant differences between them (Fig. 5E),

and IBA was induced by treatment with 0.1 mM melatonin (Fig. 5F), regardless of the sampling time.

Melatonin Supplementation Induced the Accumulation of tJA in Valencia Sweet Orange Leaves

The impact of treatment with melatonin on tJA was investigated and is presented in Figure 6A. The endogenous tJA contents increased slightly at 12 hpt, then began to increase at 24 hpt until they reached their highest peak at 72 hpt (for the lowest melatonin treatment [0.05 mM]), but it decreased thereafter. The trees treated with 0.05 mM melatonin had the highest tJA levels throughout the experiment, particularly at 72 hpt (1.52-fold increase; Fig. 6A).

The nonlinear relationship between exogenous melatonin concentrations and the endogenous tJA content was fitted using a second-degree polynomial regression model and is presented in Figure 6B. Generally, the relationship between supplementary melatonin rates and tJA content followed a positive and quadratic model and could be described by the equation $tJA = -50.5x^2 + 32.2x + 337.6$ ($R^2 = 0.061$, $R^{2\text{adj}} = 0.004$, and $P = 0.3537$). In addition, the fit means model for each value showed that the accumulation of tJA was induced by treatment with 0.05 mM melatonin (Fig. 6B), regardless of the sampling time.

Melatonin Supplementation Increased the ABA Content of Valencia Sweet Orange Leaves

The effect of supplementary melatonin on ABA content was studied and is presented in Figure 7A. Like SA and auxins, the application of exogenous melatonin did not affect the ABA content at 12 hpt; however, the ABA endogenous levels increased significantly at 24 hpt, reaching the highest peak at 96 hpt. The trees treated with 0.05 or 0.1 mM melatonin had the highest ABA levels during the experiment (Fig. 7A), without significant differences between the treatments in all sampling points. At 120 hpt, a slight reduction in ABA content was recorded in the trees treated with 0.05 or 0.1 mM melatonin.

Results from the second-degree polynomial regression analysis of the nonlinear relationship between exogenous melatonin concentrations and the endogenous ABA content showed a positive and quadratic relationship (Fig. 7B). The relationship between exogenous melatonin rates and ABA is described by the equation $ABA = 12.6x^2 - 25.6x + 342.3$ ($R^2 = 0.093$, $R^{2\text{adj}} = 0.038$, and $P = 0.1988$). In addition, the fit means model for each value showed that the accumulation of ABA was

Figure 4. (Continued.)

confidence intervals for the estimated regression are gray shaded and edged by dotted lines. Regression equations, R^2 , $R^{2\text{adj}}$, and P value based on the F test ($P \leq 0.05$) were also obtained and are presented within the graphs. FW, Fresh weight.

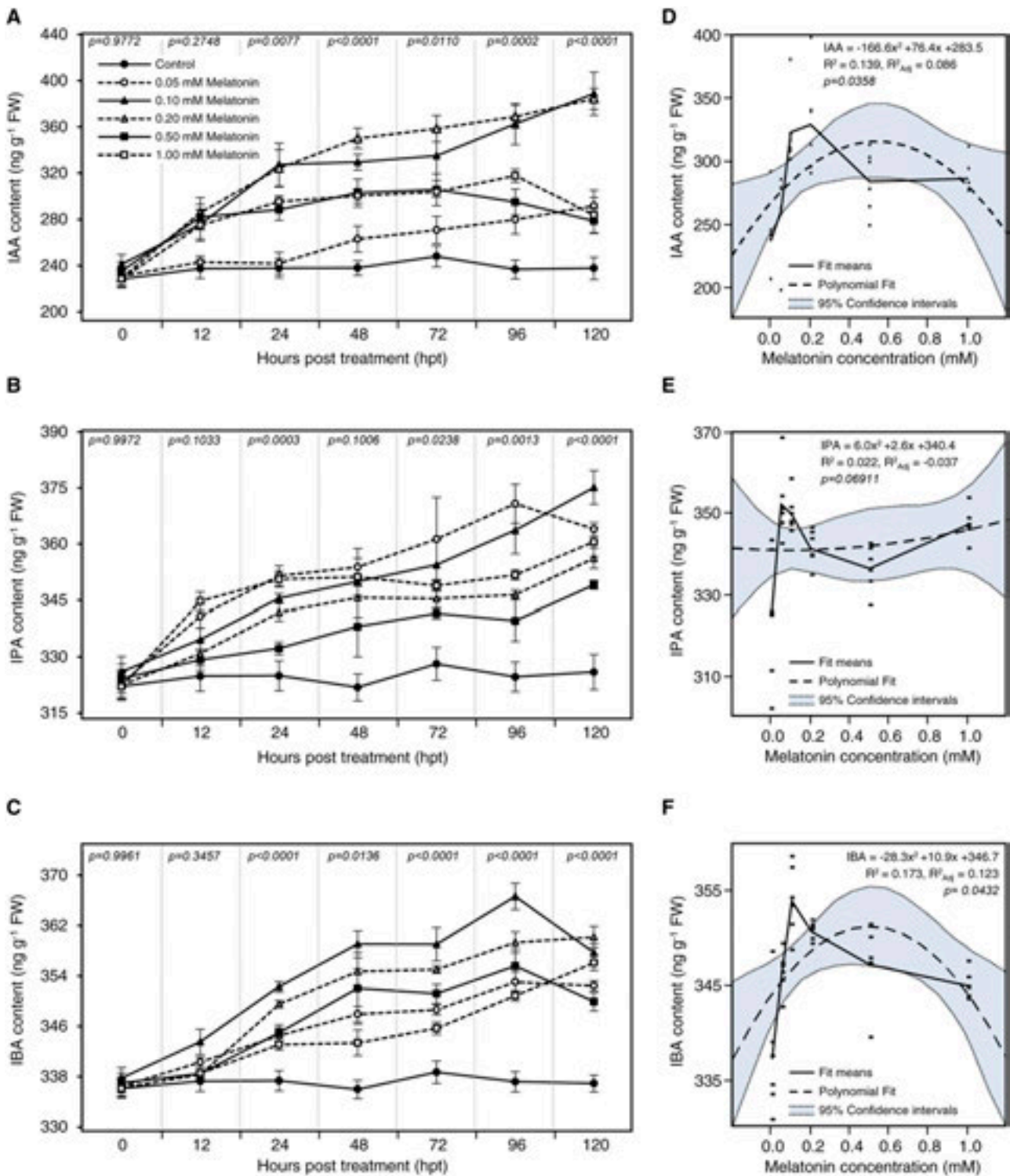


Figure 5. Effects of exogenous melatonin supplementation on the endogenous auxins content of Valencia sweet orange. A to C, Endogenous levels of IAA (A), IPA (B), and IBA (C) after the exogenous melatonin treatments. Valencia sweet orange trees were treated with 150 mL of Milli-Q water (control; 0 mM melatonin) or 0.05, 0.1, 0.2, 0.5, or 1 mM melatonin. Data shown are means \pm SD ($n = 5$). $P < 0.05$ indicates statistically significant differences among treatments, while $P > 0.05$ indicates no significant differences among them using Tukey’s HSD. D to F, Fit means models and quadratic polynomial regression analysis between exogenous melatonin concentrations and endogenous contents of IAA (D), IPA (E), and IBA (F). Black dots represent the raw data ($n = 6$). The fit means line is presented as a solid line, while polynomial regression models are presented as dashed lines. The 95%

induced by treatment with 0.05 mM melatonin (Fig. 7B), regardless of the sampling time.

Melatonin Supplementation Differentially Altered the Expression of the Phytohormone Biosynthetic Genes

In total, we investigated the transcript levels of 38 genes involved in four phytohormone biosynthesis pathways in Valencia sweet orange trees at 72 hpt with melatonin. These genes included 11 genes of SAs biosynthesis (Fig. 8A), 11 genes of auxin biosynthesis (Fig. 8B), 10 genes of *t*JA biosynthesis (Fig. 8C), and six genes involved in the ABA biosynthesis pathway (Fig. 8D). Gene expression data were normalized using four reference/housekeeping genes (*CsEF1*, *CsF-box*, *CsGAPDH*, and *CsSAND*), as described above. The normalized transcript levels using the four reference genes were very similar to each other. Generally, the expression levels of most of the phytohormone biosynthetic genes increased after melatonin supplementation (Fig. 8), which supported our GC-MS findings.

Generally, all SAs biosynthetic genes were expressed at higher levels after melatonin supplementation, with a greater effect of the low melatonin concentrations (0.05 and 0.1 mM), which were clustered together (Euclidean distance was approximately 18; Fig. 8A). Chorismate synthase (*CsCS*) had the highest gene expression among all SAs biosynthetic genes (about 12.6-fold increase) when Valencia sweet orange trees were treated with 0.1 mM melatonin, followed by arogenate dehydratase/prephenate dehydratase1 (*CsADT1*) and tyrosine aminotransferase (*CsTAT*) at the same treatment (approximately 11.5-fold; Fig. 8A). Interestingly, *CsCS* catalyzes the last step in the shikimate pathway to produce chorismate, which is used for the biosynthesis of aromatic amino acids such as Phe (the precursor of SA) and Trp (the precursor of auxins and melatonin).

Likewise, the transcript levels of all auxin biosynthetic genes were induced after supplementation with 0.1 and 0.2 mM melatonin, which were clustered together (Euclidean distance was less than 2.5; Fig. 8B). The total HCA dendrogram among auxin biosynthetic genes showed that the 11 genes separated into three distinct clusters. Cluster I includes five genes that were highly expressed after melatonin supplementation, but less than 4-fold (Fig. 8B). Cluster II included three genes that were expressed up to 6-fold higher. Cluster III included another three genes that were expressed more than 6-fold higher (Fig. 8B). Interestingly, cluster III includes two Trp:pyruvate aminotransferase genes (*CsTAA2* and *CsTAA4*) and the indole-3-pyruvate monooxygenase (*CsYUC2*). All of these genes are involved in the biosynthesis of IAA from Trp via indole-3-pyruvate as an intermediate compound. Furthermore,

CsYUC2 had the highest transcript levels among all IAA biosynthetic genes (up to a 7.2-fold increase) when Valencia sweet orange trees were treated with any melatonin concentration except 1 mM.

