Involvement of abscisic acid in the resistance of citrus fruit to *Penicillium digitatum* infection

María T. Lafuente*, Ana-Rosa Ballester, Luis González-Candelas

Instituto de Agroquímica y Tecnología de Alimentos, IATA-CSIC, Calle Agustín Escardino
7, 46980 Paterna, Valencia, Spain

Ana-Rosa Ballester, ballesterar@iata.csic.es

Luis González-Candelas, lgonzalez@iata.csic.es

* Corresponding author. Tel.: +34 900 022; fax: +34 963 636 301

E-mail address: mtlafuente@iata.csic.es

ABSTRACT

The involvement of hormone abscisic acid (ABA) in citrus fruit resistance to *Penicillium digitatum* infection was investigated. To this end, the effect of both exogenous ABA and inhibitors of ABA biosynthesis in plants on the resistance of sweet oranges to be infected by this major postharvest pathogen, and also on *in vitro* fungal growth, was examined. The ability of both *P. digitatum* to infect wild-type Navelate orange fruit (*Citrus sinensis* (L.) Osbeck) and its spontaneous ABA-deficient mutant Pinalate was compared. The results showed that the Pinalate orange was more susceptible than its parental to infection, and exogenous ABA reduced the percentage of infected fruits and also the maceration zone of this mutant without affecting *in vitro* fungal growth. The global results indicated that the ABA of the fruit plays a defensive role in citrus fruit against *P. digitatum* infection. This work demonstrates, for the first time, the ability of *P. digitatum* to produce ABA. The hormone decreased in fruit in early stages as a consequence of infection. Thereafter when tissue maceration became evident, ABA increased, and this rise seemed related to the *P. digitatum*-induced rise in ethylene production. This was suggested by the fact that ABA increased in Navelate orange, but not in its ABA-deficient mutant Pinalate in this infection stage, while exogenous ethylene barely increased ABA in the mutant. Finally, the sharp marked rise in free ABA in later stages was likely related to free ABA being released from conjugated ABA, and also to fungal ABA. However, fungal ABA was not a primary virulence factor in citrus fruit infection.

KEYWORDS

Abscisic acid-deficient orange mutant; free and conjugated abscisic acid; fungal ABA; postharvest; resistance; stress.

1. INTRODUCTION

Abscisic acid (ABA) is a plant hormone that plays an important role in plant stress responses, but can be also synthesised by some fungal species (Chanclud and Morel, 2016; Ding et al., 2016). Upon pathogen attack, infected plant cells induce signalling molecules to initiate mechanisms in the surrounding cells to reduce pathogen spread. Plenty of information is available about the role of plant hormones, like jasmonic acid (JA), salicylic acid (SA) and ethylene, in the interaction between pathogens and plants (Robert-Seilanianantz et al., 2011; Romanazzi et al., 2016; Zhou et al., 2018). Fewer studies are available about the involvement of ABA in the susceptibility of plants, and especially of fruit crops, to be infected by phytopathogenic fungi. However, it is known that ABA may affect the outcome of plant-microbe interactions either positively or negatively (Robert-Seilanianantz et al., 2011; Chanclud and Morel, 2016). The results obtained by using the ABA-deficient mutants of tomato plants (Audenaert et al., 2002) and strawberry fruit (Li et al., 2013) have shown that ABA promotes the infection caused by the necrotrophic fungus *Botrytis cinerea*. Similarly, ABA may lead to enhanced pathogen susceptibility in different fruit crops like pepper (Hwang et al., 2008), banana (Li et al., 2017) and tomato (Gong et al., 2017; Wilkinson et al., 2018). In contrast, a positive correlation between ABA and disease resistance has been found in grapes infected by *Glomerella cingulata* (Wang et al., 2015), and also in *Brassica napus* and *Arabidopsis* infected by *Leptosphaeria maculans* (Kaliff et al., 2007). Such differences in the effect of ABA on the resistance to pathogens may depend on the ABA-induced mechanisms in a specific plant-pathogen interaction or crop.

Penicillium digitatum (Pers.:Fr.) Sacc. is the major pathogen that causes postharvest disease in the citrus fruit grown under Mediterranean conditions. Previous research has

demonstrated that ethylene (Porat et al., 1999; Marcos et al., 2005) and JA (Droby et al., 1999) are involved in the defence of citrus fruit against this pathogen. The role played by ABA in the response of this important agronomic fruit crop to *P. digitatum* infection remains unknown, but endogenous ABA levels are known to increase in response to ethylene in citrus peel (Lafuente et al., 1997).

This work aimed to determine variations in ABA levels during the fruit infection process and to elucidate whether the hormone is involved in the defence response of citrus fruit to infection by *P. digitatum*. To this end, the spatio-temporal changes in the ABA levels in citrus fruit infected with this pathogen were examined. We also determined the effect of both exogenous ABA and inhibitors of the hormone on the resistance of sweet oranges to *P. digitatum* infection. We compared the ability of the fungus to infect wild-type Navelate orange fruit (*Citrus sinensis* (L.) Osbeck) and its ABA-deficient spontaneous mutant Pinalate. The availability of artificially generated mutants is not very common in woody plants. Therefore by considering the limitations of pharmacological experiments, this ABA-deficient yellow sweet orange mutant characterised by Rodrigo et al. (2003) has been a valuable tool to investigate the involvement of ABA in the response of citrus fruit to infection. This mutant has a fruit-specific biochemical blockage in the carotenoid biosynthetic pathway that results in a yellow-pigmented fruit and a marked reduction in ABA levels following the biosynthesis of carotenoids (Rodrigo et al., 2003). The putative involvement of conjugated-ABA, ethylene and fungal ABA in the pathogen-induced changes in ABA are also discussed.

