Phytohormones in the *Penicillium digitatum*-citrus fruit interaction

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**Abstract**

Early- and long-term variations in indole-3-acetic acid (IAA), salicylic acid (SA), and jasmonic acid (JA)-related metabolites occurring during *Penicillium digitatum* infection in the flavedo (outer peel part) and albedo (inner peel part) of Navelate orange (*Citrus sinensis* (L.) Osbeck), and in the flavedo of its mutant Pinalate, which is deficient in abscisic acid (ABA), were compared. The levels of these metabolites were also examined in the fungal spores either obtained from in vitro grown cultures or isolated from infected fruit. The hormones were determined by ultra-high performance chromatography (UHPLC) connected to a Qtrap triple quadrupole mass spectrometer. The results showed that IAA barely changed either in the albedo of Navelate or in the flavedo of the mutant, but sharply and transiently increased in the flavedo of the parental. This highlights the relevance of IAA in very early infection perception. Moreover, results showed that IAA levels were higher in zones clearly showing mycelium or spores than in the healthy or macerated zones. They also indicate that SA participates in the initial signalling of both the flavedo and the albedo upon fungal attack and demonstrated the relevance of the interaction between this hormone and ABA in the citrus fruit-*P. digitatum* interaction. The concentration of cis-(-)-12-oxo-phytodienoic acid (OPDA) dropped during the initiation of maceration and at early infection stages. The fungus redirected jasmonate metabolism towards the synthesis of methyl jasmonate (MeJA) and jasmonyl-isoleucine (JAIle) from OPDA in a later infection stage. Finally, the results demonstrated for the first time that *P. digitatum* is able of producing SA, IAA, OPDA, JA, MeJA and JAIle, and that the fungus’ ability to produce OPDA was reduced, and that of IAA and JA stimulated, because of the fruit influence.

1. Introduction

A substantial amount of work has been performed to demonstrate the function of hormones in the interaction between pathogens and plants, but there are fewer studies related to the hormones’ involvement in fruit resistance to the fungi that cause postharvest diseases (Flors et al., 2008; Robert-Seilaniantz et al., 2011; Chanclud and Morel, 2016; Romanazzi et al., 2016; Zhou et al., 2018; Wang and Bi, 2021). While late hormonal responses are affected by the interaction among hormones (Verhage et al., 2016; Zhou et al., 2018; Wang and Bi, 2021), there are fewer studies related to the hormones’ action in early infections.

Different reports have demonstrated that the application of jasmonic acid (JA), methyl jasmonate (MeJA) or salicylic acid (SA) may lower rots produced by *Penicillium digitatum* (Pers.:Fr.) Sacc. in citrus fruit (Droby et al., 1999; Iqbal et al., 2012; Moscoso-Ramirez and Palou, 2013; Zhou et al., 2018; Wang et al., 2015, 2021), and that JA levels are increased by MeJA application in this crop (He et al., 2018). Likewise, it is worth mentioning that postharvest blue light and UV-C irradiations reduce disease caused by *P. digitatum* in this crop fruit and favour the synthesis of JA and of oxylipins’ metabolism (Lafuente et al., 2021c; Phonyiam et al., 2021), although cis-(-)-12-oxo-phytodienoic acid (OPDA), MeJA and JAIle contents were not determined during treatments or infection. The protective effect of ethylene and abscisic acid (ABA), and that of their interaction, against *P. digitatum* development in citrus fruit have been described (Chalutz et al., 1978; Marcos et al., 2005; González-Candelas et al., 2010; Lafuente et al., 2019, 2022; Ballester et al., 2018; Fresno and Munné-Bosch, 2021).

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The relevance of Pinalate in the study of the interaction between synthesis inhibitors either damage the fruit peel, which should favour P. digitatum OXS3 protein, whose repression in plants is important for the activation which those encoding a bifunctional inhibitor/LTP, a thaumatin or a pher different mechanisms that underpin the beneficial effects of ABA reducing disease caused by P. digitatum in citrus fruit. They involve phospholipases (Lafuente et al., 2021a), and different genes among which those encoding a bifunctional inhibitor/LTP, a thaumatin or a OXS3 protein, whose repression in plants is important for the activation of ABA-responsive genes, are remarkable (Lafuente and Romero, 2022). The relevance of Pinalate in the study of the interaction between P. digitatum and citrus fruit is reinforced by the fact that different ABA synthesis inhibitors either damage the fruit peel, which should favour disease incidence, or have a direct antimicrobial effect against P. digitatum (Lafuente et al., 2019). Transcriptomic analyses have also helped to decipher differences between responses to infection of outer (flavedo) citrus peel tissue and its inner part (albedo) (Lafuente et al., 2021b), which is less resistant to be infected (Ballester et al., 2006). Among them is noteworthy results suggesting the specific flavedo ability to favour the accumulation of H$_2$O$_2$, phenylpropanoids, and stress-related proteins; and that of the albedo to synthetize indole glucosinolates, cutin, or oxylipins (Lafuente and González-Candelas, 2022).

