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Comparative Study of Antimicrobial Peptides To Control Citrus Postharvest Decay Caused by *Penicillium digitatum*

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The objective of this study was to investigate and compare the in vitro efficacy and in vivo potential of eight distinct short antimicrobial peptides to control the postharvest green mold disease of oranges caused by the fungus *Penicillium digitatum*. The L-amino acid versions of the four peptides PAF26, PAF38, PAF40, and BM0, previously obtained by combinatorial approaches, were examined. The study included two antibacterial peptides formerly identified by rational design, BP15 and BP76, and it is demonstrated that they also have in vitro antifungal properties. The natural antimicrobial peptides melittin and indolicidin were also selected for comparison, due to their well-known properties and modes of action. In vitro and in vivo results indicated differential behaviors among peptides, regarding the inhibitory potency in growth media, selectivity against distinct microorganisms, fungicidal activity towards nongerminated conidia of *P. digitatum*, and efficacy in fruit inoculation assays. Interestingly, a high in vitro inhibitory activity did not necessarily associate with an effective control of fruit infection by *P. digitatum*. The short tryptophan-rich cationic peptides PAF26, PAF38, PAF40, and BM0 were lethal to conidia of *P. digitatum*, and this property is correlated with better protection in the decay control test.

KEYWORDS: Antifungal peptides; postharvest pathology; citrus fruit; *Penicillium digitatum*

INTRODUCTION

Fungicides are the prime method to control postharvest diseases caused by fungal phytopathogens in fruits and vegetables (*1*–*3*). However, both the public and the health authorities have become increasingly concerned about the presence of fungicides in food and the release of residues to the environment. As a direct result, research efforts to develop alternative methods for the control of postharvest decay have been intensified (*2*–*6*). The potential of antimicrobial peptides (AMP) as novel antibiotics is widely recognized (*7*, *8*), and some of them are well-known as food-grade preservatives (*9*–*11*). The use of AMP to control plant disease in agriculture (*12*–*14*) and postharvest conservation (*15*) has been proposed. The de novo design of new AMP could avoid some of the problems associated with certain peptides, leading to the reduction of unwanted toxicity or an increase in stability. There is an increasing number of examples of novel AMPs designed towards plant pathogens (*16*–*20*). For instance, a chimerical peptide hybrid of cecropin and melittin has been transgenically expressed in potato, and tubers were protected against the soft rot causing bacteria *Erwinia amylovora* (*18*). Another such hybrid that showed antifungal properties (*17*) was produced in *Saccharomyces cerevisiae*, which was able to inhibit fungal infection in tomato fruits (*21*).

Combinatorial chemistry is a powerful tool for the identification of novel bioactive peptides (*11*, *22*), including AMP against phytopathogens (*23*–*25*). Previously, a combinatorial approach on a D-amino acid hexapeptide library was used to identify a group of cationic AMP (so-called PAFs), active towards phytopathogenic fungi that cause postharvest decay in fruits (*24*). The D-amino acid hexapeptide PAF26 has in vitro antifungal activity against the citrus fruit pathogen *Penicillium digitatum*, and it retards infection on citrus fruit and is active against strains resistant to commercial fungicides (*24*, *26*). Recently, a set of D-amino acid heptapeptides derived from PAF26 was designed by N-terminal amino acid addition, and in vitro screening for microbial inhibition allowed the identification of AMP with improved activity and variations in their specificity and toxicity against nontarget microorganisms, being PAF38 and PAF40 among the most promising candidates for further study (*27*).

The objective of this study was the parallel in vitro and in vivo evaluation of eight distinct AMP (**Table 1**) as alternatives to control postharvest citrus decay caused by *P. digitatum*. We assayed the L-amino acid versions of the previously identified peptides PAF26, PAF38, and PAF40 (**Table 1**). Additionally, the related peptide BM0 had been identified from a octapeptide combinatorial library through a dual screen for inhibition of yeast growth and activity of a *S. cerevisiae* membrane ATPase

