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Penicillium digitatum infection mechanisms in citrus: What do we know so far?

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ABSTRACT

Penicillium digitatum is the major source of postharvest decay in citrus fruits worldwide. This fungus shows a limited host range, being able to infect mainly mature fruit belonging to the Rutaceae family. This highly specific host interaction has attracted the interest of the scientific community. Researchers have investigated the chemical interactions and specialized virulence strategies that facilitate this fungus's fruit colonization, thereby leading to a successful citrus infection. There are several factors that mediate and affect the interaction between *P. digitatum* and its host citrus, including hydrogen peroxide modulation, secretion of organic acids and consequently pH control, and other strategies described here. The recently achieved sequencing of the complete *P. digitatum* genome opened up new possibilities for exploration of the virulence factors related to the host-pathogen interaction. Through such techniques as RNAseq, RT-PCR and targeted gene knockout mediated by *Agrobacterium tumefaciens*, important genes involved in the fungal infection process in citrus have been reported, helping to elucidate the molecular mechanisms, metabolites and genetic components that are involved in the pathogenicity of *P. digitatum*. Understanding the infection process and fungal strategies represents an important step in developing ways to protect citrus from *P. digitatum* infection, possibly leading to more productive citriculture.

1. Introduction

Penicillium digitatum is a species within the Ascomycota division of fungi with considerable importance to the environment as well as to the food industry (Frisvad and Samson, 2004). This species is the major source of postharvest decay in citrus fruits worldwide, followed by *Penicillium italicum* (Poppe et al., 2003). In addition, *P. digitatum* was surprisingly reported as a plum postharvest pathogen, but this host-pathogen interaction has not been thoroughly elucidated to date (Louw and Korsten, 2019).

Economically, citrus fruits have the largest production in the world compared to other fruits, with a global citrus production of approximately 98.3 million tons, including oranges, tangerines, lemons and grapefruit (Citrus: World Markets and Trade), indicating their global economic importance (Moltó et al., 2010).

Various postharvest pathogens are reported to cause significant losses at different storage stages after harvest, accounting for nearly 50 % of the wastage in citrus fruits (Ladanyia, 2010). In particular, *P. digitatum* is one of the most severe postharvest pathogens, causing green mold disease (Ghooshkhaneh et al., 2018), which results in damage to citriculture and commerce. In arid zones and tropical subclimates, *P. digitatum* has been reported to contribute to 90 % of the total post harvest losses in citriculture (Macarisin et al., 2007).

P. digitatum is able to invade and infect the fruit through wounds that can originate from environmental factors, such as wind, hail and insects, or during the harvest process, transport and subsequent treatments (Perez et al., 2017). This fungus spreads on a number of skin oil glands through pores and wounds (Ghooshkhaneh et al., 2018), in which nutrients are available to stimulate spore germination. The initiation of the infection area appears as a soft watery spot (sometimes referred to as clear rot) on the fruit peel, and if suitable temperature and conditions are available, it becomes a white mycelium that later turns an olive

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color with spore production (Vu et al., 2018; Ismail and Zhang, 2004).

Currently, the postharvest control is carried out by the mass application of fungicides in commercialized fruits. A number of synthetic fungicides, such as prochloraz, imazalil, and pyrimethanil, have been used worldwide as postharvest treatments to control the pathogen in citrus fruits (Hao et al., 2011). Nevertheless, the repeated use of certain fungicides has led to the appearance of fungicide-resistant populations of *P. digitatum*, representing a significant obstacle to postharvest conservation (Kanetis et al., 2010). In addition to the serious implications for product toxicity, environmental risk and low consumer confidence (Hao et al., 2011).

Alternative strategies to control *P. digitatum* infection have been investigated. Recently, Lafuente et al. (2018) verified the effect of light-emitting diode (LED) blue light (LBL) at a 450-nm wavelength reducing the viability and the capacity of the infection by *P. digitatum* in citrus fruits. The application of noncontinuous LBL is a promising alternative to decrease the capacity of postharvest decay since it is responsible for anomalous fungal spore morphologies and darkening of the cytoplasm. In addition, a significant decrease in ethylene production in plates that were exposed to the continuous LBL in relation to plates under darkness was observed over eight days.

Studies concerning the citrus–*Penicillium* system have mainly focused on new alternatives for controlling the disease or on the analysis of the mechanisms involved in induced resistance to fungal infection. However, the recently sequenced complete genome opened up new possibilities for investigating the virulence factors related to the host-pathogen interaction. Here, we describe the known mechanisms and important genes related to citrus–*Penicillium* interactions. Understanding the infection process and fungal strategies may represent an important step in developing ways to protect citrus from *P. digitatum* infections, leading to more productive citriculture worldwide.

2. P. digitatum – *citrus interaction*

There are several factors that mediate and affect the interaction between *P. digitatum* and its host during infection. In this section, we briefly describe the molecules and variables that directly or indirectly influence the infection process.

2.1. Hydrogen peroxide production as a plant response

Hydrogen peroxide production by the host has been described as an important defense mechanism, which is bypassed by *P. digitatum* due to increased catalase production. Hydrogen peroxide is mostly an initial consequence of plant defense in response to pathogen infection, functioning as a signaling molecule (Levine et al., 1994; Mellersh et al., 2002; Qin et al., 2011), and it has been reported to play a role as a signal in the induction of systemic acquired resistance (SAR) in plants (Neuencchwander et al., 1995; Hunt et al., 1996; Conrath, 2006). Hydrogen peroxide also participates in lignification processes of the cell wall, oxidative cross-linking of proteins (Olson and Varner, 1993; Brisson et al., 1994), phytoalexin biosynthesis (Apostol et al., 1989) and induction of host defense genes (Nathues et al., 2004), inhibiting pathogen growth (Lu and Higgins, 1999; Kuźniak and Urbanek, 2000; García-Olmedo et al., 2001).