Transcriptomic analyses have also revealed the participation of some hormone-related transcripts in the elicited resistance against P. digitatum (González-Candelas et al., 2010; Ballester et al., 2011; Deng et al., 2018; He et al., 2018; Chen et al., 2021; Lafuente et al., 2021b, 2021c). Nevertheless, knowledge about the dynamics of relevant hormones associated with such a process (He et al., 2018) or the fungal-derived plant hormones and their putative role in virulence are still scarce (Chanclud and Morel, 2016; Liu et al., 2021). In the context of the present report, it is worth mentioning works by Ballester and González-Candelas (2020) and by Lafuente et al. (2019) demonstrating the ability of P. digitatum to produce ethylene and ABA, respectively, and that both fungal hormones are not critical virulence factors but might delay citrus fruit colonisation by fungi. Moreover, it is noteworthy that there is only one work that links JA production and virulence of a filamentous fungus (Liu et al., 2021). There is still also much to know about the specific hormonal responses to infection in inner and outer peel parts, as well as the putative connection between the protective effect of ABA against this fungus and jasmonates, SA and/or indole-3-acetic acid (IAA). We hypothesised that there are differential tissue-specific, JA, jasmonate-related metabolites and IAA content dynamics against infection, and that such dynamics may be regulated by ABA and be conditioned by the infection stage. Therefore, we determined these hormones’ levels in the short and long terms after infection in both the outer and inner peel parts of Navelate orange, as well as the flavedo of its mutant Pinalate. The flavedo was chosen to examine the influence of ABA because differences in ABA levels in the albedo between Navelate and Pinalate fruit were less evident than in the flavedo. Moreover, and considering that there is a knowledge gap for P. digitatum’s ability to produce these metabolites, we investigated whether P. digitatum is able to produce SA, IAA or jasmonate-related metabolites OPDA, JA, MeJA, and jasmonoyl-isoleucine (JAILE), which is the active form of JA (Fonseca et al., 2009), and whether this ability is affected by the fruit-pathogen interaction.

2. Material and methods

2.1. Fruit material

Fully mature sweet oranges of the Lane Late, Navelate and Pinalate (Citrus sinensis (L.) Osbeck) cultivars were used in this study. Fruit of the Navelate and Pinalate cultivars were harvested at random on the same day from trees grown in the experimental orchards of the ‘The Spanish Citrus Germplasm Bank’ located at the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). Fruit of the Lane Late cultivar were also harvested at random from the trees cultivated in commercial orchards in Llíria (Valencia, Spain). The fruit free of damage of each cultivar were shortly transported to the laboratory, sterilised and washed as described by Lafuente and González-Candelas (2022).

The fruit of both Navelate and Pinalate oranges were assigned to two different groups. All the oranges in the first group were inoculated with a P. digitatum conidial suspension (10 µL 10$^5$ conidia ml$^{-1}$) prepared as described in Section 2.2. Fruit in the second group were inoculated with the same volume of water to be used as control samples. Two other groups of Pinalate oranges were employed to determine whether ABA application would, to some extent, reverse changes in hormones profiles occurring because P. digitatum infection and, thus, whether ABA application may mimic the hormones profiles found in the Navelate fruit in Pinalate. Before being inoculated, these two Pinalate groups were dipped in ABA (Sigma-Aldrich, St. Louis, MO, USA) 1 mM for 2 min because this treatment was effective in reducing the disease severity caused by P. digitatum in Pinalate orange (Lafuente et al., 2019). To dissolve the hormone, 0.7% ethanol was used. Therefore, the above-mentioned groups of the ABA-untreated Navelate and Pinalate oranges were dipped for 2 min in 0.7% ethanol. We did not determine the effects of applying ABA to the parental fruit (Navelate) because, as we previously showed, applying ABA 1 mM barely affects the disease severity in this cultivar (Lafuente et al., 2019). This was explained by the fact that ABA concentration in the flavedo of Navelate was already very high and, therefore, such concentration is high enough to induce protective mechanisms against this or other stresses (Romero et al., 2012a; Lafuente et al., 2019). As a consequence, the effects of such stresses would not be affected by exogenous ABA. Two fruit subgroups were formed in each group, which were used for tissue sampling or to determine the severity of the disease. For each sample type (Navelate, Pinalate and Pinalate treated with ABA), the first subgroup contained five fruit per biological replicate to estimate the evolution of disease, and the second subgroup contained 10 fruit per replicate and sampling period to measure the concentration of the different hormones. Three biological replicates were used for each determination.

