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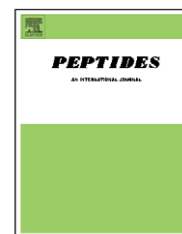

First isolation of a novel thermostable antifungal peptide secreted by *Aspergillus clavatus*

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ABSTRACT

A novel antifungal peptide produced by an indigenous fungal strain (VR) of *Aspergillus clavatus* was purified. The antifungal peptide was enriched in the supernatant after heat treatment at 70 °C. The thermostable character was exploited in the first purification step, as purified peptide was obtained after ultrafiltration and reverse phase-HPLC on C18 column application. The purified peptide named "AcAFP" for *A. clavatus* antifungal peptide, has molecular mass of 5773 Da determined by MALDI-ToF spectrometry. The N-terminal sequence showed a notable identity to the limited family of antifungal peptides produced by ascomycetes fungi. The AcAFP activity remains intact even after heat treatment at 100 °C for 1 h confirming its thermostability. It exhibits a strong inhibitory activity against mycelial growth of several serious human and plant pathogenic fungi: *Fusariuym oxysporum*, *Fusarium solani*, *Aspergillus niger*, *Botrytis cinerea*, *Alternaria solani*, whereas AcAFP did not affect yeast and bacterial growth.

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1. Introduction

One of the big challenges nowadays is to find substitutes for the classical antibiotics and antifungal molecules that are used in medicine or spread in the nature. Usually, they are chemically synthesized. Their major drawbacks are the development of resistant pathogen strains and the appearance of some side effects on users. Peptides with antibacterial or antifungal activities are becoming a valuable alternative. They are very small molecules, biologically synthesized, very specific and therefore would have fewer side effects. Moreover, they should contribute to the reduction of resistance since they enlarge the panel of applied molecules. Antifungal peptides are produced throughout all kingdoms from Prokaryotes [28,37] to lower and higher Eukaryotes [4–8,10,15,14,21,23,30–32,34]. Among them, a restraint family of antifungal peptides are secreted by filamentous fungi. AFP, was the firstly isolated one from *Aspergillus giganteus* [16,27,35]. Then PAF was isolated from *Penicillium chrysogenum* [25,26]. In addition, Geison isolated NAF from *Penicillium nalviogense* that was identical to PAF [9]. Finally AnAFP was identified from the mould *Aspergillus niger* [19]. These peptides act against numerous plant pathogenic fungi but only the AnAFP inhibited the growth of some yeast strains. Nevertheless they do not show any antibacterial activity. Several other molecules of interest are produced by the cited filamentous fungi. For instance α-sarcin and gigantin a ribosome inactivating proteins were isolated from *A. giganteus* [18,27,36]. *Aspergillus clavatus* mould secretes also ribonuclease proteins namely c-sarcin and clavin [12,29]. However, no antifungal peptide was described for *A. clavatus*.

The aim of this study was to identify for the first time an antifungal peptide from an indigenous strain of *A. clavatus* (AcAFP). The antifungal peptide production, purification and N-terminal sequence were performed. The antimicrobial activity of the antifungal peptide was evaluated against

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several pathogenic fungi. Finally, more in deep investigations were carried on its effect against *Fusarium oxysporum*, the responsible fungus of the ''Bayoudh'' disease which has devastated the palm plantation in North Africa.

2. Materials and methods

2.1. Strains and media

The AcAFP producing strain named VR1, was locally isolated and was identified by the Central Bureau voor Schimmelcultuur (CBS-Netherlands) as *A. clavatus*. Antifungal activity was evaluated against different microbial species: *A. niger* (F38), *Aspergillus nidulans* (F8), *Penicillium occitanis* (CT1), *Stacchybotris microspora* (N1), *Trichoderma reesei* (RutC30), *Beauveria bassiana* (P1), *Saccharomyces cervisiae* (W303-1B) and *Pichia pastoris* (X33) from Laboratoire de Génétique Moléculaire des Eucaryotes (LGME, CBS, Tunisia) collection [1]; *Botrytis cinerea*, *F. oxysporum*, *Fusarium solani* were kindly gifted by Atef Jaouani (Laboratoire de Physiologie et d'Ecologie Microbiennes, Bruxelles-Belgium) and *Alternaria solani* was gifted by Mohamed Ali Triki from The Institut de l'Olivier, Sfax-Tunisia. All fungi were grown on potato dextrose agar (PDA) plates: 39 g l^{-1} (Difco), except for yeast strains which were cultivated on yeast peptone glucose (YPG) medium: 1% bacto-peptone (Difco), 1% yeast extract (Difco), 2% glucose, 17 g l^{-1} agar (Difco) and were grown at 30 °C. *A. clavatus* was grown on LBG liquid medium (Lauria Broth LB medium: 1% yeast extract, 0.5% peptone (Pastone-Pasteur), 0.5% NaCl, pH 7 containing 2% glucose) at 30 °C.

