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Comparative analysis of the sensitivity to distinct antimicrobials among *Penicillium* spp. causing fruit postharvest decay

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Summary. The postharvest fungal pathogens *Penicillium digitatum*, *P. italicum* and *P. expansum* are an increasing problem for the Mediterranean orchards and fruit industry. This study was designed to gain knowledge on factors affecting susceptibility of *Penicillium* spp. to antimicrobial peptides (AMPs) as new antifungal compounds for plant protection. The previously characterized PAF26 is a novel penetratin-type AMP with activity against phytopathogenic fungi. Comparative analyses were conducted on the sensitivity of *Penicillium* spp. to PAF26, to the cytolytic peptide melittin and to other antimicrobials. The research included microscopic observations, chitin quantification, virulence assays on citrus and apple fruits, and molecular phylogenetic relationships within *Penicillium* isolates from citrus fruit. Virulence analysis and phylogenetic reconstruction confirmed the host specificity and monophyletic origin for *P. digitatum*, contrary to the closely-related species *P. expansum* and *P. italicum*. A parallelism was found between sensitivity to PAF26 of *Penicillium* isolates and to the chitin dye calcofluor white (CFW). No such correlation was found between sensitivity to PAF26 and to the membrane perturbing compound SDS or the oxidizing agent H_2O_2. Microscopy studies showed that mycelium and conidia from the PAF26-sensitive fungi were also prone to CFW staining, but no direct correlation with the mycelial chitin content was found. The data are consistent with the fact that fungal cell walls influence the outcome of the interaction of AMPs with fungi, and that PAF26 is more active towards *Penicillium* citrus fruit pathogens. In this context, CFW could help both to elucidate AMPs mode of action and in studies of the mechanisms of virulence and host specificity within *Penicillium* spp.

Key words: post-harvest, antimicrobial peptides, fungal cell wall, calcofluor white.

Introduction

The genus *Penicillium* comprises more than 150 species but only a minor proportion of them are economically important phytopathogens (Samson and Pitt, 2000; Barkai-Golan, 2001). Among these, *Penicillium* spp. causing postharvest diseases are of high relevance to agriculture and the fruit-tree industry. Green and blue mould caused by *Penicillium digitatum* Sacc. and *Penicillium italicum* Wehmer, respectively, are among the main postharvest diseases of citrus fruits responsible for about 80% of postharvest losses. *P. digitatum* is a necrotrophic wound pathogen that requires injured peel to penetrate the host tissue, and colonizes mostly through the maceration enzymes. Interestingly, and despite these rather unspecific properties, *P. digitatum* is host specific and has not been described as naturally-occurring in other pathosystems outside citrus fruits. Although *P. italicum* is found mostly

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in association with citrus, it can be isolated from other commodities and can infect other fruits such as stone or pome fruits and grapes under laboratory conditions. *P. expansum* Link is more polyphagous, although its main hosts are pears and apples. Also importantly, other non-pathogenic *Penicillium* strains are increasingly important, due to their ability to produce secondary metabolites and contaminants in the fruit postharvest industry, e.g. *Penicillium brevicompactum* (Overy and Frisvad, 2005; Patino *et al*., 2007). While the etiology of most *Penicillium* fruits rots is well established, the physiological and biochemical bases of host specificity are not understood.

The use of fungicides is the main control strategy to manage plant diseases caused by fungi, including postharvest diseases of fruits and vegetables (Knight *et al*., 1997; Barkai-Golan, 2001; Narayanasamy, 2006). However, health authorities and consumers have become increasingly concerned about the presence of fungicide residues in foods and their release in the environment. Thiabendazol (TBZ), imazalil (IMZ) and sodium *o*-phenylphenate (SOPP) are the most commonly used fungicides for managing green and blue mould of citrus (Smilanick *et al*., 2006). Nowadays, resistance to these fungicides is very common, compromising their efficacy and becoming an important factor in limiting their use. In this scenario, extensive molecular studies have been conducted to explain the mechanisms leading to fungicide resistance in phytopathogenic fungi, including *P. digitatum* (Ma *et al*., 2007; Fernández-Ortuño *et al*., 2008; Sánchez-Torres and Tuset, 2011). Also, research efforts to develop alternative methods for the control of postharvest decay have been intensified (Barkai-Golan, 2001; Narayanasamy, 2006). The potential of antimicrobial peptides (AMPs) as novel antibiotics is widely recognized (Hancock and Sahl, 2006), and has been extended to agriculture (Montesinos, 2007; Marcos *et al*., 2008;) and food preservation (Cotter *et al*., 2005; Rydlo *et al*., 2006). As for other antimicrobials, a detailed knowledge of AMP mode of action is essential for their potential application to plant protection (Marcos *et al*., 2008). Research is needed for the identification of biochemical/molecular determinants related to susceptibility to peptides in phytopathogenic microorganisms.

We are interested in the identification and characterization of short AMPs active against fungal phytopathogens, with a focus on citrus fruit pathogens. Combinatorial and rational design approaches have led to a group of synthetic hexa- and heptapeptides (called PAFs) with varying degrees of specificity and potency against phytopathogenic fungi (López-García *et al*., 2002; Muñoz *et al*., 2007b). PAF26 is a representative hexapeptide with activity against *P. digitatum*. Contrary to the cytolytic peptide melittin, PAF26 does not induce quick permeation of the fungal plasma membrane but rather is a penetratin-like AMP which enters fungal mycelium and conidia (Muñoz *et al*., 2006). PAF26 also induces changes in fungal morphology, such as alteration of normal patterns of fungal growth (polar growth and branching), swelling of hyphal cells and tips, and abnormal chitin deposition as determined by calcofluor white (CFW) staining (Muñoz *et al*., 2006).

Increased knowledge of the factors that determine susceptibility to AMPs is relevant. The present study compared *Penicillium* spp. infecting citrus and apple fruits, assessed their susceptibility to PAF26, melittin and other antimicrobial compounds of known mode of action, and determined their cell wall properties, phylogeny and pathogenicity.

Materials and methods

Fungal isolates and culture conditions

In this study we used the *Penicillium* field isolates PHI-1, PHI-8, PHI-26 and PHI-65, all of them related to fruit postharvest diseases, which were previously collected from rotten fruits and a contact plate from a packing-house (López-García *et al*., 2000; López-García *et al*., 2003). All fungi were routinely cultured on potato dextrose agar (PDA) (Difco-BD Diagnostics, Sparks, MD, USA) plates for 7 to 10 days at 24°C. Conidia were collected by scraping the agar surfaces with a ster-

ile spatula and transferring conidia to sterile water. Conidia were then filtered and titrated with a heamatocytometer.

