Identification and Characterization of a Hexapeptide with Activity Against Phytopathogenic Fungi That Cause Postharvest Decay in Fruits

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A hexapeptide of amino acid sequence Ac-Arg-Lys-Thr-Trp-Phe-Trp-NH₂ was demonstrated to have antimicrobial activity against selected phytopathogenic fungi that cause postharvest decay in fruits. The peptide synthesized with either all D- or all L-amino acids inhibited the *in vitro* growth of strains of *Penicillium italicum*, *P. digitatum*, and *Botrytis cinerea*, with MICs of 60 to 80 μ M and 50% inhibitory concentration (IC₅₀) of 30 to 40 μ M. The inhibitory activity of the peptide was both sequence- and fungus-specific since (i) sequence-related peptides lacked activity (including one with five residues identical to the active sequence), (ii) other filamentous fungi (including some that belong to the genus *Penicillium*) were insensitive to the peptide's antifungal action, and (iii) the peptide did not inhibit the growth of several yeast and bacterial strains assayed. Experiments on *P. digitatum* identified conidial germination as particularly sensitive to inhibition although mycelial growth was also affected. Our findings suggest that the inhibitory effect is initially driven by the electrostatic interaction of the peptide with fungal components. The antifungal peptide retarded the blue and green mold diseases of citrus fruits and the gray mold of tomato fruits under controlled inoculation conditions, thus providing evidence for the feasibility of using very short peptides in plant protection. This and previous studies with related peptides indicate some degree of peptide amino acid sequence and structure conservation associated with the antimicrobial activity, and suggest a general sequence layout for short antifungal peptides, consisting of one or two positively charged residues combined with aromatic amino acid residues.

Fungicide application is a usual practice in the fight against plant diseases (Knight et al. 1997), as exemplified in the control of the decay of fruits and vegetables during postharvest handling and storage (Eckert and Ogawa 1985). Fruit rot accounts for about 10% of those plant diseases that can be controlled by fungicides (Schwinn 1992). However, growing public health and environmental concerns have resulted in the de-registration of some important fungicides. In addition, selection for resistance among fungal populations has been re-

ported and could result in loss of effectiveness of these compounds (Eckert 1988; Knight et al. 1997). This scenario has driven the search for alternative disease management strategies and/or safer antifungal agents that could substitute for the current use of fungicides. Biological control (Thomashow and Weller 1996), the induction of natural plant defenses (Lyon et al. 1995), and crop biotechnology (Lamb et al. 1992) are promising alternatives to fungicide treatment. The application of these approaches to postharvest pathology has been addressed (Wilson and Wisniewski 1989; Wilson et al. 1994), although they either have not performed consistently under commercial criteria or require further development.

Both plants and animals produce a wide variety of small proteins (i.e., <100 amino acids) and peptides possessing antimicrobial activity (antibacterial and antifungal) that are thought to be part of constitutive or inducible defense mechanisms against pathogen infection (Rao 1995; Broekaert et al. 1995). Microorganisms also produce peptide antibiotics with very diverse biochemical properties and specificities (Sahl and Brierbaum 1998). Most plant antimicrobial peptides contain disulfide bonds and their sequence/structure similarities allow them to be grouped into several classes (Broekaert et al. 1997; García-Olmedo et al. 1998). The smallest antimicrobial plant peptides isolated to date are a family of 20 amino acid peptides found in the seeds of *Impatiens balsamina* (Tailor et al. 1997). 15-mer synthetic peptides whose sequences were derived from a radish defensin have also been shown to have antifungal activity (De Samblanx et al. 1996). The use of antimicrobial peptides for engineering crop protection has been proposed (Rao 1995; Broekaert et al. 1997) and examples have been reported of decreased susceptibility to pathogen infection resulting from transgenic production of thionins (Carmona et al. 1993), defensins (Terras et al. 1995), and lipid transfer proteins (LTP) (Molina and García-Olmedo 1997) in plants. Similar observations have been made with antimicrobial peptides from animals (Huang et al. 1997). The use of this wide class of peptides seems a promising approach in plant protection and their application could potentially be extended to postharvest pathology, since some are active against fungi that cause postharvest decay. For instance, the polypeptide bacteriocin nisin, used as a food preservative, was shown to act synergistically with a biocontrol agent to protect against apple rots (El-Neshawy and Wilson 1997).

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Targeted sequence modifications of antimicrobial peptides have been demonstrated to have antifungal (Powell et al. 1995; De Samblanx et al. 1997; Cavallarin et al. 1998), antibacterial (Powell et al. 1995), and antiviral (Marcos et al. 1995) properties against plant pathogens, demonstrating that it is feasible to expand and improve the repertoire of natural antimicrobials for use in plant protection. In the search for new bioactive peptides, the combinatorial chemistry approach provides methods for the efficient synthesis and screening of large collections (i.e., "libraries") of related compounds (Blondelle et al. 1995). Examples have been reported of peptide combinatorial library use facilitating the identification of drug lead compounds and antimicrobial agents of medical interest (Dörner et al. 1996; Al-Obeidi et al. 1998). Recently, a synthetic peptide library in an iterative format was screened for the identification of a hexapeptide with in vitro antifungal activity against selected phytopathogenic fungi (Reed et al. 1997), although the effect of the peptides on disease development was not reported. For these approaches to be exploited to their full potential in plant protection, studies are needed to confirm, extend, and characterize the activity of defined short peptides against phytopathogens.