2.2. Production of AcAFP peptide

A. clavatus conidia (2×10^7 conidia ml^{-1}) were inoculated into 1 l Erlenmeyer flasks containing 200 ml of LBG culture medium pH 6.5. Then it was incubated at 30 °C in a shaking incubator (150 rpm). After 4 days incubation, the culture supernatant was obtained by filtering through four layers of cheesecloth and centrifugation at 8000 rpm during 20 min. It was then stored at 4 °C. This supernatant containing extra cellular antifungal activity was used as the crude preparation and no loss of activity was detected after 24 months of conservation at 4 °C, without any additives.

2.3. Assay of thermal stability of antifungal activity

In order to evaluate the thermal stability of the antifungal activity, the crude culture supernatant was incubated at 50 and 70 °C for 5, 10 and 15 min, for each heating temperature, respectively. After cooling the treated sample on ice for 10 min, the residual antifungal activities was measured as described below. Moreover, the thermal stability of the purified antifungal peptide was essayed for 60 min at 100 °C.

2.4. Purification of AcAFP peptide

The purification procedure involved three steps, the thermostable character of the antifungal activity was exploited in the first step of purification. The supernatant was incubated at 70 °C for 10 min, then thermolabile and insoluble materials were removed by centrifugation at 6000 rpm for 20 min. The pooled supernatant was desalted by ultrafiltration using Centricon (MW 3.000 kDa cut-off) and chromatographied on C18 reverse phase column (μ-Bondpack C18: 300 mm $\times$ 4.6 mm) equilibrated with aqueous 0.05% trifluoroacetic acid (TFA). Elution was carried out using a linear gradient of 0–80% acetonitrile in aqueous 0.05% TFA at a rate of 0.5 ml min^{-1}. Purified samples were concentrated by speed-vac and acetonitrile was totally evaporated. Before measuring the antifungal activity, these samples were brought to the required concentration with sterilized Milli-Q water. All purification steps were performed at room temperature.

2.5. Characterization of the AcAFP by SDS-PAGE

Analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmeli and Favre [17], the polyacrylamide concentration in the separating gel was 15% (w/v). Proteins were stained with Coomassie Brillant Blue R 250 (Sigma). Protein concentration was determined using the method of Bradford [3] with BSA as a standard.

2.6. Mass spectrometry

Matrix-assisted lazer desorption ionization-time of flight mass spectrometry (MALDI-ToF MS) was employed to determine the molecular mass of the purified protein using an Autoflex™ (Bruker Daltonics, Bermen, Germany)

2.7. N-terminal amino acid sequence

Samples for sequencing were electro-blotted on to polyvinyl difluoride membranes (PVDF) according to Bergman and Jörnvall [2]. Proteins transfer was performed during 1 h at 1 mA cm^{-2} at room temperature. The NH_2-terminal end of the antifungal protein was sequenced by automated Edman's degradation, using an applied biosystems Protein Sequencer Procise 492cLC protein sequencer equipped with 140C HPLC system [11].

2.8. Antifungal activity assay

In order to determine antifungal activity, 30 μl of a conidia suspension (10^6 conidia ml^{-1}) of the target fungus were spread out on PDA plates. An aliquot (100 μl) of several diluted-peptide solutions were deposited in wells made in agar medium. The concentrations of the peptide mixture varied from 1 to 200 μg ml^{-1} as estimated by Bradford reagent. The plates were incubated for 72 h at 30 °C. The inhibitory effect of the peptide was estimated by measuring the clear zone (mm) for each tested fungi minus the size of the central well. The minimal inhibitory concentration (MIC) value for the antifungal activity against our target fungi *F. oxysporum* was determined according to the method described by Zhao et al. [38]. MIC was estimated as the χ intercept calculated from the semi-logarithmic plot by the least mean squares method [20]. To determine the 50% growth inhibition concentration (IC_{50}) value for the antifungal activity, different concentrations of

