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Antimicrobial peptides and immunolocalization of a LTP in *Vigna unguiculata* seeds

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Abstract – Two cysteine-rich antimicrobial peptides (6.8 and 10 kDa) were isolated from cowpea (*Vigna unguiculata*) seeds and shown to deter development, in an in vitro assay, of the phytopathogenic fungi *Fusarium oxysporum* and *F. solani* and the yeast *Saccharomyces cerevisiae*. The peptides purified by RP-HPLC were submitted to automated N-terminal amino acid sequencing. Sequence analysis of these peptides showed the presence of a defensin and a lipid transfer protein (nsLTP) with high degree of homology to other antifungal peptides isolated from plants. LTP was detected in both cotyledon and embryonic axes of the seeds. Immunofluorescence assays also indicated that LTP was localized in the cell wall and in cytosolic compartments. In addition the presence of nsLTP was detected in seeds of different cultivars of cowpea and in three other leguminous seeds (*Vigna vexillata*, *Canavalia ensiformis* and *Phaseolus vulgaris*). © 2001 Éditions scientifiques et médicales Elsevier SAS

cowpea seeds / defensins / defence proteins / LTP / *Vigna unguiculata*

BSA, bovine serum albumin / nsLTP, non-specific lipid transfer protein / PBS, phosphate buffered saline / RP-HPLC, reverse phase high protein liquid chromatography / TFA, trifluoroacetic acid

1. INTRODUCTION

In the last years, an increasing number of cysteine-rich antimicrobial peptides have been isolated from plants and particularly from seeds [3]. Based on amino acid sequence homology, these peptides are classified in at least six different classes: peptides isolated from seeds of *Mirabilis jalapa* [6], *Amaranthus caudatus* [4], and *Zea mays* [9], members of the thionin family [11], members of the lipid transfer proteins [7], and members of the plant defensins [27]. More recently it has become increasingly clear that these peptides have an important role to play in the protection of plants against microbial infection and could prove to be useful tools for the genetic engineering of fungal resistance in transgenic plants [5, 26].

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Plant defensins are 5-kDa cysteine-rich cationic peptides and are structurally related to insect and mammalian defensins [27]. They exhibit potent antimicrobial activity in vitro and have now been isolated from over twenty different plant species from various monocot and dicot species or identified via the sequencing of cDNAs [23]. Another group of small cysteine-rich, highly basic peptides (9 kDa), that are thought to play a role in the protection of plants against microbial infections, are the non-specific lipid transfer proteins (nsLTP) [17]. A defence role for these peptides is supported by evidence of their preferential cell-wall location in epidermal cells throughout the plant and by the increased expression of their genes in response to pathogens [7, 19, 31].

Cowpea (*Vigna unguiculata*) is a tropical legume originating from Africa which is cultivated in most tropical regions of the world [24]. In Brazil, cowpea is mostly cultivated in the north-eastern region and is the

main protein source for most of the poor population in this region. Cowpea seeds are heavily attacked both in the field and in stores by the cowpea weevil (*Callosobruchus maculatus*) and by a great number of pathogens and it is thought that this high susceptibility to predation is associated with low levels of defences, either constitutive or induced [12].

In the last years, several proteins like trypsin and papain inhibitors, glucan hydrolases and variant vicilins were found in seeds of cowpea and these are traditionally linked to plant defence mechanisms or have recently been so [32]. Two recently isolated glucan-hydrolases, a chitinase of Mr 22 000 and a β-1,3-glucanase of Mr 26 000, were shown to inhibit the growth of several phytopathogenic fungi [15]. Recent results have also shown that vicilins isolated from cowpea seeds exert detrimental effects on the development of the cowpea weevil, as well as an inhibitory effect in different phytopathogenic fungi and yeast [13, 14]. Defensins, as another antimicrobial peptides such as nsLTPs, are present in cowpea seeds [3, 17]. The identification of these peptides which are associated with defence mechanisms in cowpea seeds is of great importance for the establishment of appropriate techniques for their manipulation and general utilization through classical breeding techniques or through the more recent techniques of genetic manipulation.