We have chosen fungal postharvest pathogens as a working system to target the identification of novel antimicrobial peptides. The fungus *Botrytis cinerea* provides an excellent model in this context; it has a wide host range (over 200 plant species) and infects fruits (both in the field and after harvest), flowers, and vegetative tissues. Several groups of fungicides are used to fight *B. cinerea* and resistances to some of them have appeared in natural populations (Leroux et al. 1999). On the other hand, the two major postharvest diseases of citrus are green and blue molds, caused by *Penicillium digitatum* and *P. italicum*, respectively (Murata 1997). Fungicides belonging to the benzimidazole (thiabendazole and benomyl) and thiazol (imazalil) groups are used for control, and resistant strains have been described (Eckert and Brown 1986; Bus 1992). In the present study, we have identified and characterized a short, synthetic hexapeptide that shows specific antifungal activity against these three fungi.

RESULTS

Identification of a hexapeptide with antifungal activity.

Our groups are involved in the utilization of peptide libraries to identify peptide sequences with activity against plant

#1:	Ac-	d	n	t	w	f	w	-NH ₂
#2:	Ac-	d	n	t	p	f	w	-NH ₂
#8:	Ac-	d	q	t	p	f	w	-NH ₂
#19:	Ac-	r	k	t	w	f	w	-NH ₂
#20:	Ac-	r	k	t	p	f	w	-NH ₂
#19L:	Ac-	R	K	T	W	F	W	-NH ₂
#19Lr:	Ac-	W	F	W	T	K	R	-NH ₂

Fig. 1. Peptide amino acid sequences analyzed in this study. Following standard rules, D-stereoisomers of amino acids are noted in lowercase and L-stereoisomers in uppercase. For D-amino acid peptides, residues identical to those of hexapeptide #19 are shaded. All peptides were acetylated at the N terminus (Ac) and amidated at the C terminus (NH₂).

pathogens. A synthetic hexapeptide combinatorial library constructed with D-amino acids has been used to search for peptides that inhibit the infection caused by plant viruses (Pérez-Payá et al. 1999) and, following this trend, different sets of all D-amino acid hexapeptides with defined sequences were synthesized. A routine screening procedure was conducted to test whether any of these short peptides were effective against phytopathogenic fungi. Figure 1 shows the sequences of some of the hexapeptides analyzed.

A microtiter plate assay was set up to test the effect of the peptides on the growth of fungi in a synthetic medium by measuring the increase in OD at 492 nm over time (see Materials and Methods). Using this quantitative assay, we identified a hexapeptide (#19; Fig. 1) that showed remarkable antifungal activity against some postharvest pathogens. Experiments involving growth of an isolate of *P. digitatum* in the presence of increasing concentrations of hexapeptide #19 showed that concentrations of 20 to 40 μ M partially affected the OD₄₉₂ increase over time and that the minimum concentration at which no growth was observed (MIC) was 60 μ M (Fig. 2A). Above this concentration there was no detectable growth under our experimental conditions. These results were consistently reproduced; only occasionally was a small in-

Fig. 2. Effects of hexapeptides (A) #19 and (B) #20 on in vitro growth of *Penicillium digitatum* PHI-26. Results are shown as mean values of optical density (OD) at 492 nm $\pm$ SD over time (in hours) for control samples in the absence (black circles) and presence of (white squares) 20, (white triangles) 40, (white diamonds) 60, and (white circles) 80 μ M concentrations of peptide.

crease in OD₄₉₂ observed at 60 µM peptide but it did not exceed the standard deviation of the measurements (as in the experiment shown in Figure 2A). Quantitative comparison with the OD₄₉₂ values of the control (with no added peptide) at 48 and 72 h (and taking into account several independent experiments) allowed the calculation of the peptide concentration required to obtain 50% inhibition of fungal growth (IC₅₀), which was found to be 32 ± 7 µM (Table 1).

Specific antifungal activity of hexapeptide #19.

Experiments were conducted to determine the specific nature of the amino acid sequence that provided antifungal activity. It was observed that none of the other hexapeptides analyzed from our peptide collection affected fungal growth. Sequence-related hexapeptides #2 and #8 (Fig. 1) did not show antifungal activity under the same assay conditions described for hexapeptide #19 (data not shown). Hexapeptide #1 was difficult to dissolve in MOPS buffer, and the maximum concentration at which it could be assayed was 25 µM. In a representative experiment, 25 µM hexapeptide #1 did not inhibit the growth of *P. digitatum* PHI-26 after 72 h of incubation (i.e., 116% growth, compared with control), while hexapeptide #19 showed 61% inhibition (i.e., 39% growth). Of particular note in these experiments was hexapeptide #20, in which a single amino acid residue substitution of Trp for Pro at position 4 (Fig. 1) completely abolished antifungal activity (Fig. 2B and Table 1).