Macarisin et al. (2007) performed a detailed study on the level of hydrogen peroxide during the infection process of citrus fruits with *P. digitatum* (compatible interactions) and with *Penicillium expansum* (nonhost interactions). The authors presented clear evidence that *P. digitatum* is able to avoid the H_2O_2-oxidative burst during the first 25 h after inoculation in host cells, while *P. expansum* triggers a considerable accumulation of H_2O_2 in the host (Macarisin et al., 2007). In addition, the authors correlate the effect of organic acids and low pH buffer with H_2O_2 host production based on the evaluation of whether the accumulation of certain organic acids might result in inhibition of the oxidative burst. Treatment with citric acid, oxalic acid and ascorbic acid contributed the most to the increase in pathogen virulence and infection severity (Macarisin et al., 2007).

2.2. Catalase production as a fungal infection strategy

Macarisin et al. (2007) also evaluated the effects of catalase addition (CAT) on the infection incidence and lesion diameters. These researchers reported that 500 U/mL catalase was sufficient to promote a significant increase in the lesion diameter of samples infected with *P. digitatum* and, to a lesser extent, with *P. expansum* (Macarisin et al., 2007). Catalase is an antioxidant enzyme produced by fungi (Chaga et al., 1992; Aguirre et al., 2006; Ballester et al., 2006), and it decomposes hydrogen peroxide to water and oxygen. Therefore, it is suggested that the H_2O_2 that is produced by the plant as a defense mechanism is decomposed by the catalase secreted by *P. digitatum* (Macarisin et al., 2007; Ballester et al., 2006) as an infection strategy.

2.3. Organic acid secretion by the pathogen for pH modulation

An interesting strategy used by the pathogen to infect the host is the secretion of organic acids resulting in pH modulation. Controlling the pH has been considered a factor and a regulatory cue used by *P. expansum*, *P. digitatum*, and *P. italicum* for increased pathogenicity. The authors verified a pH difference of 1.65 between the healthy oranges (pH 4.77 ± 0.45) and decayed oranges (pH 3.12 ± 0.07) infected by *P. digitatum*. Prusky et al. (2004) suggested that *P. digitatum* could enhance its pathogenicity through local pH modulation of hosts, leading to an optimal pH for specific cell wall-degrading enzymes, such as polygalacturonases (PG) (Barmore and Brown, 1981; Prusky et al., 2004; Zhang et al., 2013).

2.4. Flavonoid production in citrus fruits as a natural defense barrier

Another factor that influences the *P. digitatum*-citrus interaction is the production of flavonoids by the host as defense barriers. Flavonoids are well-documented as part of plant defense in *Citrus* spp. as reported by different studies (Ortuño and Del Río, 2009; Ortuño et al., 2011; Kim et al., 2011). Flavonoids act as a chemical barrier in the outermost citric tissue and can be considered phytoalexins against *P. digitatum*, such as the polymethoxyflavones localized at the flavedo level of fruits and the flavanones localized at the albedo level (Fig. 1) (Ortuño and Del Río, 2009; Ortuño et al., 2011). Studies indicate changes in the levels of flavanone glycoside - hesperidin (decreased approximately 7.8 %) and its corresponding aglycon - hesperetin (increased approximately 7.2 %) at five days after inoculation with *P. digitatum*. Similar results were also found in lemons and grapefruits and can be associated with the hydrolyzing action of *P. digitatum* (Ortuño and Del Río, 2009; Ortuño et al., 2011).

Ortuño et al. (2006) described polymethoxyflavones 5,6,7,3′,4′-pentamethoxyflavone (sinensetin), 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin), 3,5,6,7,8,3′,4′-heptamethoxyflavone and 5,6,7,8,4′-pentamethoxyflavone (tangeretin) as important compounds in citrus defense against pathogens. Furthermore, these researchers observed *in vitro* that nobiletin followed by hesperidin and naringin were the flavonoids that exhibited higher activities against *P. digitatum* growth on potato dextrose agar (PDA) medium

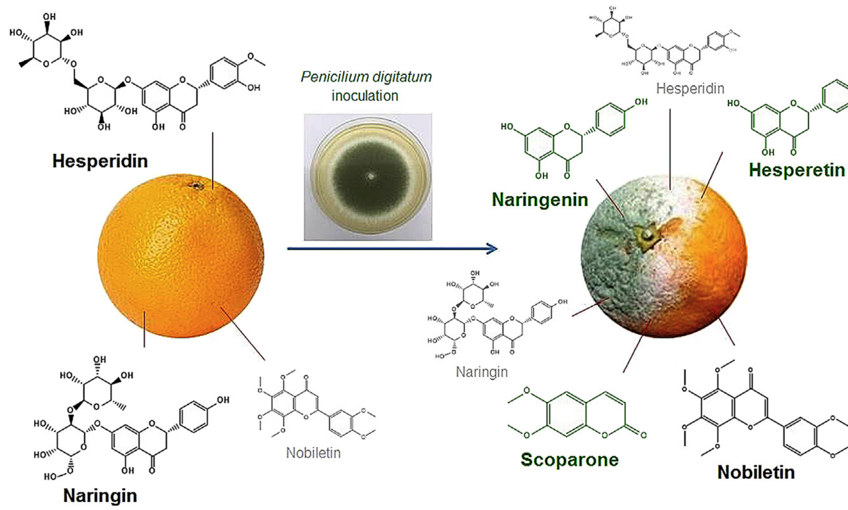

Fig. 1. Natural product (NP) levels on citric fruits before and after interaction with *Penicillium digitatum* - NP shown in black are from citric fruits, which decrease the level in approximately 7–8 % (hesperidin and naringin) before *P. digitatum* inoculation or increase the level in approximately 7 % (nobiletin) after *P. digitatum* inoculation. NP shown in gray color are at a low level. NP shown in green color (hesperetin, naringenin and 6,7-dimethoxy coumarin) were detected only after inoculation of *Penicillium digitatum* in fruit, which was in keeping with previously published findings (Ortuño et al., 2011). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

compared to 5,6,7,3′,4′-pentamethoxyflavone and tangeretin (Ortuño et al., 2011). Interestingly, the corresponding aglycones are more active as fungistatic agents against *P. digitatum* than the glycosylated flavanones (Ortuño and Del Río, 2009), which is in contrast to the hydrolyzing action of *P. digitatum* and requires further investigation.