The comparison of the effect of infection on hormone profiles between the albedo and flavedo discs (0.7 cm diameter) centred at the point of inoculation was done using the Navelate cultivar. Both peel tissues were carefully separated with a razor blade, homogenised in liquid nitrogen and kept at -80°C until hormones’ analyses. Considering that the differences in ABA concentration between the fruit of Navelate and Pinalate cultivars are mainly evident in the outer peel part, only the flavedo samples were utilised to evaluate the putative effect of ABA on the changes that occur in the profiles of other hormones during fungal infection. To that end, the tissue samples were periodically taken from the wounded (control) and infected oranges of the Pinalate and Navelate cultivars, and of Pinalate orange treated with exogenous ABA, stored at -80°C. Samples were taken at fruit harvest, and from the control and infected fruit stored for 1, 2, 3 and 7 d. Samples were also taken by day 14. These last samples were completely rotten, and the albedo was almost non-existent so, on day 14, we did not measure the hormones in the albedo sample.

In order to know whether the spores of P. digitatum obtained from the infected citrus fruit produced the same hormones/metabolites as the fruit, another Navelate fruit group was included to isolate spores from
rotten fruit at 12 and 18 d post-inoculation (dpi). Per each sampling period, five oranges were included in each replicate to ensure that sufficient spores could be obtained to detect and determine the metabolites. The spores were isolated by carefully tapping the fruit with a spatula and passing them through a sieve to avoid possible traces of fruit tissue.

Lane Late oranges were used to determine the spatial changes in metabolites upon infection, as described by Lafuente et al. (2006) and by using the Penicillium digitatum (Pers.:Fr.) Sacc. isolate Ps1 (CECT 20795). P. digitatum was grown on potato dextrose agar (PDA, Becton, Dickinson and Company, Sparks, MD, USA) for 7 d at 24 ºC. Quantification of spores was performed by using a hemocytometer and the conidia concentrations were adjusted to either 10^6 (for Navelate and Pinalate oranges) or 10^7 conidial mL^-1 (for Lane Late oranges). This higher inoculum was employed to ensure that enough sporulated tissue would be obtained from the same fruit from which the healthy and macerated tissues were taken, and also the tissue with mycelium, to analyse hormones.

Before inoculating the oranges with water (the mock control sample) or with the conidial suspensions, they were wounded (4 mm depth) because P. digitatum is a wound pathogen. The incidence of the disease was determined by estimating the percentage of infected wounds, and disease severity by determining the lesion area (mm²) as described by Ballester et al., 2006 during fruit incubation at 90–95% RH and 20 ºC. The diameters of the zones showing mycelium or sporulation were also determined. Four wounds were performed in the equatorial area of each orange and, therefore, 20 wounds per replicate were used to estimate disease evolution.

2.2. Determination of jasmonates, SA and IAA in both fruit and fungus

Jasmonates, SA and IAA concentrations were measured in three biological replicates of the previously frozen tissues and also in the fungal spores isolated from either the infected fruit or potato dextrose agar (PDA) plates. A total of 80 flavedo or albedo discs were taken in each replicate (10 fruit x 8 discs per fruit). The hormone-related metabolites were determined as described by Lafuente and Romero (2022). Briefly, IAA, SA and the jasmonate-related metabolites (OPDA, JA, MeJA and JAile) were extracted from the previously frozen ground samples with 70% methanol containing 1% glacial acetic acid and prostaglandin B1 (Cayman Chemical Co., Ann Arbor, Michigan, USA) was used as an internal standard (Flors et al., 2008). Extracts were centrifuged and filtered through 0.2 µm Nylon filters prior to UHPLC determinations. All the samples from the same experiment were extracted and filtered on the same day and stored overnight at ~80 ºC before being injected. Hormones’ analysis was performed by ultra-high performance chromatography (UHPLC) (ExionLC AD system, Framingham, MA, USA). The chromatograph was connected to a Sciex Qtrap 6500 plus triple quadrupole mass spectrometer and equipped with a refrigerated ExionLC AD Autosampler at the SCSIE-UV Chromatography Facility (Valencia, Spain). All the analytical conditions and the software used to process quantitative data were the same as previously reported (Lafuente and Romero, 2022).