All *t*JA biosynthesis genes were upregulated after melatonin supplementation with greater effect for the lowest concentration (0.05 mM melatonin), which clustered alone (Euclidean distance was more than 6; Fig. 8C). The total HCA dendrogram among the 10 *t*JA biosynthetic genes showed that nine genes separated into three distinct clusters, in addition to 3-ketoacyl-CoA thiolase (*CsKAT*), which clustered alone in the bottom of the HCA dendrogram. Cluster I included four genes, cluster II included three genes, and cluster III included acetate/butyrate-CoA ligase (*CsAAE7*) and enoyl-CoA hydratase2 (*CsAIM2*) only (Fig. 8C). Interestingly, these three genes (*CsAAE7*, *CsAIM2*, and *CsKAT*) catalyze the late steps in the JA biosynthesis pathway.

Furthermore, all ABA biosynthetic genes were highly expressed after melatonin supplementation, with greater effect for the low concentrations (0.05 and 0.1 mM melatonin), which clustered together (Euclidean distance was approximately 1.5; Fig. 8D). The six ABA biosynthetic genes separated into three distinct clusters. Cluster I included zeaxanthin epoxidase (*CsZEP*) and violaxanthin deepoxidase (*CsVDE*), cluster II included 9-cis-epoxycarotenoid dioxygenase (*CsNCED*) and abscisic aldehyde oxidase (*CsAAO3*), and cluster III included neoxanthin synthase (*CsNSY*) and short-chain alcohol dehydrogenase (or xanthoxin dehydrogenase [*CsABA2*]; Fig. 8D).

Melatonin Supplementation Diminished the *Ca. L. asiaticus* Population within the Infected Valencia Sweet Orange Leaves

The effect of melatonin supplementation on the bacterial population of *Ca. L. asiaticus* within the detached leaves of Valencia sweet orange was investigated using quantitative PCR (qPCR) and expressed as cycle threshold (C_T) values, which negatively reflect the bacterial population within the infected tissues. In agreement with our findings above, data presented in Figure 9B showed that the low melatonin concentrations (0.05 and 0.1 mM melatonin) significantly increased the C_T values, particularly at 72 hpt, indicating a lower bacterial population of *Ca. L. asiaticus*. Furthermore, results from the second-degree polynomial regression analysis of the nonlinear relationship between exogenous melatonin concentrations and the C_T values showed a positive and quadratic relationship (Fig. 9C). The relationship between exogenous melatonin rates and C_T is described by the

Figure 5. (Continued.)

confidence intervals for the estimated regression are gray shaded and edged by dotted lines. Regression equations, R^2 , R^{2adj} , and P value based on the F test ($P \leq 0.05$) were also obtained and are presented within the graphs. FW, Fresh weight.

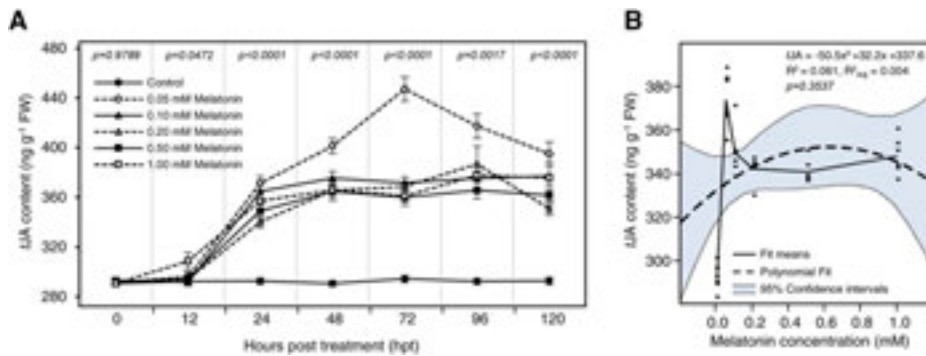


Figure 6. Effects of exogenous melatonin supplementation on the endogenous flA content of Valencia sweet orange. A, Endogenous levels of flA after the exogenous melatonin treatments. Valencia sweet orange trees were treated with 150 mL of Milli-Q water (control; 0 mM melatonin) or 0.05, 0.1, 0.2, 0.5, or 1 mM melatonin. Data shown are means \pm SD ($n = 6$). $P < 0.05$ indicates statistically significant differences among treatments, while $P > 0.05$ indicates no significant differences among them using Tukey’s HSD. B, Fit means models and quadratic polynomial regression analysis between exogenous melatonin concentrations and endogenous flA content. Black dots represent the raw data ($n = 6$). The fit means line is presented as a solid line, while polynomial regression models are presented as dashed lines. The 95% confidence intervals for the estimated regression are gray shaded and edged by dotted lines. Regression equations, R^2 , R^2_{adj} , and P value based on the F test ($P \leq 0.05$) were also obtained and are presented within the graph. FW, Fresh weight.

equation $C_T = -6.54x^2 + 6.63x + 30.64$ ($R^2 = 0.0442$, $R^2_{adj} = 0.0266$, and $P < 0.0001$). Besides, the fit means model for each value showed that the C_T value was higher when the detached leaves were treated with 0.1 mM melatonin (Fig. 9C), regardless of the sampling time.

Without melatonin supplementation, the SLR analysis showed a negative correlation between C_T values and time posttreatment (Fig. 9D), which indicates higher bacterial titer. On the other hand, the linear regression of the C_T values versus the time posttreatment showed a significant positive relationship between them after melatonin supplementation (Fig. 9, E–I). The lower

melatonin concentrations (0.05 and 0.1 mM) showed a stronger positive correlation between C_T values and the time posttreatment. For instance, the relationship between C_T values and time posttreatment could be described by the equation $C_T = 0.075x + 29.17$ ($R^2 = 0.4493$, $R^2_{adj} = 0.4399$, and $P < 0.0001$) after the treatment with 0.05 mM melatonin (Fig. 9E), whereas it could be described by the equation $C_T = 0.087x + 29.6$ ($R^2 = 0.5783$, $R^2_{adj} = 0.571$, and $P < 0.0001$) after the treatment with 0.1 mM melatonin (Fig. 9F). However, the higher melatonin concentrations (0.2, 0.5, and 1 mM) showed a lower positive correlation between C_T values and the time posttreatment (Fig. 9, G–I). In other words, there was

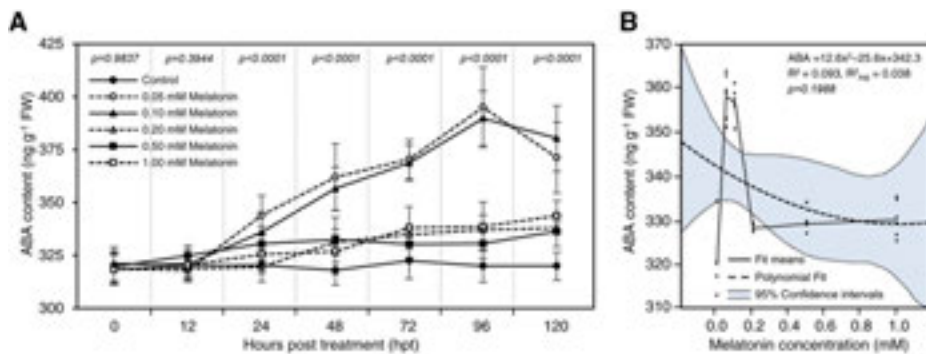


Figure 7. Effects of exogenous melatonin supplementation on the endogenous ABA content of Valencia sweet orange. A, Endogenous levels of ABA after exogenous melatonin treatments. Valencia sweet orange trees were treated with 150 mL of Milli-Q water (control; 0 mM melatonin) or 0.05, 0.1, 0.2, 0.5, or 1 mM melatonin. Data shown are means \pm SD ($n = 5$). $P < 0.05$ indicates statistically significant differences among treatments, while $P > 0.05$ indicates no significant differences among them using Tukey’s HSD. B, Fit means models and quadratic polynomial regression analysis between exogenous melatonin concentrations and endogenous ABA content. Black dots represent the raw data ($n = 6$). The fit means line is presented as a solid line, while polynomial regression models are presented as dashed lines. The 95% confidence intervals for the estimated regression are gray shaded and edged by dotted lines. Regression equations, R^2 , R^2_{adj} , and P value based on the F test ($P \leq 0.05$) were also obtained and are presented within the graph. FW, Fresh weight.

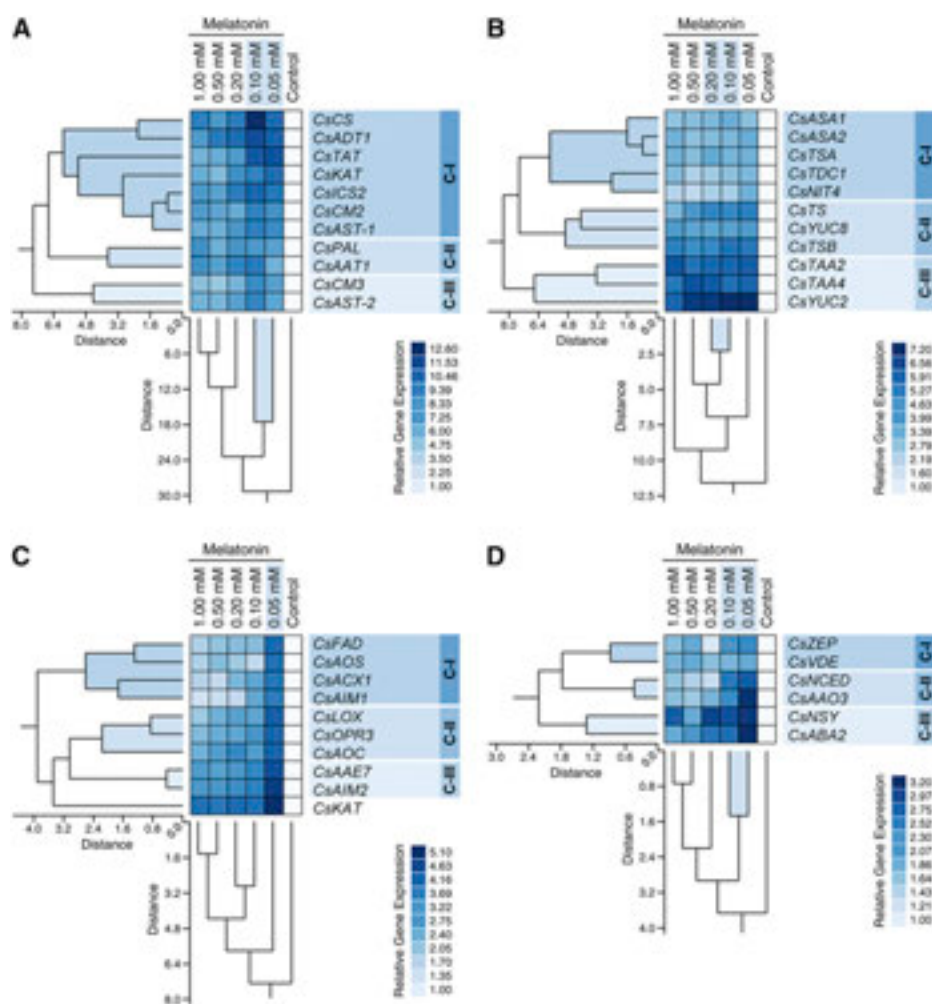


Figure 8. Relative expression of genes involved in the phytohormone biosynthesis pathways in Valencia sweet orange. Two-way HCA and its associated heat map diagrams of differential non-standardized gene expression patterns of SAs (A), auxins (B), *t*JA (C), and ABA (D) after exogenous melatonin supplementation are shown. Rows represent the genes, whereas columns represent different treatments. Low expression levels are colored light blue and high expression levels are colored dark blue (see the scale at the right bottom corner of the heat maps). Treatments and genes in the HCA were organized using Ward's minimum variance method (Ward, 1963). The full list of expressed genes, names, accession numbers, and primers is available in Supplemental Table S2.