Molecular identification and phylogenetic analysis

A PCR approach was followed to amplify and sequence the ITS region of the fungal ribosomal DNA. Genomic DNA was purified from mycelia as previously described (Lee and Taylor, 1990). Oligonucleotide primers ITS1 and ITS4 (White *et al*., 1990) were used to amplify a region of approximately 600 bp that covers ITS1, 5.8S rRNA and ITS2. PCR products were analyzed through gel electrophoresis to confirm the presence of a single amplicon. PCR products from three equivalent, but independent, PCR reactions were mixed together in order to spot and eliminate possible amplification mistakes, and directly sequenced by an external service (IBMCP, CSIC-UPV, Valencia, Spain).

Additional ITS *Penicillium* sequences were obtained from public databases. Multiple alignments of rDNA sequences were obtained using ClustalW (Thompson *et al*., 1994). Phylogenetic analyses were performed using the following programs of the PHYLIP package (v3.6) (Felsenstein, 1996): DNAPARS was used to carry out unrooted parsimony, coupled to SEQBOOT (1000 repetitions) and CONSENSE to perform bootstrap analyses. TreeView (v1.6.6) was used to draw the resulting phylogenetic tree.

Fruit decay test

Experiments were carried out as described previously (Marcos *et al*., 2005) on freshly harvested oranges or apples. Briefly, fruits were submerged for 5 min in a 5% commercial bleach solution (equivalent to 0.25% free chlorine), extensively washed with tap water and allowed to air-dry. Fruits were wounded by making punctures (approximately 3 mm depth) with a sterile nail at four sites around the equator of each fruit. An equal volume of conidia from a 10^4 conidia mL^{-1} solution was applied onto each wound. For each fungus, three replicates (five fruits per replicate, four wounds per fruit) were prepared in each experiment. Fruits were maintained at 20ºC and 90% RH. Symptoms were scored at different days post-inoculation (dpi) as the number of infected wounds per replicate, and the mean values ± SD for each treatment were subsequently calculated.

Antimicrobial assays

The antimicrobial peptide PAF26 (Ac-RKKWFW-NH_2) was purchased at >95% purity from GenScript Corporation (Piscataway, NJ, USA), wherein it was synthesized by solid phase using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. Melittin peptide (GIGAVLKVLTTGLPALISWIKRKRQQ) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Fungal growth inhibition by AMPs was assessed using a microtiter plate assay as described previously (López-García *et al*., 2000). The growth medium was potato dextrose broth (PDB) (Difco-BD Diagnostics) containing 2.5×10^4 conidia mL^{-1}, 0.003% (w:v) of chloramphenicol and the different peptide concentrations (2, 4, 8, 16, 32, 64 and 128 μM). Three replicates were prepared for each treatment. Growth was determined by measuring the OD at 492 nm in a Multiskan Spectrum microplate spectrophotometer (Thermo Electron Corp., Finland). Fungicidal activity on non-germinated conidia was assayed by preparing fungal conidial suspensions (2.5×10^4 conidia mL^{-1}) with different concentrations of PAF26 and melittin (from 2 to 64 μM) in water and incubated 24 h. Subsequently, samples were diluted two fold, and 5 μL was each applied onto peptide-free PDA plates that were incubated for 4 days at 24ºC to monitor fungal growth recovery (Muñoz *et al*., 2006).

Activity of other antimicrobials was assayed on PDA plates amended with the different compounds: Calcofluor White (Fluorescent Brightener 28, Sigma-Aldrich) at 50–300 μg mL^{-1} of final concentration, hydrogen peroxide (H_2O_2) (Sigma-Aldrich) at 0.5–6 mM, and SDS (Sigma-Aldrich) at 100–500 μg mL^{-1}. In all assays, a 5 μL droplet of a serial two fold dilution from a $2.5–10^4$ conidia mL^{-1} solution was applied onto each amended plate, and the plates were incubated at 24ºC for 3–4 days. Additionally, PDA and 1.2 M D-

sorbitol amended PDA plates were incubated at 30ºC for 5–7 days to test the thermotolerance and osmotic remediability of the fungal strains.

Microscopy

Microscopy analysis was carried out with a fluorescence microscope (Eclipse 90i, Nikon Instruments Europe), using differential interference contrast (DIC) for bright field images and with the filter set at an excitation wavelength of 395 nm and an emission wavelength of 440 nm for CFW fluorescence. Either conidia or 24h-old mycelium were stained with 0.1% (w/v) CFW for 5 min in the dark and subsequently washed in distilled water (Pringle 1991). Samples were mounted in 20% (v/v) glycerol immediately before visualization.

CFW fluorescence images of different fungi were taken under the same gain (2.0) and time exposure (300 ms) conditions and subsequently processed using the Image-Pro Plus 7.0 software (Media Cybernetics Inc., MD). A minimum of ten different conidia, hyphal tips or cell wall areas from independent images were selected for quantitative analyses of Fluorescence Intensity (FI) using Image-Pro draw tools. Average ± SD FI from each group of fungal structures was calculated.

Measurement of chitin content

Chitin content was measured essentially as previously described by Din *et al*. (1996) and Martín-Urdíroz *et al*. (2004). Briefly, conidia (1×10^6 conidia mL^{-1}) were grown in PDB at 24ºC with soft shaking. Mycelium was collected after 48 h, washed twice and lyophilized. Ten to 20 mg dry weight was treated with 6% (w/v) KOH for 90 min at 80ºC, followed by glacial acetic acid addition. Insoluble material was washed and suspended in 0.5 mL sodium phosphate buffer (50 mM, pH 6.3), and digested with 100 µL of a 5 mg mL^{-1} chitinase suspension (Sigma-Aldrich) at 37ºC for 20 h. Following centrifugation at 13,000×*g* for 15 min, 475 µL of the supernatant were treated with 25 µL of a 1×10^4 units mL^{-1} β-glucuronidase Type H-5 (Sigma-Aldrich) at 37 C for 2h. Aliquots of 100 µL were assayed for *N*-acetylglucosamine (GlcNAc) content (Reissig *et al*., 1955). Statistical analyses of data were carried out with the software package StatGraphics Plus 5.1 (StatPoint, Hemdon, VA).