The activity of peptide #19 was maintained when other postharvest fungi were assayed, such as a field isolate of another *Penicillium* pathogen of citrus, *P. italicum*, and collection strains of *P. digitatum* and *B. cinerea* (Table 1 and Fig. 3A). The IC₅₀ of hexapeptide #19 for all these fungi was around 40 µM, and the MIC was 80 µM. Significantly, a collection strain of *P. italicum* (CECT2294) was found that was not sensitive to hexapeptide #19. As an internal control to validate our assays, we also conducted experiments with the 26 amino acid peptide melittin, a well-known lytic peptide

from the honeybee. Under our experimental conditions, melittin was more potent than hexapeptide #19 (as concluded from its lower IC₅₀ and MIC) against all fungi challenged (Table 1), but unlike hexapeptide #19 it was also toxic to bacteria and yeasts.

Further evidence for the specificity of hexapeptide #19 arose from experiments with other filamentous fungi. *Aspergillus nidulans* was not sensitive to the peptide nor were strains of other, unrelated, phytopathogenic fungi, *Fusarium oxysporum* (Fig. 3B), *P. expansum*, and *Rhizopus stolonifer* (Table 1). Our experiments also showed that the two fungi from the genus *Penicillium* that were resistant to the peptide exhibited a slight increase in growth in the presence of 80 to 100 µM hexapeptide #19. At present, we cannot explain this behavior. Previous studies identified a hexapeptide of amino acid sequence Ac - f r l k f h - NH₂ (peptide 66-10) as being active against phytopathogenic fungi (Reed et al. 1997). We have demonstrated activity of 66-10 against all the fungi tested in our experiments (Table 1), including fungi that were either sensitive (Fig. 3A) or resistant (Fig. 3B) to hexapeptide #19, thus indicating a broader antifungal spectrum. No correlation was observed between the relative antifungal activities of peptides #19 and 66-10. For example, 66-10 was more active against *F. oxysporum* than *B. cinerea*, while #19 was more active against *B. cinerea* (Fig. 3).

Hexapeptide #19 was also assayed with other microorganisms, including laboratory strains of the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae*, and two field isolates of species of yeast epiphytes of fruits, *Rhodotorula* and *Metschnikowia* (Table 1 and Fig. 3C). Peptide #19 was not inhibitory to any of these, since values close to 100% growth were observed at all the peptide concentrations tested. By contrast, peptide 66-10 was inhibitory to *S. cerevisiae*, with an IC₅₀ of 45 µM (Fig. 3C), suggesting a broader toxic activity that affects non-filamentous fungi. Taken together, these data suggest that the antifungal activity of hexapeptide #19 is rather specific toward certain filamentous fungi.

Table 1. Effect of selected synthetic peptides on the in vitro growth of fungi, yeast and bacteria

Medium ^a	Microorganism	Peptides									
		#19			#20		66-10			Melittin	
		IC ₅₀ ^b	MIC ^b	Growth (80 µM) ^c	IC ₅₀ ^b	MIC ^b	IC ₅₀ ^b	MIC ^b	Growth (80 µM) ^c	IC ₅₀ ^b	MIC ^b
PDB	<i>Penicillium digitatum</i> PHI-26	32 ± 7	60	0%	NI ^d		<10	20	0%	6	20
	<i>P. digitatum</i> CECT2954	41 ± 7	80	0%	NI		12	20	0%	16	40
	<i>P. italicum</i> PHI-1	41 ± 13	80	0%	NI		30	60	0%	8	20
	<i>P. italicum</i> CECT2294		NI	125%	NI		41	80	0%	25	40
	<i>P. expansum</i> CMP-1		NI	>100%	NI		31	80	0%		
	<i>Botrytis cinerea</i> CECT2100	43 ± 14	80	0%	NI		27	60	0%	24	60
	<i>Rhizopus stolonifer</i> CECT2672		NI	106%							
	<i>Fusarium oxysporum</i> CECT2866		NI	106%			24	40	0%		
	<i>Aspergillus nidulans</i> biA1	> 80	> 80	89%	NI						
PDB (Ca ²⁺ , K ⁺)	<i>P. digitatum</i> PHI-26		NI	98%							
	<i>P. italicum</i> PHI-1		NI	108%							
LB	<i>Escherichia coli</i> DH5α		NI	97%	NI		NI		93%	10	20
YPD	<i>Saccharomyces cerevisiae</i> W303-1A		NI	93%	NI		45	>100	20%	19	60
	<i>Metschnikowia pulcherrima</i> isolate 4.4		NI	102%			NI		106%		
	<i>Rhodotorula glutinis</i> isolate #81		NI	107%							

^a PDB = potato dextrose broth; LB = Luria-Bertani; YPD = 1% yeast extract, 1.5% peptone, 2% dextrose.

^b Fifty percent inhibitory concentrations (IC₅₀) and MICs are given in µM for the microorganism in which a significant effect was observed.

^c Growth observed (expressed as percentage of control) at 80 µM peptide.

^d Not inhibitory (i.e., no significant effect observed on growth) at 80 µM peptide.

Mode of action of hexapeptide #19.

Experiments were conducted to gain some insight into the mode of action of hexapeptide #19. The antimicrobial properties of some plant peptides have been shown to be sensitive to the ionic strength of the growth medium (Broekaert et al. 1997). Our data demonstrated that the toxicity of hexapeptide

#19 was abolished when PDB medium was supplemented with 1 mM CaCl_2 and 50 mM KCl (Table 1); under these conditions there was >95% growth of *P. italicum* and *P. digitatum* at 100 μM peptide, compared with controls with no peptide added.