Metabolites were also analysed in the spores taken from fruit at 12 and 18 dpi and from the cultures grown on PDA (7 d, 24 ºC) to determine whether P. digitatum produces them and whether this ability would be altered by fruit. The spores from fruit were carefully separated with a spatula and sieved to remove putative contamination by fruit tissues before freezing them in liquid nitrogen for future analyses. The spores from the PDA plates were recovered with a spatula and frozen as those that came from fruit for later analyses. The amount of spores was measured to express the results as ng metabolite per spore. Each of the three biological replicates herein used included the spores isolated from five different fruit (in vivo experiment) or from three different PDA plates (in vitro experiment). The hormones of all the samples were simultaneously extracted twice from conidia with 1 mL of the same solvent employed when extracting them from the flavedo or the albedo. The combined supernatants were centrifuged (5 min at 13,000 x g), filtered by using Nylon filters (0.2 µm) and 5 µL of the filtered supernatants were analysed by UHPLC as described above for the determination of the hormones in peel tissues.

2.4. Statistical analysis

The values shown in the figures are the mean of three replicate biological samples ± standard error. Significant differences (P < 0.05) for the same storage time were determined by applying the analysis of the variance (ANOVA) and a Tukey’s test the Statgraphics Plus 4.0 Software (Manugistics, Inc.).

3. Results

3.1. ABA deficiency accelerates the disease and sporulation development

Disease symptoms were evident in some wounds in the Pinalate orange by day 3, albeit their macerated area was very small (Fig. 1). By day 5, disease was hardly detected in Navelate orange and in its mutant treated with ABA. These fruit showed 12 and 6 times smaller maceration areas around the inoculated wounds, respectively, than those of the mutant not treated with ABA (Fig. 1A). By day 7, the proportion of macerated wounds was similar and higher than 80% in all the samples (data not shown), but the area of maceration of the Pinalate fruit was still larger than that of the ABA-treated Pinalate oranges, followed by that of Navelate (Fig. 1A). This pattern was consistent with the results obtained when comparing the area of the fruit containing mycelium (Fig. 1B) or spores (Fig. 1C).

3.2. Hormones’ basal levels and the ability to induce hormone signalling molecules upon infection differs between albedo and flavedo

For the same storage period, the SA, IAA (Fig. 2) and JAile (Figs. 3G and 3H) levels were significantly higher in the flavedo than in the albedo of Navelate oranges, and in both the mock control wounded and the infected orange. The OPDA (Figs. 3A and 3B), JA (Figs. 3C and 3D) and MeJA (Figs. 3E and 3F) contents were much higher in the albedo at fruit harvest. Nevertheless, OPDA and JA sharply decreased immediately after fruit harvest in this peel tissue (Figs. 3A and 3C), and rose in the flavedo (Fig. 3B and D), which resulted in higher values in the flavedo after 1 dpi in both the control and infected wounds. On the contrary, MeJA concentration in the inner peel part was always higher than in the outer part irrespectively of wounding or infection (Figs. 3E and 3F).