a significant negative density-dependent relationship between the bacterial titer of *Ca. L. asiaticus* and melatonin content over time, which confirms the antibacterial role of melatonin against *Ca. L. asiaticus* within the infected tissues.

Endogenous Melatonin and SA Levels Are Higher in *Ca. L. asiaticus*-Tolerant Citrus Varieties

Our findings showed that the endogenous levels of melatonin and SA, but not *t*JA, were higher in *Ca. L. asiaticus*-tolerant varieties (Fig. 10). Briefly, the tolerant variety *C. latipes* had the highest melatonin content ($941.56 \pm 130.35 \text{ pg g}^{-1}$ fresh weight), followed by Dancy tangerine, Mexican lime, and *C. macrophylla* (783.68 ± 100.52 , 611.51 ± 29.91 , and $393.93 \pm 30.43 \text{ pg g}^{-1}$ fresh weight, respectively; Fig. 10, A and B). On the other hand, the susceptible varieties, Valencia sweet orange and Duncan grapefruit (*Citrus paradise*), were lower in melatonin without significant differences between them (327.62 ± 23.19 and $254 \pm 49.32 \text{ pg g}^{-1}$ fresh weight, respectively; Fig. 10, A and B). Furthermore, the SLR showed that the endogenous melatonin content

was positively correlated with the tolerance degree ($\text{Mel} = 285.91x - 19.76$; $R^2 = 0.8181$, $R^{2\text{adj}} = 0.8127$, and $P < 0.0001$; Fig. 10D).

Likewise, SA was associated with citrus tolerance to *Ca. L. asiaticus* (Fig. 10, D and E). *C. latipes* had the highest SA content ($1,453.51 \pm 185.62 \text{ ng g}^{-1}$ fresh weight) followed by Dancy tangerine, *C. macrophylla*, and Mexican lime ($1,338.37 \pm 131.29$, $1,215.27 \pm 176.51$, and $1,116.28 \pm 165.79 \text{ ng g}^{-1}$ fresh weight, respectively), without significant differences between them (Fig. 10, D and E). On the other hand, the susceptible varieties, Duncan grapefruit and Valencia sweet orange, had the lowest SA content without significant differences between them (840.27 ± 59.59 and $774.65 \pm 52.34 \text{ ng g}^{-1}$ fresh weight, respectively; Fig. 10, D and E). Moreover, the SLR showed that the endogenous SA content was positively correlated with the tolerance degree ($\text{SA} = 294.24x + 534.59$; $R^2 = 0.7485$, $R^{2\text{adj}} = 0.7411$, and $P < 0.0001$; Fig. 10F).

On the other hand, no significant differences were observed in the endogenous content of *t*JA between all studied varieties (Fig. 10, G and H). Additionally, no significant correlation was observed between endogenous *t*JA content and tolerance levels ($t\text{JA} = -6.76x +$

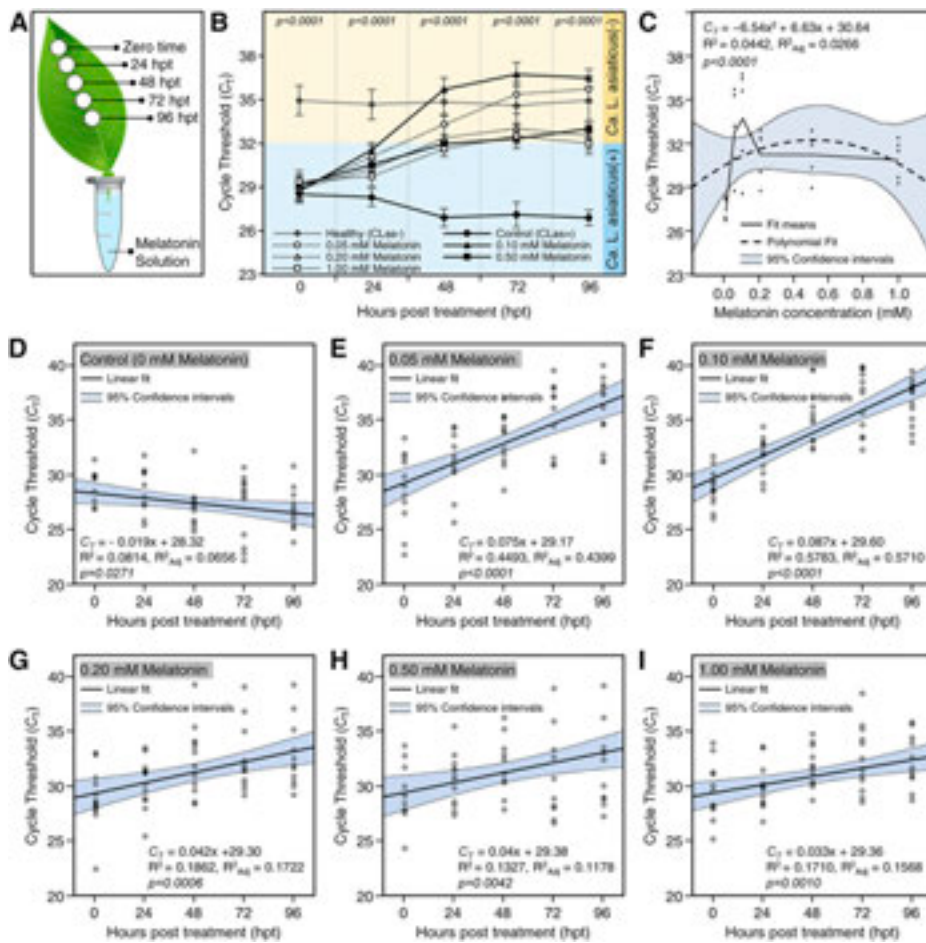


Figure 9. Effects of melatonin supplementation on the bacterial population of *Ca. L. asiaticus* in HLB-symptomatic detached leaves of Valencia sweet orange. A, Fully mature HLB-symptomatic detached leaves, with their complete petioles, were incubated in one of five concentrations of melatonin (0.05, 0.1, 0.2, 0.5, and 1 mM aqueous solution), in addition to the control (mock; Milli-Q water with 0 mM melatonin). A single leaf disc was collected from each leaf before the treatment (zero time) and at 24, 48, 72, and 96 hpt for *Ca. L. asiaticus* quantification. B, C_T of reverse transcription quantitative PCR (RT-qPCR) for the detection of *Ca. L. asiaticus* in HLB-symptomatic detached leaves of Valencia sweet orange after treatment with different concentrations of melatonin. Data shown are means \pm sd ($n = 12$). $P < 0.05$ indicates statistically significant differences among treatments, whereas $P > 0.05$ indicates no significant differences between them using Tukey’s HSD. C, Fit means model and quadratic polynomial regression analysis between exogenous melatonin concentrations and C_T of RT-qPCR for the detection of *Ca. L. asiaticus*. Black dots represent the raw data ($n = 6$). The fit means line is presented as a solid line, while polynomial regression models are presented as dashed lines. The 95% confidence intervals for the estimated regression are gray shaded and edged by dotted lines. Regression equations, R^2 , R^{2adj} , and P value based on the F test ($P \leq 0.05$) were also obtained and are presented within the graph. D to I, Simple linear regression plots of hpt versus the C_T of RT-qPCR for the detection of *Ca. L. asiaticus* in detached leaves after treatment with different concentrations of melatonin. White dots represent the raw data ($n = 12$). The fitted regression line is presented as a solid line, whereas the 95% confidence intervals for the estimated regression are gray shaded and edged by dotted lines. Regression equations, R^2 , R^{2adj} , and P value based on the F test ($P \leq 0.05$) were also obtained and are presented within the graphs.

272.82; $R^2 = 0.0179$, $R^{2adj} = 0.0109$, and $P = 0.4363$; Fig. 10C).

Melatonin and SA Supplementation Enhanced the Endogenous Content of Melatonin and SA of *Ca. L. asiaticus* Plants

The impact of exogenous treatment with SA, tJA, and melatonin on their endogenous levels was investigated

(Fig. 11). Generally, there were no significant differences between all treatments at the beginning of the experiment (zero time). However, at 72 hpt, the endogenous melatonin level was increased after the treatment with 0.1 mM melatonin ($610.5 \pm 79.87 \text{ pg g}^{-1}$ fresh weight) or 0.25 mM SA ($363.94 \pm 47.79 \text{ pg g}^{-1}$ fresh weight) but not tJA ($238.98 \pm 55.32 \text{ pg g}^{-1}$ fresh weight), which was significantly similar to the control ($246.45 \pm 55.62 \text{ pg g}^{-1}$ fresh weight; Fig. 11, A and B).