Conidia incubated with the peptide in PDB (for up to 18 h), subsequently washed three times consecutively and finally incubated in PDB, germinated and grew as did controls (data not shown), demonstrating that the peptide had been washed away and that its mechanism of action was not fungicidal. Microscopic observations of conidia incubated in the presence of peptides for 18 to 20 h (the time point by which germination had occurred) showed a significant reduction in the percentage of germination for conidia incubated in 100 μM hexapeptide #19 (Table 2). This concentration is clearly above the MIC limit (Table 1) and accordingly no OD_{492} increase was observed in these experiments at 100 μM hexapeptide #19 (not shown); it is noteworthy, however, that germinated spores were found at this peptide concentration (2 to 28% in experiments shown in Table 2). These observations demonstrated that the peptide has a major effect as an inhibitor of conidial germination, and also suggested that subsequent mycelial growth must also be affected. In fact, microscopic observations showed a much smaller size of germ tube elongated from conidia maintained in the presence of peptide #19, but remarkably not from those maintained in the presence of hexapeptide #20 (Fig. 4).

Effects on hyphal elongation were further demonstrated in assays in which the peptide was added at different time points after the initiation of incubation (Fig. 5); 100 μM peptide retarded, but did not stop, ongoing fungal growth, showing that mycelial growth was less sensitive to hexapeptide #19 than was germination (i.e., 100 μM was not inhibitory). In addition, there was a significant OD_{492} "jump" if the peptide was added to 39- to 48-h fungal cultures (Fig. 5). We attributed this increase in optical density to hyphal aggregation, although we could not confirm this conclusion by microscopic observations due to the massive mycelial accumulation (data not shown).

Antifungal activity of hexapeptide #19 synthesized with L-amino acids.

At least one previous study has shown that the biological activity of short peptides constructed with D-amino acids is maintained in all L-amino acid peptides of the same sequence (Wade et al. 1990). We used L-stereoisomers to synthesize the corresponding hexapeptide of the same amino acid sequence as #19 (Fig. 1; peptide #19L) and, in addition, one peptide of the reversed sequence (Fig. 1; peptide #19Lr). These peptides were assayed to compare their activities with that of hexapeptide #19 (Fig. 6). *P. digitatum* was less sensitive to peptide #19L than to #19, as concluded from the inhibition curves at 50 μM peptide (Fig. 6) and also from the IC_{50} values obtained after 72 h of growth at different peptide concentrations: 34 μM for #19L and 28 μM for #19 in the experiment shown. Both peptides were completely inhibitory at 100 μM (data not shown). It must be noted, however, that #19L and #19 had the same levels of activity over the initial 48 h of incubation (the time by which almost 50% of the final growth of the control was reached), as no OD_{492} increase was observed for either of them (Fig. 6 and data not shown). This observation suggests that the relative decrease in #19L activity that occurred at later

Fig. 3. Effects of hexapeptides #19 and 66-10 on in vitro growth of (A) *Botrytis cinerea*, (B) *Fusarium oxysporum*, and (C) *Saccharomyces cerevisiae*. Results are shown as mean values of percentage of growth of control (100% = growth with no peptide added) $\pm$ SD, at each peptide concentration and after (A, B) 72 or (C) 24 h of incubation. Experiments were conducted with peptide #19 (white symbols) and peptide 66-10 (black symbols).

time points was due to the greater proteolytic degradation to which peptides constructed with natural amino acids are prone, unlike those constructed with D-amino acids. That peptide #19 was more resistant to degradation was confirmed in vitro by chromatographic analyses of peptides incubated with supernatant fractions of fungal cultures. In a representative experiment, 94% of #19L was no longer detectable after 2 h of incubation with a supernatant fraction from a 48-h PHI-26 culture, while 84% of #19 was still detectable after 21 h of incubation.

Interestingly, the reverse sequence peptide #19Lr was less inhibitory than #19L, as concluded from its lower IC₅₀ (47 µM) and growth inhibition curve (Fig. 6). Moreover, fungal growth in the presence of #19Lr was observed over the initial 48 h of incubation, as opposed to #19L (Fig. 6, inset). These differences in activity between #19L and #19Lr indicate that antifungal toxicity is not simply a property of the additive effect of the amino acid side chains and that, despite their short length (6 amino acids) there is some structural conformation involved. In any case, #19Lr was inhibitory at higher concentrations (i.e., 100 µM) (data not shown). Additional control experiments demonstrated that the corresponding #20L and #20Lr did not affect fungal growth even at the highest concentration assayed, 100 µM (data not shown).

Effect of peptide #19 on infection of fruits by *Penicillium* spp. and *Botrytis cinerea*.

All the fungi against which we found that hexapeptide #19 was inhibitory cause postharvest decay of fruits. Laboratory controlled fruit inoculations were conducted to determine whether the observed in vitro antifungal properties of the peptide correlated with an inhibition of fruit rot caused by fungal infections. We used a bioassay in which conidial suspensions (in the presence or absence of peptides) were inoculated onto the wounded outer peel of either citrus or tomato fruits.