In addition, citrus defense mechanisms studies against the phytopathogen *P. digitatum* demonstrated that scoparone, an antifungal phenolic compound derived from phenylpropanoid metabolism, plays an important role as a citric phytoalexin present in oil glands (Kim et al., 1991; Ben-Yehoshua et al., 2008). Other studies on the role of flavonoids in the defense mechanisms of citrus fruits against the *P. digitatum* can be checked in the following references (Del Río et al., 1998, 2004; Kim et al., 2011; Ballester et al., 2013).

2.5. Metabolomic and transcriptomic analysis of citrus fruits infected by *P. digitatum*

Tang et al. (2018) performed a search for the mechanism of deterioration of Powell orange pulps from *Citrus sinensis* infected by *P. digitatum* after 40 h and 60 h using GC–MS to determine the levels of 26 polar primary metabolites. The authors also used the headspaced solid phase microextraction and the gas-chromatography-mass spectrometry (HS-SPME-GC-MS) technique for the determination of 48 volatile organic compounds (VOCs). The authors verified a change in the levels of these VOCs, similar to octanoic acid ethyl ester, ethanol and aldehydes, which increased the level in citrus fruits infected, suggesting that there is metabolic reprogramming during *P. digitatum* infection. It was also observed that jasmonates and ethylene pathways are involved in the plant defense response for this interaction, as reported previously in other pathogen-host interactions (Tang et al., 2018). In addition, Tang et al. (2018) performed a transcriptomic analysis of *P. digitatum* infection and verified that there was activation of some transcription factors: 29 MYB, 25 WRKY and 33 AP2/ERF were upregulated in fruits after 60 h of infection, suggesting their involvement in the fruits' defense against the pathogen (Tang et al., 2018).

2.6. Production of natural products by the fungi and pathogenicity

There is evidence that several phytopathogenic fungi have the ability to produce small molecules able to decrease plant defense responses, causing necrotic reactions in the infected cells (Morrissey and Osbourn, 1999). For instance, the virulence of several fungi (*Cochliobolus heterostrophus, C. miyabeanus, Fusarium graminearum* and *Alternaria brassicicola*) on their respective host plants has been reported to be mediated by particular siderophores, a class of secondary metabolites involved in iron uptake (Fox and Howlett, 2008).

In this context, during *P. digitatum* infection, Ariza et al. (2002) identified secondary metabolites produced in *P. digitatum* biomass: the indole alkaloids tryptoquialanine A **(1)** and tryptoquialanine B **(2)** and the steroids cholesterol **(3)**, ergosta-7,22-dien-3β-OH **(4)**, ergosta-7,22,24(28)-trien-3β-OH **(5)**, episterol **(6)**, and eburicol **(7), with (1)** and **(2)** being reported as major secondary metabolites in *P. digitatum* (Fig. 2). Secondary metabolites produced by various microorganisms can contribute to the pathogenicity of several pathogenic fungi (Scharf et al., 2014). However, tryptoquialanine A **(1)**, tryptoquialanine B **(2)** and other *P. digitatum* secondary metabolites have not been directly related to the pathogenicity of *P. digitatum* (Zhu et al., 2017); therefore, the exact biological role of these secondary metabolites has not been determined to date.

Attempting to investigate the biological role of tryptoquialanine A **(1)**, Costa et al. (2019) recently analyzed the surface of oranges with green mold disease through mass spectrometry imaging and observed that tryptoquialanines are the major metabolites produced during the decay process on the orange surface. Costa et al. (2019) performed insecticidal bioassays using tryptoquialanine A and observed high toxicity against *Aedes aegypti* larvae. Larvae exposed to tryptoquialanine A presented a mortality rate of 37 % within 24 h and of 81 % within 96 h, indicating an important insecticidal action during the infection process. These insecticidal assays suggest that the tryptoquialanines are involved in the protection of the pathogen and the rotten citrus against insects, which explains the production of the tryptoquialanines by *P. digitatum* on the citrus surface (Costa et al., 2019). Based on these results, it is