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Table 1. Amino Acid Sequences, Properties, and Growth Inhibitory Activity of Peptides

peptide	sequence [a]	source	MW	net charge [b]	GRAVY [c]	MIC (μM) [d]		
						P. digitatum PHI-26	*S. cerevisiae* FY1679	*E. coli* DH5α
PAF26	Ac-RKKWFW-NH_2	*24*	991.2	3+	−1.883	4	32	32
PAF38	Ac-RRKKWFW-NH_2	*27*	1147.4	4+	−2.257	4	16	8
PAF40	Ac-HRKKWFW-NH_2	*27*	1128.3	3.1+	−2.071	4	16	16
melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	*30*	2847.4	5+	0.273	2	16	8
BM0	Ac-RFWWFRRR-NH_2	*28*	1350.6	4+	−1.775	4	32	8
indolicidin	Ac-ILPWKWPWWPWRR-NH_2	*32*	1948.3	3+	−1.069	16	ND [e]	16
BP15	KKLFKKILKVL-NH_2	*19*	1356.8	5+	0.309	4	16	4
BP76	KKLFKKILKFL-NH_2	*19*	1404.9	5+	0.182	4	16	4

[a] The L-amino acid sequence is shown as single letter code. Where indicated, the peptides are acetylated at the N terminus (Ac) and/or amidated at the C terminus (NH_2). [b] Estimated at pH 7. [c] Peptide grand average of hydropathicity index (GRAVY) was calculated using a web-based tool (http://www.expasy.ch/). [d] MIC is the minimum concentration that was completely inhibitory in all experiments. [e] ND, not determinined.

(*28*). BM0 has remarkable sequence similarities to PAF peptides (**Table 1**) and belongs to the same class of tryptophan-rich cationic AMPs (*29*). Other de novo-designed peptides are BP15 and BP76 (**Table 1**), which are hybrid derivatives of cecropin and melittin and have been identified in a screen towards the phytopathogenic bacteria *E. amylovora*, *Pseudomonas syringae*, and *Xanthomonas vesicatoria* (*19*). These peptides showed high bactericidal activities and low hemolysis and sensitivity to protease degradation. The activity of BM0, BP15, and BP76 against fungal phytopathogens has not been evaluated previously. Finally, two AMPs found in nature were also included. The well-known toxic peptide melittin (*30*), which was used in studies on the mode of action of PAF26, showed growth inhibition activity against *P. digitatum* but a lack of fungicidal activity towards conidia (*31*), and the peptide indolicidin, which belongs to the cathelicidin family of AMP, is also an arginine- and tryptophan-rich peptide with broad spectrum and potent antimicrobial activity (*29*, *32*).

MATERIALS AND METHODS

Microorganisms. *P. digitatum* PHI-26 is a natural isolate with high virulence towards citrus fruits (*15*), and it was cultured on potato dextrose agar (PDA) (Difco-BD Diagnostics, Sparks, MD) plates for 7–10 days at 24 °C. Conidia were collected, filtered, and titrated with a hematocytometer, adjusted to the appropriate concentration, and used. *S. cerevisiae* FY1679 was grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 30 °C. *Escherichia coli* DH5α was grown in Luria–Bertani (LB) medium at 37 °C. Yeast and bacterial cultures were grown to stationary phase, diluted to the appropriate concentration, and used.

Peptides. BP15 and BP76 (*19*) were gifts from Drs. E. Montesinos and E. Bardají (Institut de Tecnologia Agroalimentària and LIPPSO, Universitat de Girona, Spain). Melittin was purchased from Sigma (St. Louis, MO). Other custom-ordered peptides (**Table 1**) were synthesized at >90% purity from GenScript Corp. (Piscataway, NJ) by solid-phase methods using *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry. Some peptides were acetylated at the N terminus (Ac) and/or amidated at the C terminus (NH_2). Stock solutions of each peptide were prepared at 1 mM concentration in 5 mM 3-(*N*-morpholino)propanesulfonic acid pH 7 buffer and stored at −20 °C.

In Vitro Antimicrobial Activity Assay. The in vitro antimicrobial activities of the peptides were determined by dose–response curves by using a microtiter plate assay as previously described (*31*, *33*). In all experiments, three replicates were prepared for each peptide concentration and the means and standard deviations (SDs) of the optical density (OD) at 492 nm were calculated. The minimum inhibitory concentration (MIC) of a peptide for a given microorganism was defined as the lowest peptide concentration that showed no growth at the end of the experiment (after 4 days of incubation) in all of the independent experiments carried out.