Results

Molecular identification, phylogeny and pathogenicity of *Penicillium* isolates

Taxonomic identification by molecular techniques was performed for the different *Penicillium* isolates used in this study. A PCR approach to isolate and sequence the ITS region of the ribosomal DNA (White *et al*., 1990) was used. A phylogenetic tree re-construction was conducted with these sequences and representative homologs from public databases using parsimonious methods of the PHYLIP package (Felsenstein, 1996) (Figure 1). An ITS sequence from *Eupenicillium shearii* (accession number AJ004893) was arbitrarily defined as the out-group. Several phylogenetic clusters were noteworthy. Firstly, all the analyzed *P. digitatum* strains were grouped together with a 100% confidence as defined by bootstrap analyses, which indicates a monophyletic origin (Figure 1). An additional cluster with high significance (91%) included most, but not all, of the *P. italicum* and *P. expansum* sequences. However, the sub-branches within this group had much lower significance, and one *P. expansum* isolate (AF455466) was separated from the rest. Therefore, the tree reflects a more diffuse separation between the species *P. italicum* and *P. expansum*. Additional *P. italicum* or *P. expansum* sequences were located at distinct positions within the tree, and those branches that included them were conserved in less than 50% of the replications. Another exception was the *Penicillium* sp. CECT2294, previously classified as *P. italicum* (Alaña *et al*., 1989), which has an ITS sequence identical to that of several *P. crustosum* and *P. commune* isolates. The isolate *P. brevicompactum* PHI-8, which was recovered from the equipment of a citrus packinghouse, was the most distinct isolate in its phylogeny of the *Penicillium* spp. isolates tested in this work (Figure 1).

We carried out fruit decay assays under laboratory controlled conditions. The patho-

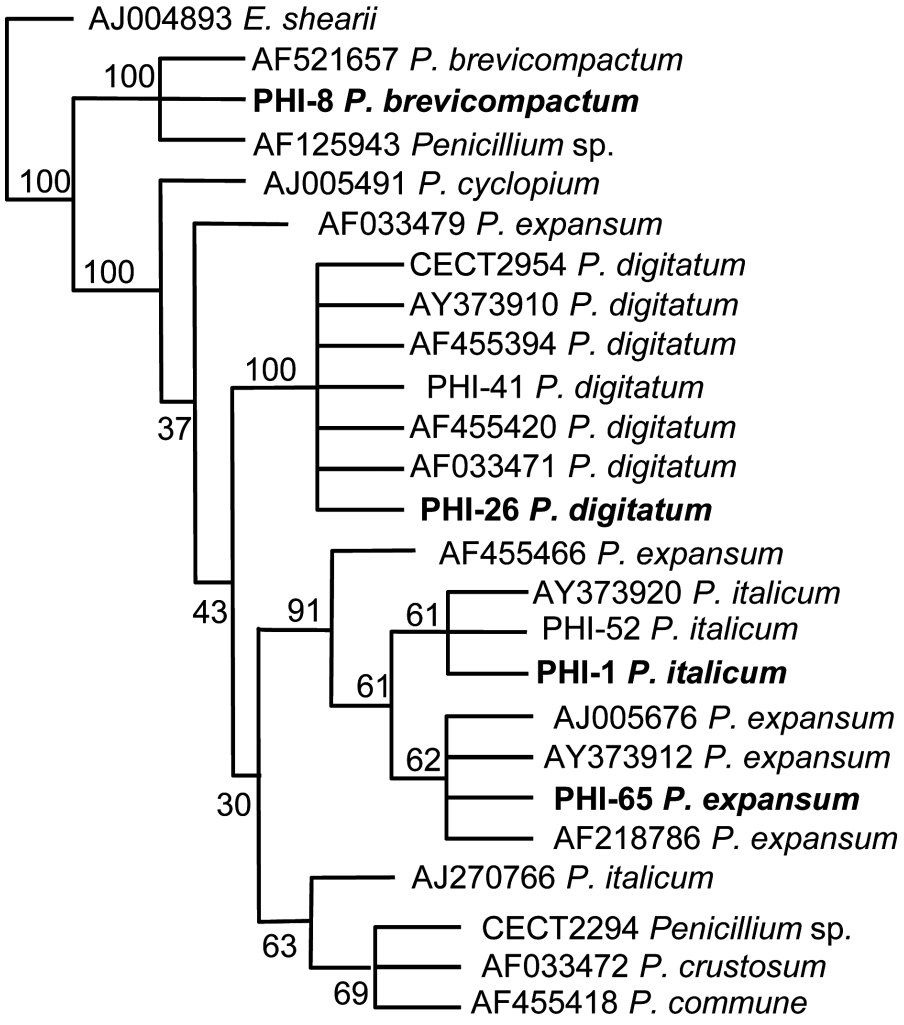

Figure 1. Phylogenetic tree reconstruction of *Penicillium* isolates based on the ITS ribosomal DNA sequence. Isolate sequences obtained from public databases are labelled with the accession number of the sequence. Isolates characterized in this study are indicated in bold. Additional fruit isolates PHI-41 and PHI-52, and strains CECT2294 and CECT2954 from "Colección Española de Cultivos Tipo" (CECT, Spanish National Collection of Type Cultures) were included as references. Numbers at nodes indicate the percentage of replicates in which the node was found, as determined by bootstrap analysis (1000 replicates). Accession numbers of the resulting sequences are: *P. digitatum* PHI-26: AJ250547; *P. italicum* PHI-1: AJ250549; *P. brevicompactum* PHI-8: JN036722; and *P. expansum* PHI-65: JN036723.

genicity and virulence of the different isolates were studied in oranges (Figure 2a) and apples (Figure 2b) as previously described (Marcos *et al*., 2005). The *P. digitatum* isolate caused typical green mould on citrus which reached approximately 100% of infected wounds after 7 dpi. This fungus gave no tissue maceration or fungal sporulation on apples, confirming absence of pathogenicity to this host. *P. italicum* displayed usual blue mould symptoms on citrus, albeit with overall lower incidence than the *P. digitatum* isolate at the same inoculum concentration. On apples, *P. italicum* gave limited tissue maceration around the inoculation zone in the few wounds that became infected. Other laboratory isolates such