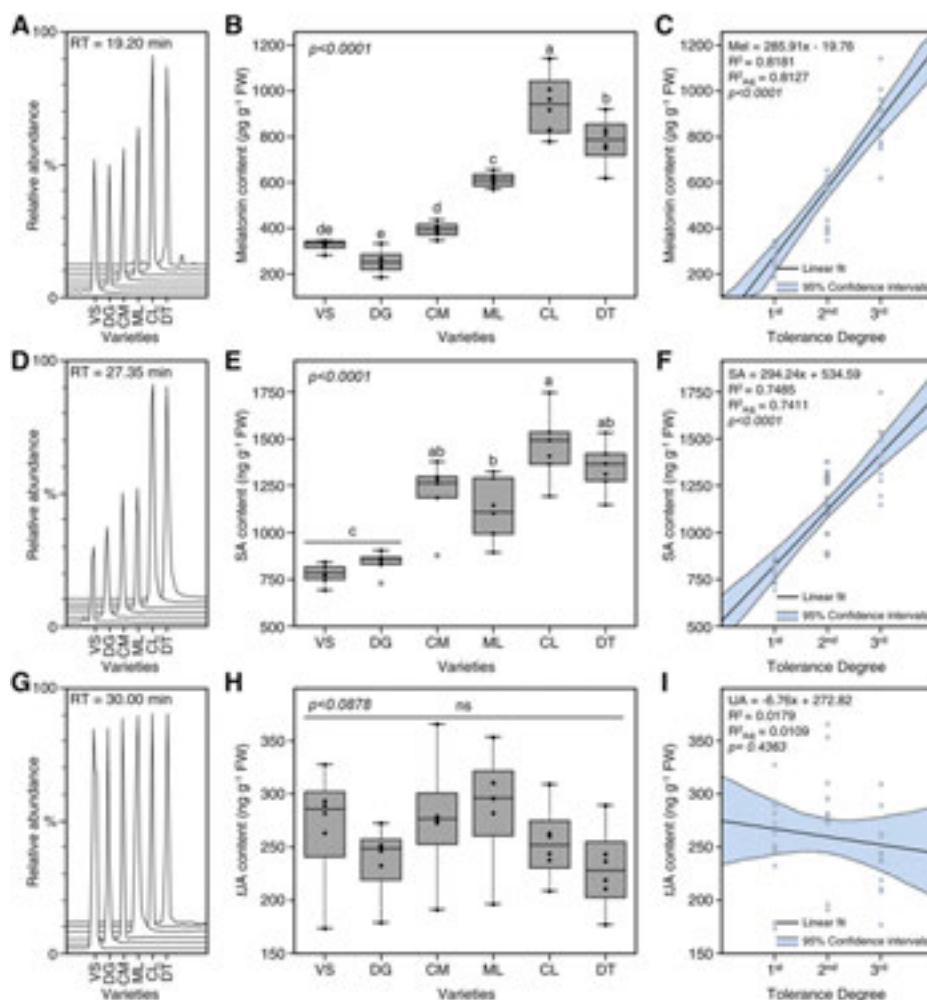


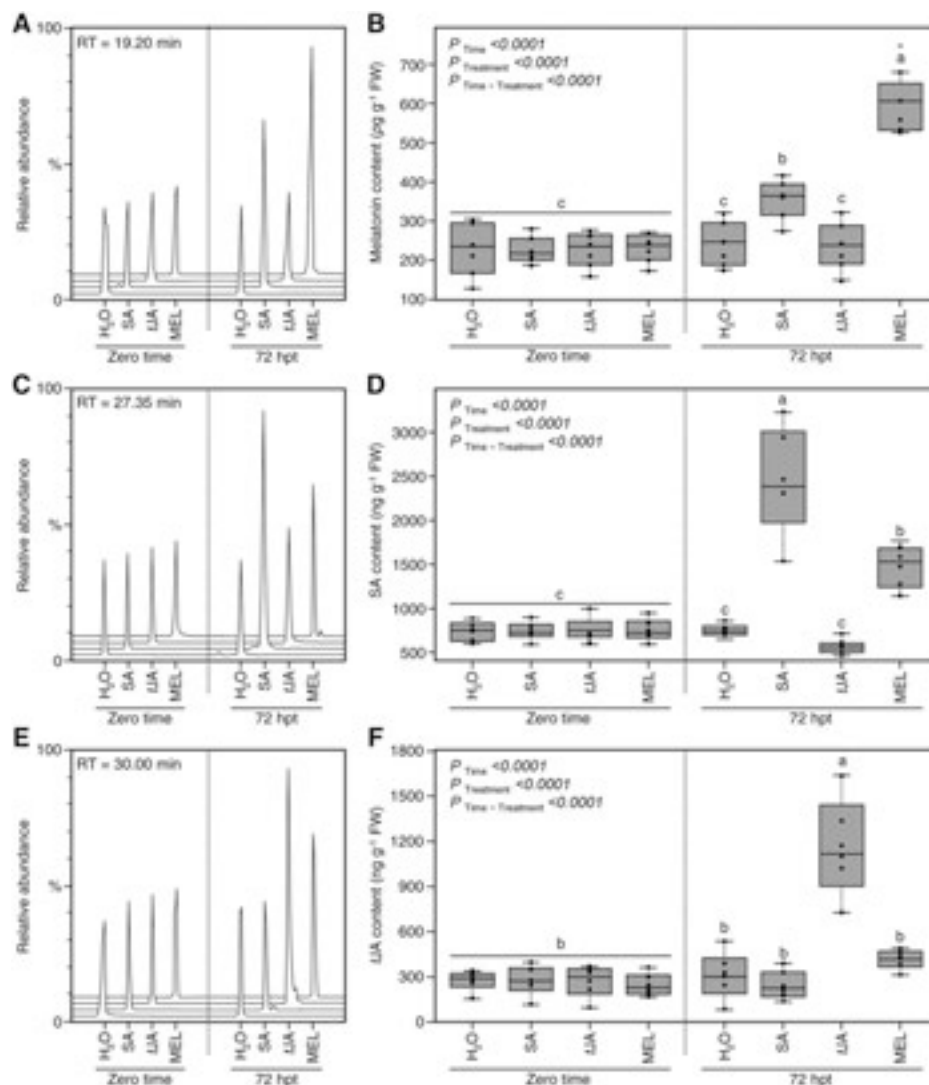
Figure 10. Endogenous contents of phytochemicals detected in leaves of some citrus varieties with different degrees of tolerance to *Ca. L. asiaticus* using targeted GC-MS-SIM. A, D, and G, Representative chromatograms of melatonin (A), SA (D), and *t*JA (G) from different varieties. RT, Retention time. B, E, and H, Endogenous contents of melatonin (pg g^{-1} fresh weight; $n = 6$), SA (ng g^{-1} fresh weight; $n = 6$), and *t*JA (ng g^{-1} fresh weight; $n = 6$), respectively, from different varieties. Whiskers indicate the minimum and maximum values of the data, horizontal thick lines show the medians, black dots indicate the raw data, and boxes show the interquartile ranges (25th to 75th percentiles of the data). Different letters indicate statistically significant differences among varieties, while the same letter signifies no significant differences between them using Tukey's HSD ($P < 0.05$). C, F, and I, Simple linear regression analysis between the HLB tolerance degree and endogenous content of melatonin (C), SA (F), and *t*JA (I). Dots represent the raw data. The fitted regression line is presented as a solid line. The 95% confidence intervals for the estimated regression are gray shaded and edged by dotted lines. Regression equations, R^2 , $R^{2\text{adj}}$, and P value based on the F test ($P \leq 0.05$) were also obtained and are presented within the graphs. FW, Fresh weight. Citrus varieties are as follows, from left to right: VS, Valencia sweet orange; DG, Duncan grapefruit (*Citrus paradisi*); CM, alemow (*Citrus macrophylla*); ML, Mexican lime (*Citrus aurantifolia*); CL, Khasi papeda (*Citrus latipes*); DT, Dancy tangerine (*Citrus tangerina*).

Likewise, the endogenous levels of SA were elevated by 3.26-fold after treatment with 0.25 mM SA and about 2-fold after treatment with 0.1 mM melatonin. Interestingly, SA content slightly dropped after treatment with 0.1 mM *t*JA ($564.02 \pm 85.11 \text{ ng g}^{-1}$ fresh weight); however, this reduction was not significant compared with the control ($757.41 \pm 62.32 \text{ ng g}^{-1}$ fresh weight; Fig. 11, C and D). On the other hand, the exogenous application of *t*JA (0.1 mM) only induced the accumulation of endogenous *t*JA but not SA or melatonin (Fig. 11, E and F).

Melatonin and SA Supplementation Diminished the *Ca. L. asiaticus* Population within the Infected Citrus Plants

To confirm our findings of the antibacterial role of melatonin against *Ca. L. asiaticus* from the detached leaves experiment and to investigate if SA or *t*JA has a direct or indirect effect on the *Ca. L. asiaticus* infection, the effect of SA, *t*JA, and melatonin supplementation on the bacterial population of *Ca. L. asiaticus* within the infected Valencia sweet orange plants was investigated. In agreement with our findings above, data presented

Figure 11. Effects of exogenous application of melatonin, SA, and β A on the endogenous contents of melatonin, SA, and β A of *Ca. L. asiaticus*-infected Valencia sweet orange trees using targeted GC-MS-SIM. Citrus plants were treated with 200 mL of Milli-Q water (control), melatonin (0.1 mM), SA (0.1 mM), or β A (0.1 mM) through foliar application. A, C, and E, Representative chromatograms of melatonin (A), SA (C), and β A (E) at zero time and at 72 hpt. RT, Retention time. B, D, and F, Endogenous content of melatonin (pg g⁻¹ fresh weight; $n = 6$), SA (ng g⁻¹ fresh weight; $n = 6$), and β A (ng g⁻¹ fresh weight; $n = 6$), respectively, from *Ca. L. asiaticus*-infected Valencia sweet orange trees at zero time and at 72 hpt. Whiskers indicate the minimum and maximum values of the data, horizontal thick lines show the medians, black dots indicate the inter-quartile ranges (25th to 75th percentiles of the data). Different letters indicate statistically significant differences among varieties, while the same letter signifies no significant differences between them using Tukey's HSD ($P < 0.05$). FW, Fresh weight.



in Figure 12 showed that the exogenous application of melatonin (0.1 mM) or SA (0.25 mM) significantly increased the C_T values at 72 hpt without significant differences between them, indicating a lower bacterial population of *Ca. L. asiaticus* within the infected plants.