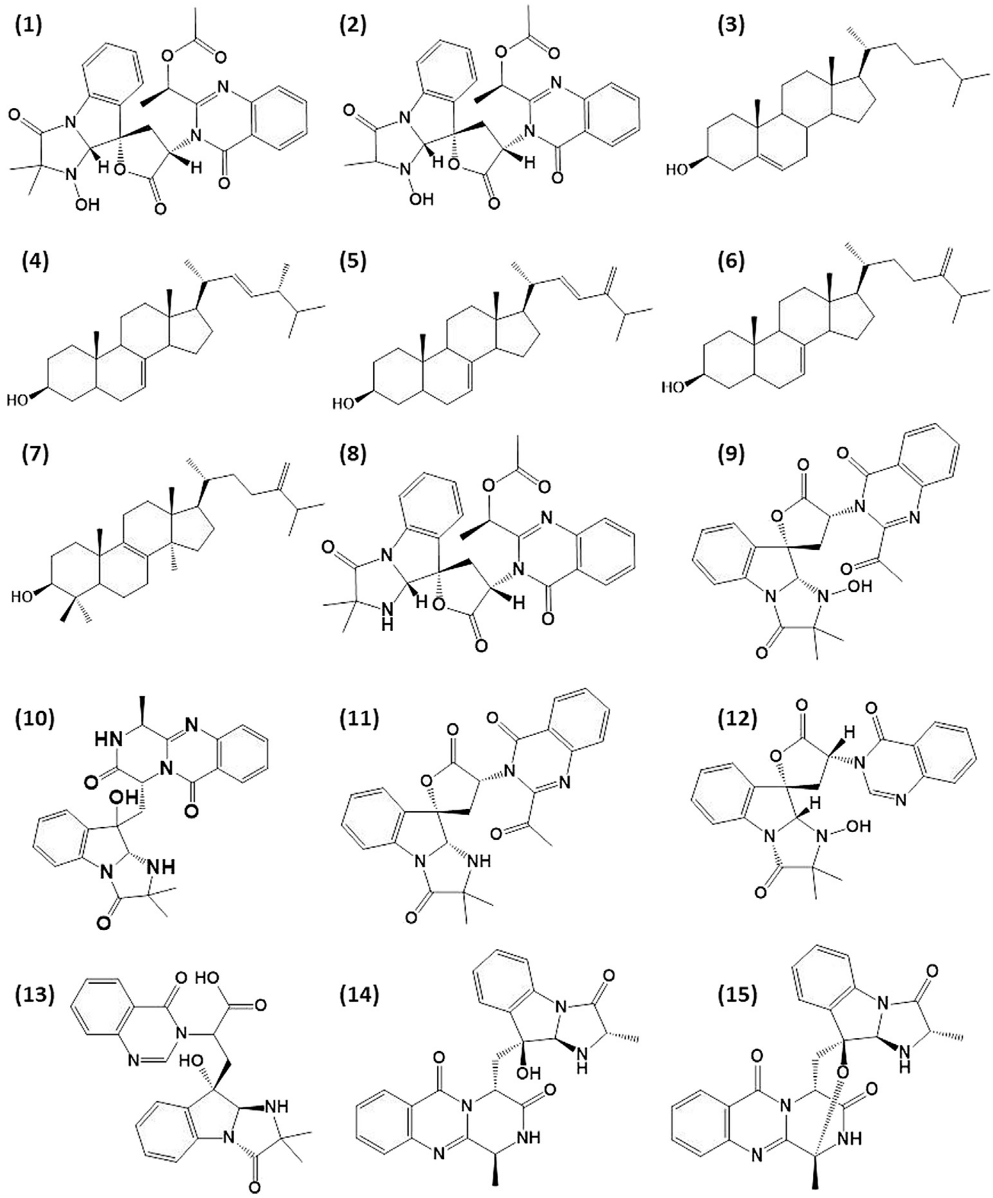

Fig. 2. Chemical structure of tryptoquialanine A (**1**), tryptoquialanine B (**2**), cholesterol (**3**), ergosta-7,22-dien-3β-OH (**4**), ergosta-7,22,24(28)-trien-3β-OH (**5**), episterol (**6**), and eburicol (**7**), tryptoquialanine C (**8**), tryptoquialanone (**9**), 15-dimethyl-2-epi-fumiquinazoline A (**10**), deoxytryptoquialanone (**11**), tryptoquivaline L (**12**), tryptoquivaline Q (**13**), fumiquinazoline A (**14**) and fumiquinazoline C (**15**) isolated from *Penicillium digitatum* biomass.

possible to provide the first insight into the biological role of these indole alkaloids involved in the *P. digitatum*-citrus interaction, suggesting that tryptoquialanine A is an effective biocontrol agent against insects during orange decay.

In addition, Costa et al. (2019) reported for the first time for *P. digitatum* the production of a new tryptoquialanine, tryptoquialanine C (**8**), and of intermediates involved in the tryptoquialanine A biosynthetic pathway, such as tryptoquialanone (**9**), 15-dimethyl-2-epi-fumiquinazoline A (**10**), and deoxytryptoquialanone (**11**). Other secondary metabolites, such as tryptoquivaline L (**12**), tryptoquivaline Q (**13**), fumiquinazoline A (**14**) and fumiquinazoline C (**15**), have also been reported (Costa et al., 2019).

The discovery of metabolites produced by *P. digitatum* and the understanding of their biological role can provide more information on the infection process and how this fungus may survive to environmental pressures in the host. Once the *P. digitatum* genome has been entirely sequenced, there is abundant information on the great potential for secondary metabolite production. However, notably few studies have been able to correlate these metabolites to a biological role in the infection process.

During this process, the volatiles emitted from the ruptured oil glands in wounded peel tissue can facilitate infection by promoting spore germination and germ tube elongation of *P. digitatum* (Droby et al., 2008).

Ariza et al. (2002) aimed to understand the citrus-pathogen interaction by comparing the volatile profile of healthy oranges, oranges submitted to mechanical damage and oranges infected with *P. digitatum*. These researchers observed higher amounts of limonene and other known citrus monoterpenes that were released (instead of sesquiterpenes typical of healthy fruits) in mechanically injured fruits than healthy ones. Other studies concerning the role of limonene in the infection process and spore germination can be checked in the following references (Rodríguez et al., 2015; Tao et al., 2019). Regarding oranges contaminated with *P. digitatum*, the pattern of volatile metabolites released was similar to the situation in which the citrus fruit was mechanically damaged, with the exception of the following volatile metabolites also identified in the infected fruits: ethanol, methyl acetate, and ethyl acetate, as well as an unknown volatile compound likely to have a molecular weight of 114 amu. This unknown compound was primarily responsible for the characteristic moldy odor experienced from cultures of *P. digitatum* (Ariza et al., 2002).