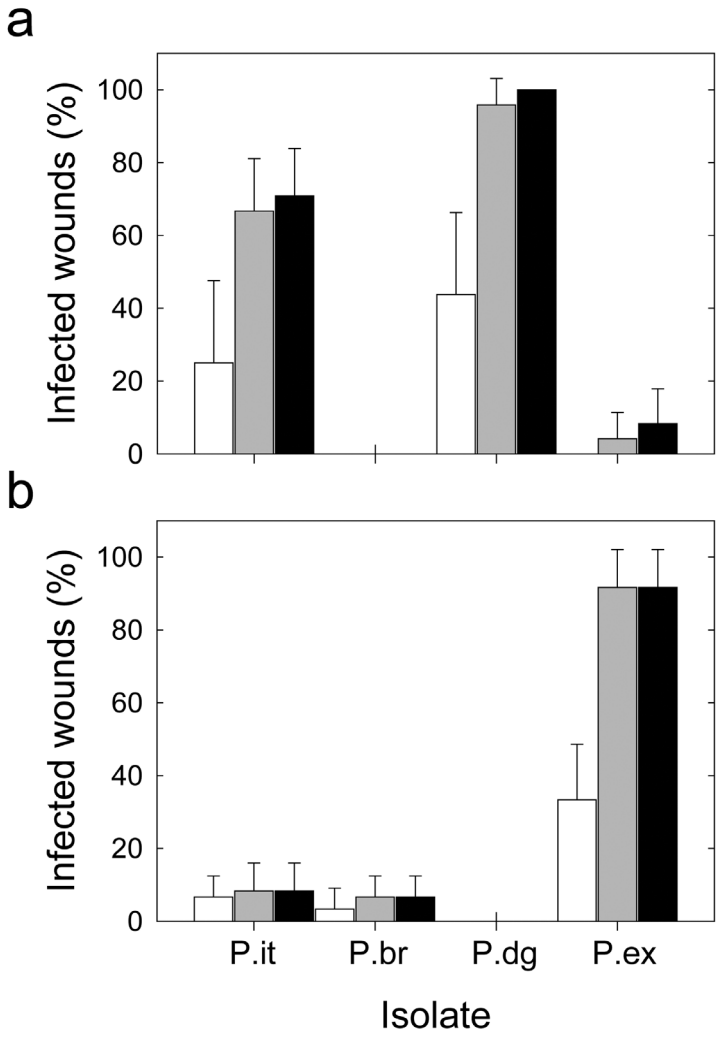

Figure 2. Fungal infection on fruits. Conidia of *Penicillium* italicum PHI-1, *P. digitatum* PHI-26, *P. brevicompactum* PHI-8, and *P. expansum* PHI-65 were inoculated onto orange (a) or apple (b) fruits, and kept at 20ºC and 90% RH. Graphs show the percentage of infected wounds ± SD recorded every day post inoculation (dpi) at 3 dpi (white bars), 5 dpi (grey bars) and 7 dpi (black bars). Abbreviations: P.it: *P. italicum* PHI-1; P.dg: *P. digitatum* PHI-26; P.br: *P. brevicompactum* PHI-8; P.ex: *P. expansum* PHI-65.

as *P. digitatum* PHI-41 or *P. italicum* PHI-52 confirmed their phylogeny adscription (Figure 1) and virulence on citrus fruits (data not shown), having similar behaviour to the above described fungi.

On the contrary, low incidence of infection on citrus was found for the *P. expansum* isolate (Figure 2a), although the limited infected area showed blue sporulation at the inoculation point. The low virulence of *P. expansum* onto citrus was not due to a lack of virulence factors since in its natural host (apple) it gave typical blue mould symptomatology, with the highest incidence among the analysed isolates (Figure 2b). The *P. brevicompactum* isolate gave very limited infection in either of the two hosts (Figure 2). *P. brevicompactum* is a rather ubiquitous species that contaminates diverse substrates and commodities, wherein it can produce toxic compounds that compro-

mise food safety (Overy and Frisvad, 2005; Patino *et al*., 2007); however, pathogenicity to plant tissues has not been described as confirmed in the present study.

Susceptibility to PAF26 and melittin peptides

In previous research, we have reported that susceptibility to the growth inhibition activity of the hexapeptide PAF26 varied among *Penicillium* spp. responsible for post-harvest fruit decay (López-García *et al*., 2002; López-García *et al*., 2003; López-García *et al*., 2007). This differential susceptibility was maintained with both D- and L-stereoisomers of the peptide and also in assays conducted with different dilutions of PDB in growth medium. In addition, we have shown that growth inhibition and fungicidal activities of AMPs towards *P. digitatum* are not necessarily linked, as there are examples of peptides such as melittin which are inhibitory to mycelium but not fungicidal to non-germinated conidia of *P. digitatum* even at high peptide concentrations (Muñoz *et al*., 2006; Muñoz *et al*., 2007a). In the present study, we extend these previous observations in the context of the comparative analysis over the *Penicillium* isolates studied here. The susceptibility of isolates *P. digitatum* PHI-26, *P. italicum* PHI-1, *P. expansum* PHI-65 and *P. brevicompactum* PHI-8 to two AMPs with distinctive modes of action (López-García *et al*., 2010) and their response to stress conditions or to antimicrobial compounds of known mode of action were further analyzed.

Parallel experiments shown in this work confirmed the differential sensitivity to PAF26 and melittin of the representative fungal isolates. *P. digitatum* and *P. italicum* were the more sensitive to both AMPs (Figure 3a). Within peptides, melittin was the AMP with the highest fungistatic activity (Figure 3a, bottom graphic), whereas PAF26 showed a greater specificity to inhibit the growth of *P. digitatum* and *P. italicum* (Figure 3a, top graphic). Assays of fungicidal activity of PAF26 confirmed the previous inhibitory data showing high fungicidal activity towards *P. digitatum* and *P. italicum* conidia (Figure 3b, top plates). On the contrary, melittin showed non-fungicidal activity against *P. digitatum* conidia, as reported previously for 30 μM of peptide (Muñoz *et al*., 2006), or other *Penicillium* spp. as well (Figure 3b, bottom plates). The *P. brevicompactum* isolate which showed the lowest sensitivity to PAF26 was the fungus with limited virulence to citrus and apple fruits (Figure 2), opposite to what occurred with the highly PAF26-sensitive *P. digitatum* strain which was highly virulent to citrus fruits.

Comparative analyses of thermotolerance and sensitivity to other antimicrobials

In order to explore other phenotypical aspects of the fungal biology among isolates with differential susceptibility to AMPs, additional treatments were assayed. Thermotolerance was examined by the incubation of fungi at 30ºC for 5–7 days on PDA plates (Figure 4a). *P. expansum* and *P. brevicompactum* were the most affected by the temperature stress, *P. digitatum* was slightly affected, while macroscopic growth of *P. italicum* did not change significantly. Osmotically remediable thermosensitivity is likely due to defects or impairment in cell wall structure or composition (Momany *et al*., 1999). Addition of the osmoprotectant sorbitol partially restored the phenotype and growth of *P. expansum* and *P. brevicompactum* isolates at restrictive temperature (Figure 4a).