Citrus fruits (oranges) were inoculated with the *P. digitatum* PHI-26 field isolate. Under our conditions and inoculum concentration, tissue maceration appeared at the end of day 2 (not shown), and by day 3 all wounds were infected (Fig. 7A). A short delay in the appearance of symptoms was observed when hexapeptide #19 was included in the inoculum (i.e., only 30% of the wounds were infected by day 3 in the experiment shown in Figure 7A), although all the wounds became infected by later time points. The delay was correlated with smaller lesion size (Fig. 7A) and retarded sporulation in the lesion (not shown). As occurred with the in vitro experiments, the effect of hexapeptide #19 was sequence specific since it

was not observed when sequence-related peptides were assayed (Fig. 7A). The level of green mold control obtained with hexapeptide #19 was reproduced on inoculated mandarin fruits, and also for blue mold caused by *P. italicum* PHI-1 on oranges. In all cases, a short delay and a decrease in the size of the affected surface were observed (data not shown).

Tomato fruits were inoculated with *B. cinerea* at two different conidial concentrations (Fig. 7B). At 10⁶ conidia per ml, all wounds were infected after 3 days of incubation; if the inoculum was reduced by 10-fold, 100% infection was not achieved even after 7 days (not shown). When conidia were inoculated in the presence of hexapeptide #19, we observed a delay in the appearance of symptoms (Fig. 7B and C; inoculated wounds in fruits 4 and 5 are not infected) and reduced size of the infected areas (Fig. 7C; the affected surface of fruit 6 is smaller than that of fruits 1 to 3).

In these in vivo experiments, fungi were able to infect fruits even at 100 µM hexapeptide #19, a concentration above the in

Fig. 4. Effects of hexapeptides on germ tube elongation from germinated conidia of *Penicillium digitatum* PHI-26. Conidia were incubated in vitro in (A) absence of peptide, or in the presence of 100 µM (B) hexapeptide #19 or (C) hexapeptide #20. Micrographs were taken at 20 h and show representative observations of the many germinating conidia in the (A) absence of peptide or presence of (C) hexapeptide #20, and (B) of one of the few conidia that germinated in the presence of hexapeptide #19.

Table 2. Effect of selected synthetic peptides on the in vitro germination of fungal conidia observed in three independent experiments

Experiment	Fungus	Control	Peptides ^a				
			#8	#19		#20	Melittin
			100 µM	50 µM	100 µM	100 µM	50 µM
Experiment 1	<i>Penicillium italicum</i> PHI-1 (2.5 × 10 ⁵) ^b	26/40 (65) ^c	25/52 (48)	67/97 (69)	19/68 (28) ^d		
Experiment 2	<i>P. italicum</i> PHI-1 (2.5 × 10 ⁵)	46/103 (45)	75/96 (78)		23/109 (21) ^d		
	<i>P. digitatum</i> PHI-26 (2.5 × 10 ⁵)	19/50 (38)			3/87 (4) ^d		
Experiment 3	<i>P. digitatum</i> PHI-26 (2.5 × 10 ⁴)	52/126 (41)		6/79 (8) ^d	2/99 (2) ^d	49/117 (42)	0/103 (0) ^d

^a Peptide concentration (µM) used in each experiment is indicated below the peptide.

^b Conidial concentration (as conidia/ml) used in each experiment is indicated in parentheses.

^c Number of germinated conidia/total number of conidia observed (percentage of germinated conidia).

^d Values statistically lower than the control at 95% significance (χ² test).

vitro MIC, and there was no difference between the size of the infected areas produced at either 50 or 100 μ M peptide (data not shown).

DISCUSSION

Small, antimicrobial peptides have drawn attention in relation to their potential for application in plant disease control (Rao 1995). Their potential application in the field of postharvest pathology is unexplored. So far, these approaches have relied mostly on natural peptides (or their modifications) and little on non-natural amino acid sequences. In this work, we have identified a hexapeptide sequence (Ac-Arg-Lys-Thr-Trp-Phe-Trp-NH₂) that exhibits antifungal properties against specific phytopathogenic fungi (Figs. 2A, 3, and 6, and Table 1) but not against other fungi, bacteria, and yeasts, and retards the postharvest decay caused by fungal infection (Fig. 7). Our study confirms the potential for the application of very short peptides in plant protection.

Reed et al. (1997) showed two synthetic D-amino acid peptides, the hexapeptide 66-10 (Ac - f r l k f h - NH₂) and a pentapeptide (Ac - f r l h f - NH₂), that exhibited activity in vitro against fungi from the genera *Fusarium*, *Rhizoctonia*, *Ceratocystis*, and *Pythium*. The reported MIC values of 66-10 for *F. oxysporum*, *R. solani*, and *P. ultimum* were of the same order of magnitude as the values shown in the present study for hexapeptide #19-sensitive fungi, while those for *C. fagacearum* were lower. Since hexapeptide #19 was not active against our isolate of *Fusarium* we synthesized 66-10 and tested it for activity against our isolates in direct comparison experiments. Our data indicate that 66-10 is more potent than #19, but also quite non-specific since we did not find any fungus to be insensitive to it. Unlike peptide #19, 66-10 was toxic to *S. cerevisiae* (Fig. 3C), providing further evidence for its non-selective toxicity. It should also be noted that in the previous work (Reed et al. 1997) many peptides with single residue changes (i.e., at position 6) still had substantial activity, while we have demonstrated that the single residue substituted

hexapeptide #20 and other very similar sequences (Fig. 1) lack activity (Table 1, and not shown). Besides some general features (see below), there are no sequence identities between the amino acid sequences identified in the two studies.