DISCUSSION

One of our goals in this study was to develop a GC-MS-SIM-based method to determine the endogenous melatonin content in citrus leaf tissues after derivatization with MSTFA. This derivatization procedure produces a bis-TMS-melatonin derivative as a final product, with no indications of the presence of the mono-TMS-melatonin, and these results are in agreement with our previous study on *D. citri* (Nehela and Killiny, 2018) as well as the study of Nùñez-Vergara et al. (2001) on pharmaceutical tablets. Interestingly, the mass spectrum of the bis-TMS-melatonin derivative does not exist in the entries of the two mass spectral

libraries employed in our lab: NIST 2011 (National Institute of Standards and Technology) and Wiley 9th edition (John Wiley and Sons). Although the citrus leaf extract is a complex matrix, our method was efficient in the simultaneous qualitative and quantitative determination of melatonin in Valencia sweet orange leaves. Melatonin was reported previously in citrus fruits (Johns et al., 2013) and juice (Fernández-Pachón et al., 2014); however, to the best of our knowledge, melatonin was never reported in Valencia sweet orange leaves previously. Herein, we detected melatonin from Valencia sweet orange leaves under different biotic stressors (*Ca. L. asiaticus* infection and *D. citri* infestation).

Our findings showed that *Ca. L. asiaticus* infection induced the accumulation of melatonin and upregulated the expression of its biosynthetic genes in the leaves of infected trees. These findings are in agreement with previous studies that showed that the endogenous melatonin level was elevated in Arabidopsis leaves upon infection with the virulent bacterial pathogen

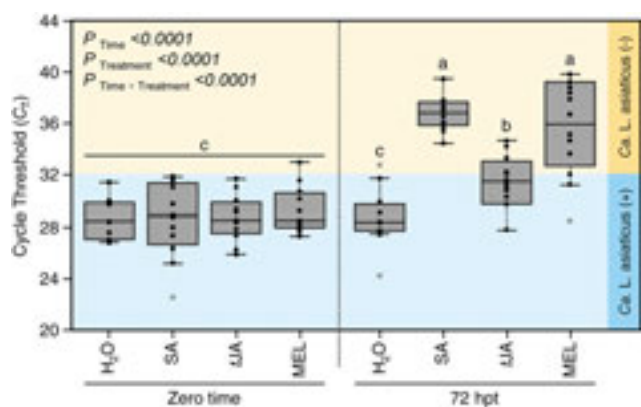


Figure 12. Exogenous application of melatonin, SA, and IJA on the bacterial population of *Ca. L. asiaticus* in HLB-infected Valencia sweet orange trees. C_T of RT-qPCR for the detection of *Ca. L. asiaticus* in HLB-infected Valencia sweet orange trees after treatment with 200 mL of Milli-Q water (control), melatonin (0.1 mM), SA (0.1 mM), or IJA (0.1 mM) through foliar application is shown. Whiskers indicate the minimum and maximum values of the data, horizontal thick lines show the medians, black dots indicate the raw data, and boxes show the interquartile ranges (25th to 75th percentiles of the data). Different letters indicate statistically significant differences among varieties, while the same letter signifies no significant differences between them using Tukey's HSD ($P < 0.05$).

Pst-DC3000 (Shi et al., 2015) and the avirulent pathogen *P. syringae* pv *tomato* DC3000 expressing the elicitor *avrRpt2* (*Pst-avrRpt2*; Lee et al., 2015; Lee and Back, 2016, 2017a). Likewise, the melatonin level was increased in watermelon (*Citrullus lanatus*) plants in response to the fungal powdery mildew pathogen *Podosphaera xanthii* (Mandal et al., 2018).

Exogenous melatonin application enhanced plant resistance against fungi and fungi-like organisms. For instance, exogenous melatonin treatment enhanced the resistance of apple trees to Marssonina apple blotch disease caused by *D. mali* (Yin et al., 2013), improved banana (*Musa acuminata*) responses to the *Fusarium* wilt disease caused by *Fusarium oxysporum* f. sp. *cubense* (Wei et al., 2017), and protected strawberry (*Fragaria × ananassa*) fruits against the postharvest decay caused by *Botrytis cinerea* and *Rhizopus stolonifer* (Aghdam and Fard, 2017). Furthermore, exogenous application of melatonin showed a significant reduction in hyphal growth and conidia development of the powdery mildew pathogen *P. xanthii* on watermelon, zucchini (*Cucurbita pepo*), and summer squash (*Cucurbita pepo*) leaves (Mandal et al., 2018). Likewise, melatonin significantly reduced the mycelial growth, sporulation intensity, and zoospore release of the soil-borne oomycete *Phytophthora capsici* in watermelon and other cucurbit plants (Mandal et al., 2018). Moreover, the addition of melatonin to the standard medium showed growth inhibition activity against several phytopathogenic fungi, including *Alternaria* spp., *Botrytis* spp., and *Fusarium* spp., and decreased the infection rate of *Penicillium* spp. on nonsterilized *Lupinus albus*

seeds (Arnao and Hernández-Ruiz, 2015). In terms of fungi-like organisms, melatonin supplementation mitigated the negative effects of *Phytophthora infestans*, the causal agent of potato late blight (Zhang et al., 2017), and the soil-borne oomycete *P. capsica*, the causal agent of *Phytophthora* crown rot on watermelon and other cucurbits (Mandal et al., 2018). It has also been shown recently that melatonin supplementation might play a key role in the enhancement of host resistance against virus infection of numerous plants such as apple (*Malus domestica*; Chen et al., 2019a), rice (*Oryza sativa*; Lu et al., 2019), and tobacco (*Nicotiana tabacum*; Zhao et al., 2019). Taken together, these findings suggest that melatonin itself might be a defense molecule that can act as a biocide or as a biotic antistressor against phytopathogenic bacteria and fungi (Arnao and Hernández-Ruiz, 2015).

Our findings showed that melatonin has an antibacterial effect against *Ca. L. asiaticus*, since the low concentrations (between 0.05 and 0.1 mM) significantly reduced the bacterial titer within the HLB-symptomatic detached leaves of Valencia sweet orange. These findings are in agreement with our previous study, where we showed that exogenous melatonin reduced the *Ca. L. asiaticus* titer within the *D. citri* body (Nehela and Killiny, 2018). Although there is still debate concerning the antibacterial role of melatonin, previous in vitro studies in Animalia demonstrated that melatonin is effective against both gram-positive and gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* (Tekbas et al., 2008). In plants, previous studies showed that melatonin supplementation triggered the defense responses of Arabidopsis against the phytopathogenic gram-negative bacterium *P. syringae* pv *tomato* (Lee et al., 2014, 2015; Qian et al., 2015; Shi et al., 2015; Zhao et al., 2015; Lee and Back, 2016, 2017a) and cassava (*Manihot esculenta*) against *Xanthomonas axonopodis* pv *manihotis* (Wei et al., 2018a).

Furthermore, it has been shown recently that exogenous melatonin inhibited the growth of *Xanthomonas oryzae* pv *oryzicola*, the causal agent of rice bacterial leaf streak disease, in a dose-dependent manner (Chen et al., 2019b) and enhanced rice resistance against the bacterial pathogen (Xian et al., 2020). Melatonin application has been reported previously to inhibit the proliferation of several bacteria and fungi species (Atroschi et al., 1998; Öztürk et al., 2000; Wang et al., 2001; Tekbas et al., 2008). This inhibitory role might be due to the indole in its structure (Chen et al., 2019b). Previously, it was reported that indoles and their derivatives have an antibacterial effect (Raut et al., 2012; Yang et al., 2014; Chen et al., 2019b) through the inhibition of dimorphism (Raut et al., 2012) and biofilm formation (Raut et al., 2012; Yang et al., 2014) or the modulation of Ca^{2+} efflux (Yang et al., 2014). We suggest that the antibacterial role of melatonin against *Ca. L. asiaticus* might be because of its indole properties. However, more investigations are required to understand the molecular mechanisms behind the antibacterial role of melatonin

against *Ca. L. asiaticus*, which is currently difficult due to the challenges associated with growing the bacterium in pure cultures.

Generally, melatonin is biosynthesized from the amino acid L-Trp via four enzymatic steps (Arnao and Hernández-Ruiz, 2006, 2018; Hardeland, 2008; Tan et al., 2014; Back et al., 2016; Nawaz et al., 2016; Dhole and Shelat, 2018; Lee and Back, 2019). In plants, phyto-melatonin is synthesized in different subcellular locations, where L-Trp is first decarboxylated to form tryptamine by the cytosolic *TDC* (De Luca et al., 1989; Park et al., 2008; De Masi et al., 2017; Zhao et al., 2018; Facchiano et al., 2019). After Trp decarboxylation, tryptamine is hydroxylated by *T5H* (a cytochrome P450 monooxygenase), leading to the synthesis of serotonin, the key intermediate in melatonin synthesis (Kang et al., 2007; Fujiwara et al., 2010; Park et al., 2011, 2012). Subsequently, serotonin is converted into *N*-acetylserotonin via the activity of *SNAT* (Kang et al., 2013; Byeon et al., 2014; Lee et al., 2015; Back et al., 2016). The last step is to methylate *N*-acetylserotonin into phyto-melatonin by either *ASMT* or *COMT* (Byeon et al., 2015; Back et al., 2016; Zheng et al., 2017; Yan et al., 2019). Interestingly, our findings showed that the transcript levels of two *CsTDCs*, *CsT5H* (or *CsCYP71A1*), two *CsSNATs*, seven *CsASMTs*, and three *CsCOMTs* were significantly increased after *Ca. L. asiaticus* infection more than for other treatments and after melatonin supplementation.

TDC was previously reported to be involved in plant defense responses against fungal diseases such as rice brown spot disease, caused by *Bipolaris oryzae* (Ishihara et al., 2008), and nematode diseases such as cereal cyst nematode, *Heterodera avenae* (Huang et al., 2018). Furthermore, the *snat* knockout mutant lines of *Arabidopsis* exhibited lower endogenous content of melatonin and SA and were more susceptible to the avirulent strains of *Pst-avrRpt2* (Lee et al., 2015), whereas overexpression of *OsSNAT1* or human *SNAT* elevated the endogenous levels of *N*-acetylserotonin and melatonin in transgenic rice plants and conferred resistance against abiotic stress and senescence (Kang et al., 2010; Lee and Back, 2017b). Likewise, the contribution of *ASMT* and *COMT* to host defense against abiotic stress has been reported previously (Park et al., 2013; Zuo et al., 2014; Byeon and Back, 2016; Xu et al., 2016; Zheng et al., 2017; Yan et al., 2019). However, the potential roles of melatonin biosynthetic genes in plant defense responses against phytopathogenic bacteria are poorly understood. Our findings showed that melatonin biosynthetic genes, particularly *CsTDCs*, *CsSNATs*, and *CsASMTs*, are involved in the citrus response to *Ca. L. asiaticus* through the induction of melatonin biosynthesis, which was positively correlated with SA content and its biosynthetic genes but not *tJA*. However, further studies are required to clarify the functional and regulatory roles of these genes in citrus in general and to better understand how they regulate SA-based defensive mechanisms.