In Vitro Fungicidal Activity Assay. The assessment of peptide fungicidal activity was conducted by incubation of *P. digitatum* conidia (10^4 conidia mL^{-1}) with peptides at different concentrations in sterile distilled water, for 16 h at room temperature. Treatments were prepared in triplicate. After treatment, samples were serially diluted and spread onto peptide-free PDA plates that were incubated to count colony forming units (CFU). Data were used to calculate the number of viable conidia after each peptide treatment.

Fruit Decay Test. Experiments were carried out on freshly harvested orange fruits (*Citrus sinensis* L. Osbeck) from different cultivars of the navel group, as described (*15*, *34*). Briefly, freshly harvested fruits were surface sterilized in a commercial bleach solution, washed, and allowed to air dry. Fruits were wounded by making punctures (approximately 3 mm in depth) with a sterile nail at four sites around the equator. Inocula contained 10^4 conidia mL^{-1} of *P. digitatum* PHI-26 and peptides at different concentrations in water, and 5 μL was applied onto each wound. In some experiments, as described in the Results section, different times of incubation of conidia with peptides prior to inoculation were evaluated. For each treatment, three replicas (five fruits per replica, four wounds per fruit) were prepared. Fruits were maintained at 20 °C and 90% realtive humidity. Symptoms were scored at different days postinoculation (dpi) as the number of infected wounds per replica, and mean values ± SDs for each treatment were calculated.

Statistical Analyses. Statistical analyses were carried out with the software package StatGraphics Plus 5.1 (StatPoint, Herndon, VA). If necessary, data were log transformed to fulfill the equal variance criteria of the analysis of variance tests. The *F* test was applied to test if the difference between the treatment means was significant. The Tukey's honestly significant difference procedure was used for mean separation between treatments.

RESULTS

Selection of AMPs. All of the peptides analyzed were synthesized with L-amino acids (**Table 1**). PAF26 synthesized with the L-enantiomers has been described previously as having in vitro properties comparable to the D-amino acid counterpart (*31*); however, its activity under fruit inoculation assays has not been evaluated before. We assessed in this study the L-amino acids counterparts of PAF38 and PAF40 (**Table 1**). Other de novo-designed peptides that were included are BM0, described as having activity against human pathogenic yeasts (*28*), and BP15 and BP76, which are antibacterial (*19*). The peptides melittin and indolicidin were used as examples of natural broad spectrum AMP (*29*, *30*).

The peptides used in this study have a positive net charge at neutral pH (**Table 1**) and are therefore considered cationic AMPs. Negative hydropathic values of five peptides indicate that they are hydrophilic, while melittin, BP15, and BP76 are considered hydrophobic. With the exception of melittin, which is 26 amino acids long, the remaining peptides are rather short ranging from six to 13 residues.

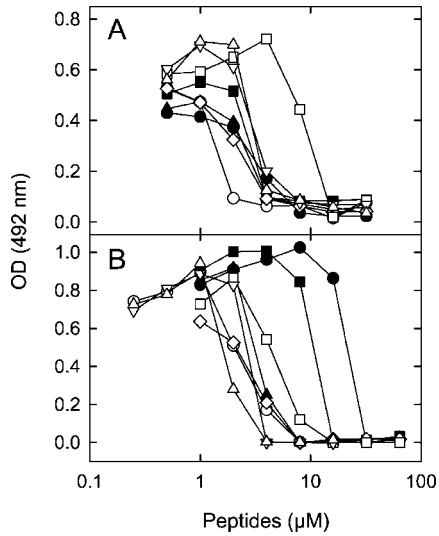

Figure 1. In vitro inhibitory activity of synthetic peptides on the growth of *P. digitatum* (**A**) and *E. coli* (**B**). Microorganisms were grown in the presence of increasing concentrations of peptides PAF26 (black circles), PAF38 (black upward triangles), PAF40 (black squares), BM0 (white diamonds), melittin (white circles), indolicin (white squares), BP15 (white downward triangles), and BP76 (white upward triangles). Samples were prepared in triplicate, and data shown are the mean values of the OD measurements at each peptide concentration, after 48 h of incubation for *P. digitatum* (**A**) or at 24 h for *E. coli* (**B**).