2. MATERIAL AND METHODS

2.1. Fruit material

Full mature Navelina and Lane Late sweet oranges (*Citrus sinensis* (L.) Osbeck) were harvested from the trees grown in commercial orchards in Liria (Valencia, Spain). The fruit of the Navelate (*Citrus sinensis* (L.) Osbeck) sweet orange and its spontaneous ABA-deficient yellow mutant (Pinalate) were randomly harvested from the adult trees grown in experimental orchards according to normal cultural practices in the ‘The Spanish Citrus Germoplasm Bank’ at the Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Valencia) in Spain.

Oranges from all the cultivars were immediately delivered to the laboratory. The fruit with no damage or visual defects were surface-sterilised with commercial bleach and then rinsed with abundant tap water as previously described (Ballester et al., 2010). After drying at room temperature, fruit were divided into groups to be immediately inoculated with *P. digitatum* conidia or treated with chemicals (see Section 2.3) before being inoculated with the fungus.

Two groups of oranges of each cultivar were used to compare changes in disease incidence, and in free and conjugated ABA and ethylene production. The fruit in the first group were inoculated with 10 µL of the conidial suspension, while those of the second group were inoculated with the same volume of water (control fruit). The oranges in each group were divided into two subgroups. Three replicates of five fruit were included in the first subgroup to determine disease evolution. The second subgroup included three replicates of at least five fruit per incubation period. These fruit were used to periodically determine ABA and ethylene levels in peel or flavedo (outer coloured part of peel) discs (7 mm in diameter) taken around the inoculation site. The ethylene production of discs was determined immediately as described below (Section 2.7), while the discs used for the free and

conjugated ABA analyses were immediately frozen, homogenised in liquid nitrogen and kept at -80°C for later determinations.

To test whether the application of ABA, and of the inhibitors of its synthesis (Section 2.3), modified the response of oranges to *P. digitatum*, fruit were divided into four groups. Two groups were treated with either ABA or the solutions of the ABA inhibitors (Groups 1 and 2). The other two groups (3 and 4) were treated only with water containing the same volume of ethanol used to dissolve the hormone, or the inhibitors, and were used as the controls of the chemically treated fruit. After drying at room temperature, the oranges in Groups 1 and 3 were inoculated with the fungus, whereas those in Groups 2 and 4 were inoculated only with water. The oranges in each group were divided into two subgroups, as described above to evaluate disease incidence and for the ABA analysis.

To examine the spatial changes in the free and bound ABA, the flavedo samples were taken from several well-defined concentric zones of flavedo, which included tissue taken: 1) around the inoculation point with abundant spores; 2) in the contiguous area with abundant mycelium; 3) from the macerated zone; 4) from the healthy non-infected area. In order to obtain these samples on the same day, fruit were infected and kept in plastic boxes at 20°C and 90-95% relative humidity (RH) for 6 d. To obtain enough tissue from the different zones for the ABA analysis, 15 fruit were included in each replicate and three biological replicates were used in the experiment.

In order to understand whether the differences observed in the changes in ABA during the infection of Pinalate and Navelate fruit could be related to the rises in the hormone ethylene, two additional groups of fruit of each cultivar were used. The fruit from the first group were treated with ethylene (2 or 40 $\mu\text{L L}^{-1}$, see Section 2.3) and those of the second group with air (control samples). During the treatments, all the fruit were maintained at 20°C

and 90-95% RH to avoid dehydration. In this experiment, the flavedo samples were taken from the total fruit surface, and were frozen and ground as described above for the ABA analysis. Three biological replicates of five fruit per replicate and cultivar were collected during each sampling period.

2.2. Fungal material, and disease incidence and severity

To infect fruit, *P. digitatum* conidia suspensions were prepared in sterile distilled water from the 7-day-old cultures grown on potato dextrose agar (PDA) at 24°C. Experiments were performed using the *Penicillium digitatum* (Pers.:Fr.) Sacc. isolate Pd1 (CECT 20795) (Marcet-Houben et al., 2012). The conidia concentration was measured with a haemocytometer. The conidial suspensions were adjusted to concentrations ranging between 10^4 and 10^6 conidia mL⁻¹. Fruit were inoculated by wounding peel tissue with a flame sterilized needle (4 mm depth) to reach the albedo (inner white part of peel), or at a depth of 2 mm to inoculate fruit in the limit between flavedo and albedo tissues. Ten µL of conidial suspensions were used to inoculate each wound. The wounds in the control samples were inoculated with the same volume of water.

To determine disease incidence and severity, the percentage of infected wounds and lesion diameter (cm) of the fruit macerated zone were periodically determined in two perpendicular directions during fruit incubation in plastic boxes, kept in the dark at 90-95% RH and 20°C. For each treatment, determinations were made in three replicate samples of five fruit and with four equidistant wounds in the equatorial zone per fruit.

2.3. Chemical treatments.

To test whether ABA was involved in the resistance of citrus fruit to *P. digitatum* infection, fruit were treated with either ABA or the inhibitors of ABA biosynthesis in plants. The selected inhibitors were: 1) Tungstate (Tungs) (PubChem CID: 24465), which inhibits the last ABA biosynthesis step by inhibiting ABA aldehyde oxidase; 2) Nordihydroguaiaretic acid (NDGA) (PubChem CID: 453483489), an inhibitor of 9-cis epoxycarotenoid dioxygenase (NCED), which is a key enzyme in the biosynthesis of ABA in citrus fruit; 3) Norflurazon (NFZ) (PubChem CID: 33775), which inhibits phytoene desaturase at the beginning of carotenoid biosynthesis (Supplementary Fig. S1). All the compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fruit were treated by dipping them for 2 min in aqueous solutions containing concentrations of each compound (indicated in the Results section) and 0.5% ethanol to dissolve them. The control fruit were simply treated with water containing the same proportion of ethanol according to the same procedure. Treatments were performed before wounding the fruit. These solutions were also used to test whether either ABA or the inhibitors may affect *in vitro* fungal growth.