The current literature concerning the strategies used by *P. digitatum* during the infection process reveals the fungus's capacity to modulate the acidity of the environment to achieve the degradation of the cell wall, to prevent oxidative bursts and to inhibit enzymes that participate in plant defense mechanisms. Other studies revealed the response of citrus against the infection. Fig. 3 shows the main infection strategies known in the literature for *P. digitatum* and the responses of citrus during host-pathogen interaction. In addition, current studies have focused on the development of new plant defense methods through the addition of microorganisms that act as biocontrol agents (Talibi et al., 2014; Parveen et al., 2016). However, metabolites secreted by this pathogen and its metabolome have not been determined to date.

3. Exploring gene function in host-pathogen interaction: unveiling infection mechanisms

To the best of our knowledge, *P. digitatum* was the first phytopathogenic *Penicillium* species for which the complete genome has been entirely sequenced (Marcet-Houben et al., 2012; Sun et al., 2013). Additionally, biosynthetic analysis of *P. digitatum* PHI26 gene clusters indicated 24 putative biosynthetic gene clusters, including 13 involved in peptide biosynthesis by nonribosomal peptide synthases (NRPS), 14 involved in polyketide biosynthesis by polyketide synthases (PKS) and 3 gene clusters encoding the production of hybrid NRPS/PKS metabolites (Marcet-Houben et al., 2012).

Julca et al. (2015) analyzed the genome of four isolates of *P. digitatum* and seven of *P. expansum* from different geographical locations to help determine why the infection process of *P. digitatum* occurs specifically in citric fruits, while *P. expansum* is able to infect a wide range of fruits. Julca et al. (2015) observed low genetic variability in *P. digitatum*, in contrast with the higher genetic variability of *P. expansum*. Similar results were reported by Marcet-Houben et al. (2012): the complete genome sequence of two *P. digitatum* strains isolated from infected orange (PHI26) and grapefruit (Pd1) revealed high similarity, sharing on average 99.96 % identity at the protein level. Julca et al. (2015) concluded that for *P. digitatum*, a single lineage has been cloned and reached worldwide distribution, and the contrasting patterns of genomic variation between the two species reflect the difference in host specificities.

The genome of *P. digitatum* opened up new study possibilities, and many genes were studied concerning their involvement in the pathogen-host interaction and host specificity. Understanding the infection process and fungal strategies is a way to develop alternatives to the use of fungicides and to new control strategies (Ramón-Carbonell and Sánchez-Torres, 2017a; Zhang et al., 2013b). Plant biotechnology, for instance, makes use of special techniques where genes can be introduced into plants to improve resistance against fungi. The introduced genes can express proteins, peptides or antimicrobial compounds that are toxic to pathogens or can directly inhibit pathogen virulence products (Wani, 2010).

Through molecular techniques, such as RNAseq, RT-PCR and targeted gene knockout (Wang and Li, 2008), important genes involved in the fungal infection process in citrus have been reported, helping to characterize the molecular mechanisms, metabolites and genetic components related to the pathogenicity of *P. digitatum*. *Agrobacterium tumefaciens*-mediated transformation (ATMT) and the marker genes red fluorescent protein (DsRed) from the reef coral *Discosoma* sp. and green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* have been applied in many filamentous fungi to understand host-pathogen interactions (Vu et al., 2018). Vu et al. (2018) expressed the genes DsRed and GFP in *P. digitatum* and observed that the green fluorescent color of GFP can be used to visualize *P. digitatum* during the interaction process with the host citrus. Expression of fluorescent markers and gene knockout through ATMT are promising ways to characterize and

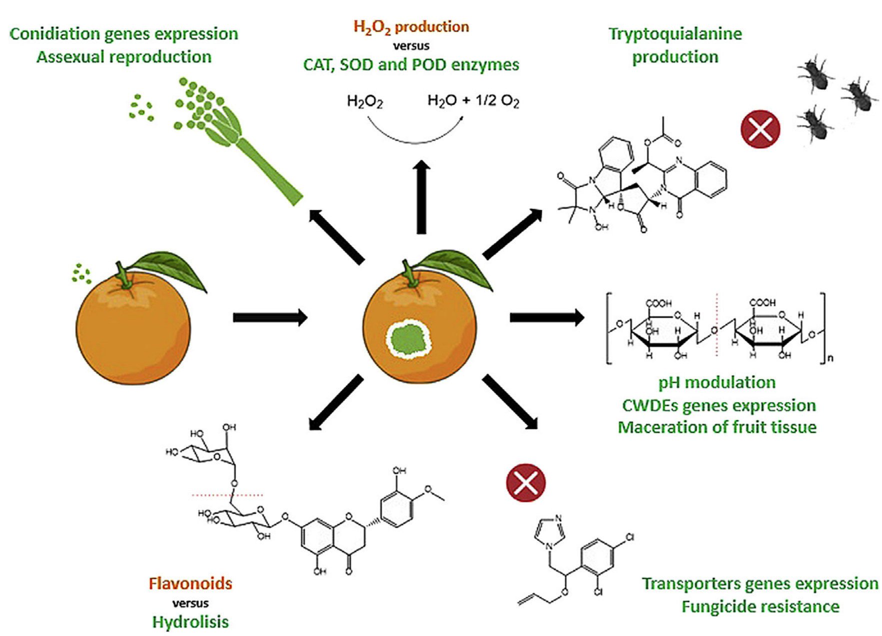

Fig. 3. Overview on host-pathogen interaction between *P. digitatum* and citrus. Figure created in the Mind the Graph platform (www.mindthegraph.com).

understand citrus colonization by *P. digitatum* since the whole mechanism of infection has not been determined to date (Vu et al., 2018; Zhu et al., 2017; Zhang et al., 2013b).