In Vitro Growth Inhibition of Microorganisms by Peptides. The in vitro growth inhibition properties of the eight peptides were compared against the phytopathogenic fungus *P. digitatum*, the unicellular model yeast *S. cerevisiae*, and the Gram-negative bacteria *E. coli*. Importantly, assays on a given microorganisms were conducted in parallel with the eight peptides so as to faithfully reflect differences in antimicrobial potency and repeated at least three times. We conducted antimicrobial assays against the nontarget microorganisms (yeast and bacteria) as a mean to evaluate the specificity of peptides. **Figure 1** shows examples of dose–response curves and illustrates the relative antimicrobial potency of peptides. **Table 1** shows the MIC values of each combination of peptide microorganism.

Melittin showed the best inhibitory activity towards *P. digitatum*, although as a general trend all of the remaining peptides except indolicidin were also highly inhibitory to the fungus with in vitro MIC values of 4 μM (**Table 1** and **Figure 1**). A lower antimicrobial activity was found against the laboratory strain of the yeast *S. cerevisiae*, with values similar for all of the peptides (**Table 1**). However, the more significant differences among peptides were found in the antibacterial assays; BP15 and BP76 were the most inhibitory to *E. coli* (MIC of 4 μM) (**Table 1** and **Figure 1**), as expected since these peptides were primarily identified by screening against bacteria (*19*). From our growth inhibition data, it is concluded that indolicidin, BP15, and BP76 were similarly active against *E. coli* and *P. digitatum*, while the remaining five peptides were more active towards *P. digitatum* than to *S. cerevisiae* or *E. coli*.

Effect of AMPs on Fruit Infection by *P. digitatum*. Experiments were designed to evaluate and compare peptide capability to control infection caused by *P. digitatum*. We have demonstrated that D-amino acid PAF peptides (*24, 26*) as well as lactoferricin-derived peptides (*34*) are able to retard the fungal infection in experiments where conidia of the fungus were coinoculated with the peptides at concentrations higher than 50 μM and up to 100 μM. Likewise, we have also observed that the L-amino acid PAF26 at 100 μM has a high protective effect (data not shown). In the present study, lower concentrations of peptides were used to better discriminate the most active peptides. When all of the peptides were assayed in parallel at 32 μM, a modest retard in disease progression was observed for some peptides, and only BM0 reached significance in the control of fruit decay (a representative is shown in **Figure 2A**, top panel).

We have recently observed that control of green mold by PAF26 in laboratory fruit inoculations is improved by extending the time of incubation of conidia with the peptide, prior to inoculation (unpublished data). To broaden and report this observation, experiments were carried out in which the inocula were prepared 16 h before inoculation; therefore, conidia of the fungus were incubated with peptides in sterile water for an extended period of time, and the data were compared with those of short time incubations (**Figure 2A**).

Statistical analyses demonstrated that the control of infection by PAF26, PAF38, PAF40, BM0, and indolicidin increased significantly with the longer incubation of conidia with peptides (Student's *t* test, 95% confidence). Noteworthy, in the case of melittin, no significant difference was found between the short and the long incubation times at any of the dpi (Student's *t* test), and even no reduction of disease was observed at the end of the experiment (7 pdi, **Figure 2A**) despite the high growth inhibitory properties of the peptide (**Figure 1** and **Table 1**). Mean separation analyses indicated a higher reduction of disease after treatment of conidia with peptides PAF38, PAF40, and BM0 for 16 h (**Figure 2A**, bottom panel).

Fungicidal Activity of Peptides against *P. digitatum* Conidia. We have previously reported that PAF26 and melittin have similar in vitro growth inhibition activity against *P. digitatum* but differ in their killing capacity towards nongerminated conidia; PAF26 is markedly fungicidal (*31*). It was addressed whether differences in fungicidal properties could account for the differences in the fruit inoculation assays. An estimate of fungicidal activity was obtained by spreading aliquots from the inocula of the experiment shown in **Figure 2A** onto peptide-free plates, to test viability of conidia after exposure to peptides (**Figure 2B**). The growth recovery of fungus showed differences among peptides; PAF26, PAF38, PAF40, and BM0 were the most fungicidal. Indolicidin, BP15, and BP76 showed intermediate activity, while melittin had no observable activity under these conditions.