The existence of inactive, closely related peptides and the identification of fungi sensitive and insensitive to hexapeptide #19, even within the same genus and species (e.g. the *P. italicum* collection strain), strongly point to some component(s) providing specificity in both the peptide and the fungi tested that would be responsible for the observed activity. This hypothetical component could vary with the collection strain. This observation could be useful in future studies aimed at the elucidation of the mode of action of the peptide.

The mechanism of action of many natural and artificially designed antimicrobial peptides is presumed to involve the interaction of the amphipathic cationic peptide with the target cell membrane, followed by membrane disruption (Blondelle and Houghten 1992; Shai 1995). Previously, peptides active against phytopathogenic fungi have been demonstrated to disrupt fungal membranes (Reed et al. 1997) and/or cause hyphal aggregation and distortion (Cavallarin et al. 1998). Our data showed that the action of the cationic peptide #19 is abolished in a high ionic strength medium, suggesting potential interaction(s) that could be disrupted by ionic charge; for instance, with the fungal cell wall and/or cell membrane. This interaction is unlikely to be mediated by protein receptors since it was demonstrated that D- and L-enantiomers of peptide #19 share similar biological activity (Fig. 6). Peptide #19 primarily inhibits conidial germination and germ tube elongation (Table 2 and Fig. 4), with hyphal growth being less sensitive to its action (Fig. 5). It does not seem to have rapid fungicidal activity since conidia are able to resume normal germination and growth if the peptide is removed within 18 h of exposure. The ease with which the peptide can be removed by simple washing further supports the notion that it interacts reversibly with conidia, as has been observed with peptides reported pre-

Fig. 5. Antifungal effect of hexapeptide #19 on growing in vitro cultures of *Penicillium digitatum* PHI-26. Results are shown as in Figure 2 for control samples in absence of peptide (black circles) or samples at which 100 μ M hexapeptide #19 was added after (white circles) 18, (white triangles) 24, (white squares) 39, or (white diamonds) 48 h of incubation. Arrows denote time points of peptide addition and for those samples data are shown only from the time of addition of peptide.

Fig. 6. Effects of hexapeptides synthesized with either L- or D-amino acids on in vitro growth of *Penicillium digitatum* PHI-26. Results are shown as in Figure 2 for control samples in the absence of peptide (black circles), or samples in the presence of hexapeptides #19 (white circles), #19L (white triangles), and #19Lr (white squares). Data are shown only for 50 μ M peptide concentration for better discrimination of effect of different peptides. Insert shows percentage of growth in the presence of each peptide at three time points (compared with control with no added peptide).

viously (Reed et al. 1997). Future research will focus on further elucidating the mechanism of peptide action as an approach to improve toxicity to fungi.

IC₅₀ values of plant antimicrobial peptides against bacterial and fungal pathogens generally range from 0.1 to 5 µM for thionins, plant defensins, LTP, and snakins (Broekaert et al. 1997; Segura et al. 1999), and on this basis are stronger inhibitors than the peptide identified in our work (IC₅₀ 30 to 40 µM, for fungal pathogens). Differences in molecular size should be considered for comparison since hexapeptide #19 is 5 to 10 times smaller than known natural plant defense peptides. 11- to 12-mer cecropin A-derived peptides were also assayed against a *Penicillium* sp. and *B. cinerea*, exhibiting MICs of 20 to 60 and 70 to 90 µM, respectively (Cavallarin et al. 1998), which are in the same range as the values reported herein. It is conceivable that the activity of peptide #19 could be improved by incorporating additional antimicrobial motifs in a modular approach, as demonstrated for cecropin-derived peptides (Cavallarin et al. 1998), provided that it can be shown to function in a larger polypeptide.

Some properties are noticeable when the amino acid sequences of short (i.e., 5 to 20 residues), non-natural antimicrobial peptides are compared (Blondelle and Houghten 1992; Powell et al. 1995; Kang et al. 1996; Reed et al. 1997; Cavallarin et al. 1998; and this work). In general, the only charged residues are basic (Arg and Lys) and provide a net positive charge at neutral pH. This is probably a sign of the interaction with the negatively charged cell wall and/or membrane, which can be abolished in the presence of inorganic cations, as seen for peptide #19 (Table 1). Furthermore, the requirement for basic residues is demonstrated by the loss of activity when the residues are substituted in both short (as concluded from hexapeptide #1 data) and longer peptides (Powell et al. 1995). In addition, antimicrobial peptides contain residues with aromatic rings (Trp and Phe). However, the lack of activity of hexapeptide #20 demonstrated that these two properties (positive charge and aromatic residues) are not sufficient for antimicrobial toxicity. The antimicrobial center of the peptide lactoferricin B (Arg-Arg-Trp-Gln-Trp-Arg-NH₂) contains two Trp residues that structural analyses have shown become buried in a membranelike environment upon interaction with sodium dodecyl sulfate micelles (Schibli et al. 1999). These residues anchor and position the peptide in the micelles to adopt an unconventional amphipathic secondary structure. This peptide and hexapeptide #19 share a Trp-Xaa-Trp motif and we have shown that mutation of one of the two Trp residues of hexapeptide #19 completely abolished the activity (Fig. 2 and Table 1). Taken together, these results suggest that two Trp residues are needed for activity and/or that hexapeptide #19 could adopt, by analogy, some degree of structure upon interaction with the fungal membrane. Although it would seem unusual for a peptide of only six residues to fold, we have also demonstrated the involvement of some degree of structural information since the reversed peptides #19L and #19Lr differ in their toxicity (Fig. 6).