AcAFP were added separately to four aliquots. Each one containing 4 ml PDA at 42 °C, mixed rapidly and poured into four small Petri dishes. The same amount of spores of the test fungus was added to each plate. After incubation at 30 °C for 72 h, the area of the mycelial colony was measured and the inhibition of antifungal peptide determined. IC_{50} is defined as the concentration of the antifungal peptide that produces 50% reduction in the area of the mycelial colony. The peptide concentrations used for the determination of MIC and IC_{50} were ranged from 3.5 to 100 $\mu g\ ml^{-1}$ and sterilized water was used as negative control. The inhibitory activity of AcAFP against different microorganisms (fungi, yeasts and bacteria) was evaluated by plate inhibition assays. Ten fungal strains were tested for hyphal growth inhibition in the presence of 50 $\mu g\ ml^{-1}$ of AcAFP by measuring the diameter of the inhibition zone.

3. Results

3.1. *Purification of the antifungal peptide AcAFP*

An antifungal peptide named "AcAFP" was purified after few steps, essentially after having checked its thermo stability in culture supernatant (Fig. 1). Indeed, the simplest and efficient way was the heat treatment for 10 min at 70 °C. Heat was very effective and suitable to remove a large amount of inactive and thermo sensitive proteins from the concentrated crude supernatant. After removing the precipitated proteins, the obtained supernatant contained essentially a small protein with a MW of about 6 kDa as estimated by SDS-PAGE (Fig. 2). This fraction, which conserved the entire antifungal activity, was desalted using Centricon (MW cut-off; 3.000). The active fraction was deeply purified through the reverse-phase HPLC on a C18 column. Two peaks were obtained (Fig. 3) and tested. Antifungal activity was only found in peak II in which the AcAFP was purified to homogeneity as confirmed by SDS-PAGE (Fig. 4).

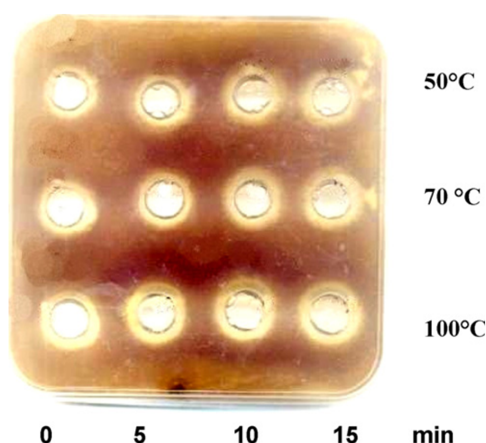

Fig. 1 – Thermostability of the antifungal activity within the crude supernatant culture of VR. C: control crude supernatant without heat treatment. Same amount of supernatant culture heated for 5, 10 and 15 min at 50, 70 and 100 °C.

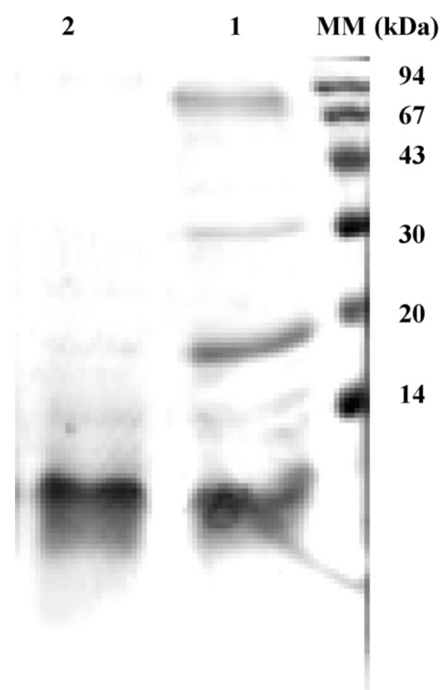

Fig. 2 – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; 15% (w/v). Lane 1: 20 μg of crude supernatant extract; lane 2: 20 μg of protein in the supernatant after centrifugation (6000 rpm/20 min) of heated proteins (70 °C for 10 min). Right lane: molecular mass marker from Amersham Biosciences including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