2. RESULTS AND DISCUSSION

2.1. Purification of the seed antifungal peptides

Several defence proteins such as trypsin and papain inhibitors, glucan hydrolases and variant vicilins were found in seeds of cowpea [12]. In this work, we investigate the presence of antimicrobial peptides in seeds of different cultivars of cowpea. Our working hypothesis is that leguminous seeds especially cowpea contain different defence proteins as well as cysteine-rich antimicrobial peptides like defensins and non-specific lipid transport protein (nsLTP). Broekaert et al. [5] and Kader [17] reported the amino acid sequence of several plant defensins and nsLTP respectively derived from N-terminal protein sequencing or deduced from cDNA clones. In these two articles, there are reports of defensins and nsLTP cDNA present in cowpea seeds.

The purification of cysteine-rich antimicrobial peptides (a defensin and a nsLTP) from cowpea seeds was performed basically by chromatographic methods. A DEAE-Sepharose was initially used for the separation of basic peptides after ammonium sulphate fractionation (*figure 1A*). The non-adsorbed fraction (FI) from the ion-exchanger step was further fractionated by gel-filtration in Sephadex G-50 that showed the presence of four different peaks named P1, P2, P3 and P4 (*figure 1B*). In a subsequent purification step, a fraction containing antifungal activity (P3) was submitted to reverse-phase chromatography (HPLC) and separated

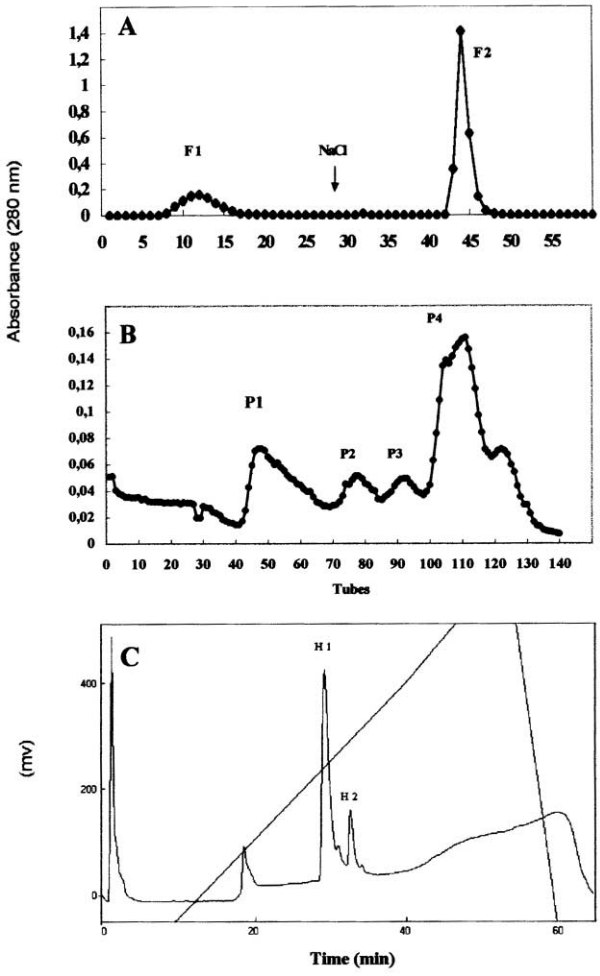

Figure 1. Purification of the antifungal peptides from cowpea seeds. **A**, DEAE-Sepharose chromatography of the F/30-70 fraction. Column previously equilibrated with 50 mM Tris-HCl buffer (pH 8.0). Elution with the 1-M sodium chloride. The flow rate was 50 mL·h^{-1} and 1 mL fractions were collected. **B**, Sephadex G-50 chromatography of the DEAE fraction F1. Column equilibrated and eluted with 20 mM Tris-HCl buffer (pH 8.0). The flow rate was 30 mL·h^{-1} and 1 mL fractions were collected. **C**, RP-HPLC chromatography. Gel-filtration fraction (P3) was applied to a C4 reverse-phase column and run in a Shimadzu apparatus. Elution was carried out as described in section 2. The line represents the acetonitrile gradient and the dark one the protein elution profile at 220 nm.

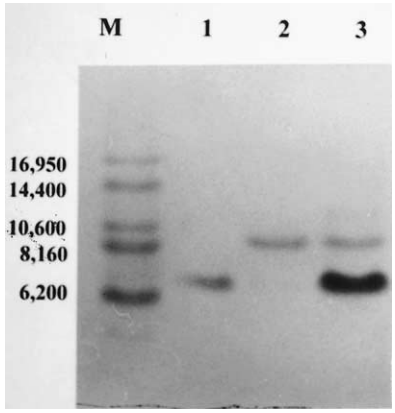

Figure 2. SDS-tricine-gel electrophoresis of purified peptides from cowpea seeds. 1, H1 peak from the HPLC chromatography; 2, H2 peak from the HPLC chromatography; 3, P3 fraction from Sephadex G-50. Side numbers in lane (M) refer to molecular mass (Da) markers.