Quantification of fungicidal activity was obtained by dose–response assays and determination of CFU (**Figure 3**). Differences in losses of viability were observed in conidia treated with distinct peptides. PAF26, PAF38, PAF40, and BM0 (**Figure 3** and data not shown) diminished viability by two log units even at 4 μM. Indolicidin (**Figure 3**) and BP15 and BP76 (data not show) showed intermediate fungicidal activity, while melittin data were not significantly different from the control with no peptide treatment. Noteworthy, no statistically significant differences were found among the different concentrations of a given peptide.

Effect of Peptide Concentration on Control of *P. digitatum* Infection. Different concentrations of selected peptides were tested in subsequent experiments, in an attempt to better define the most active AMP with potential application. Also, and considering the variability occurring in the fruit inoculation bioassays among different citrus cultivars, fruit seasons, and

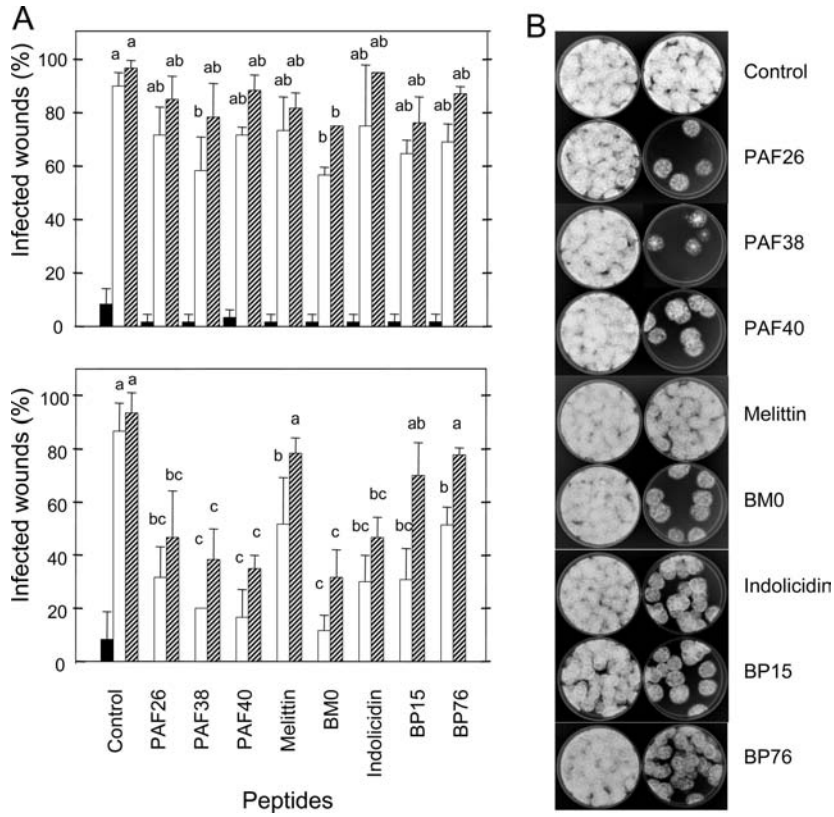

Figure 2. Effect of synthetic peptides on the infection and viability of conidia of *P. digitatum*. (**A**) Effect on the fungal infection of citrus fruits. Fruits (*C. sinensis* L. Osbeck cv. Navelina) were inoculated with *P. digitatum* alone (control) or in the presence of the peptides (as indicated at the bottom) at 32 μM. Conidia were mixed with peptides in sterile water and either immediately inoculated (top panel) or incubated for 16 h at room temperature prior to inoculation (bottom panel). Results are shown as the mean of the percentage of infected wounds $\pm$ SD for each treatment at 3 (black bars), 5 (white bars), and 7 (hatched bars) dpi. Bars within the same dpi and panel labeled with the same letter do not differ at the 95.0% confidence. (**B**) Effect on conidia viability. Aliquots from the inocula of the experiment shown in panel **A** were spread onto peptide-free PDA plates that were incubated to monitor CFU from *P. digitatum*. Left and right columns show samples from the top and bottom panels in panel **A**, respectively.