Collectively, our findings showed that melatonin and its biosynthetic genes are involved in citrus response against the phytopathogenic bacterium *Ca. L. asiaticus*. Recently, the protective roles of melatonin against various phytopathogens have been reviewed (Moustafa-Farag et al., 2019). Although the antibacterial role of melatonin against several phytopathogenic bacteria has been reported previously (Lee et al., 2014, 2015; Qian et al., 2015; Shi et al., 2015; Zhao et al., 2015; Lee and Back, 2016, 2017a; Wei et al., 2016, 2018a), the molecular and biochemical mechanisms of melatonin-based defense are poorly characterized. Previous studies showed that melatonin defensive mechanisms involve the activation of the NO-dependent pathway (Shi et al., 2015; Lu et al., 2019), the modulation of carbohydrate, sugar, and glycerol metabolism (Qian et al., 2015; Zhao et al., 2015), the induction of hydrogen peroxide- and NO-mediated defense signaling (Lee and Back, 2017a), upstreaming transcription factors such as MAPK cascades and RAV transcription factors (Lee and Back, 2016; Wei et al., 2018a), the induction of pathogenesis- and defense-related genes, such as *ENHANCED DISEASE SUSCEPTIBILITY1*, *PHYTOALEXIN DEFICIENT4*, and *PR1*, *PR2*, and *PR5* (Lee et al., 2014; Shi et al., 2015; Lee and Back, 2017a), and/or the modulation of stress-associated phytohormones, particularly SA, JA, and ET, as well as their biosynthetic genes (Lee et al., 2014, 2015; Lee and Back, 2017a).

Changes in host-derived phytohormones upon phytopathogen infection and/or insect infestation vary based on their feeding activity and trophic types (Glazebrook, 2005; Bari and Jones, 2009). For instance, the SA-mediated pathway is associated with defense response for biotrophic and hemibiotrophic phytopathogens, whereas the JA/ET-mediated pathway is associated with defense against necrotrophic phytopathogens and insect herbivory (Hatcher et al., 2004; Glazebrook, 2005; Bari and Jones, 2009). Our previous studies showed that *Ca. L. asiaticus* infection significantly induced the accumulation of SA, its precursor L-Phe, and its biosynthetic genes compared with *D. citri*-infested plants. On the other hand, the infestation with *D. citri* increased the level of *tJA*, its precursor linolenic acid, and its biosynthetic genes compared with *Ca. L. asiaticus*-infected plants (Nehela et al., 2018). The cross talk between these two pathways is possible and complicated. However, the interaction between SA and JA/ET is controversial. Some studies suggested that the SA- and JA/ET-mediated pathways are antagonistic (Bari and Jones, 2009), and the activation of one usually suppresses the other (Robert-Seilaniantz et al., 2007; Bari and Jones, 2009), while other studies have demonstrated that the SA-JA interaction is mutually synergistic (Kunkel and Brooks, 2002). Although several previous studies demonstrated the association between melatonin and SA-activated defense genes (Lee et al., 2014, 2015; Vielma et al., 2014; Qian et al., 2015), the cross talk between melatonin, SA, and/or JA is poorly studied. To the best

of our knowledge, the interaction between these three compounds has not been reported previously.

In this study, we reported that phytomelatonin is involved in citrus response against HLB via modulation of stress-associated phytohormones and their biosynthetic genes. To summarize our findings, a hypothetical model for the cross talk between phytomelatonin and phytohormones in citrus was suggested and is presented in Figure 13. In this model, we propose that infection with the *Ca. L. asiaticus* bacterium and infestation with the *D. citri* vector, as well as exogenous melatonin supplementation, induced the biosynthesis of phytomelatonin and several phytohormone groups, including SAs, *t*JA, ABA, and auxins. Moreover, melatonin supplementation diminished the *Ca. L. asiaticus* population within the infected leaves and whole plants, which supports our hypothesis that melatonin might play an antibacterial role against *Ca. L. asiaticus* particularly and gram-negative bacteria in general.

Furthermore, melatonin supplementation triggered the gene expression of all SAs biosynthetic genes, particularly CsCS. This enzyme catalyzes the last step in the shikimate pathway to produce chorismate, which is used for the biosynthesis of aromatic amino acids such as Phe (the precursor of SA) and Trp (the precursor of auxins and melatonin). Interestingly, our previous

study showed that CsCS was highly upregulated after *Ca. L. asiaticus* infection (Nehela et al., 2018). On the other hand, even though the SLR modeling of endogenous melatonin and *t*JA content did not show a significant correlation between them, melatonin supplementation slightly induced the accumulation of *t*JA in Valencia sweet orange leaves and upregulated all *t*JA biosynthesis genes, particularly CsKAT. CsKAT is involved in the metabolism of fatty acids, which is part of JA biosynthesis. Interestingly, our previous study showed that CsKAT was highly upregulated only after *D. citri* infestation (Nehela et al., 2018). Taken together, these findings indicate that exogenous melatonin plays a key role in the activation of the SA-mediated pathway against pathogen infection, which is the most common defensive pathway in vascular plants (Glazebrook, 2005; Robert-Seilaniantz et al., 2007; Bari and Jones, 2009). Besides these well-known pathways, we suggest that melatonin might be involved in the activation of both ABA and auxin pathways. ABA and auxins play key roles in the citrus defense response against HLB (Nehela et al., 2018). The activation of four phytohormone pathways leads to complex cross talk among them, which interact synergistically or antagonistically and feeds back into the SA-mediated pathway and JA-mediated pathway. However, further research focusing on melatonin-ABA-auxin interactions and their roles in the activation of SA- and JA-mediated

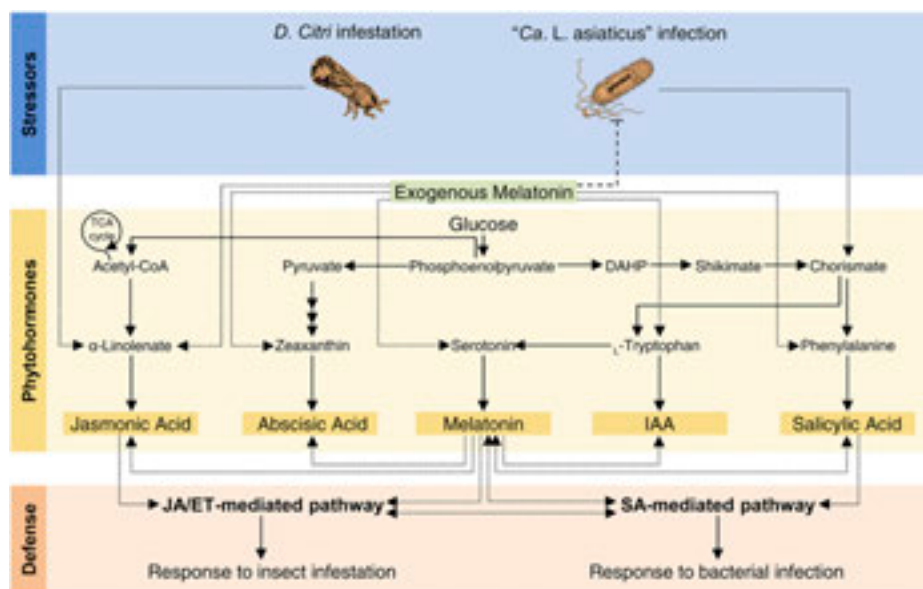


Figure 13. Hypothetical model of the cross talk between melatonin and different phytohormones of Valencia sweet orange leaves. In this model, we propose that infection with *Ca. L. asiaticus* bacterium and infestation with *D. citri* vector, as well as exogenous melatonin supplementation, might induce the biosynthesis of phytomelatonin and several phytohormone groups, including SAs, *t*JA, ABA, and auxins. Briefly, exogenous melatonin plays a dual role in the activation of both the SA-mediated pathway against pathogen infection and the JA-mediated pathway against herbivory, which are the most common defensive pathways in vascular plants. Besides these well-known pathways, our findings show that melatonin is involved in the activation of both ABA and auxin pathways. ABA and auxins play a key role in citrus defense response against HLB. The activation of four phytohormone pathways leads to complex cross talk among them, which interact synergistically or antagonistically together, and link back to the SA-mediated pathway and the JA-mediated pathway. Solid lines with arrows indicate well-established/confirmed pathways, dashed lines with whiskers signify negative reactions, and dotted lines represent hypothetical mechanisms or uncharacterized elements. DAHP, 3-Deoxy-D-arabino-heptulosonic acid 7-phosphate; TCA, tricarboxylic acid.

pathways is required for a better understanding of citrus phytohormonal response to HLB disease.

Our findings showed that the endogenous levels of melatonin and SA, but not *tJA*, were higher in *Ca. L. asiaticus*-tolerant varieties such as *C. latipes* and Dancy tangerine compared with susceptible varieties such as Valencia sweet orange and Duncan grapefruit. These findings suggest that both melatonin and SA biosynthesis pathways are more active in HLB-tolerant varieties. Our previous study showed that the phloem sap chemical composition of most of the tolerant cultivars such as *C. latipes* was higher in most of the metabolites, especially amino acids that are precursors for most of the secondary metabolites implicated in plant defense (Killiny and Hijaz, 2016). For instance, the phloem sap of the HLB-tolerant variety *C. latipes* had a higher L-Trp content compared with the moderately tolerant and susceptible varieties (Killiny and Hijaz, 2016). Interestingly, L-Trp is the precursor of auxins and melatonin (Arnao and Hernández-Ruiz, 2006, 2018; Hardeland, 2008; Tan et al., 2014; Back et al., 2016; Nawaz et al., 2016; Dhole and Shelat, 2018; Lee and Back, 2019), which supports the suggestion that the melatonin biosynthesis pathway is more active in HLB-tolerant varieties.