3.2. *Determination of inhibition spectrum*

Among 10 fungal strains tested for hyphal growth inhibition 7 were sensitive, whereas the rest, i.e. *A. nidulans*, *P. occitanis and B. bassiana*, remained unaffected even in the presence of higher concentrations of AcAFP reaching 200 $\mu g\ ml^{-1}$ (Table 1). Sensitive fungi showed increasing inhibition with increasing AcAFP concentrations. The MIC and IC_{50} values were determined only toward the target mold *F. oxysporum*, they were estimated to 3.4 and 23.3 $\mu g\ ml^{-1}$, respectively (Fig. 5). The antifungal activity behaved in a dose-dependant manner up to 90 $\mu g\ ml^{-1}$. On the other hand, all yeasts and bacterial strains were not sensitive to AcAFP.

3.3. *Molecular mass determination and N-terminal sequence of AcAFP*

The accurate molecular mass of the AcAFP was determined by MALDI-ToF MS was 5773 Da (Fig. 6). The NH_2-terminal sequencing, allowed the identification of 24 residues. Explicitly it was NH_2-A-T-Y-D-G-C-K-C-Y-K-K-D-N-I-C-K-Y-K-A-Q-S-G-K-T. Findings showed that N-terminal sequence of AcAFP is very close AFP [16,35]. A single change at the fourth residue was detected: Asp in AcAFP against Asn in AFP. It exhibits also a moderate degree of homology

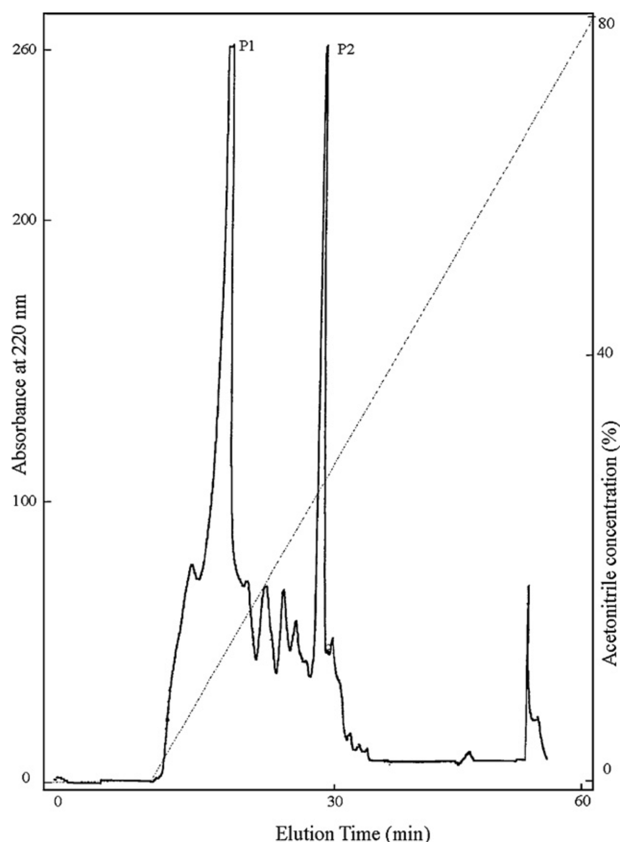

Fig. 3 – Chromatogram of the VR1 extracellular proteins on the reverse-phase HPLC C18 column. Two major peaks are indicated, the active one being the peak P2.

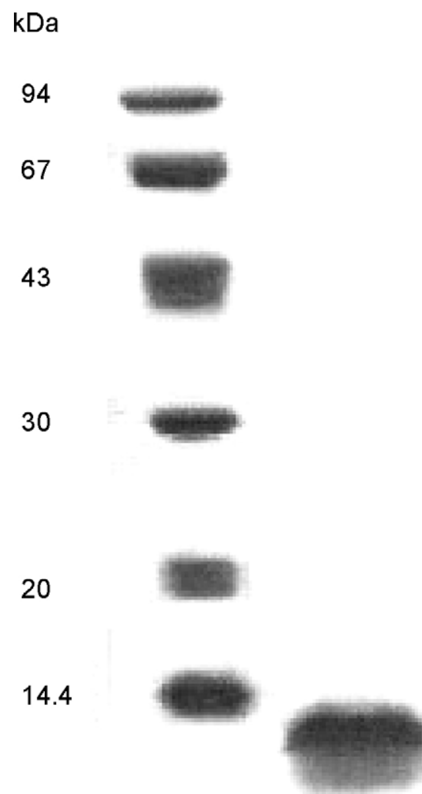

Fig. 4 – Sodium dodecyl sulphate-polyacrylamide gel 15% (w/v) electrophoresis of the purified AcAFP. Lane 1: molecular mass marker (Amersham Biosciences); lane 2: 10 μg of purified AcAFP obtained after RP-HPLC (C18).