Ethylene treatments were performed in Navelate and Pinalate fruit as previously described (Lafuente et al., 2014). The fruit of both cultivars were treated for 7 d with 2 $\mu\text{L L}^{-1}$ ethylene at 90-95% RH and 20°C. Thereafter, the effect of ethylene on the ABA levels of the mutant and parental fruit was further examined by treating the fruit for 3 d with a higher ethylene concentration (40 $\mu\text{L L}^{-1}$). The control fruit were air-treated under the same experimental conditions used for the ethylene treatments.

2.4. *In vitro* experiments

The *in vitro* experiments were performed as previously described (Marcet-Houben et al., 2012; Ballester et al., 2015) in PDB (potato dextrose broth) and/or PDA to test whether ABA and inhibitors Tungs, NDGA and NFZ affected *P. digitatum* growth. Briefly, the experiments in PDB were performed in 96-well microtiter plates. Wells contained 10^4 conidia mL^{-1} in the PDB medium supplemented with either ABA or the inhibitors of ABA biosynthesis in plants. These chemicals were assayed at final concentrations ranging between 0.1 and 100 mM. Three replicate wells per compound and concentration were used. Plates were incubated at 24°C and fungal growth was measured as A_{492} for up to 6 d. The PDA experiments were also conducted by centrally inoculating 9-cm PDA plates containing the desired concentration of the tested chemical, with a 5 μL drop of conidia (10^6 conidia mL^{-1}), and by incubating plates at 24°C in the dark. Chemicals were added after autoclaving PDA when the medium was still warm. Colony diameter (cm) was recorded. The percentage of *in vitro* growth inhibition was also calculated as previously described (Ballester and Lafuente, 2017) according to this formula:

$$\text{Percentage of growth inhibition} = 100 \times (\text{GC} - \text{GSL}) / \text{GC}$$

GC is the diameter of the fungal colony of the control plates and GSL is that of the plates treated with the ABA-inhibitors.

2.5. Analysis of the free ABA in both fruit and fungus

ABA was determined in three biological replicate samples of previously frozen peel tissues (whole peel or flavedo, as indicated in each experiment) taken from different fruit lots, and also in *P. digitatum* spores. To determine the temporal changes in ABA that occurred in response to infection, the free ABA was determined in the peel or flavedo discs

(7 mm) taken around the inoculation zone. A minimum of eight discs per fruit and of five independent fruit (40 discs) were used in each replicate. ABA was analysed as previously reported (Lafuente et al., 1997; Romero et al., 2012) in the representative homogenised samples taken from the infected fruit and their respective control samples inoculated only with water. ABA was extracted from 1 g of fresh weight tissue samples with 80% acetone containing 0.1 g L⁻¹ butylated hydroxytoluene and 0.5 g L⁻¹ citric acid. After 5 min of centrifugation at 3000 x g, the supernatants of each sample were diluted with cold TBS (6.05 g L⁻¹ Tris, 0.2 mg L⁻¹ MgCl₂ and 8.8 g L⁻¹ NaCl) at pH 7.8 to reach the final ABA concentrations, which fell within the linear range of the ABA standard curve. The extracted samples were analysed in duplicate by an indirect ELISA method using the ABA-4'-BSA conjugate. This compound was synthesised as described by Weiler (1980) with some modifications (Norman et al., 1988). To determine the effect of ethylene on the ABA levels of the Navelate and Pinalate fruit, the free ABA was analysed in the flavedo of the fruit of both cultivars treated with either ethylene or air (control). In this experiment, three replicate samples containing five fruit each were used and the flavedo samples were taken from the whole fruit. ABA was expressed per kg peel or flavedo, as indicated in the figure captions, and on a fresh weight basis.

To know whether *P. digitatum* was able to produce ABA, the conidial suspensions from the cultures grown at 24°C on PDA during different periods were adjusted to 10⁸ conidia mL⁻¹. After 5 min of centrifugation at 1,3000 x g, ABA was extracted from the precipitated conidia with 1 mL of the same solvent used to extract the hormone from fruit. Extracts were concentrated with a SpeedVac and the resulting residues were diluted with 400 µL of the TBS solution used to determine the free ABA in duplicate following the same indirect ELISA method described above. Three biological replicate samples of spores were used per fungal

growth period and the results were expressed as pg ABA x 10⁸ per spore unit. To this end, the number of spores was determined before extracting the hormone. This method was validated by also analysing ABA by reverse UHPLC-MS in the same samples taken from the 7-day-old cultures, as previously reported (Seo et al., 2011), at the phytohormones quantification service of the IBMCP/CSIC (Valencia, Spain).

2.6. Conjugated ABA analysis

The conjugated ABA was analysed, as previously described (Lafuente et al., 1997), by determining the total ABA and calculating the conjugated ABA concentration by the difference between the total and free ABA. Briefly, 970 µL of the TBS solution (pH 7.8) were added to 30 µL of the diluted extracts used to determine the free ABA in glass tubes. Thereafter, pH was adjusted to pH 11 with 0.5 N NaOH and hydrolysis was performed by heating tubes at 60°C for 1 h. The extract was neutralised with 2 N HCl, diluted twice with TBS and the total ABA was analysed as described above for the free ABA.

2.7. Determination of ethylene production

Ethylene production was determined in three replicate samples of the flavedo discs taken around the inoculation site, as previously described (Lafuente et al., 2001). Discs were taken from the same fruit used to determine ABA. Six 7-mm diameter discs taken from three independent fruit (two discs per fruit) were included in each replicate and ethylene was measured during *P. digitatum* progression in the discs taken from the fruit inoculated with

either the fungus or water (control samples). Each replicate sample was incubated in sealed 15-mL glass tubes at 20 C, and 1 mL of gas was drawn from each tube with a hypodermic syringe and injected into a Gas Chromatograph (GC) (Perkin Elmer Autosampler), equipped with an activated alumina column. The chromatograph was equipped with a flame ionisation detector and the oven temperature was maintained at 140°C.