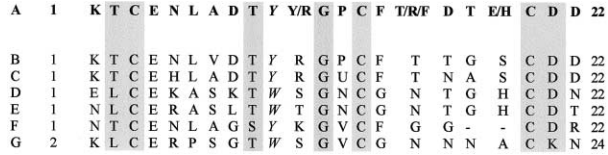

Figure 3. Comparison of defensins amino acid sequences deduced from cDNAs or protein sequencing. Cysteine and glycine residues are within boxes. Gaps were introduced for better alignment. The following sequences are presented: A, H1 (*V. unguiculata*, cv Epace-10); B, pSAS10 (*V. unguiculata*) [23]; C, Psdf1 (*Pisum sativum*) [24]; D, Dm-AMP1 (*Dhalia merckii*) [25]; E, Ct-AMP1 (*Clitoria ternatea*) [25]; F, pI230 (*P. sativum*) [26]; G, Rs-AFP1 (*Raphanus sativus*) [9].

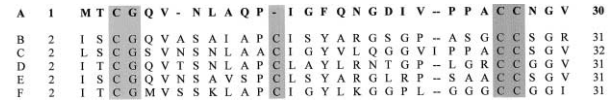

Figure 4. Comparison of lipid transfer proteins amino acid sequences deduced from cDNAs or protein sequencing. Cysteine and glycine residues are within boxes. Gaps were introduced for better alignment. The following sequences are presented: A, H2 (*V. unguiculata*, cv Epace-10); B, maize [27]; C, *Arabidopsis thaliana* [28]; D, tobacco [29]; E, rice [30]; F, spinach [31].

in two new fractions H1 and H2 (*figure 1C*). After this step the peptides were finally purified.

2.2. Characterization of the seed antifungal peptides

The isolated peptides show relative molecular masses of 6.8 (H1) and 10 kDa (H2) by SDS-tricine-gel electrophoresis respectively (*figure 2*). The values for the peptides lie in the range of molecular masses found by several authors for these antimicrobial peptides isolated from other plants [5, 17]. Purified peptides were submitted to automated N-terminal amino acid sequencing (Edman degradation using a Shimadzu liquid phase sequencer). Sequence analysis revealed that the purified peptides were a defensin (H1) and a non-specific lipid transfer protein (nsLTP) (H2) with high degree of homology to other peptides isolated from seeds. Sequence alignments of the cowpea cysteine-rich antimicrobial peptides is shown in *figures 3* [1, 8, 16, 20, 26] and *4* [2, 10, 25, 28, 30].

2.3. Effect of peptides on fungi growth

Most of the work on cysteine-rich antimicrobial peptides is based on the assumption that they are involved in defence mechanisms of plants against phytopathogenic fungi [5]. The antimicrobial activity of defensins was first reported for two isoforms isolated from radish seeds and now it has been extended for defensins from different species [16, 27]. Osborn et al. [20] showed that plant defensins isolated from many seeds cause very distinct morphological changes in some but not all fungi tested. Based on the observed antimicrobial effects on fungi, at least two groups can be distinguished: the 'morphogenic' defensins which cause reduced hyphal elongation with a concomitant increase in hyphal branching, and the 'non-morphogenic' defensins that only slow down hyphal elongation but do not induce marked morphological distortions. Antimicrobial activity of LTPs has also been reported for all tested members of the family; the relative activities of different LTPs have some degree of specificity. This was confirmed by the isolation of LTP-like proteins from cell wall preparations from the leaves of *Arabidopsis thaliana* or spinach. A synergistic effect against fungi growth was also shown when the LTPs were combined with other peptides, for example thionins [17, 22]. We tested preparations rich in both peptides for growth inhibition of phytopathogenic fungi, namely *Colletotrichum lindemuthianum* (agent of anthracnose, a disease of many plants), *Fusarium oxysporum* and *F. solani* (causing wilting in cowpea) as well as the yeast *S. cerevisiae*. *Figure 5* shows the patterns of fungi growth in the presence of P3 fractions (rich in both antimicrobial peptides) and in control medium. A high inhibitory effect on the growth of *F. oxysporum*, *S. cerevisiae* and *F. solani* was observed but growth of *C. lindemuthianum* was not inhibited. Photomicrographs of the above fungi growing in presence or absence of the P3 fraction were taken after the 44-h growth period but