Furthermore, our findings showed that the endogenous levels of melatonin and SA were increased after treatment with melatonin or SA, but not *tJA*, which was significantly similar to the control. In other words, the exogenous application of melatonin increased the endogenous SA levels, and vice versa, the exogenous application of SA increased the endogenous melatonin levels, which indicates a strong correlation between them. Several previous studies demonstrated the role of melatonin in plant immunity against *Pst*-DC3000 in *Arabidopsis* and provided evidence that it requires SA as a key downstream component (Yin et al., 2013; Lee et al., 2014, 2015; Vielma et al., 2014; Qian et al., 2015; Shi et al., 2015, 2016; Zhao et al., 2015). Moreover, it has been reported that the melatonin-dependent defense response against *Pst*-DC3000 in *Arabidopsis* plants was associated with the SA-mediated defense signaling pathway (Lee et al., 2014, 2015; Vielma et al., 2014; Qian et al., 2015).

For instance, exogenous application of melatonin on the leaves of *Arabidopsis* and *N. benthamiana* induced various PR genes as well as a series of SA-activated defense genes compared with mock-treated plants (Lee et al., 2014). Likewise, melatonin activated numerous PR genes and other defense-related genes through the modulation of the levels of both SA and NO in infected plants (Yin et al., 2013; Shi et al., 2015, 2016; Zhao et al., 2015). Additionally, the SNAT (a key melatonin biosynthetic gene) knockout mutants not only exhibited decreased levels of melatonin but also had lower SA content along with a greater susceptibility to the bacterial pathogen (Lee et al., 2015). On the other hand, melatonin supplementation failed to induce defense-related genes in the *nahG* mutants of *Arabidopsis* but restored the induction of defense-related

genes in the *snat* knockout mutants (Lee et al., 2015). Taken together, the evidence indicates the existence of cross talk between melatonin and other phytohormones and that the melatonin-elicited defense response that facilitates fine-tuning of resistance against phytopathogenic bacteria is SA dependent.

In conclusion, melatonin and SA biosynthesis pathways share a common precursor (chorismate; Fig. 13), which is generated from shikimic acid, and both play a relevant role in citrus physiology, particularly in aspects related to the response to biotic and abiotic stress. Our findings showed that both *Ca. L. asiaticus* infection and/or *D. citri* infestation significantly increased the endogenous melatonin and SA content and their biosynthetic genes of Valencia sweet orange leaves, with a greater effect of *Ca. L. asiaticus* alone. Moreover, *Ca. L. asiaticus*-tolerant and -moderately tolerant varieties had higher levels of both melatonin and SA but not *tJA*. All of these pieces of evidence support the potential role of melatonin in citrus response to different biotic stressors, including the phytopathogenic bacterium *Ca. L. asiaticus* and its insect vector, *D. citri*. Additionally, melatonin supplementation enhanced the endogenous content of stress-associated phytohormones, including SAs, auxins, *tJA*, and ABA and their biosynthetic genes. Likewise, the endogenous level of melatonin was increased after the treatment with SA but not *tJA*. These findings highlight the molecular and biochemical mechanisms of the melatonin-mediated defensive response and demonstrate its cross talk with other phytohormones. Furthermore, melatonin supplementation diminished the *Ca. L. asiaticus* population within the HLB-infected detached leaves and whole trees of Valencia sweet orange, which confirms the antibacterial role of melatonin against *Ca. L. asiaticus* within the infected tissues. To the best of our knowledge, the potential role of melatonin against citrus bacterial diseases in general, and HLB particularly, has not been reported previously. Melatonin is considered an eco-friendly molecule, which might be a promising alternative strategy to combat the citrus greening disease in particular and plant bacterial diseases in general. Finally, a thorough understanding of the melatonin-based defense mechanism in citrus might lead to a more comprehensive image of defense responses to HLB, critical for finding immediate and sustainable management strategies for HLB.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Valencia sweet orange (*Citrus sinensis*) was used as an experimental plant in this study. All trees were about 18 months old and 75 ± 10 cm tall at sampling time. All trees were grown in a U.S. Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS)-approved secured greenhouse ($28^\circ\text{C} \pm 4^\circ\text{C}$, $60\% \pm 5\%$ relative humidity, and 16/8-h light/dark photocycle) at the Citrus Research and Education Center, University of Florida ($28^\circ10'N$, $81^\circ71'E$), in Lake Alfred. Weekly, trees were irrigated twice and fertilized once using 20-10-20 water-soluble fertilizer (Peter's Florida Special).

Preparation of *Candidatus Liberibacter asiaticus*-Infected and/or *Diaphorina citri*-Infested Trees

To obtain the *Ca. L. asiaticus*-infected trees, healthy Valencia sweet orange trees (about 10 months old) were graft inoculated with budwood from a PCR-positive *Ca. L. asiaticus*-infected source and maintained in the same conditions described above. Eight months later, the infection was confirmed using PCR as described (Tatineni et al., 2008). For *D. citri*-infested and the double-attacked trees, 18-month-old healthy or *Ca. L. asiaticus*-infected trees exhibiting 15 cm of new flush were exposed to 100 healthy adult psyllids (PCR negative) per tree and caged individually using insect-rearing cages (60 × 60 × 90 cm; Bioquip) and maintained in a USDA-APHIS/Centers for Disease Control-approved secured growth room under the same conditions described above. *Ca. L. asiaticus*-free psyllids were obtained from our laboratory colony reared on *Berbera koenigii* plants, a nonhost for the pathogen. For all treatments, three leaves were collected from different positions on each tree. The collected leaves were chopped and mixed together and then kept at -80°C for further analysis.

Exogenous Application of Melatonin on Healthy Citrus Plants

Due to the lack of previous studies about melatonin application on citrus plants, our selection of tested concentrations for the exogenous application was based on the previous literature on other fruit trees such as apple (*Malus domestica*; Wang et al., 2012, 2013; Yin et al., 2013; Liang et al., 2018; Wei et al., 2018b; Zhang et al., 2019), pear (*Pyrus communis*; Liu et al., 2018, 2019; Zhai et al., 2018), wild jujube (*Zizyphus acidojuba*; Ren et al., 2018), tamarillo (*Cyphomandra betacea*; Lin et al., 2018), and sweet cherry (*Prunus avium*; Tijero et al., 2019). Treatments with five concentrations of melatonin (powder, purity $\geq 98\%$; Sigma-Aldrich) consisted of 0.05, 0.1, 0.2, 0.5, and 1 mM, in addition to the control (Milli-Q water with 0 mM melatonin). Melatonin solutions were applied through root drench under low light intensity to avoid any degradation of melatonin under light conditions. Briefly, Valencia trees were irrigated with 150 mL of aqueous melatonin solution per tree. The treatments in excess of soil capacity were collected using plastic cups and added back to the soil the following day. All treatments (six biological replicates per each) were kept in the same growth room conditions as described previously. For leaf sampling, three leaves were collected from each tree from different positions before melatonin treatment (zero time) and at 12, 24, 48, 72, 96, and 120 hpt. The samples were stored at -80°C until analysis.

Melatonin Analysis Using GC-MS-SIM

Melatonin was analyzed using our reported method (Nehela and Killiny, 2018), developed from the published methods (Aboul-Enein et al., 1999; Hernández-Ruiz et al., 2004, 2005; Wójciak-kosior and Woźniak, 2008). Briefly, about 300 mg of leaf tissues was ground to a fine powder using liquid nitrogen. Melatonin was extracted with 1.5 mL of ethyl acetate containing 100 mg L⁻¹ butylated hydroxytoluene (as an antioxidant) as described in our previous work (Nehela and Killiny, 2018). Subsequently, samples were derivatized with 50 μL of MSTFA (Thermo Fisher Scientific) containing 1% (v/v) chlorotrimethylsilane (98%; Acros Organics, Thermo Fisher Scientific) by incubating at 85°C for 45 min in a multiblock aluminum heater as described (Núñez-Vergara et al., 2001). For the GC-MS analysis, 1 μL was injected into the GC-MS apparatus running in SIM mode using the same chromatographic conditions as described in our previous study (Nehela and Killiny, 2018). Peaks were identified by comparing their retention times, linear retention indices, and mass spectra with those of the authentic melatonin standard (powder, purity $\geq 98\%$; Sigma-Aldrich). In the SIM mode, seven characteristic ions were used for peak identification.

Phytohormone Analysis Using GC-MS-SIM

Citrus phytohormones were extracted using an extraction mixture of methanol:water:HCl (6 N; 80:19.9:0.1, v/v/v) as described (Nehela et al., 2016). After extraction, the supernatant was concentrated to 50 μL under a gentle nitrogen stream and derivatized using methyl chloroformate as described previously (Hijaz and Killiny, 2014; Nehela et al., 2016, 2018). After derivatization, the chloroform extract was concentrated to 20 μL under a nitrogen stream and a few milligrams of sodium sulfate (two to three crystals) was added to dry the organic phase. For GC-MS analysis, 1 μL was injected in SIM mode

using the published thermal conditions, and three to five ions were used to identify each compound (Nehela et al., 2016). The abundances and ratios of the characteristic ions were similar to those of the authentic standards. TurboMass software version 6.1 (Perkin-Elmer) was used to analyze chromatograms. Identification of all phytohormones was performed by comparing their retention times, linear retention indices, and characteristic ions with those of authentic phytohormone standards.

Gene Expression Analysis Using RT-qPCR

Total RNA was extracted using Trizol reagent (Ambion, Life Technologies). Subsequently, we analyzed the expression of 15 genes involved in the melatonin biosynthetic pathway (Supplemental Table S1) and 38 genes involved in phytohormone biosynthetic pathways (Supplemental Table S2) in triplicate for each biological replicate for each treatment ($n = 30$), as described (Nehela et al., 2018; Nehela and Killiny, 2018). The relative expression of the consensus sequence among PCR products was done according to the 2^{- $\Delta\Delta\text{CT}$} method (Livak and Schmittgen, 2001). Four reference genes were used for normalization of gene expression: *CsEF1*, *CsF-box*, *CsGAPC1*, and *CsSAND* (Mafra et al., 2012; Wei et al., 2014a, 2014b).