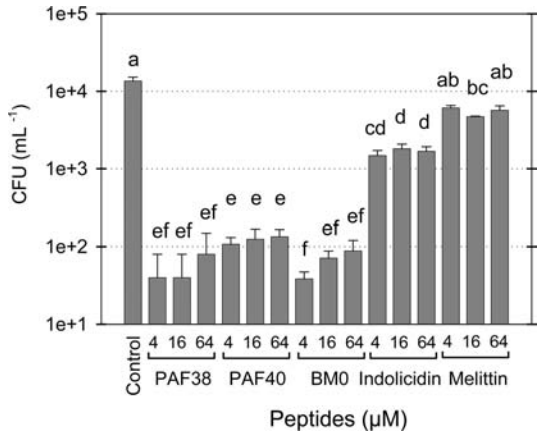

Figure 3. Dose–response effect of synthetic peptides on the viability of conidia of *P. digitatum*. Conidia of *P. digitatum* were incubated in sterile water for 16 h at room temperature alone (control) or in the presence of the peptides at 4, 16, or 64 μM (as indicated at the bottom). Data are shown as bars with the mean value of recovered CFU mL^{-1} $\pm$ SD for each treatment, in a logarithmic scale. Bars labeled with the same letter do not differ at 95.0% confidence.

growing and harvest conditions, these experiments took into account all of these factors. **Figure 4** shows results of two distinct representative examples from different orchards and cultivars. Consistently, peptides PAF38, PAF40, and BM0 showed the best control of experimental green mold infections when used at concentrations as low as 4 μM (**Figure 4**). Other peptides such as melittin showed no significant protection at such low concentrations, while indolicidin showed intermediate results.

DISCUSSION

There is an increasing number of AMP with in vitro activity against fungal and bacterial plant pathogens (*20*), which have been proposed as an alternative to develop novel plant disease control strategies (*12–15*). Comparison of in vitro antimicrobial properties of distinct peptides from data obtained in different laboratories should be taken cautiously. Microorganism strain variations, culture conditions, assay formats (microtiter, liquid or solid media), and peptide modifications (acetylation, amidation, or synthesis with D- or L-amino acids) always sum up to make differences in reported activities difficult to evaluate. Moreover, in vitro quantification of growth of filamentous fungi by spectrophotometric methods can be imprecise (*35*). Our study provides a comparison of in vitro and in vivo activities of up to eight different peptides. The activity of some of them against some of the three microbes tested is well-known (i.e., melittin against *E. coli*), but they have been included to carry out more precise side-by-side comparisons. This parallel evaluation allows (i) selection of candidate AMP to further development as alternatives to control citrus green mold caused by *P. digitatum* and also (ii) several more general conclusions to be drawn.

First, and regarding the chemical structure of the AMP analyzed, it has been shown that PAF38 and PAF40 peptides