(12 identical amino acids out of 21) with PAF peptide. Moreover it has a much lower homology with AnAFP. Table 2 shows the N-terminal sequences of AFP, PAF and AnAFP [19,23,24,35].

3.4. *Thermal stability*

The purified AcAFP exerted prominent antifungal activity when tested on *F. oxysporum*. The antifungal activity remained unchanged even after 1 h at 100 °C.

Table 1 – Growth inhibition of filamentous fungi and yeasts, treated with AcAFP.

Microorganism	Zones of inhibition (mm)		Sensitivity
	50 μg ml^{-1}	200 μg ml^{-1}	
Fungi			
Fusarium oxysporum	7	ND	+++
Fusarium solani	4	–	++
Botrytis cinerea	10	–	+++
Trichoderma reesei (RutC30)	8	–	+++
Aspergillus niger	5	–	++
Stacchybotris microbiospora (A1)	12	–	+++
Alternaria solani	5	–	++
Beauveria bassiana	0	0	–
Penicillium occitanis (CT1)	0	0	–
Aspergillus nidulans (F8)	0	0	–
Yeasts			
Candida albicans	0	0	–
Saccharomyces cerevisae	0	0	–
Pichia pastoris (X33)	0	0	–

(+++) sensible; (++) moderate sensitivity; (–) insensitive.

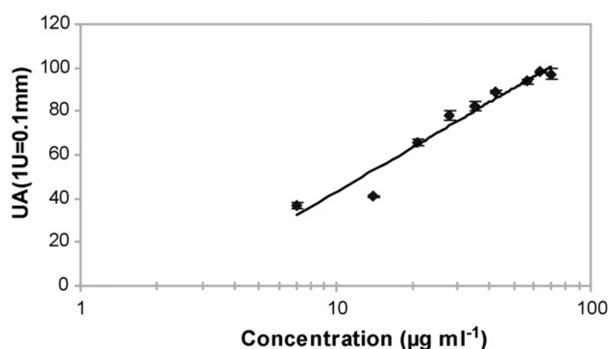

Fig. 5 – Antifungal activity of AcAFP against *Fusariuym oxysporum*. A unit corresponds to the size of the clear zone (mm) measured for each sample concentration minus the size of the central well assuming 1 unit = 0.1 mm. AcAFP was used at a concentration of 3–100 μg ml^{-1}. The date presented are representative of three independent experiments. Results are the mean ± S.E.M. of three replicates. The regression lines are calculated by the least mean squares method.