2.8. Statistical analysis

All the values are provided as the mean of three replicate samples±standard error. A mean comparison using the Tukey's test was made to determine whether the mean values significantly differed ($P \leq 0.05$). The analyses were performed with the Statgraphics Plus 4.0 Software (Manugistics, Inc.).

3. RESULTS

3.1. Free ABA decreases in sweet oranges in early *P. digitatum* infection stages

In the first experiment run to know whether citrus fruit showed changes in ABA in response to *P. digitatum* infection, the Navelina oranges harvested in November were infected with the Pd1 strain. A high conidia concentration (10^6 conidia mL⁻¹) was applied to induce a rapid marked response to infection. Fruit were also inoculated at a depth of 4 mm. In this way, infection started in albedo, which is more susceptible than flavedo (Ballester et al., 2006). In this experiment, ABA was analysed in the frozen samples of peel discs, which included both albedo and flavedo tissues. Under these experimental conditions, the ABA

levels in the control samples barely changed, but an early decrease in the free ABA was observed in the infected fruit, which lasted until day 2 (Fig. 1A). An approximate 2-fold increase in ABA occurred when peel maceration started (Fig. 1B), and 10 % of the wounds inoculated with the pathogen showed disease symptoms. This pattern of changes in ABA was also observed in the fruit of the same cultivar harvested in January which, in this cultivar, corresponded to the latest commercial maturity stage during the citrus season (Supplementary Fig. S2).

In a subsequent experiment, performed in Lane Late sweet oranges (*Citrus sinensis* L. Osbeck), the changes that occurred only in flavedo at the inoculation depth of 2 mm, at the limit between flavedo and albedo tissues, were examined. This approach was performed because of: 1) the poor penetration of exogenous compounds in citrus fruit peel, which is very hydrophobic as the aim was to evaluate the effect of both exogenous ABA and inhibitors of its synthesis in plants; 2) the ABA deficiency in the Pinalate mutant was evident mainly in flavedo; 3) the ABA levels were much higher in flavedo than in albedo (Lafuente et al., 1997). Under these experimental conditions, infection progression (Fig. 2B) was much slower than in the Navelina fruit infected at a depth of 4 mm (Fig. 1B). By day 3, the percentage of wounds showing disease symptoms was only 22% (Fig. 2B). As shown in Fig. 2A, the ABA levels in the flavedo of the infected fruit were lower than the ABA levels of the wounded control fruit by day 3, which was 2 d later than in the above-mentioned experiments. Therefore, by considering that the susceptibility of flavedo to be infected was lower than that of albedo, the ABA sampling period was extended in subsequent experiments when ABA was determined in this outer part of peel and/or when inoculation was performed between albedo and flavedo at a depth of 2 mm.

3.2. ABA is involved in the resistance of citrus fruits to *P. digitatum* infection

The above results suggested that ABA is involved in the susceptibility of citrus fruit to *P. digitatum* infection. Therefore, the effect of both exogenous ABA and the inhibitors of ABA synthesis in plants was examined on the incidence of *P. digitatum* infection in citrus fruit. The effect of Tungs was first evaluated because it inhibits the last ABA biosynthesis step in plants and does, therefore, not affect other precursors of the hormone in the biosynthetic pathway (Supplementary Fig. S1). A high (10 mM) Tungs concentration was applied to fruit because, as mentioned above, penetration of exogenous compounds in the peel of citrus fruit is poor. Tungs reduced disease in the mature Lane Late sweet oranges (Supplementary Fig. S3A) but, according to the *in vitro* experiments, Tungs also had a strong effect as it reduced fungal growth in a dose-dependent manner (Supplementary Figs S3B and S3C). Therefore, the effect of NDGA, an inhibitor of NCED, was assayed. NCED is a key enzyme in ABA biosynthesis in citrus fruit (Supplementary Fig. S1). Like Tungs, NDGA affected both *in vitro* and *in vivo* growth (data not shown). In view of these results, the *in vitro* effect of NFZ, which inhibits phytoene desaturase, was evaluated at the beginning of carotenoid biosynthesis (Supplementary Fig. S1). This inhibitor did not modify fungal growth (data not shown). Similarly, the application of 1 mM ABA did not alter the growth of the fungus (Supplementary Fig. S4).

After considering these results, and as the application of 1 mM ABA significantly increased ABA levels in citrus fruit (Romero et al., 2012), the effect of both 1 mM ABA and 1 mM NFZ was examined on the resistance of Navelate and its ABA-deficient mutant (Pinalate) to *P. digitatum* infection. In this experiment, a low conidia concentration was used and inoculation was performed at a depth of 2 mm to make the putative differences in *P. digitatum* infection after the ABA or NFZ application more evident, as well as the differences

between cultivars in their resistance to the fungus. Under these experimental conditions, the ABA-deficient mutant (Pinalate) was more susceptible to infection than the parental fruit (Figs 3A and 3B). The percentage of wounds showing disease by the end of the experiment was about 3-fold higher in the ABA-deficient mutant (Fig. 3C). The results also showed that ABA and NFZ had a mild effect on infection in Navelate fruit (Fig. 3A), which already had high ABA levels in flavedo (Fig. 3D). However, in the fruit of the ABA-deficient mutant, which presented about one fourth of the ABA levels compared to the parental (Fig. 3D), the exogenous ABA had a clear effect by reducing infection (Fig. 3B). It was noteworthy to find that NFZ favoured infection in the ABA-deficient mutant (Fig. 3B), but barely affected disease severity in the parental fruit (Fig. 3A). However, this compound also favoured peel damage in the Pinalate fruit, which was manifested as tissue darkening while fruit were kept in the dark (Supplementary Fig. S5). The NFZ-related tissue darkening was not observed in the Navelate fruit. Therefore, this inhibitor was also ruled out and research focused on comparing the behaviour of both the mutant and the parental fruit rather than using pharmacological approaches in subsequent experiments.