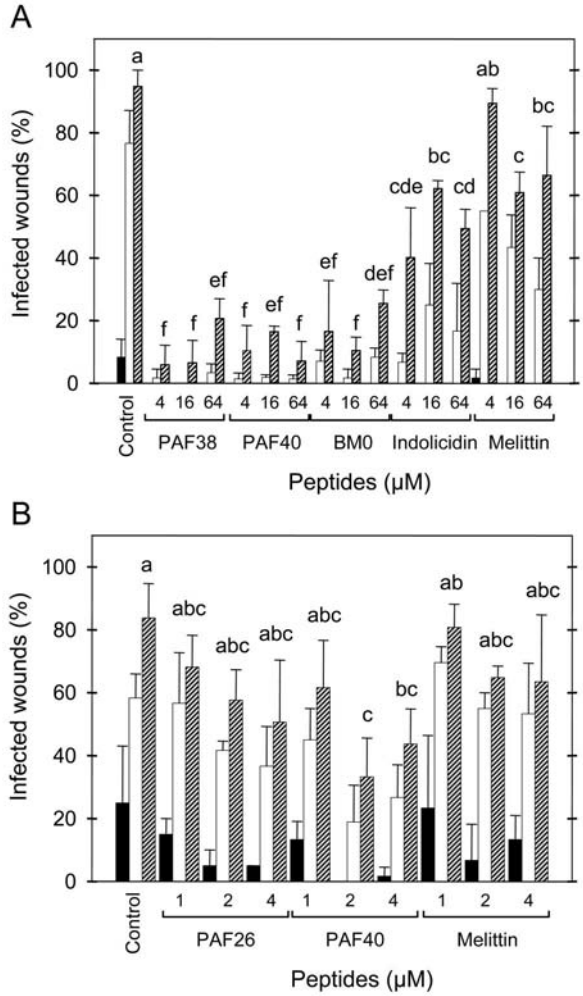

Figure 4. Dose–response effect of synthetic peptides on the infection of *P. digitatum*. Fruits from distinct cultivars were inoculated with *P. digitatum* alone (control) or in the presence of peptides at different concentrations (as indicated at the bottom). (**A**) Citrus fruit (*C. sinensis* L. Osbeck cv. Navelate) were used with peptide concentrations from 4 to 64 μM. (**B**) Citrus fruit (*C. sinensis* L. Osbeck cv. Lanelate) were used with peptide concentrations from 1 to 4 μM. In both **A** and **B**, conidia were incubated for 16 h at room temperature prior to inoculation. Results are shown as the mean of the percentage of infected wounds ± SD for each treatment at 3 (black bars), 5 (white bars), and 7 (hatched bars) dpi. For simplicity, only the mean separation result from 7 dpi is shown. Bars labeled with the same letter do not differ at 95.0% confidence.

synthesized with the L-amino acids do not differ substantially from the corresponding D-peptides in their in vitro activity against *P. digitatum* (**Table 1** and data not shown) (*27*). The use of the L-stereoisomers is a requisite if the production of AMP is to be achieved by expression in genetically modified organisms (see below), but it poses a potential problem related to the presumed higher sensitivity to degradation. In fact, BP15 and BP76 peptides included a criteria of stability against degradation in their selection procedure (*19*). All of our previous fruit bioassays had been conducted with the D-peptides PAF19 and PAF26 (*15*, *24*, *26*). We have shown for the first time significant protection in fruit with the L-stereoisomer derivatives of PAF26, PAF38, PAF40, and also BM0 (**Figures 2** and **4**), thus indicating that potential susceptibility to degradation seems not to be relevant, at least for this class of AMPs and under our assay conditions. Future investigations will explore the reasons for this behavior.

In this study, we used acetylated and amidated derivatives in the case of PAF peptides, BM0, and indolicidin (**Table 1**). We have shown previously that acetylation and amidation of PAF26 do not change substantially the in vitro activity towards *P. digitatum* (*36*). Although indolicidin is found in nature with the free N terminus and amidated at the C terminus (*29*), we used an acetylated derivative for a better comparison with other peptides. Because BP15 and BP76 were characterized with the free N termini (*19*), they were used as such, since the aim was to determine the putative antifungal properties of highly bactericidal peptides. Interestingly, BM0 was originally selected as a D-amino acid peptide with a free amino terminus, not acetylated (*28*). It was previously shown that either the nonacetylated L-counterpart of BM0 or the acetylated D-peptide had both a 4-fold reduction in inhibitory activity against *S. cerevisiae* (*28*). We have used an acetylated L-version of BM0 (**Table 1**) to better compare with the other peptides in the study. As expected, this derivative of BM0 had low activity towards our *S. cerevisiae* strain. It demonstrated an activity towards *P. digitatum* comparable to PAF38 and PAF40, with which it shares size, hydrophilicity, and net charge (**Table 1**).

We have also shown that differences can be observed between in vitro growth inhibition and experimental inoculation data. In our pathosystem, the high inhibitory activity of melittin (**Figure 1** and **Table 1**) does not correspond with a significant protection level (**Figure 2A** and **4**). To a minor extent, it also occurs in the case of BP15 and BP75. Therefore, we have shown convincingly that screening peptides for plant disease control should incorporate bioassays to confirm the in vitro data. Postharvest diseases are a good model system since screenings can be made in a relatively stable environment, which in fact reflects the industry storage conditions.