The antimicrobial effect of additional compounds was also assayed. The anionic dye CFW is known to interact with chitin chains of cell walls interfering with fungal development and cell wall formation (Ram and Klis, 2006). Calcofluor white has been used to screen mutant collections and the effects of specific genes on chitin biosynthesis or cell wall structure. *P. digitatum* was the most sensitive to CFW of the fungi assayed, closely followed by *P. italicum*, and *P. expansum* and *P. brevicompactum* were relatively more resistant (Figure 4b). As the latter were more sensitive to growth at relatively high temperature (Figure 4a), their CFW resistance is hypothesized to be due to lower chitin content or accessibility within the fungal cell walls (see below). Notably, the relative susceptibil-

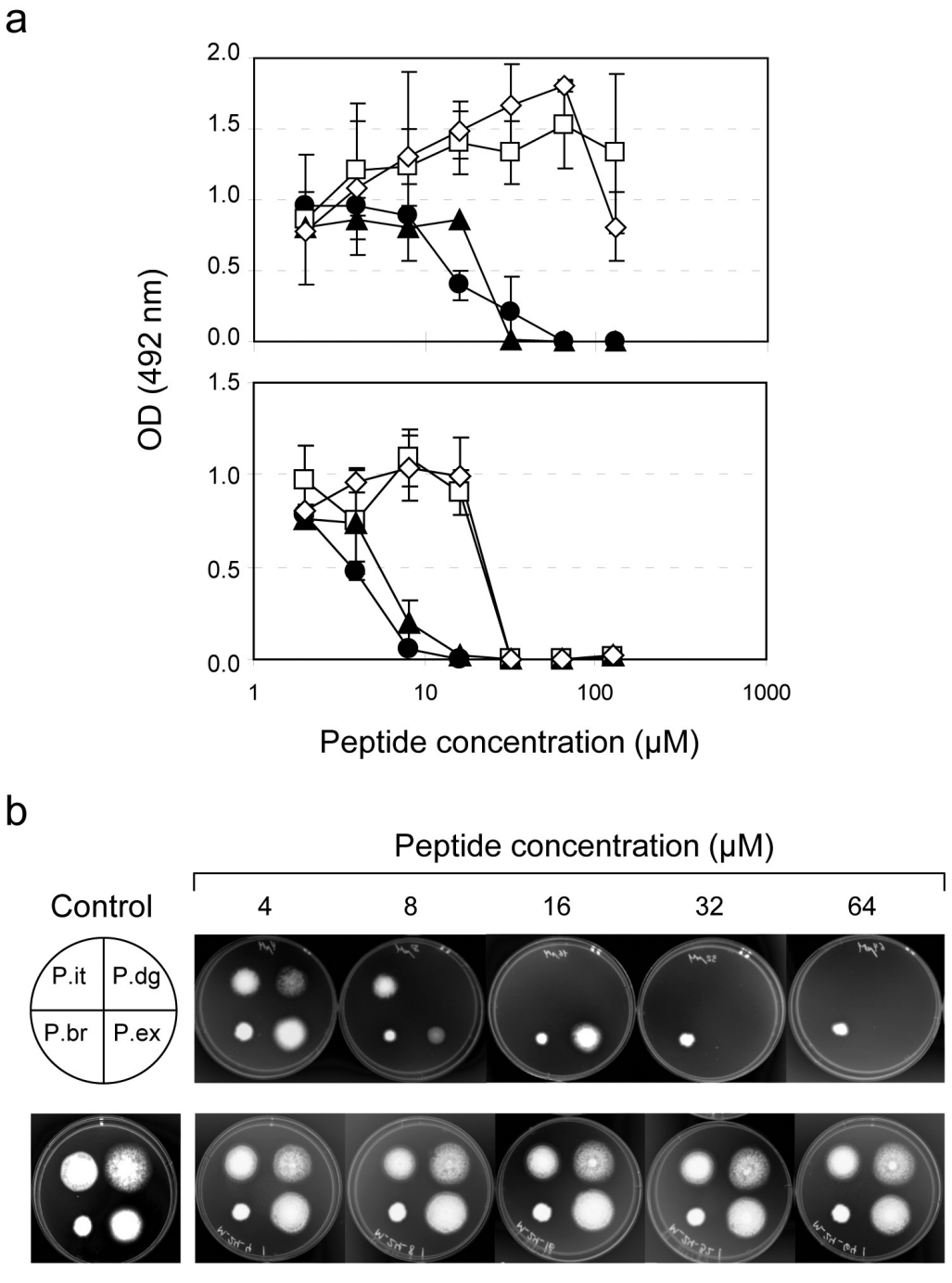

Figure 3. *In vitro* inhibitory and fungicidal activity of antimicrobial peptides towards *Penicillium* isolates. (a) Conidia of *P. italicum* PHI-1 (●), *P. digitatum* PHI-26 (▲), *P. brevicompactum* PHI-8 (□) and *P. expansum* PHI-65 (◊) were cultured in PDB in the presence of increasing concentrations of PAF26 (top panel) and melittin (bottom panel). Samples were prepared in triplicate, and data show the mean values ± SD of the OD at 492 nm at each peptide concentration, at 48 h of incubation. (b) Conidia of each fungus in a water solution were exposed for 24 h to the different peptide concentrations shown of PAF26 (top panel) and melittin (bottom panel), diluted and applied onto peptide-free PDA plates to monitor fungal growth.

ity differences of these fungi to the presence of CFW were very similar to those found after exposure to the antimicrobial peptide PAF26 (Figure 3).

Hydrogen peroxide is produced in plants as a response to pathogen infection and has an important role in plant defence mechanisms due to its antimicrobial activity (Lamb and Dixon, 1997). *P. brevicompactum* and *P. digitatum* were the most resistant fungi to H_2O_2, while *P. expansum* was the most susceptible (Figure 4b). Thus, correlations between sensitivity to H_2O_2 and either sensitivity to AMPs or pathogenicity towards citrus or apples were not found.

SDS is a lipophylic detergent known to interact with membranes and affect cellular membrane integrity. Although *P. digitatum* was the most sensitive fungus to SDS (Figure 4b), no correlation was found between sensitivity to AMPs and SDS when the other three fungi were considered. Under our assay conditions, *P. italicum* and *P. brevicompactum* were indistinguishable regarding their resistance to SDS while *P. italicum* was clearly more susceptible to PAF26 (Figure 3).