3.1. Transporters

The putative sucrose uptake transporter (SUT) gene *PdSUT1* was disrupted in *P. digitatum* (Ramón-Carbonell and Sánchez-Torres, 2017b). Initially, the SUTs were considered specific to plants; however, *Schizosaccharomyces pombe* was the first fungus found with the homologous gene *Sut1p* (Ramón-Carbonell and Sánchez-Torres, 2017b; Reinders and Ward, 2001). SUTs have also been reported in other fungi and related to virulence, but the main function and regulation of fungal sucrose uptake transporters have not been thoroughly elucidated to date (Ramón-Carbonell and Sánchez-Torres, 2017b). Δ*PdSUT1* mutants had lower virulence than the wild type in citrus infection, while mutants with overexpression in *PdSUT1* did not present an increase in virulence (Ramón-Carbonell and Sánchez-Torres, 2017b).

Expression of the genes involved in the major facilitator superfamily (MFS) transporters was also analyzed by Ramón-Carbonell and Sánchez-Torres (2017b) and revealed that this expression is enhanced in the Δ*PdSUT1* mutants, which may explain the increase in resistance to fungicides since the MFS carriers are powered and capable of carrying a wide variety of compounds, such as toxic products and drugs (Ramón-Carbonell and Sánchez-Torres, 2017b, Law et al., 2008; Reddy et al., 2012). Ramón-Carbonell and Sánchez-Torres (2017b) concluded that the *PdSUT1* gene is not directly involved in virulence and resistance to fungicides in *P. digitatum*. Instead, this gene only contributes to these factors because it activates the expression of the MFS transporter genes.

The ATP-binding cassette (ABC) and MFS transporters are involved in virulence by mediating the secretion of host-specific toxins or providing protection against plant defense components (Stergiopoulos et al., 2002). ABC transporter genes in *P. digitatum* are conserved and directly involved in drug resistance (Sánchez-Torrez and Tuset, 2011; Sun et al., 2013). In contrast, two MFS transporters were studied and related to fungicide resistance, although there are more than one hundred MFS genes in *P. digitatum* (Sun et al., 2013). Disruption of the MFS genes *PdMfs1* (Wang et al., 2012) and *PdMfs2* (Wu et al., 2016) in *P. digitatum* leads to mutants that are more sensitive to fungicides and less virulent in citrus fruits.

The mechanism by which MFS plays a role during *P. digitatum* infection requires further investigation (Wang et al., 2012). One of the possibilities is that a toxin transported by MFS acts as a virulence factor (Wang et al., 2012) or that MFS plays a role in infection through a different way. For example, Liu et al. (2017) recently described that the *ChMfs1* gene of the phytopathogenic fungus *Colletotrichum higginsianum* is involved in intrahyphal hyphae formation, demonstrating a novel function of MFS transporters that plays a key role in infection.

3.2. Cell wall-degrading enzymes

Phytopathogenic fungi need to pass through the plant cell wall, an important barrier, to attack. Thus, phytopathogenic fungi produce cell wall-degrading enzymes (CWDEs) to depolymerize plant cell wall structures, such as cellulose and pectin (Kubicek et al., 2014). The ability to degrade the host cell wall has long been thought to play a key role in *P. digitatum* infection such that CWDEs are upregulated during the infection process (Zhang et al., 2013c).

Zhang et al. (2013c) verified the importance of the *PdSNF1* gene for virulence. In microorganisms, *SNF1* regulates the derepression of glucose-repressible genes, controlling the use of carbon sources and, in the case of plant pathogenic fungi, the expression of CWDE (Zhang et al., 2013c; Nadal et al., 2010; Yi et al., 2008). Concerning virulence, Zhang et al. (2013c) observed that the Δ*PdSFN1* mutants were still pathogenic to citrus, but the symptoms took much longer to develop in the fruits, and the production of conidia was affected. Expression of the CWDE genes was upregulated for the wild-type when induced with pectin, but in the Δ*PdSNF1* mutants, the expression levels almost did not change (Zhang et al., 2013c). Zhang et al. (2013c) showed that the *PdSNF1* gene is involved in the regulation of CWDE gene expression in *P. digitatum* and, consequently, in virulence.

CWDEs are also affected in *P. digitatum* by the mitogen-activated protein kinase B gene *PdMPkB*. Through qRT-PCR analyses, Ma et al. (2016) verified that CWDE genes were downregulated in ΔPdMPkB mutants. Therefore, in fruits, Δ*PdMPkB* mutants showed fewer lesions than the wild type (Ma et al., 2016). The effect of pH signaling transcription factor gene *PdpacC* deletion in *P. digitatum* was studied by Zhang et al. (2013a), and the disruption of this gene also leads to fewer virulence mutants since the expression levels of the polygalacturonase gene *Pdpg2* and pectin lyase gene *Pdpnl1* were not upregulated or were weakly upregulated compared to the wild-type strain. These results clearly reinforce that CWDEs are essential for *P. digitatum* virulence as for other phytopathogens. Additionally, genes that are involved in CWDE gene regulation are consequently involved in pathogenicity.

3.3. Signaling pathway components

Studies on some signaling pathway genes demonstrated that they are required for virulence but not directly involved in the pathogenic mechanisms of *P. digitatum*. For example, *PdpacC* is a signaling pathway gene that is essential for virulence because it regulates CWDE gene expression (Zhang et al., 2013a).