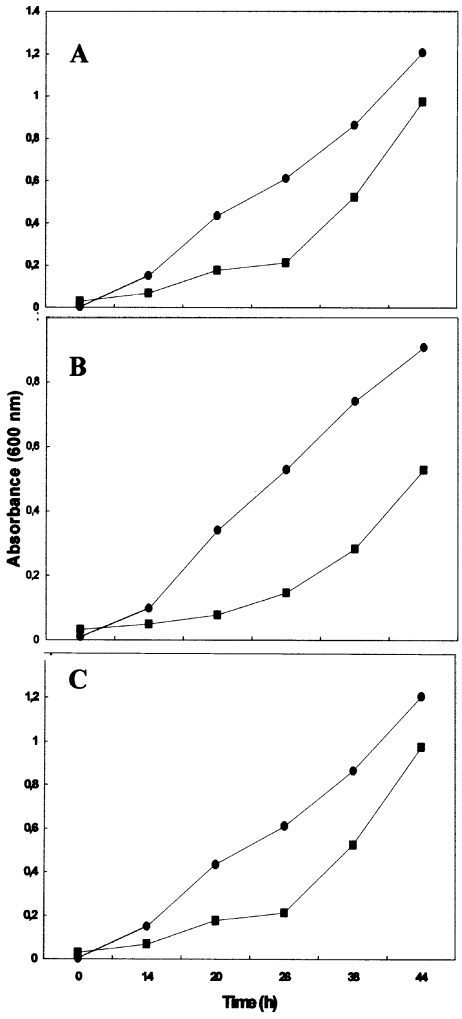

Figure 5. The effect of peptides (P3 fraction) on the growth of fungi. The absorbance at 600 nm was taken as a measure of fungal growth. (●), Control; (■), defensin plus nsLTP (20 μg·mL^{-1}). **A**, *Fusarium oxysporum*; **B**, *Fusarium solani*; **C**, *Saccharomyces cerevisiae*. Experiments were run in triplicate and the standard errors (coefficients of variation were less than 20 %) were omitted for clarity.

only those for *F. oxysporum* and *F. solani* are shown (*figure 6*). Light photographs show many hyphal morphological alterations for fungi growing in the presence of P3 fraction (*figure 6A*, part b and *6B* part b) as compared with growth on control medium (without peptides) that shows normal hyphal development (*figure 6A* part a and *6B* part a). Probably these hyphal morphological changes are attributable to the early inhibitory phase.

2.4. Organ-specific expression of antifungal peptides for indirect immunofluorescence assay in cowpea seeds

The spatial expression pattern of LTP genes has been studied in a wide range of plant species including arabidopsis, barley, broccoli, carrot, tobacco and maize. LTPs have been found to be expressed in a variety of plant organs and tissues including embryos, cotyledons, leaves, stems and flower organs [3]. Localization studies by immunocytochemical electron microscopy have also shown that LTPs are located in the cell walls, at least in various arabidopsis organs and in broccoli leaves [3, 17]. In order to determine the pattern of spatial expression of nsLTP in cowpea seeds, indirect immunofluorescence was carried out in different parts of the seeds. LTP was detected in both cotyledons (*figure 7A–D*) and embryonic axes (*figure 7E–H*). In addition, immunofluorescence assays indicated that LTP was localized not only in the cell wall (arrow) but also within the protein storage vacuoles (protein bodies) (*figure 7C, D, G, H*). The majority of LTPs are most often extracellular proteins; however, it cannot be excluded that some LTP isoforms are addressed to cytosolic compartments. For example, on the basis of immunocytochemical evidence, LTP from castor been appears partly located within the glyoxysomes [17]. However, it remains to be explained whether an LTP isoform is truly located in the cytosol and how it is addressed to this compartment.

2.5. The presence of antifungal peptides in different leguminous seeds

The presence of LTP in different cowpea cultivars (*figure 8*) and other legume seeds (*V. vexillata*, *C. ensiformis*, *P. vulgaris*) (*figure 9*) was also determined by using an anti-nsLTP serum by western blotting. On the other hand, the presence of nsLTPs in *A. pavonina*, *Albizia* sp. and *G. max* seeds was not detected (*figure 9*). The cowpea culture is strongly limited by diseases and pests. Pests and disease producing agents like fungi, bacteria and insects cause losses both in yield and quality of cowpea products [12]. The knowledge of the basis of the defence mechanisms through which these plants respond to attack by pests and pathogens is a fundamental step towards the development of suitable control methods as well as towards the understanding of the interactions between host and pathogens.