Calcofluor white staining and chitin content analysis

The results shown above indicate a parallelism between sensitivity to CFW, virulence to citrus fruits, and susceptibility to the antifungal peptides, particularly PAF26. Since CFW sensitivity is partly related to the chitin content within the fungal cell wall (Ram and Klis, 2006), we carried out chitin quantification analysis of the different isolates. Cell wall digestion and colorimetric quantification of the GlcNAc released was used to estimate the content of chitin in mycelia of the four isolates. Mean values of GlcNAc showed very limited variation among the fungi (Table 1). Statistical analyses demonstrated a significant difference only between *P. brevicompactum* and *P. expansum*. These two isolates were among the fungi tested that showed the least sensitivity to AMPs and to CFW in our previous assays (Figures 3 and 4).

Nevertheless, staining of fungal preparations with CFW and visualization by microscopy demonstrated differences in fluorescence intensity among the *Penicillium* strains (Figure 5), which presumably are related to distinct chitin accessibility within the cell walls rather than content. Conidia of *P. brevicompactum* (Figure 5b) and *P. expansum* (Figure 5d) gave very low CFW fluorescence in images obtained from equivalent preparations at equivalent exposure time and gain conditions, as opposed to the clearly stained *P. italicum* (Figure 5a) and *P. digitatum* (Figure 5c). This same qualitative difference was observed in actively growing fungal mycelium. *P. digitatum* (Figure 5c2) and *P. italicum* (Figure 5a2) mycelium showed the greatest CFW staining, allowing the clear identification of fungal cell walls, hyphal tips, and septum separations. On the other hand, the mycelium of *P. brevicompac-*

Table 1. Chitin content in mycelium of fungal isolates.

Isolate	GlcNAc μg mg^{-1} dry weight [a]	*n* [b]	Group [c]
P. brevicompactum PHI-8	6.84 ± 1.03	6	a
P. digitatum PHI-26	5.97 ± 1.02	7	ab
P. expansum PHI-65	5.37 ± 0.85	7	b
P. italicum PHI-1	6.27 ± 0.63	7	ab

[a] Mean values ± SD.
[b] *n* value shows the number of replicated samples from independent experiments.
[c] Homogeneous group labelled with the same letter do not differ at 95.0% confidence (Tukey's honestly significant difference procedure).

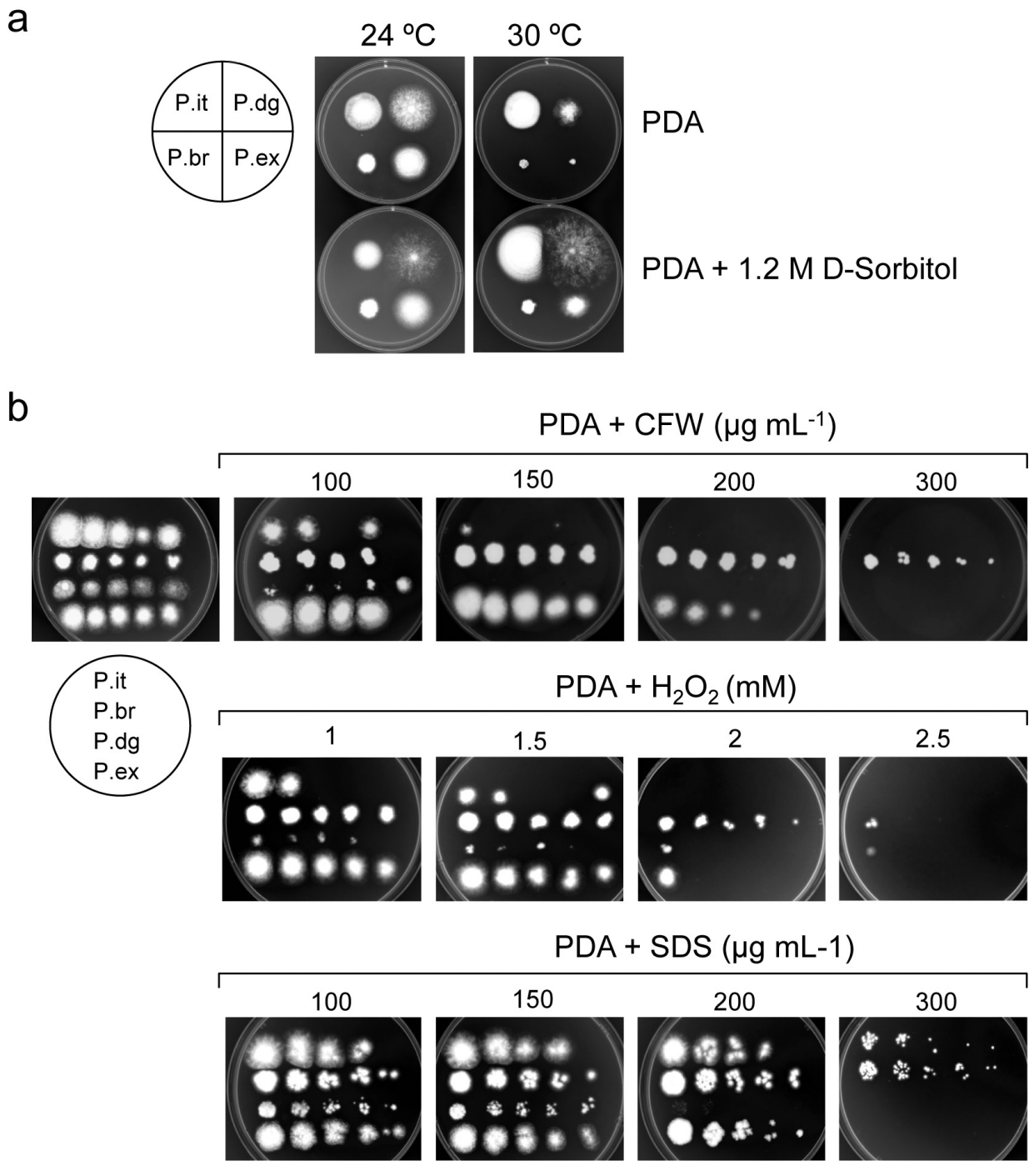

Figure 4. Thermosensibility and susceptibility of *Penicillium* strains to CFW, SDS and H_2O_2. Conidia of *P. italicum* PHI-1 (P.it), *P. brevicompactum* PHI-8 (P.br), *P. digitatum* PHI-26 (P.dg) and *P. expansum* PHI-65 (P.ex) were spotted onto PDA plates containing the compounds at the final indicated concentrations in order to test (a) their thermosensibility and osmotic remediability and (b) their susceptibility to antimicrobials with different modes of action. Plates were incubated for 3-4 days at 24ºC or 5-7 days at 30ºC to monitor fungal growth.