The SA levels increased for up to 2 days in response to wounding in both the albedo (Fig. 2A) and flavedo (Fig. 2B). This increase was greater in the infected samples in early infection stages, but SA was lower in the infected than in the control wounds by 7 d in both tissues. A comparison of the SA levels between the control and infected flavedo samples at 14 dpi, when peel tissue structures were disorganised, the albedo was almost non-existent and abundant spores colonised fruit, revealed that the SA levels were much higher in the infected than in the mock-
inoculated oranges (Fig. 2B). Moreover, the SA content markedly increased from 7 to 14 d by *P. digitatum* colonisation (Fig. 2B). In early infection stages, IAA transiently rose in the control and infected flavedo samples (Fig. 2D). Maximum levels peaked by 3 d and were 2.5-fold higher in the infected wounds (Fig. 2D). The IAA content did not increase in response to wounding and was initially lower in the infected wounds in the albedo. However, it was much higher in the infected tissue over longer periods (7 dpi) (Fig. 2C). Like SA, IAA clearly increased from 7 to 14 d (Fig. 2D). Infection markedly lowered the OPDA levels by 7 dpi in the albedo, and after 2 dpi in the flavedo (Figs. 3A and 3B). Infection also lowered JA content after 2 dpi in the flavedo, and barely changed from 7 to 14 d (Fig. 3D). In contrast, the levels of this metabolite were at least 2 times smaller in the control than in the infected albedo samples after 2 dpi (Fig. 3C). The most marked effect of infection on MeJA content occurred at 7 dpi in the flavedo, but MeJA lowered by 14 dpi (Fig. 3F). During this
levels of this metabolite continued to increase in the albedo until day 7, but the final JAIle content was lower in this tissue than in the flavedo. The rise in JAIle was observed later in the flavedo (7 dpi) (Fig. 3H). The JAIle level lowered from 7 to 14 dpi in this peel tissue, but it was still higher in the infected than in the non-infected sample (Fig. 3H).

3.3. Hormone levels differ between healthy and non-healthy peel zones

Examination of the spatial changes in the hormone profiles by analysing the samples of the different healthy and non-healthy tissue areas demonstrated that the levels of the hormones differed among the different zones and that the trend of changes varied among hormones (Fig. 4). Small differences appeared in the SA and MeJA contents among the different zones (Figs. 4A and 4E). The IAA content increased from the healthy zone to the zone surrounding the inoculation site (Fig. 4B), which clearly showed spores and mycelium. OPDA levels were higher in the healthy than in the macerated areas, which in turn showed higher OPDA levels than the mycelium-containing areas. (Fig. 4C). The JA (Fig. 4D) and JAIle (Fig. 4F) contents sharply increased from the healthy to the macerated tissue. The huge increase in JAIle was observed only in this zone despite the small differences found in JA content between the macerated zone and the zones showing mycelium or sporulated tissues.

3.4. P. digitatum spores are able to produce SA, IAA, OPDA, JA, MeJA and JAIle

The results shown when examining the evolution in the levels of the hormones during Navelate fruit infection (Figs. 2 and 3), or the differences found among the various zones (Fig. 4), suggested that P. digitatum could produce SA and IAA, and some jasmonate-related metabolites. Therefore, we examined whether this necrotrophic fungus was able to produce them, and whether its ability could be modified during the interaction with fruit. To do so, the levels of the hormones were measured in spores obtained from PDA medium after 7 d of incubation and in the spores isolated at 12 and 18 dpi from the infected fruit (Fig. 5).

The results indicated that P. digitatum produces all of them and that their production was influenced by citrus fruit in some cases (Fig. 5). The data obtained from the spores collected from PDA demonstrated that this fungus is able of producing jasmonates’ precursor OPDA at a much greater extent than that of SA, IAA or MeJA, followed by JA and JAIle (Fig. 5, black bars). The OPDA levels in the spores isolated from fruit at 12 and 18 dpi were about 2.6 and 12 times smaller, respectively (Fig. 5, white and grey bars). In contrast, the JA and IAA levels in the spores sampled at 12 dpi were higher than in those isolated from PDA, whereas no differences were found in SA, MeJA or JAIle when comparing both spore types (Fig. 5). The concentration of all these metabolites, except that of JA, lowered with time to different extents in the spores collected from the infected fruit (Fig. 5).

3.5. ABA deficiency affects the capability of oranges to induce hormone signalling molecules when they are infected by P. digitatum

Our group showed that ABA plays a protective role against P. digitatum infection in oranges (Lafuente et al., 2019), and that Pinalate orange, which is deficient in ABA in the flavedo compared to its parental Navelate, is an useful resource to better understand the role of this hormone in the infection of oranges (Lafuente et al., 2021a; Lafuente and Gonzalez-Candelas, 2022). Here, we compared the effect of infection on the levels of SA and IAA, and of the jasmonate-related metabolites between either ABA-treated and non-treated Pinalate oranges (Figs. 6 and 7), and its parental (Figs. 2 and 3). To that end, Pinalate and Navelate oranges were collected on the same date and in the same experimental orchard.