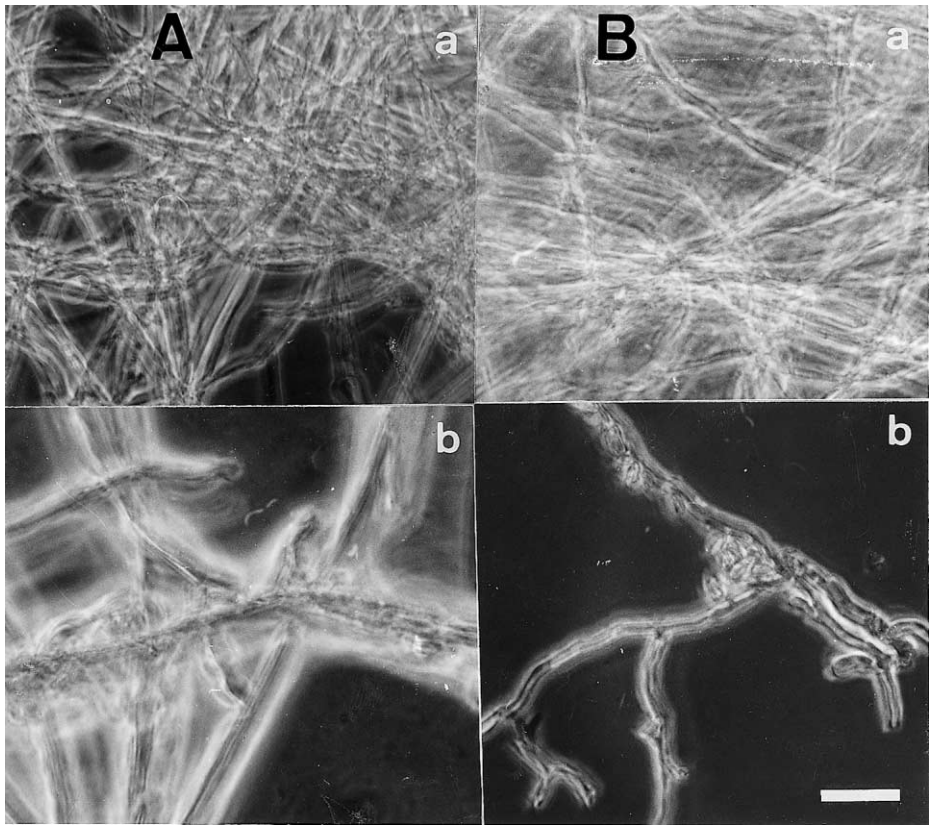

Figure 6. Light micrographs of *Fusarium oxysporum* (**A**) and *F. solani* (**B**) mycelia after 60 h of fungi growth in the presence of control medium (part a). (part b), P3 fraction (defensin plus nsLTP (20 μg·mL^{-1}). Magnification bars = 15.6 μm (a), 30 μm (b).

3. CONCLUSION

In conclusion, it is shown that *Vigna unguiculata* contains defensin and lipid transfer proteins (LTP) which inhibit the early growth of several fungi and cause many hyphal morphological alterations for fungi growing in the presence of these peptides as compared with growth on control medium. The LTPs were immunolocalized in the cell walls and in intracellular compartments like protein storage vacuoles of the cotyledons and embryonic axes. The presence of LTP in different cowpea cultivars and other legume seeds was also determined. The presence of nsLTPs in other important Brazilian seeds such as *V. vexillata*, *Canavalia ensiformis* and *Phaseolus vulgaris* was clearly detected. The findings reported in this paper on the presence of defence peptides in legume especially cowpea seeds suggest an important role for these peptides in constitutive host defence mechanisms against microbial pathogens. This may contribute to the development of biological control of fungal pathogens typical of the crop.