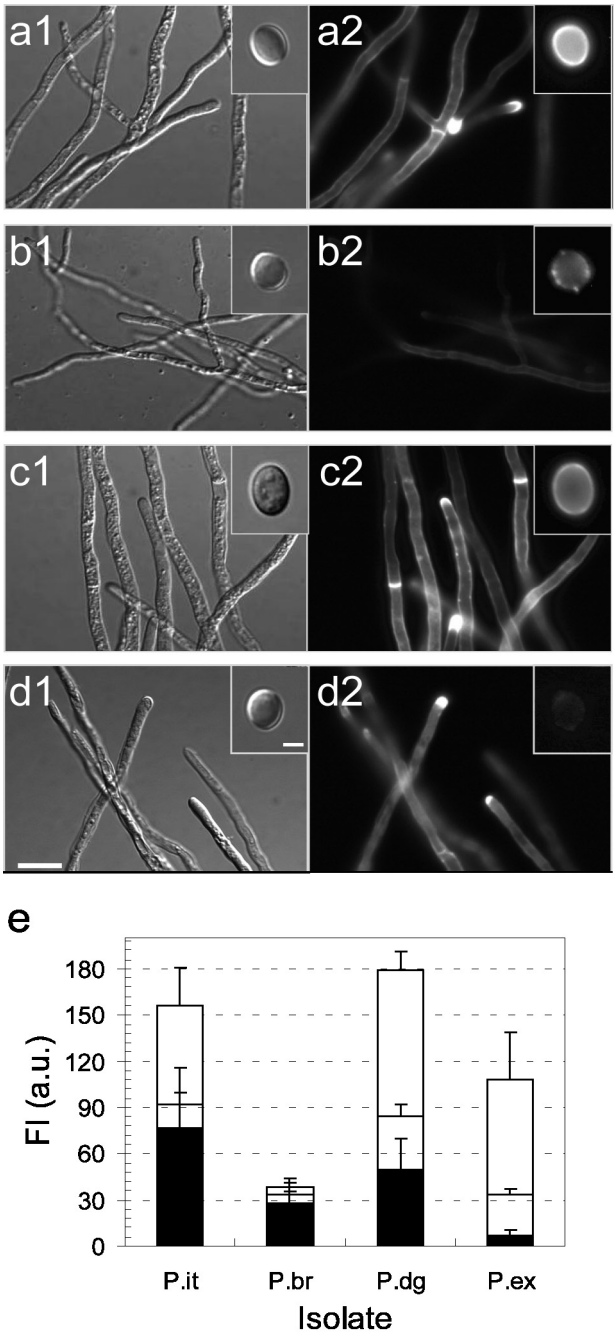

Figure 5. Microscopical visualization and fluorescence intensity quantification of conidia and mycelium of fungi stained with CFW. Samples correspond to *Penicillium* italicum PHI-1 (a), *P. brevicompactum* PHI-8 (b), *P. digitatum* PHI-26 (c) and *P. expansum* PHI-65 (d). Panels show the same area under DIC bright field (panel suffix 1) or fluorescence emission from CFW (panel suffix 2). Inset panels show magnified conidia of each fungus. Histogram in (e) represents the fluorescence intensity (FI) values obtained after processing of micrographs by the Image-Pro Plus 7.0 software. Piled columns show FI values ± SD for conidia (black bar), mycelium walls (striped bar) and hyphal tips (white bar) of *P. italicum* PHI-1 (P.it), *P. brevicompactum* PHI-8 (P.br), *P. digitatum* PHI-26 (P.dg) and *P. expansum* PHI-65 (P.ex) isolates. u.a. arbitrary units. Bar: 20 μm (2 μm inset bar).

tum had the least affinity for CFW (Figure 5b2). These observations were confirmed by quantifying fluorescence intensity of a number of micrographs obtained across replicated experiments. *P. digitatum* and *P. italicum* were the isolates with greatest affinity to CFW in terms of global values (Figure 5e). Regarding the three different fungal structures analyzed, the mycelium cell walls as well as the hyphal tips of isolates of the three fruit pathogens *P. digitatum, P. italicum* and *P. expansum* were stained more strongly by CFW than those of the *P. brevicompactum* isolate. However, conidia of *P. expansum* were the least prone to CFW binding. In addition, irregular staining in *P. brevicompactum* conidia was observed as brighter spots along conidial walls (see Figure 5b2, inset). These results indicate that different accessibility/organization of chitin rather than chitin content could influence the differential affinity and susceptibility to CFW.

Discussion

Our results show that the fungus *P. digitatum* has high susceptibility to antimicrobial compounds with different modes of action, including the peptide PAF26, the chitin dye CFW and the detergent SDS, but remarkably not to H_2O_2 (Figures 3 and 4). A previous report has demonstrated the high susceptibility of *P. digitatum* to the antimicrobial peptide aureobasidin A, when compared to other phytopathogenic fungi (Liu *et al*., 2007). We have previously reported a similar relative susceptibility of the distinct *Penicillium* spp. to additional antimicrobial peptides derived from bovine lactoferrin (Muñoz and Marcos, 2006). Similar observations were obtained in the case of melittin (Figure 3). Therefore, *P. digitatum* (and to some extent also *P. italicum*) seems to be a fungus with high susceptibility to distinct antifungal peptides with distinctive properties.

We used ITS sequence analysis to confirm the assignment of isolates to *Penicillium* species. Clustering based on β-tubulin gene sequences has indicated that *P. digitatum*, *P. italicum* and *P. expansum* have strong relationships to over 180 species belonging to the subgenus *Penicillium* (Samson *et al*., 2004). The parsimony phylogeny based on the ribosomal ITS showed that our *P. italicum* and *P. expansum* isolates are closely-related species (Figure 1) which differ in their host range, citrus fruits and apples, respectively, on which they produce a similar disease, so-called blue mould. In fact, the tree indicates a diffuse separation between these two species. Similarly, previous phylogenetic analysis based on amplified fragment length polymorphisms showed that *P. expansum* is a genus dispersed in separated clusters (Oliveri *et al*., 2007). Besides host adaptation, another distinctive property between the two species is that *P. italicum* showed reproducible greater sensitivity to PAF26 than *P. expansum* (Figure 3). *P. italicum* and *P. digitatum*, pathogenic to citrus, were the most sensitive to fungistatic and fungicidal PAF26 activity and to fungistatic activity of melittin. Although *P. brevicompactum* has been associated with pre- and postharvest fruit infections and storage (Overy and Frisvad, 2005; Patino *et al*., 2007), we confirmed that our strain is the most distant according to the phylogenetic tree (Figure 1), is non-pathogenic on oranges and apples (Figure 2) and also showed a much lower sensitivity to the tested antimicrobials (Figures 3 and 4).