Temporal evolution of SA in the Pinalate flavedo during infection (Fig. 6) was similar to that of its parental (Fig. 2). Initially, SA content...
was slightly, but significantly, higher in the infected samples of the mutant, but were lower by day 3 (Fig. 6A vs. 2B). The content of this hormone considerably increased when adding ABA to Pinalate fruit in early infection stages in both the control and infected samples (Fig. 6B). The IAA levels were always significantly much lower in the mock and infected samples of the mutant compared to the parental fruit (Fig. 6C vs. 2D), but adding ABA barely modified the IAA content (Fig. 6D). The ODPA profile (Fig. 7A) followed the same trend as in the flavedo of Navelate oranges (Fig. 3B), but these levels were always significantly lower in Pinalate (Fig. 7A). A similar overall pattern was found in Pinalate for the jasmonate-related metabolites, with significantly lower levels in relation to its parental fruit (Fig. 7 vs. 3). Adding ABA had a slight effect on increasing the OPDA levels in Pinalate (Fig. 7B vs. 7A), which favoured the early transient rise in JA in the control wounded samples and the rise that occurred at late infection times (Fig. 7D). A slight effect on increasing the MeJA and JAIle levels was also observed after ABA application in Pinalate (Figs. 7F and 7H).

At 14 dpi, the OPDA levels were significantly lower, whereas the SA, IAA, JA, MeJA and JAIle were higher in the infected than in the control wounds of the mutant orange (Figs. 6 and 7). During this time period, adding ABA rose the SA concentration in the control wounded sample (Fig. 6B), but lowered it in the infected tissue (Fig. 6D). In contrast, exogenous ABA favoured the rise in IAA in the infected tissue and lowered the IAA content in the control sample at 14 dpi. Moreover, exogenous ABA led to higher JA content in the infected, but not in the control, oranges (Fig. 7D). Finally at 14 dpi, ABA had no effect on OPDA, MeJA or JAIle in either the control or infected fruit (Fig. 7).

4. Discussion

Results from the current work demonstrate that both the content and ability of the flavedo and the albedo to induce changes in SA and IAA, and in all the jasmonate-related metabolites when the oranges are infected by *P. digitatum* differ, and ABA deficiency influences both events. The basal levels of SA, IAA and JAIle were much lower in the inner than in the outer peel part. Those of all the studied metabolites were lower in the ABA-deficient mutant than in Navelate. These findings parallel the lower susceptibility of the flavedo than the albedo (Ballester et al., 2006), and of the ABA-deficient mutant than its wild-type fruit, to infection (Fig. 1), which may suggest the participation of SA, IAA and JAIle in protecting the oranges against this necrotrophic fungus.

The examination of the early responses to the fungus (0–3 dpi), which occurred before visual disease development, indicate that the ability of the Navelate flavedo to increase IAA in response to the pathogen is very high; the IAA levels lowered in the albedo and barely changed in the flavedo of the ABA mutant (Figs. 2C and 6B). These results suggest a link between the capability of *P. digitatum* to infect the oranges and to lower IAA levels in the albedo in early infection stages. They also support the notion that IAA may be a relevant player in very early citrus flavedo defence responses to cope with the fungus, which would agree with reports that propose auxins to be a prime early plant defence against fungal pathogens in plants (Qi et al., 2012; van den Berg et al., 2004).
In line with this, Lu et al. (2015) found that Rhodosporidium paludigenum-mediated resistance elicitation against 

*P. digitatum* involves genes participating in IAA metabolism in citrus fruit. IAA increased later in the albedo (7 d), but the IAA levels were still lower than those of the flavedo by day 3. Moreover, the increase in IAA in the albedo occurred when tissue maceration was already visible (Fig. 2), which would suggest that the fungus is able of producing the hormone. The present findings also indicate that SA participates in the early-defence response in citrus fruit, which agrees with the results found by van den Berg et al., 2018 in their study into the *Persea americana-Phytophthora cinnamomomi* interaction. Thus, significant increases in SA occurred when the oranges were infected by *P. digitatum*, first in the flavedo (1 dpi), followed by rises in the albedo (2 dpi) (Figs. 2A and 2B). Moreover, the SA concentration in the infected oranges was much lower in the more susceptible tissue to infection (albedo). Given these results, it cannot be discarded the contribution of SA in the different adaptive responses of the flavedo and the albedo to *P. digitatum*, which is in concordance with Rodriguez et al., 2014; He et al., 2018. Therefore, the relevance of SA in citrus fruit defence against *P. digitatum* varies among cultivars and, consequently, it appears that SA may be important, but is not critical in the defence of this crop to such a process. In this regard, it is worth mentioning that the proportional change found by day 2 in IAA was much higher than that of SA.