Effect of Melatonin Supplementation on *Ca. L. asiaticus* Titer in Citrus Detached Leaves

The antibacterial activity of melatonin against *Ca. L. asiaticus* was examined using the detached leaves assay, which was used previously to study the effects of exogenous melatonin on abiotic and biotic stressors in several plant species, including *Arabidopsis thaliana* (Weeda et al., 2014), apple (Yin et al., 2013; Wei et al., 2018b), grape (*Vitis vinifera*; Shi et al., 2019), and perennial ryegrass (*Lolium perenne*; Zhang et al., 2016). Briefly, fully mature HLB-symptomatic leaves were detached with their complete petioles from 18-month-old trees of *Ca. L. asiaticus*-infected Valencia sweet orange. Collected leaves were covered immediately with wet absorbent gauze, placed on ice, and transferred to the laboratory. To investigate the effect of melatonin supplementation on *Ca. L. asiaticus* titer, the detached leaves were incubated separately in one of five concentrations of melatonin (0.05, 0.1, 0.2, 0.5, and 1 mM aqueous solution), in addition to the control (mock; Milli-Q water with 0 mM melatonin). All treatments (12 biological replicates per each) were kept in the same growth conditions as described above. For sampling, a single leaf disc (~31 mm²) was collected from each leaf using a paper hole puncher before the treatment (zero time) and at 24, 48, 72, and 96 hpt (Fig. 9A). Leaf discs were placed individually into 2-mL screw-cap Lysing Matrix tubes (MP Biomedicals) and stored at -80°C until analysis.

Determination of Endogenous Levels of Melatonin, SA, and tJA in Some Citrus Varieties with Different Degrees of Tolerance to *Ca. L. asiaticus*

Seedlings from six *Citrus* species with different degrees of tolerance to *Ca. L. asiaticus* were used to investigate the endogenous levels of melatonin, SA, and tJA. These species included two susceptible varieties (Valencia sweet orange and Duncan grapefruit [*Citrus paradisi*]), two moderately tolerant varieties (alemow [*Citrus macrophylla*] and Mexican lime [*Citrus aurantifolia*]), and two tolerant varieties (Khasi papaya [*Citrus latipes*] and Dancy tangerine [*Citrus tangerina*]; Folimonova et al., 2009; Hijaz et al., 2020). Seedlings were grown from seeds as described in our previous study (Hijaz et al., 2020) and maintained under greenhouse conditions as described above. All seedlings were about 1 year old. Leaf samples from all varieties (six biological replicates per each) were collected from each tree from various positions as described above. The samples were stored at -80°C until analysis. Subsequently, the endogenous levels of melatonin were analyzed using our developed method (Nehela and Killiny, 2018), whereas SA, and tJA were extracted and analyzed as described by Nehela et al. (2016).

Exogenous Application of Melatonin, SA, and tJA on *Ca. L. asiaticus*-Infected Plants

Ca. L. asiaticus-infected Valencia sweet orange was used as an experimental plant in this study. Trees were ~18 months old, 75 ± 10 cm tall, and maintained in a USDA-APHIS-approved secured greenhouse as described above. Aqueous

solutions of 0.1 mM melatonin, 0.25 mM SA, or 0.1 mM tJA (Sigma-Aldrich) containing 0.1% (v/v) Tween 80 (Sigma-Aldrich) were applied, in addition to the control (Milli-Q water + 0.1% [v/v] Tween 80). Trees were treated using 200 mL of these aqueous solutions through foliar application to the point of runoff using a 2-gallon chemical sprayer (RYOBI One World Technologies). After the application, all treatments (six biological replicates per each) were kept under greenhouse conditions as described above. For leaf sampling, three leaves were collected from each tree from various positions before the treatment (zero time) and at 72 hpt. The samples were stored at -80°C until analysis. Subsequently, the endogenous levels of melatonin, SA, and tJA were analyzed as described previously (Nehela et al., 2016; Nehela and Killiny, 2018).

Ca. L. asiaticus Quantification Using qPCR

Ca. L. asiaticus quantification was carried out using the leaf disc assay as described recently (Etxeberria et al., 2019; Attaran et al., 2020). Briefly, individual leaf discs (12 biological replicates per treatment) were frozen by submerging in liquid nitrogen for 10 min, then homogenized twice with one metal ball using a TissueLyser II (Qiagen) without lysis buffer for 1 min of each run at 30 Hz. Subsequently, DNA was isolated from a single leaf disc using a manual extraction protocol developed in our laboratory. Briefly, 1,500 μL of the lysis buffer (0.1 mM NaCl, 10 mM Na-EDTA, and 50 mM Tris base, pH 9) along with dithiothreitol (0.16 g per 100 mL) was added, and the tubes were inverted to mix. Samples were centrifuged at 2,500g for 1 min, then an aliquot of 1,300 μL of the supernatant was recovered into a new 2-mL Eppendorf tube. A total of 90 μL of SDS (10%, w/v) was added before incubation in a water bath at 65°C for 30 min. After incubation, 500 μL of 5 M potassium acetate (490.8 g of potassium acetate, 115 mL of glacial acetic acid, and Milli-Q water for a final volume of 1 L) was added, then samples were incubated on ice for 20 min. Samples were centrifuged at 15,000g for 10 min at 5°C , then an aliquot of 1,000 μL of the supernatant was recovered into a new 2-mL Eppendorf tube. The DNA was then precipitated by adding 500 μL of ice-cold isopropanol. Finally, DNA was collected by centrifugation at 16,000g for 10 min at 5°C , washed using 500 μL of ice-cold 70% (v/v) ethanol, dried, and resuspended in 20 μL of ultra-filtered DNase- and RNase-free water. The quantity and quality of extracted DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and adjusted to 100 ng μL^{-1} . DNA was used for RT-qPCR amplification using 16S rRNA primers HLBasf and HLBbr, the probe HLBp, TaqMan PCR master mix, and SYBR green PCR master mix. The qPCR assay was performed on a 96-well plate using an ABI 7500 RT-qPCR system, and supplies were from Applied Biosystems as described (Killiny et al., 2017; Etxeberria et al., 2019; Attaran et al., 2020). The bacterial titer of *Ca. L. asiaticus* was expressed as C_T values, which negatively reflect the bacterial population within the infected leaves.

Statistical Analysis

A completely randomized design was used as the experimental design throughout the study. In all experiments and based on the type of the experiment, six to 12 biological replicates and two technical replicates per treatment were analyzed. The technical replicates themselves were not used in the statistical analysis to avoid the possibility of pseudoreplication. All data were statistically analyzed according to the ANOVA technique, followed by posthoc pairwise comparisons between them using Tukey's HSD ($P \leq 0.05$). In addition, based on the assumptions of linearity, SLR analysis was performed to model the relationship between phytomelatonin content (as an independent variable) and the endogenous content of different phytohormones (as dependent variables) as well as to model the linear relationship between hpt (as an independent variable) and C_T (as a dependent variable). The fitted regression line was expressed as a significant equation, as determined by the F test ($P \leq 0.05$). Both the coefficient of determination (R^2) and the adjusted coefficient of determination (R^{2adj}) were also obtained. Furthermore, due to the observed nonlinear phenomena between exogenous melatonin concentrations (as an independent variable) and endogenous phytohormone content (as a dependent variable), and to understand the curvilinear relationship between them, data were fitted with a second-degree polynomial regression model (quadratic model). Likewise, the curvilinear relationship between exogenous melatonin concentrations (as an independent variable) and C_T (as a dependent variable) was fitted using a second-degree polynomial regression model (quadratic model). For polynomial regression models, the 95% confidence intervals for the estimated regression, quadratic equation, R^2 , R^{2adj} , and P value based on the F test ($P \leq 0.05$) were obtained. The transcript levels of various genes involved in the biosynthesis of melatonin and different phytohormone groups are presented as heat maps

combined with two-way HCA. HCA was performed using nonstandardized and standardized means of the matrices for all studied treatments. Distance and linkage were done using Ward's minimum variance method (Ward, 1963).

Accession Numbers

Sequence data used for gene expression analysis from this article can be found in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/gene/>) data libraries under the following accession numbers: XM_006469752.3 (CsTDC1/CsAADC-like); XM_006479363.3 (CsTDC2); XM_015531938.2 (CsCYP71P1-like); XM_006481177.3 (CsSNAT-1); XM_006484231.3 (CsSNAT-2); XM_006467651.3 (CsASMT1-1, isoform 1); XM_006467653.3 (CsASMT1-2); XM_025094656.1 (CsASMT1-3); XM_006469074.3 (CsASMT2-1); XM_006469074.3 (CsASMT2-2); XM_006480390.3 (CsASMT2-3); XM_006486606.3 (CsASMT); XM_006478027.3 (CsCOMT-1); XM_006477837.2 (CsCOMT-like-1); XM_006477776.3 (CsCOMT-like-2); AY498567.1 (CsEF1); XM_006482390.1 (CsF-box); XM_006483974.2 (CsGAPC1); XM_006488024.2 (CsSAND); XM_006485798.2 (CsCS); XM_006483415.2 (CsCM2); XM_006482655.2 (CsCM3); XM_006467360.2 (CsADTI); XM_006469841.1 (CsTAT); XM_006476023.2 (CsAST-1); XM_006476024.2 (CsAST-2); XM_006481431.2 (CsPAL); NM_001288910.1 (CsAAT1); XM_006489736.1 (CsKAT); XM_006476586.2 (CsIC52); XM_015532734.1 (CsASAI); XM_006469235.2 (CsASA2); XM_006482255.1 (CsTS); XM_006470948.2 (CsTSA); XM_006493882.2 (CsTSB); XM_015529685.1 (CsTAA2); XM_006473060.2 (CsTAA4); XM_006479363.2 (CsTDC1); XM_006466708.2 (CsYUC2); XM_006480095.2 (CsYUC8); XM_006487697.2 (CsNIT4); XM_006480990.2 (CsFAD); XM_006483993.1 (CsLOX); NM_001288906.1 (CsAOS); NW_006260521.1 (CsAOC); XM_006488806.2 (CsAAE7); XM_006475468.2 (CsOPR3); XM_006477083.2 (CsACX1); XM_006488772.2 (CsAIM1); XM_006473681.2 (CsAIM2); XM_006480138.2 (CsKAT); XM_006466537.2 (CsZEP); NM_001288881.1 (CsVDE); NM_001288932.1 (CsNSY); NM_001288935.1 (CsNCED); NM_001288867.1 (CsABA2); and XM_006487736.2 (CsAAO3).

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. Primers used for gene expression analysis of melatonin biosynthetic genes by RT-qPCR.

Supplemental Table S2. Primers used for gene expression analysis of genes involved in the biosynthesis of four major groups of phytohormones in Valencia sweet orange by RT-qPCR.

ACKNOWLEDGMENTS

We thank the members of our laboratory for helpful discussions and comments.

Received March 31, 2020; accepted August 6, 2020; published August 25, 2020.

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