Another example is the gene *Pdos2,* which encodes the mitogen-activated protein kinase Hog1 studied by Wang et al. (2014) and is part of the high-osmolarity glycerol pathway. Δ*Pdos2* mutants are more sensitive to hyperosmotic stress and cell wall-disturbing agents and are more resistant to fludioxonil once this fungicide acts on the HOG pathway (Wang et al., 2014). Wang et al. (2014) concluded that *Pdos2* is associated with positive regulation of glycerol synthesis and negative regulation of ergosterol synthesis, and the decrease in virulence may result from the defect in vegetative growth and sensitivity to osmotic stress.

The role of the calcineurin-responsive transcription factor gene *PdCrz1* in *P. digitatum* was investigated by Zhang et al. (2013b). Δ*PdCrz1* mutants were observed to be defective in conidiation, virulence, and hypersensitivity to stress caused by Ca^{2+} and DMI fungicides. In addition, the expression of cell wall synthase genes (*CHS2, CHS3* and *FKS1*) leads to defective cell wall integrity (Zhang et al., 2013). However, the mechanism by which *PdCrz1* controls *P. digitatum* virulence requires further investigation (Zhang et al., 2013).

Asexual spores (conidia) are the inocula of green mold disease produced by *P. digitatum* during fruit-pathogen interaction, and they play an important role in the disease cycle since germination of conidia occurs in the surface wounds of citrus (Zhang et al., 2013c; Ramón-Carbonell and Sánchez-Torres, 2017a). The *PdSNF1* gene, as mentioned before, is required for *P. digitatum* conidiation (Zhang et al., 2013c). *PdSNF1* plays a role in the expression of *PdBrlA* and *FadA*, which are genes involved in conidiation and growth signaling (Zhang et al., 2013c). *PdMPkB* and *PdSlt2* are other genes also involved in conidial formation (Ma et al., 2016; Ramón-Carbonell and Sánchez-Torres, 2017c). Thus, Δ*PdSNF1,* Δ*PdMPkB* and Δ*PdSlt2* mutants showed less virulence compared to the wild-type strain (Zhang et al., 2013c; Ma et al., 2016; Ramón-Carbonell and Sánchez-Torres, 2017c) because conidiation was affected in these mutants. The *PdSte12* transcription factor is functionally conserved in *P. digitatum* for asexual reproduction and is another example of a signal pathway gene that contributes to the infection process once it controls invasive growth and asexual reproduction as the major virulence function (Ramón-Carbonell and Sánchez-Torres, 2017a).

3.4. Structural components

Zhu et al. (2014) reported the function of glucosylceramides (GlcCers) in *P. digitatum* through knockout of the glucosylceramide synthase gene *PdGcs1*. For decades, GlcCers were considered only structural components of the cell membrane; however, recent studies describe that fungal glycosylceramides play important roles in vital processes such as growth, spore germination, secretion, cell wall assembly, and regulation of virulence (Mouyna et al., 2010; Nimrichter et al., 2008). Deletion of *PdGcs1* resulted in a decrease in sporulation, growth and virulence and a delay in conidia germination of *P. digitatum* (Zhu et al., 2014). Lesions caused by the infection appear at 1 dpi for the wild-type strain and at 2 dpi for *ΔPdGcs1* mutants. At 5 dpi, the lesion diameter was approximately 11 cm for the wild-type strain and 5 cm for the deficient *ΔPdGcs1* mutants (Zhu et al., 2014). For some fungi, the role of GlcCers in virulence is well-established (Rittershaus et al., 2006); however, for *P. digitatum,* this role has not been determined (Zhu et al., 2014).

Gandía et al. (2014) analyzed the effect of a Chitin synthase (Chs) knockout in *P. digitatum* mutants. Chitin is a long linear homopolymer made of N-acetylglucosamine bound by β-1,4 linkage (Gandía et al., 2012, 2014, Ruiz-Herrera et al., 2002). This polysaccharide is a structural component of the fungal cell walls, giving rigidity to the cell and accounting for up to 20 % of the dry weight of the cell wall of filamentous fungi (Gandía et al., 2012, 2014, Ruiz-Herrera et al., 2002). *ΔPdChsVII* mutants showed reduced growth and conidia production and higher sensitivity to cell wall-disturbing agents and fungicides (Gandía et al., 2014). The virulence was reduced, but the mutants were still able to infect and macerate the citrus, and there was no production of visible mycelium or green conidia in fruit (Gandía et al., 2014).

As a structural component, chitin synthases seem to be essential for cell wall integrity, and the reduced virulence is a consequence. The knockout of the O-mannosyltransferase protein gene *Pdpmt2* by Harries et al. (2015) is another example. *ΔPdpmt2* mutants were more sensitive to cell wall breakers, showed a reduction in growth and number of conidia and were less sensitive to PAF26 (Harries et al., 2015). The slow growth and defective cell wall caused by *Pdpmt2* knockout limit fruit colonization and explain the lower virulence of the mutants (Harries et al., 2015).

3.5. Genes not required for virulence

There are studies in which genes deleted in *P. digitatum* did not alter virulence. It is the case of the *PdMit1* gene encoding a mannose inositol-phosphorylceramide synthase. The deletion of *PdMit1*, evaluated by Zhu et al. (2015), altered growth, delayed conidial germination, decreased conidial production and increased Ca^{2+} sensitivity.

Table 1
Main genes studied and their role in *P. digitatum* metabolism.