Using an infection assay on fruits, we have demonstrated that hexapeptide #19 delayed fungal growth and sporulation. The effectiveness of the peptide in preventing fruit decay caused by fungi was limited, despite use of peptide concentrations that were inhibitory under in vitro fungal growing conditions. Fungicide concentrations used to stop fruit decay

usually exceed those required for in vitro inhibition (Bus 1992). We performed our fruit decay test by inoculating small volumes of conidial suspensions containing peptide on wounded fruits; a 1- to 2-day delay in the appearance of symptoms and smaller infected areas was observed (Fig. 7). Since it has been demonstrated that the peptide only partially inhibits conidial germination (Table 2), we postulate that mycelium from germinated spores grows freely to infect surrounding tissue once it progresses away from the inoculation site. This result indirectly suggests that the peptide does not activate systemic defense responses in fruit tissue. The potential practical use of the peptide relies therefore on ensuring its presence throughout the tissues to be protected.

We have demonstrated that hexapeptide #19 synthesized with the L-stereoisomers of amino acids is also active against the fungal pathogens described in this work (Fig. 6). One of the objectives of studies aimed at identifying peptide antimicrobials is their production in genetically modified organisms. Some plant defense peptides have been produced in transgenic plants and conferred protection against disease (Carmona et al. 1993; Terras et al. 1995; Molina and García-Olmedo 1997). Ongoing research is exploring the use of biotechnological approaches (i.e., recombinant DNA technology and genetic transformation) for the production and/or targeted delivery of the antifungal short peptide identified in our study. Due to the short length of peptide #19, alternative strategies such as fusion to (or cleavage from) longer polypeptides will be evaluated. Once this peptide is produced, its use in combination with other disease control approaches could be tested. Indeed, the peptide nisin has been demonstrated to act synergistically with biocontrol agents to reduce postharvest rots (El-Neshawy and Wilson 1997). Recently, enhanced bioactivity of biocontrol microorganisms has already been achieved through the transgenic production of proteins having antimicrobial properties (Migheli et al. 1998; Limón et al. 1999), and postharvest biocontrol yeasts were genetically transformed and remained stable under field conditions (Chand-Goyal et al. 1999). A necessary property of antimicrobial peptides if they are to be used in conjunction with, or be produced by, biocontrol microorganisms is the absence of toxicity against non-target microorganisms, including the biocontrol agents. We have shown that hexapeptide #19 does not affect the growth of two different yeasts that have biocontrol properties (Table 1): *R. glutinis* (L. González-Candelas and J. F. Marcos, *unpublished observations*) and *M. pulcherrima* (Piano et al. 1997). Future research projects will couple combinatorial chemistry and targeted peptide modifications to identify antimicrobials exhibiting improved properties, together with the use of biotechnology for the production of the newly designed peptides in host plants or biocontrol agents.

MATERIALS AND METHODS

Microbial strains and isolates.

The fungi used in this study were *Penicillium digitatum* CECT2954 (Colección Española de Cultivos Tipo [Spanish National Collection of Type Cultures]; originally isolated from *Citrus sinensis* fruits), *Penicillium italicum* CECT2294, *Botryotinia fuckeliana* CECT2100 (anamorph *Botrytis cinerea*; originally isolated from leaves of *Vicia faba*), *Rhizopus stolonifer* CECT2672, *Fusarium oxysporum* f. sp. *lycopersici*

Fig. 7. Effects of hexapeptides on rot caused by fungal infections of fruits. **A**, Orange fruits were infected with 10^6 conidia of *P. digitatum* PHI-26 per ml either alone (black bars) or in the presence of 100 µM hexapeptide #20 (striped bars) and hexapeptide #19 (white bars). Bars show mean values of infected surface area $\pm$ SD at 3 and 5 days post inoculation (dpi). Numbers below bars indicate percentage of infected wounds for each treatment and dpi. For each treatment, four fruits (four wounds per fruit) were inoculated. **B**, Tomato fruits were infected with *Botrytis cinerea* CECT2100 at 10^5 conidia per ml, either alone (gray bars) or in the presence of 100 µM hexapeptide #19 (cross-hatched bars), or at 10^6 conidia per ml, either alone (black bars) or in the presence of 100 µM hexapeptide #19 (white bars). Bars show mean of percentage of infected wounds $\pm$ SD for each treatment and dpi. For each treatment, two replicas of four fruits (four wounds per fruit) were inoculated. **C**, Representative results showing six tomato fruits from experiment shown in B. Fruits were inoculated with 10^6 conidia per ml, either alone (fruits 1 to 3) or in the presence of 100 µM hexapeptide #19 (fruits 4 to 6). Pictures were taken at 3 dpi; point of inoculation is indicated with an arrowhead.