3.3. The *P. digitatum*-induced rise in ethylene production precedes the rise in free ABA in the infected citrus fruits

It has long since been known that ethylene increases in citrus fruit in response to *P. digitatum* infection (Chalutz et al., 1978), and that this hormone increases ABA in citrus fruit flavedo (Lafuente et al., 1997). To know whether a link exists between ABA and the infection-induced rise in ethylene, changes in ABA and ethylene production were measured in parallel in the flavedo of both the Navelate and the Pinalate fruit. Ethylene production of

the flavedo discs taken from the infected fruit of both cultivars transiently increased after 2 d (Fig. 4A and 4B). ABA initially decreased in response to infection (depth of 4 mm) in the Navelate fruit flavedo (Fig. 4C). Then it increased after a rise in ethylene. In the Pinalate fruit, the ABA levels rapidly increased in both the wounded and infected fruit (Fig. 4D). Then the ABA levels slightly dropped in the control and infected samples, despite the rise in ethylene observed in the infected fruit (Fig. 4B). Thus the effect of exogenous ethylene on ABA content in the flavedo of the fruit of both cultivars was determined in a subsequent experiment. Ethylene ($2 \mu\text{L L}^{-1}$) induced a sharp transient increase in ABA in the Navelate fruit (Fig. 5A). This increase occurred by day 3, but the ABA levels were not maintained after subsequently exposing fruit to ethylene (Fig. 5A). A slight increase in ABA was observed in the ethylene-treated Pinalate fruit by day 3, but ABA levels were negligible compared to those found in the parental flavedo (Figs 5A and 5B). It was also confirmed that applying a 20-fold higher ethylene level ($40 \mu\text{L L}^{-1}$) did not induce higher ABA concentrations (Supplementary Fig. S6).

3.4. The release of ABA from its conjugate might be responsible for the marked rise in the free ABA in a later P. digitatum infection stage.

The preliminary experiments revealed that the free ABA levels were very high in fruit, and displayed abundant sporulation. Such high free ABA levels could be related to a new hormone synthesis in later fruit infection stages, but also to the release of the free ABA from its conjugated form, or even to putative fungal ABA. The experiment shown above in Fig. 3 ended by day 7, when flavedo tissue was taken to determine ethylene, and ABA still showed poor sporulation (Supplementary Fig. S7A). Therefore, in order to test these

hypotheses, the experimental period was extended and determined both the free and conjugated ABA at later time points after fungal inoculation.

The assay was performed in the Navelate and Pinalate fruits, which were inoculated at the limit between flavedo and albedo (2 mm). Disease symptoms were evident by day 4 (Fig. 6A). During this period, the percentage of wounds showing disease was about 3-fold higher in the ABA-deficient mutant (62% in Pinalate, 21% in Navelate), tissue maceration was barely observed in the parental fruit and the macerated zone diameter was about 8-fold bigger in the mutant (Fig. 6A). The differences between both cultivars decreased thereafter, but the Pinalate fruit showed higher disease incidence and severity for up to 7 d. Sporulation was evident only by day 6 in Pinalate and 1 d later in Navelate (Fig. 6B). By day 12, the fruit of both cultivars were completely rotten and spores were abundant in flavedo, but not in albedo (Supplementary Fig. S7B). By day 18, albedo was almost disintegrated and the peel tissue structure was already disorganised (Supplementary Fig. S7C).

Marked increases in the free ABA were observed at later times during infection in both cultivars despite the mutant's ABA deficiency (Figs. 6C and 6D). Such increases were evident only by day 12, when sporulation was obvious in flavedo, but not in albedo. Thereafter, the free ABA levels continued to increase with sporulation. To test whether the marked rise in the free ABA was a consequence of releasing the hormone from the conjugated ABA, which is very abundant in citrus fruit flavedo (Lafuente et al., 1997), the conjugated ABA was also determined in these samples. As expected, the conjugated ABA (Figs. 6E and 6F) was higher in the parental fruit. The ABA conjugated form levels were more abundant than the free ABA (Figs. 6C and 6D) in the freshly harvested fruit of both the Navelate and Pinalte fruits. Conjugated ABA lowered from 12 d to 18 d in the flavedo of both the parental and mutant fruits, and these decreases were concomitant with the rise in the free ABA (Figs

6C-F). Given this result, it was not possible to rule out that the rise in the free ABA that took place during this later period was due, at least in part, to the conjugated ABA. By day 12, when the rise in free ABA was evident, no difference in the conjugated ABA levels were observed between the flavedo of the control and infected Navelate fruit. Therefore, it is interesting to consider that fungal ABA might contribute to the rise in the free ABA observed after 12 d.

The results found when examining the spatial changes in the free and conjugated ABA by taking samples of different flavedo tissue zones (healthy, macerated, with mycelium, and sporulated) from the same fruit also revealed that the free ABA was higher in the sporulated zone (Fig. 7A). However, differences in the conjugated ABA among the several zones were negligible (Fig. 7B). Thus experiments were performed to determine whether *P. digitatum* was indeed able to produce ABA.

3.5. *P. digitatum* is able to produce ABA

ABA levels were determined in the spores obtained from the cultures grown on PDA medium during different periods. The results showed that the necrotrophic fungus *P. digitatum* is able to produce ABA, and that the ability of the fungus to produce the hormone varied with its age (Fig. 8). The maximum ABA levels were found when spores were taken from the 7-day-old cultures. Thereafter, ABA lowered with fungal age.