Group	Genes	Role in *P. digitatum*	References
Transporters	*PdSUT1*	Related to virulence and fungicide sensitivity through MFS transporters gene activation	Ramón-Carbonell and Sánchez-Torres (2017b)
	PdMfs1	Required for prochloraz resistance, conidiation and full virulence	Wang et al. (2012)
	PdMfs2	Involved in DMI resistance and pathogenicity	Wu et al. (2016)
CWDE	*Pdpg2*	Involved in virulence	Zhang et al. (2013a)
Signal Pathways	*PdpacC*	Participates in the response to Na^+ and K^+ stresses, required for mycelial growth at alkaline conditions. Involved in the regulation of CWDEs genes *Pdpg2* and *Pdpnl1*	Zhang et al. (2013a)
	Pdos2	Involved in response to hyperosmotic stress, regulation of cell wall integrity, sensitivity to fungicides and virulence	Wang et al. (2014)
	PdCrz1	Involved in conidiation, virulence, DMI resistance and expression of cell wall synthase genes	Zhang et al. (2013b)
	PdSNF1	Involved in virulence through the regulation of CWDEs, *PdBrlA* and *FadA* genes expression.	Zhang et al. (2013c)
	PdMPkB	Involved in the regulation of CWDEs genes expression, conidia formation and osmotic stress adaptation.	Ma et al. (2016)
	PdSlt2	Involved in asexual reproduction and regulation of ABC and MFS genes expression	Ramón-Carbonell and Sánchez-Torres (2017c)
	PdSte12	Controls invasive growth and asexual reproduction	Ramón-Carbonell and Sánchez-Torres (2017a); Vilanova et al. (2016)
	Pdac1	Required for vegetative growth, carbon utilization, and conidial germination	Wang et al. (2016)
Structural Components	*PdChsVII*	Involved in cell wall integrity, vegetative growth and, during host colonization, mycelium development and conidia production.	Gandía et al. (2014)
	PdGcs1	Regulate cell growth, differentiation, and virulence by controlling the biosynthesis of GlcCers	Zhu et al. (2014)
	Pdpmt2	Critical role in fungal growth, conidiation and PAF26 resistance	Harries et al. (2015)
Dispensable for virulence	*PdsreA* and *PdsreB*	Important role in DMI resistance and global gene regulation	Ruan et al. (2017)
	PdMit1	Important for the growth of mycelium, sporulation and conidial germination.	Zhu et al. (2015)
	tqaA	Only involved in tryptoquialanines production	Zhu et al. (2017)
	PdKu80	Important role in homologous integration	Xu et al. (2014)

Sterol regulatory element-binding protein genes (*Pdsre*) were evaluated by Ruan et al. (2017) using three different *P. digitatum* mutants. The lack mutants *ΔPdsreA*, *ΔPdsreB* and *ΔPdsreAB* caused macerations of similar size to the lesions induced on mandarins by the wild-type strain. *Pdsre* are principally involved in resistance to DMI fungicides because *ΔPdsre* mutants increased sensitivity to these fungicides (Ruan et al., 2017).

The mutants with deletions in *tqaA*, a gene involved in the production of tryptoquialanines, showed no growth, development, stress response or pathogenicity variations, as reported by Zhu et al. (2017), indicating that tryptoquialanines are not involved in the interaction of *P. digitatum* with citrus host. Zhu et al. (2017) suggested that tryptoquialanines may be involved in the protection of rotten citrus from insects. This hypothesis was confirmed, as mentioned before by Costa et al. (2019), through the high insecticide activity of tryptoquialanine A against *A. aegypti* larvae.

Similar results were also obtained for the deletion of *PdKu80*, a gene of the nonhomologous end-joining (NHEJ) pathway (Xu et al., 2014), and for *ndo1*, a gene coding a naphthalene dioxygenase (López-Pérez et al., 2015); none of these genes was found to be important for virulence.

Table 1 summarizes the main genes studied for *P. digitatum* found in the literature.

The functions of most of the studied *P. digitatum* genes are conserved across the species. The genes that affect virulence are generally related to growth, asexual reproduction, cell wall integrity or the expression of other genes (CWDEs or transporters).

Some genes, such as *PdCrz1*, have not had their role in infection thoroughly elucidated and warrant further investigation. As mentioned previously, the functions of the transporters on virulence have not been well-established, as no secondary metabolites have been linked to the infection process, representing a gap in the research concerning the fungus *P. digitatum*. The studies reinforced that cell wall-degrading enzymes seem to be involved in the virulence of this fungus and help to explain how *P. digitatum* modulates the acidity of the environment. However, the molecular biology of *P. digitatum* warrants a thorough investigation, and an extensive analysis of the gene clusters found in the genome of this important pathogen is necessary to better understand the entire infection process.

4. Conclusions

Despite the great economic interest in *P. digitatum*, the molecular basis of infection and specificity towards the citrus host remains largely unknown. In recent years, most of the research on *P. digitatum* has focused on treatments against infection symptoms, fungicide resistance and the biocontrol of this fungus using antagonist microorganisms. However, since 2012, with the publication of the genome of this important necrotrophic fungus, this scenario has been changing, and many genes have been determined to be virulence factors and to be important in the disease. The knowledge concerning the genes required for full virulence reviewed in this study (*PdSNF1, PdMPkB, Pdos2, PdSte12, PdCrz1, Pdpmt2, PdchsVII*) provides the first insights about pathogen-host interaction in *P. digitatum* and provides initial thoughts on potential target fungicides to be developed against such phytopathogens. The main infection strategies of *P. digitatum*, citrus responses, secondary metabolites and genes involved in host-pathogen interaction are represented in Fig. 3. In our opinion, understanding the infection process and the defense responses of the hosts, as well as the virulence mechanisms of this pathogen, is the first step in developing safer and more eco-friendly alternative strategies for controlling citrus postharvest diseases, leading to new and safer control strategies for green mold disease. In addition, the secondary metabolism of *P. digitatum* and the roles of natural products in the infection merit further research. Innumerous biosynthetic gene clusters are coded in this phytopathogen's genome, and many gene clusters are described to be upregulated during the infection process; however, only tryptoquialanine-like metabolites are described as natural products for this fungus, meaning that many products remain to be discovered.

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