Although the *P. digitatum*-induced rises in SA and IAA decreased from 3 to 7 d in the infected flavedo, the levels of both hormones markedly increased thereafter with very long-term infection (14 dpi) (Fig. 2), when peel tissue structures were completely disorganised and abundant spores colonised fruit. Such increases were concurrent but higher in IAA than in SA. These long-term increases could be related to the release of the free hormones form their conjugates, as demonstrated for ABA (Lafuente et al., 2019), because IAA and SA may form different conjugates (Lee et al., 1995; Ljun et al., 2002). It seems reasonable to think that such releases, if they actually occur, are not involved in the resistance of the oranges to the fungus because they take place when the fungus has completely colonised the fruit. Given the abundance of spores, the rises noted by 14 d in both hormones could also support the idea of the fungus’ ability to produce them. In this regard, it is remarkable that the IAA levels in the zones clearly showing the mycelium or spores were much higher than those of the healthy or macerated zones (Fig. 4B). This effect was less clear for SA (Fig. 4A), although spore content was still very low by day 6 compared to its content in fruit at 14 dpi. Therefore, the levels of these metabolites were determined in the spores isolated from the fungus and, thus, providing for the first time the evidence that *P. digitatum* produces both IAA and SA (Fig. 5). Moreover, we demonstrated that this ability may be altered in the *P. digitatum*-citrus fruit interaction, which encourages future investigation to decipher the role of both fungal hormones in fruit colonisation. As shown in Fig. 5, fungal IAA production was markedly stimulated by such an interaction, whereas the influence of fruit on the fungus’ ability to produce SA was less clear. There is increasing evidence that fungal hormones act as virulence factors (Lievens et al., 2017; Liu et al., 2021).
The possible involvement of jasmonates on the early (1–3 d) responses to infection seemed to be less relevant than that of IAA or SA. With the jasmonate-related metabolites, one remarkable finding was that the OPDA levels markedly dropped from 3 to 7 dpi upon fungal challenge, and in both the inner and outer peel parts (Figs. 3A and 3B) during the initiation of the maceration process (Fig. 1A), which is in concordance with the drop in OPDA from the healthy to the macerated peel zones (Fig. 4C). These decreases could be the consequence of citrus fruit protective mechanisms that involve the synthesis of the jasmonate derivative(s) that require(s) OPDA as a substrate. This notion is supported by the sharp rises in JAlle in both the flavedo and albedo (Figs. 3G and 3H) and a lower, but significant, MeJA rise that occurred only in the flavedo during the same period (Fig. 3E). The rise in JAlle from 3 to 7 d was also higher in the flavedo (Figs. 3G and 3H) and was concomitant with the initiation of the maceration process (Fig. 1). Moreover, JAlle sharply rose from the healthy to the macerated zone in the flavedo (Fig. 4D). Interestingly, a single peak in JAlle occurred at day 7, whereas IAA and SA showed two maximum rises (3 and 14 d). Therefore from the above results, it would appear that MeJA would contribute to flavedo defence against P. digitatum, and JAlle, the relevant metabolite in JA signalling (Fonseca et al., 2009), is an important player against this fungus and might participate in containing infection from propagating in citrus fruit. The second rise in IAA and SA, which followed the rises in MeJA and JAlle, would be more likely related to the fungal colonization. This would agree with the ideas proposed by van den Berg et al., 2018 in another pathosystem with not only the relevance of key transcription factors in JA signalling (MYC2) in the resistance of tomato fruit to another necrotrophic fungus (Botrytis cinerea) (Min et al., 2020), but also with the proposed role of jasmonates in participating in late responses to stresses aiming to contain the spread of cell damage (González-Aguilar et al., 2004; Kondo, 2022; Lafuente and Romero, 2022). Findings of the current research are also in concordance with the results of He et al. (2018) by showing that OPDA drops in the whole peel of Newhall Navel orange in later infection stages, and concomitantly with the major increase in decay development from 3 to 7 d. However, in this citrus cultivar, JA decreased and JAlle barely changed with long-term infection (He et al.; 2018). Accordingly, and in spite of the findings in the current work indicating a link between the drop in OPDA and the synthesis of JA derivative JAlle in both Navelate (Fig. 3) and Pinalate orange (Fig. 7), a putative link between the capability of P. digitatum to infect oranges and to lower OPDA levels in fruit cannot be totally ruled out because this jasmonate’s precursor can mediate itself in the responses of plants to stress cues (Taki et al., 2005; Hazman et al., 2015). OPDA and JA did not change from 7 to 14 d in the infected flavedo, and MeJA and JAlle decreased (Fig. 3); although JAlle levels were still about 3-fold lower in the control than in the infected fruit by 14 d (Fig. 3H) when they had already been colonised by abundant spores. Considering the marked tissue deterioration at day 14, the changes observed by 14 d in jasmonates would not likely be related to either the changes induced by the fungus in the fruit jasmonate metabolism or the conjugation of JA with isoleucine. Therefore, an interesting possibility could be that the different trend of changes in jasmonates, which occurred from 7 to 14 d, were due, at least in part, to differences in the fungus’ ability to produce each metabolite and how this could be affected by the P. digitatum-citrus fruit interaction. To the best of our knowledge, this is the first report showing the capability of this necrotrophic fungus to synthesize JAlle, but also OPDA, JA and MeJA (Fig. 5). Moreover, the herein presented results indicate that the fungus’ ability to produce OPDA was clearly reduced because of fruit influence, while fungal IAA and JA productions were stimulated by the P. digitatum-citrus fruit interaction (Fig. 5). Knowledge about fungal-derived plant hormones and their putative role in virulence is still scarce (Chanclud and Morel, 2016; Lafuente et al., 2019; Ballester and Gonzalez-Candelas, 2020; Liu et al., 2021). Therefore, the results of the other fungi, as suggested for ethylene in P. digitatum by Ballester and Gonzalez-Candelas (2020).