4. DISCUSSION

Few studies are available about the role of ABA in the response of fruit crops to phytopathogenic fungi infection. However, it has been demonstrated that this hormone may favour, but also reduce the infection caused by different pathogens (Kaliff et al., 2007; Wang et al., 2015; Wilkinson et al., 2018). As far as we know, there is only one report about citrus fruit which shows that the hormone may participate in reducing *Alternaria* brown spot disease (Llorens et al., 2013). Some data about evaluations made of natural disease (non-inoculated fruit) incidence in fruit stored under conditions that favour peel damage may also suggest a protective role of ABA against *P. digitatum* in citrus fruit (Alfrez et al., 2005). Deng et al. (2018) found that the expression of some genes involved in ABA biosynthesis and signalling may be up- and down-regulated in response to *P. digitatum* infection. Yet whether ABA is indeed involved in the disease resistance of citrus fruit against this pathogen remains unknown. In this work, we investigated the possible involvement of ABA in the response of orange fruit to the major postharvest pathogen *P. digitatum*.

The results showed that the drop in ABA in peel constitutes a rapid early response of citrus fruit related to *P. digitatum* infection (Fig. 1A and Supplementary Fig. S2) and, therefore, suggest that ABA is indeed involved in the susceptibility of citrus fruit to infection by this necrotrophic fungus. A faster drop in ABA and quicker infection progression were observed when profound inoculation, which affected peel tissue that is more susceptible to infection (albedo) (Ballester et al., 2006), was performed (Figs. 1 and 2; a depth of 4 mm *versus* 2 mm). The fact that ABA decreased before tissue maceration development became relevant, and given that the faster infection progression was, the earlier ABA lowered (Fig. 1 and 2), both suggest that the ability of *P. digitatum* to infect citrus fruit is related to its capacity to lower ABA levels. So these results suggest that ABA would play a defensive role in citrus fruit against *P. digitatum* infection, which would agree with findings in grapes

infected with *Glomerella cingulata* (Wang et al., 2015), and in *Brassica napus* and *Arabidopsis* infected with *Leptosphaeria maculans* (Kaliff et al., 2007). Unfortunately, very little information about the role of ABA has been obtained by pharmacological experiments when applying inhibitors of ABA biosynthesis. They have been widely used to determine the role of ABA in different physiological processes and stress cues in plants. Many authors mention them as specific ABA inhibitors, but they may have additional effects related mostly to oxidative stress (Xiong et al., 2011). Under our experimental conditions, Tungs and NDGA had a clear effect by reducing the *in vitro* growth of *P. digitatum*. According to this result, the lesser disease incidence found in the fruit treated with these inhibitors could be related, at least in part, to their direct effect on fungal viability. Consequently, we cannot fairly conclude from this pharmacological approach that ABA plays a role in the defence response. So our subsequent experiment concentrated on comparing the behaviour of the Pinalate (ABA-deficient mutant) and the Navelate (parental) fruit, and examined the effect of NFZ and exogenous ABA, which did not affect *in vitro* fungal growth. The fact that the ABA-deficient mutant was more susceptible to infection reinforces the idea that ABA plays a defensive role in citrus fruit against *P. digitatum* infection. In this context, it is worth mentioning that although ABA may have direct antifungal activities (Khedr et al., 2018), the hormone does not alter *P. digitatum* growth (Supplementary Fig. S4). Therefore, the differences in the susceptibility to infection between Navelate and the ABA-deficient oranges (Pinalate) are not related to a direct effect of ABA on the fungus. It was surprising to find that NFZ favoured infection in the ABA-deficient mutant, but not in the parental fruit. However, it caused dark areas to develop in Pinalate fruit peel, which indicated that NFZ induces cell damage in this cultivar. Moreover, the NFZ concentration used in this experiment did not modify the ABA levels compared to the control fruit, and a higher

concentration would further enhance peel damage. Therefore, by also considering that NFZ might inhibit not only ABA, but also other metabolites (Supplementary Fig. S1), this pharmacological approach was ruled out. Another finding which confirmed the hypothesis that ABA may play a defensive role against *P. digitatum* infection in citrus fruit arose from the fact that exogenous ABA had a clear effect by reducing infection in the ABA-deficient mutant (Fig. 3B), and the Pinalate ABA-treated fruit showed similar ABA levels (Fig. 3D) and a similar susceptibility to infection (Fig. 3B) as the untreated Navelate fruit (Fig. 3A). Adding exogenous ABA to the parental fruit barely affected disease progression. However, it should be taken into account that the parental fruit flavedo already has very high ABA levels, and endogenous phytohormone levels might suffice to trigger cellular processes to cope with pathogen attack. This agrees with previous findings which have shown that exogenous ABA has very little effect on dehydration in Navelate, but triggers changes in the expression profile of the ABA-perception system and reduces water loss in ABA-deficient mutant Pinalate (Romero et al., 2012; 2014). Hence the above results indicate that ABA plays a defensive role against *P. digitatum* in citrus fruit, but we ought to consider: 1) Pinalate fruit is a spontaneous ABA-deficient mutant with a blockage in the carotenoid biosynthetic pathway rather than a knockout mutant, in which the gene responsible for the last ABA biosynthesis step is inoperative; 2) the partial effect of exogenous ABA on disease reduction in Pinalate fruit. Therefore, we should contemplate that ABA could contribute to reduce disease, but additional responses should participate in the complex network of the mechanisms participating in plant disease resistance.