CECT2866, a laboratory strain of *Aspergillus nidulans* (genotype *biA1*), and field isolates of *P. expansum* (isolate CMP-1; Teixidó et al. 1998), *P. digitatum* (isolate PHI-26), and *P. italicum* (isolate PHI-1) obtained from rotten fruits. All fungi were routinely cultured on potato dextrose agar (PDA) (Difco, Detroit, MI) plates for approximately 12 to 14 days at 24°C, depending on the fungus. Spores were collected by adding sterile water to the surface of the mycelium and gently scrubbing with a sterile spatula or by scraping them from the agar and transferring to distilled water. Spores were then filtered, quantified with a hemacytometer, and adjusted to the appropriate concentration. All the postharvest pathogens were tested for symptom production on fruits of the corresponding host.

Control experiments used laboratory strains of *Escherichia coli* (DH5 α) and *Saccharomyces cerevisiae* (W303-1A), and field isolates of two yeasts obtained from fruit surfaces, *Metschnikowia pulcherrima* isolate 4.4 from apples (Piano et al. 1997) and *Rhodotorula glutinis* isolate #81 obtained from oranges. Yeasts were grown in YPD medium (1% yeast extract, 1.5% peptone, 2% dextrose) at 24°C, and bacteria in Luria-Bertani (LB) medium at 37°C.

Peptide synthesis and purification.

Peptides were synthesized by solid-phase methods following standard procedures with the base-labile N-(9-fluorenyl) methoxycarbonyl (Fmoc) group for the protection of the α -amino function (Fields and Noble 1990). Peptides were assembled on polystyrene aminomethyl-RAM resin to yield the peptide as a C-terminal amide with diisopropylcarbodiimide/hydroxybenzotriazol mediated couplings. The protecting Fmoc group was cleaved by 20% piperidine in dimethyl formamide (DMF). The N terminus of the peptides was acetylated with an excess of acetic anhydride in DMF. Finally, fully deprotected cleaved peptides were precipitated in cold t-butylmethylether, centrifuged, dissolved in acetic acid, and lyophilized. The peptides were purified by preparative reverse phase high-pressure liquid chromatography (RP-HPLC) and their identity was confirmed by MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry. Two independent chemical syntheses of hexapeptide #19 were carried out in the course of this study, and the antifungal activity of the peptide was confirmed in both cases.

Stock solutions of each peptide were prepared at 1 mM in 5 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) pH 7 buffer. Peptide concentrations were determined by measuring the absorbance at 280 nm and re-checked before each experiment.

In vitro antimicrobial activity assays.

Microorganisms were grown in vitro on sterile 96-well microtiter plates (Nunc, Roskilde, Denmark) in a final volume of 200 µl. Growth was determined by measuring optical density (OD) at 492 nm in a Titertek Multiskan PLUS (Labsystems, Helsinki, Finland) microplate reader until measurements were approximately 1 unit for the control samples, values at which a plateau was reached and, in the case of fungi, sporulation sometimes occurred. Under our assay conditions this value was reached in 3 to 4 days. For fungal growth, the assay mixture contained 2.5×10^4 conidia per ml (final concentration) and 180 µl of potato dextrose broth (PDB) (Difco, Detroit,

MI) containing 0.003% chloramphenicol per well. Peptides were added in a 20- μ l volume to reach different final concentrations from 10 $\times$ stock solutions. In some experiments, PDB medium was supplemented with 1 mM CaCl₂ and 50 mM KCl. The microtiter plates were incubated at 24°C. Fungal growth was routinely double-checked by microscopic observation to confirm the OD data. In some experiments, conidial germination was monitored microscopically after an 18- to 20-h incubation period by counting the number of germinated and non-germinated conidia in each sample.

For in vitro yeast and bacterial growth, the assay was essentially the same except that the inoculum was 50 μ l of a freshly diluted overnight culture (OD₆₀₀ = 0.01) brought to 200 μ l of the appropriate growth medium. For bacterial assays, plates were incubated at 37°C.

In all the experiments, three replicas (three wells) were prepared for each microorganism at each peptide concentration; the blank mean OD value from 1 row of mock inoculations was subtracted from each sample OD measurement, and the mean and standard deviation (SD) were then calculated for each treatment.

Fruit decay test.

Experiments were carried out on freshly harvested fruits of orange (*Citrus sinensis* (L.) Osbeck 'Navelina'), mandarin (*Citrus reticulata* Blanco 'Nules') or tomato (*Lycopersicon esculentum* Mill. 'Ailsa Craig'). Fruits were surface sterilized by incubation for 10 min in a 10% commercial bleach solution and subsequent washing in distilled water three times. Fruits were air dried and wounded by making punctures with a nail at either four sites (oranges or tomatoes) or two sites (mandarins) around the equator. Suspensions (10⁵ to 10⁶ conidia per ml) were mixed with peptides (at the desired final concentrations) and 10 μ l of the solution was applied to each wound. Fruits were maintained at 24°C and 85% relative humidity for 7 days and scored daily for symptoms. For oranges and mandarins, the diameter of the infected area was measured at selected days post inoculation and the values were used to calculate the infected surface for each wound. Results were given as mean values of infected surface $\pm$ SD for each treatment.

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