4. METHODS

4.1. Plant material

Cowpea (*Vigna unguiculata*) seeds of the Epace-10 and VITA-3 cultivars (which are resistant to *Cercospora cruenta* pathogen) and seeds of jack bean (*Canavalia ensiformis*) were supplied by the 'Centro de Ciências Agrárias', 'Universidade Federal do Ceará', Fortaleza, Brazil. Seeds of the cowpea line IT81D-1045, which are resistant to *Callosobruchus maculatus* (Coleoptera: Bruchidae), were obtained from IITA (International Institute of Tropical Agriculture), Ibadan, Nigeria through the 'Centro Nacional de Pesquisa Arroz-Feijão' (EMBRAPA), Goiânia, Goiás, Brazil. Seeds of soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) were commercially available local lines. Seeds of *Adenanthera pavonina*, *V. vexillata* and *Albizia* sp. were collected in the open fields of 'Campos dos Goytacazes', RJ, Brazil.

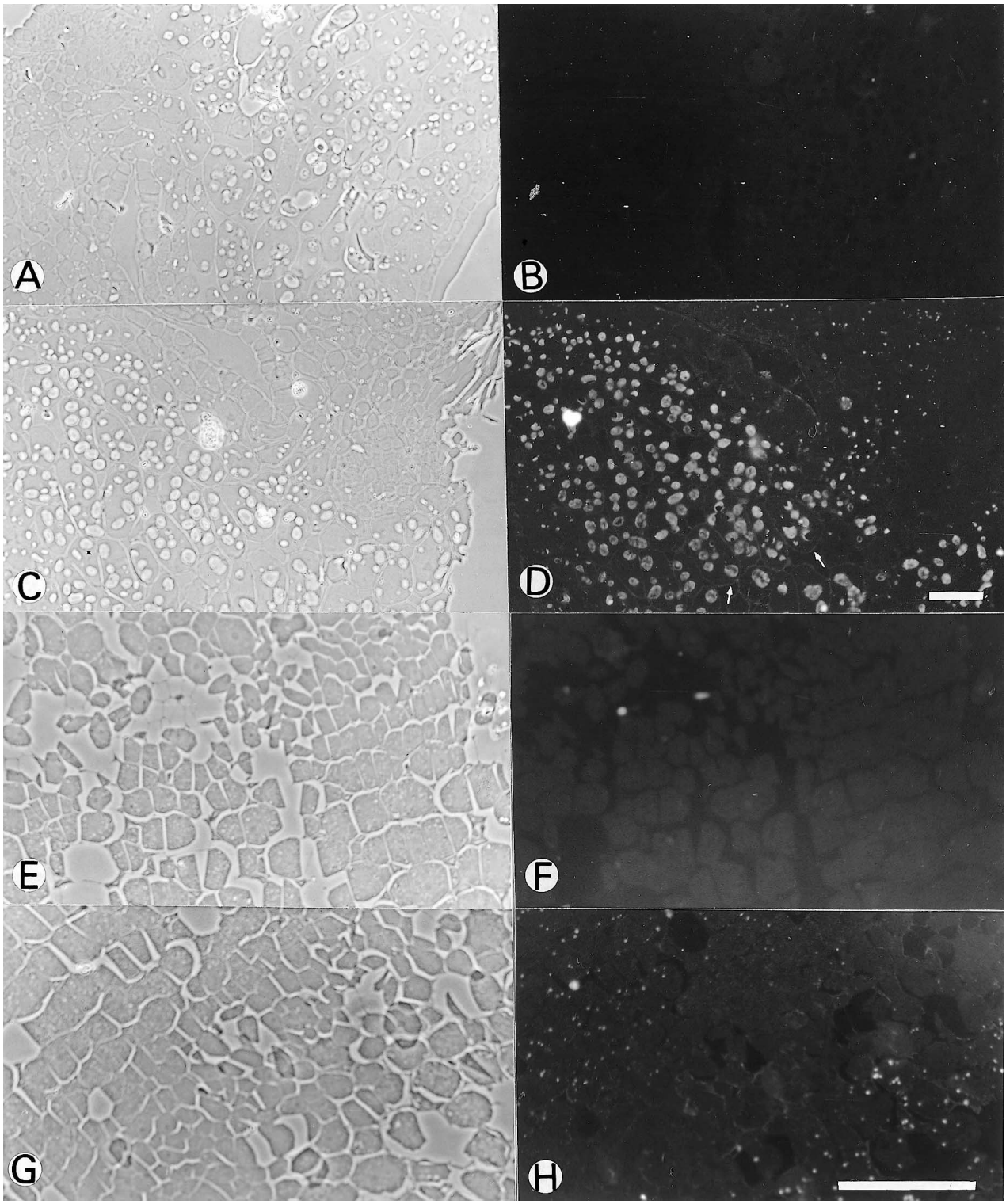

Figure 7. Immunofluorescence labelling of cotyledons (**A**–**D**) and embryonic axes (**E**–**H**) of *V. unguiculata* seeds. **A**, **C**, **E**, **G**, Section of cotyledons and embryonic axes presented in bright-field; **B**, **D**, **F**, **H**, viewed with fluorescence. **A**, **B**, **E**, **F**, Control treated with pre-immune serum; **C**, **D**, **G**, **H**, sections treated with anti-LTP serum. Bars = 25 μm.