The results of the present work further revealed different factors that influence variations in ABA content during the *P. digitatum* infection process (Fig. 9). The early ABA decrease appears indicative of a fruit infection perception response. This decrease was

490 followed by a sharp increase in hormone levels in the parental fruit, which might be mediated
491 by ethylene. Previous findings have shown that exogenous ethylene induces changes in ABA
492 metabolism (Establés-Ortiz et al., 2016) and increases hormone levels in citrus fruit
493 (Lafuente et al., 1997). As expected (Chalutz et al., 1978), ethylene increased in both the
494 Navelate and Pinalate fruit in response to infection (Fig. 4A and 4B), but the rise in ABA
495 following the rise in ethylene occurred only in the parental fruit (Fig. 4C and 4D). The peel
496 of the fruit from both cultivars presented maceration, and even sporulation, by day 7
497 (Supplementary Fig. S7A). These results, together with those indicating that the ABA levels
498 were very low in the ethylene-treated Pinalate fruit (Fig. 5B), suggest that the rise in ABA
499 following the early infection-induced reduction in ABA in the infected Navelate fruit (Fig.
500 4) is more likely related to the infection-induced rise in ethylene than to fruit reaction to the
501 tissue maceration caused by *P. digitatum*.

502 Finally, at the later fruit infection times (Fig. 6) when sporulation was evident
503 (Supplementary Figs. S7B and S7C), a marked rise in ABA occurred in both the parental and
504 ABA-deficient fruit. This result, together with the results obtained from examining the
505 spatio-temporal (Fig. 7) changes in the free and conjugated ABA, suggest that such a rise in
506 ABA is the consequence of releasing the free ABA from the conjugated ABA, which is very
507 abundant in citrus fruit flavedo (Lafuente et al., 1997). Although plant β -glucosidases have
508 been involved in this release (Lee et al., 2006), advanced tissue degradation suggests that
509 fungal β -glucosidases are more likely to be responsible for conjugated ABA hydrolysis than
510 fruit β -glucosidases, a hypothesis that deserves future research. Whether the rise in the free
511 ABA is merely a consequence of the release from its conjugate without affecting fungal
512 colonisation, or whether it plays a role in the resistance of citrus fruits against the fungus,
513 remains elusive. Nevertheless, such a release occurs too late, when the fungus has already

colonised the fruit. Another interesting idea is that *P. digitatum* is able to produce the hormone. This was confirmed by analysing ABA in the spores obtained from PDA cultures (Fig. 8), although the ABA levels produced by the fungus were much lower than those detected in rotten fruit. As far as we know, this is the first report to show the ability of *P. digitatum* to produce ABA and, therefore, the putative function of this ability is unknown. Other reports have demonstrated that ABA may be produced by other necrotrophic fungi (Nambara and Marion-Poll, 2005) through the direct mevalonic acid pathway, while ABA production in plants is derived from the indirect pathway (Lievens et al., 2017). There is converging evidence for ABA used as a virulence factor (Chanclud and Morel, 2016) and indicating that ABA may act as a pathogen effector and an immune regulator (Lievens et al., 2017). The fact that the ABA derived from *P. digitatum* mainly increased when sporulation was evident indicates that fungal ABA is not a primary virulence factor of this fungus, but might help infection progression. In line with the present work, it is worth mentioning the proposed role of ABA in altering cell wall properties that may influence necrotrophic infection outcome (Nafisi et al., 2015). Therefore, the massive rise in ABA in later *P. digitatum* infection stages might contribute to favour the pathogen entering in citrus fruit, and hence its colonisation. The aim of this paper was to understand how the fruit ABA influences the susceptibility of oranges to infection by this pathogen. Our experiments demonstrate, for the first time, that *P. digitatum* is able to produce ABA and, therefore, open up new research lines on the role of fungal ABA in infection progression.

5. CONCLUSIONS

In short, both, citrus fruit and *P. digitatum*, the main postharvest pathogen that causes rots in this important agronomic crop, produce ABA. In fruit, ABA seems to play a defensive role against infection caused by the pathogen. Fungal ABA appears not to be a primary virulence factor, but it cannot be ruled out that the fungal hormone may help fruit colonisation. The results also show, for the first time, that the trend in ABA changes varies during fruit infection because of different factors (Fig. 9). ABA decreases in early stages as a consequence of *P. digitatum* infection. Thereafter, it increases and this increase is likely related to the *P. digitatum*-induced rise in ethylene. This is demonstrated by the fact that ABA does not increase in Pinalate in this infection stage, and exogenous ethylene barely increases hormone levels in this ABA-deficient cultivar. Finally, fungal ABA, but also the release of ABA from the conjugated ABA, could be responsible for the marked rise in the free ABA in the later infection stage.

APPENDIX A: SUPPLEMENTARY DATA

The Supplementary Material related to this article can be found in the online version.

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Figure Legends

Figure 1. Changes in the free ABA in the peel discs (A) and the lesion diameter of the macerated area (B) of the Navelina fruit harvested in November and inoculated at a depth of 4 mm (●) with 10 µL of *P. digitatum* (10^6 conidia mL⁻¹). The free ABA is expressed per kg of peel. The control samples (○) were mock-inoculated with 10 µL of water. After infection, fruit were maintained in the dark at 20°C. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the infected and control fruit of the same cultivar for the same storage period.

Figure 2. Changes in the free ABA in the flavedo discs (A) and the lesion diameter of the macerated area (B) of the Lane Late fruit inoculated at a depth of 2 mm (●) with 10 µL of *P. digitatum* (10^6 conidia mL⁻¹). The free ABA is expressed per kg of flavedo. The control samples (○) were mock-inoculated with 10 µL of water. After infection, fruit were maintained in the dark at 20°C. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the infected and control fruit of the same cultivar for the same storage period.