The results described herein also broaden our previous observations on differential inhibitory and fungicidal activities of PAF26 and melittin (*31*). Both peptides had similar activity against growing *P. digitatum* but differ in their lethality towards nongerminated quiescent conidia. PAF38, PAF40, and BM0 are also fungicidal to conidia and differ in this regard from indolicidin, BP15, and BP76 (**Figure 2B** and **3**). Previously, the AMP cecropin A was described as lethal to both mycelium and conidia of *Fusarium* spp. and also to mycelium of *Aspergillus* spp. but not to nongerminated conidia of *Aspergillus* spp. (*37*). Further work related this differential behavior to breakdown of cecropin A by a conidial *Aspergillus* extracellular protease (*38*). It remains to be determined whether the higher fungicidal activity of PAF peptides and BM0 is due to a higher resistance to proteolysis or to differential interactions with conidia.

Because there are differences in the activity of peptides to conidia, the longer the time of treatment of conidia with peptides is, the higher the control of decay achieved (**Figure 2** and data not shown), also stressing the importance of experimental design in the bioassays. This observation should be considered in the future development of AMPs to control postharvest diseases, as to the time of application and strategy to deliver the peptides. It should be taken into account that most fungal infections of fruits remain quiescent until the perception of appropriate signals (*39*).

The unspecific activity against nontarget microbes and toxicity of peptides should also be considered in the selection of appropriate peptides. The distinct specificity profiles of AMP are recognized and are likely related to different compositions of microbial envelopes (*7*, *8*, *40*). BM0, PAF, and BP peptides showed similar in vitro activity against *P. digitatum* but marked

differences in antibacterial activity, being BP15 and BP76 examples of AMP with comparable activity against bacteria and fungi (**Table 1**). Previous descriptions of broad spectrum activity AMP exist (*18, 41, 42*). Clearly, the selection of peptides in each case will depend on the spectrum needed for each crop or commodity. In the case of citrus postharvest decay, bacterial pathogens do not cause significant losses; therefore, more fungal specific peptides are desirable. Following this rationale, PAF40 could be selected as having a good balance of activity and specificity (**Table 1**) and showed more than 50% decay control even at concentrations as low as 2 μM (**Figure 4B**).

The toxicity of peptides has been measured as their cytolytic activity against human red blood cells. The hemolytic activity of the D-amino acid peptides PAF26, PAF38, and PAF40 was negligible under our assay conditions (*31*). Likewise, the L-amino acid peptides PAF26 (*31*), and also PAF38, PAF40, and BM0 at the highest concentration tested (100 μM), were not hemolytic (data not shown). Indolicidin (*32*), BP15, and BP76 (*19*) show intermediate hemolytic potency. Melittin is a highly toxic peptide that induces cell killing by membrane disruption and cytolysis and accordingly is also highly hemolytic (*30, 31*).

Different strategies are envisioned to use AMPs to control postharvest decay (*15*). At present, the high cost of synthetic peptides poses an obvious limit to agricultural and food applications. The demonstration provided herein that L-versions of distinct AMP are active in fruit bioassays is of relevance if peptides are to be produced through biotechnology. There is an increasing number of examples of short AMPs effectively produced in transgenic plants (*18, 20*), which include indolicidin (*43, 44*). Alternatively, in the postharvest scenario, peptides could also be produced by cell factories and used as postharvest additives or produced in situ by microorganisms on fruit surfaces (*15, 21*). Future research will determine the feasibility of these options.

In conclusion, our study reveals differences among peptides on the activity against different microorganisms, their fungicidal action against *P. digitatum* conidia, and the citrus fruit decay control achieved by coinoculation with *Penicillium*. It also underlines the potential utility of short tryptophan-rich cationic and hydrophilic AMP as a source of specific peptides against plant and postharvest fungal pathogens. Examples are given in the case of the PAF peptides and the related BM0. These peptides are endowed with fungicidal activity against conidia of *P. digitatum*, the survival structures of the fungus, which should be taken into account in the future development of control strategies based on AMPs.

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