The fact that fungal SA and IAA were detected when spores were abundant suggests that these fungal metabolites are not primary virulence factors, but might contribute to fungal colonisation. Likewise, these fungal metabolites may participate in preventing the attack of...
present study encourage to investigate the role of fungal jasmonate-related metabolites in the capability of the necrotrophic *P. digitatum* fungus to infect citrus fruit to identify novel/critical targets for controlling green mould disease. Indeed it is worth mentioning that there is only one report that links JA production and filamentous fungi virulence by means of specific inhibitors and mutant analyses (Liu et al., 2021).

From the current results, we should point out the relevance of the interaction between ABA and SA because adding ABA to the mutant had a strong impact on increasing SA (Fig. 6B), and significantly reduced interaction between ABA and SA because adding ABA to the mutant had a strong impact on increasing SA (Fig. 6B). Moreover, different examples show that exogenous ABA does not rescue the normal phenotype in plants or fruit deficient in ABA (Busk and Pages, 1998; Sandhu et al., 2011), and that plants are much less sensitive to exogenous hormone than to rises in endogenous ABA levels induced by stress (Imay et al., 1995; Mahouachi et al., 2011).

The overall results provide an insight into the dynamic changes in hormones during fruit infection. It seems that IAA followed by SA, are the major phytohormones involved in the defense response of guava fruit (van den Berg et al., 2009). Moreover, we have noted that this is the first study showing the capability of *P. digitatum* in oranges. They also encourage further investigations into the effect of IAA or metabolites that increase JAlevels (e.g. L-isoleucine) in fruit (Kondo et al., 2021; Kondo, 2022). Improving such an understanding would be very helpful to develop more efficient methods to reduce postharvest losses caused by phytopathogenic fungi in citrus fruit and, hence, to minimize the application of traditional fungicides.

5. Conclusions

The findings of this research highlight the relevance of IAA in early infection perception when considering the different resistance of the flavedo and albedo of Naveline fruit, and that of the flavedo of this citrus cultivar and its ABA-deficient mutant (Pinalote), to develop disease caused by *P. digitatum*. They also indicate the contribution of SA in the initial fruit response against *P. digitatum*, the relevance of the ABA-SA interaction and that *P. digitatum* redirects jasmonate metabolism towards the synthesis of JAlevels in long-term infection. Finally, it has to be noted that this is the first study showing the capability of *P. digitatum* to produce SA, IAA, OPDA, JA, MeJA and JAlevels, that this capability may be specifically affected by the *P. digitatum*-citrus fruit interaction, and that these fungal metabolites do not appear to be a special factor of virulence for infection in citrus fruit. Nevertheless, their participation in fruit colonisation cannot be ruled out.

CRediT authorship contribution statement

Lafuente María T.: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. González-Candelas Luis: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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