Figure 3. Effect of ABA and NFZ on the changes in the lesion diameter of the macerated area of the Navelate (A) and Pinalate (yellow ABA-deficient mutant) (B) fruit infected at a depth of 2 mm with 10^4 conidia mL⁻¹ of *P. digitatum* (10 µL). The percentage of decay in the Navelate (■) and Pinalate (□) fruit is shown in Fig. 3C. The effect of exogenous ABA on the hormone levels (measured immediately after applying ABA) on the flavedo of the fruit of both cultivars is found in Fig. 3D. The free ABA is expressed per kg of flavedo. To visualise fruit colour in the photographs in Figs. 3A and 3B, readers are referred to the web version of

this article. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) among the various conditions evaluated for the same fruit cultivar and storage period (Fig. 3B and 3C). No significant differences in the lesion diameter were found for the same day between the control Navelate fruit and Navelate fruit treated with ABA or NFZ. Therefore, no letters are included in Fig. 3A. The significant differences ($p \leq 0.05$) between the Navelate and Pinalate fruit, treated or not with ABA, are indicated by distinct letters in Fig. 3D.

Figure 4. Changes in ethylene production (A and B) and in the free ABA levels (C and D) in the flavedo of the Navelate (A and C) and Pinalate (B and D) fruit inoculated at a depth of 4 mm with 10^4 conidia mL^{-1} of *P. digitatum* ($10 \mu\text{L}$) (●) and their respective control samples (○). Ethylene production and the free ABA are expressed per kg of flavedo. Fruit were maintained in the dark at 20°C . The error interval indicates the standard error of the estimated mean value. Distinct letters mean significant differences ($p \leq 0.05$) between the control and infected fruit for the same cultivar and storage period.

Figure 5. Effect of exogenous ethylene on the free ABA levels in the flavedo of the Navelate (A) and Pinalate (B) fruit. The free ABA is expressed per kg of flavedo. Fruit were treated for up to 7 d with $2 \mu\text{L L}^{-1}$ ethylene (grey bars) or maintained in air (control, black bars) at 20°C . The error interval indicates the standard error of the estimated mean value. Distinct letters mean significant differences ($p \leq 0.05$) between the fruit treated with air and ethylene for the same cultivar and storage period.

Figure 6. Changes in the lesion diameter of the macerated (A) and sporulated (B) zones of the Pinalate (□) and Navelate (■) fruit inoculated at a depth of 2 mm with 10 µL of *P. digitatum* (10^4 conidia mL⁻¹). The changes in the free (C and D) and conjugated (E and F) ABA levels in the flavedo of the infected (●) and control (○) Navelate (C and E) and Pinalate (D and F) fruit are also shown. The free and conjugated ABA are expressed per kg of flavedo. After infection, fruit were maintained in the dark at 20°C. The error interval indicates the standard error of the estimated mean value. Distinct letters mean significant differences ($p \leq 0.05$) between the infected and the control fruit for the same citrus cultivar and storage period.

Figure 7. The spatial changes in the free (A) and conjugated (B) ABA levels in the flavedo of the Lane Late oranges inoculated at a depth of 4 mm with 10^5 conidia mL⁻¹ of *P. digitatum* (10 µL). The free and conjugated ABA are expressed per kg of flavedo. Samples were taken at 6 dpi. The error interval indicates the standard error of the estimated mean value. Distinct letters mean significant differences ($p \leq 0.05$) among the different zones.

Figure 8. The free ABA levels of the *P. digitatum* spores obtained from the cultures grown during different periods at 24°C. The error interval indicates the standard error of the estimated mean value.

Figure 9. Schematic diagram of the factors influencing the free ABA evolution during citrus fruit infection. To visualise the figure colour, readers are referred to the web version of this article.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Supplemental Fig. S1. Schematic diagram of ABA biosynthesis in plants and the effect of NFZ, NDGA and Tungstate inhibiting its synthesis.

Supplemental Fig. S2. Changes in free ABA in peel discs of Navelina fruit harvested in January and inoculated at 4 mm depth (●) with 10 μ L of *P. digitatum* (10^6 conidia mL⁻¹). Control samples (○) were mock-inoculated with 10 μ L water after wounding the tissue at 4 mm depth. After infection, fruits were maintained under darkness at 20 °C. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the infected and the control fruit for the same storage period.

Supplemental Fig. S3. Effect of Tungs (10 mM) on lesion diameter (cm) of macerated circular zone (A) of Lane Late oranges inoculated with 10 μ L of *P. digitatum* (10^6 conidia mL^{-1}); and of different Tungs concentrations, ranging from 0.1 to 100 mM, on the *in vitro* fungal growth in PDA (B), expressed as % inhibition fungal growth (section 2.4) after determining the fungal growth diameter, and in PDB (D), expressed as optical density at A_{492} . The error interval indicates the standard error of the estimated mean value.

Supplemental Fig. S4. Effect of 1 mM ABA on the *in vitro* fungal growth in PDA, expressed as fungal growth diameter (cm), and in PDB, expressed as optical density at A_{492} . The error interval indicates the standard error of the estimated mean value. No significant differences between control and ABA-treated samples were found at any time of the experiment.

Supplemental Fig. S5. Pinalate fruit showing NFZ-induced tissue darkening.

NFZ-induced flavedo darkening

Supplemental Fig. S6. Effect of treating Navelate and Pinalate fruit for 3 d with 40 $\mu\text{L L}^{-1}$ ethylene or maintaining them in air at 20 °C on free ABA levels in the flavedo. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) among different samples for the same cultivar. FH: freshly harvested fruit.

Supplemental Fig. S7. Navelate and Pinalate flavedo disks showing poor sporulation, taken 7 d after fruit inoculation with 10 μ L of *P. digitatum* (4 mm depth, 10^4 conidia mL⁻¹) (A). Fruit were completely rotten by 12 d post-inoculation (dpi) (B); and the albedo was almost disintegrated and the peel tissue structure was disorganized by 18 dpi (C).