Our data also indicate a better correspondence between susceptibility to PAF26 and sensitivity to the chitin-binding dye CFW among the tested fungi than between any other treatments. Relative sensitivity to SDS or H_2O_2 was not associated with that to PAF26 but with noteworthy differences. For instance, *P. italicum* showed a relative resistance to SDS. SDS is a membrane-perturbing agent that affects membrane stability and elicits a stress response in fungi to reinforce cell walls, and therefore has been used to reveal cell wall modifications that result in altered accessibility of SDS to the plasma membrane (de Groot *et al*., 2001). This lack of connection between the sensitivity of *P. italicum* to PAF26 and SDS and the high sensitivity of *P. digitatum* to both SDS and PAF26, would also indirectly discard a primary mode of action of PAF26 re-

lated to perturbation of membrane integrity, even though PAF26 is capable of interacting *in vitro* with membrane-mimicking vesicles (López-García *et al*., 2004). Most AMPs also showed membrane perturbing properties when tested *in vitro* in membrane mimetics, but their antimicrobial action *in vivo* remains to be confirmed in most cases (Marcos and Gandía, 2009).

The production of H_2O_2 and other reactive oxygen species (ROS) has been demonstrated after exposure of microorganisms to specific AMPs (Narasimhan *et al*., 2001; Kaiserer *et al*., 2003; Aerts *et al*., 2007). However, the role of ROS in antimicrobial action remains controversial for specific peptides such as the cell penetrating peptide histatin-5 (Helmerhorst *et al*., 2001; Veerman *et al*., 2004). Our experiments indicate that the *P. digitatum* strain has a relatively high resistance to H_2O_2, significantly higher than *P. italicum* (Figure 4b), confirming that it has an efficient defence activity against oxidative stress. *P. digitatum* is also effective in suppressing ROS production in host tissue, and this capability has been considered a virulence factor on citrus fruit (Macarisin *et al*., 2007). The lack of correspondence between sensitivity to H_2O_2 and the peptide suggests that PAF26 would not act through ROS production within *Penicillium* spp. We did not detect presence of ROS in spores and mycelium of *P. digitatum* treated with PAF26, using diaminobenzidine (DAB) as probe (data not shown).

In fungal pathogens, cell walls are essential factors relating to morphogenesis, maintenance of cell integrity and as barriers against host defences. Not surprisingly, cell wall related compounds are considered as a broad class of targets for specific antifungal drug design (Odds *et al*., 2003). Chitin not only serves as an essential structural component of fungal cell walls but is also a plant defence signal (Kaku *et al*., 2006). It has been demonstrated in phytopathogenic fungi that disruption of chitin synthase genes diminished virulence and increased susceptibility to plant antimicrobials and compounds disrupting cell wall integrity such as CFW (Müller *et al*., 2002; Madrid *et al*., 2003; Weber *et al*., 2006; Martín-Urdíroz *et al*., 2008). *Fusarium oxysporum* and *Aspergillus oryzae* mutants in chitin synthesis genes (*ChsIII* and *ChsV*) have shown altered sensitivity to the antifungal protein AFP from *Aspergillus giganteus* (Hagen *et al*., 2007; Martín-Urdíroz *et al*., 2009). This antifungal AFP has been shown to be internalized by fungal cells and able to bind nucleic acids *in vitro* (Moreno *et al*., 2006). Fluorescently-labelled PAF26 interacted with the fungal cell wall and subsequently translocated inside the cell at subinhibitory concentrations at which no membrane permeation could be detected *in vivo* (Muñoz *et al*., 2006), being an example of an antimicrobial penetratin-like peptide (Marcos *et al*., 2008). In this previous study (Muñoz *et al*., 2006), sub-MIC concentrations of PAF26 caused morphological alterations including dichotomous tip branching and alterations of branch emergence which are similar to phenotypes of mutant filamentous fungi in which establishment and maintenance of polarity and cell wall integrity are altered (Harris and Momany, 2004; Momany, 2005). Also, swelling of hyphal cells with abnormal deposition of chitin appeared in PAF26-treated mycelium, which is indicative of alterations of cell wall structure and a phenocopy of fungal mutants in which chitin synthase genes are disrupted (Borgia and Dodge, 1992; Horiuchi *et al*., 1999; Martín-Urdíroz *et al*., 2004; Martín-Urdíroz *et al*., 2008).

Our data of fungal cell wall affinity for CFW stain (Figure 5) also paralleled those of PAF26 and CFW sensitivity (Figures 3 and 4), and indicated that susceptibility to CFW in *P. brevicompactum* or *P. expansum* results from a differential reduced chitin accessibility/organization rather than to their chitin content (Table 1). This indication is further supported by the differential capability of these fungi to grow at 30ºC (Figure 4a), which also evidences alterations in cell wall structure. Studies on filamentous fungi have shown that reduced chitin and β-glucan content or abnormal structure of cell walls increases sensitivity for growth at restrictive temperatures (Borgia and Dodge, 1992; Momany *et al*., 1999). Therefore, the parallelism in PAF26

and CFW sensitivity might be due to distinctive cell wall structure in the fungi analyzed. Taken together, available data would suggest the implication of chitin accessibility in the interaction of PAF26 and other AMPs with *Penicillium* spp. However, it is important to stress that this conclusion does not imply that PAF26 and CFW have equal (or even similar) mechanistic modes of antimicrobial action.

In conclusion, our work establishes a parallelism between the phenotype of susceptibility to distinct antimicrobials in *P. digitatum* and *P. italicum* species, which is a relatively high sensitivity to both PAF26 and CFW, and their ability to infect citrus fruits. Our work demonstrates that PAF26 shows increased antifungal activity over citrus pathogenic *Penicillium* isolates, even though they have a relatively distant phylogenetic separation. Future experiments may use CFW-sensitive and/or -resistant *Penicillium* mutants to help elucidate the mode of action of AMPs on cell walls, as well as the mechanisms of virulence in *Penicillium* species.

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