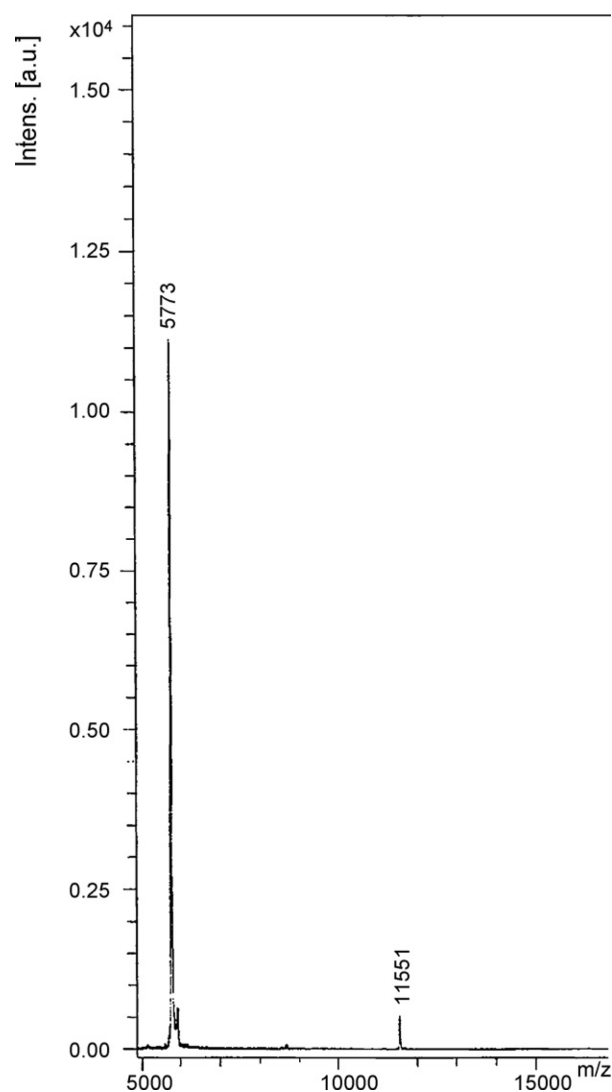

Fig. 6 – AcAFP mass spectrometry of RP-HPLC peak II.

4. Discussion

The fungal strain VR1 that showed an inhibitory activity against various fungi strains, was isolated locally as a contaminant in our laboratory. It was identified as *A. clavatus*. Its antifungal effect appears to be due to a single antifungal compound. The free cell culture supernatant heated at 70 °C for 10 min conserved its total inhibition activity towards target fungi. Moreover, the heated fraction showed an abundantly low molecular mass protein when visualized on SDS-PAGE, suggesting that *A. clavatus*, VR strain, secreted an interesting thermo-stable antifungal peptide. The antifungal peptide AcAFP was purified at homogeneity after three-step purification procedure by combination of heat treatment, ultrafiltration and RP-HPLC on C18 column. The heat treatment step was very effective since a large amount of inactive proteins were removed. The antifungal protein obtained was already highly purified after precipitation of the thermo-sensitive proteins. The purification process used was different from those previously described for the other antifungal peptide from ascomycetes fungi, which employed a cation exchange chromatography [22]. The molecular mass of AcAFP determined by MALDI-ToF spectrometry was estimated to 5773 Da which is very close to the AFP mass determined to be 5.8 kDa [16,22]. AnAFP and PAF present a molecular mass of a 6.6 and 6.3 kDa, respectively [23]. The NH_2-terminal amino acid sequence of AcAFP showed also high similarity with AFP and a moderate one with the other known antifungal peptides [19,23,24,35]. These results, suggest that our novel isolated antifungal peptide AcAFP belongs to the limited group of antifungal ascomycetes peptides. However, these antifungal peptides have many differences concerning their specificity and inhibitory spectrum towards target fungi. Indeed, AcAFP activity was not equally effective against different tested fungi as its specificity and inhibitory spectrum differed over tested fungi. No correlation of growth inhibition was observed within the members of the same genus. Indeed, *A. niger* exhibited a moderate growth inhibition, whereby *A. nidulans* was insensitive even with highest concentration of AcAFP reaching

Table 2 – N-terminal sequence comparaison of AcAFP with AFP, PAF and AnAFP.

Peptides	Microorganisms	N-terminal sequence	Reference
AcAFP	*Aspergillus clavatus*	ATYDGCKCYKKDNICKYKAQSGKT	Present work
AFP	*Aspergillus giganteus*	ATYNGCKCYKKDNICKYKAQSGKT	[26]
PAF	*Penicillium chrysogenum*	AKYTGKCTKSKNECKYKNDAG	[24]
AnAFP	*A. niger*	LSKYGGECSLEHNTCTYRKD-GKN	[19]

200 μg ml^{-1}. On the other hand, both species were shown to be moderately sensitive to the known AFP protein at 200 μg ml^{-1} [32,33] and sensitive to the PAF protein [13], only AnAFP antifungal peptide inhibited the growth of some yeast strains. No antibacterial growth was mentioned for all described antifungal peptides.

The thermostability and the wide action spectrum of the isolated peptide AcAFP constitute valuable potentialities of AcAFP to be applied in plant protection against fungi. Since *Fusarium oxisporum* was shown to be highly sensitive to AcAFP, this antifungal peptide could be considered as promising tool in the biological control of date palm against this plant pathogenic fungus responsible of the ''Bayoudh'' disease which has devastated the palm plantation in North Africa.

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