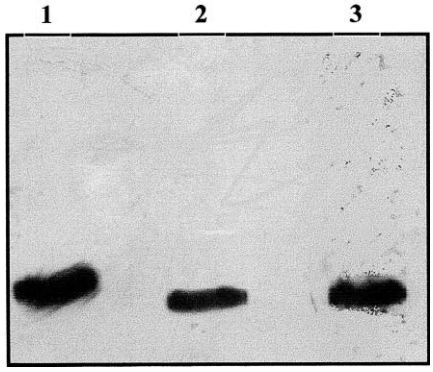

Figure 8. Western blotting of the proteins extracted from different cowpea cultivars revealed by an antiserum against purified cowpea nsLTP. Lanes 1, Epace-10; 2, VITA-3; 3, IT81D-1045.

4.2. Fungi

Fungal isolates of *Fusarium oxysporum*, *F. solani* and *Colletotrichum lindemuthianum* were kindly supplied by CNPAF/EMBRAPA, Goiania, Goiás, Brazil and *Saccharomyces cerevisiae* (1038) was obtained from the 'Departamento de Biologia', 'Universidade Federal do Ceará', Fortaleza, Brazil. The fungi were maintained on agar Sabouraud (1.0 % peptone, 2.0 % glucose and 1.7 % agar-agar). For the preparation of yeast cell cultures, an inoculum was transferred to Petri dishes containing agar Sabouraud and allowed to grow at 28 °C for 2 d; after this period, cells were transferred to sterile 0.15 M saline solution (10 mL). Yeast cells were quantified in a Neubauer chamber for further calculation of appropriate dilutions. For the preparation of conidia of *F. oxysporum*, *F. solani* and *C. lindemuthianum*, fungal cultures were transferred to Petri dishes containing agar Sabouraud for 12 d; after this period, sterile distilled water (10 mL) was added to the dishes and these were gently agitated for 1 min for spore liberation with the help of a Drigalski loop. Spores were quantified in a Neubauer chamber for appropriate dilutions.

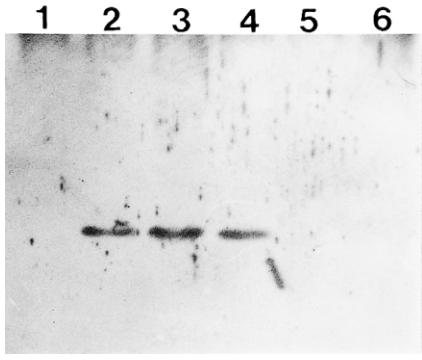

Figure 9. Western blotting of proteins extracted from some legume seeds revealed by an antiserum against purified cowpea nsLTP. Lanes 1, *A. pavonina*; 2, *V. vexillata*; 3, *P. vulgaris*; 4, *C. ensiformis*; 5, *G. max*; 6, *Albizia* sp.

4.3. Extraction and purification of the seed antifungal peptides

The purification of antifungal peptides from cowpea (Epace-10) seeds was performed basically as described by Terras et al. [27] with some modifications. Seed flours (100 g) from the different seeds were extracted for 2 h (at 4 °C) with 500 mL extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 1.5 % EDTA). Pellets formed between 30 and 70 % relative ammonium sulphate saturation were re-dissolved in distilled water and heated at 80 °C for 15 min. The resulting suspension was clarified by centrifugation and the supernatant extensively dialysed against distilled water. The dialysed solutions were recovered by freeze drying (F/30-70) and submitted to further purification by chromatographic methods. A DEAE-Sepharose column (1.5×10 cm) equilibrated with 50 mM Tris-HCl (pH 8.0) was employed for separation of proteins from the F/30-70 fraction. Elution was initially achieved by the equilibrium buffer, when we obtained fraction F1. Bound proteins (F2) were eluted with 1 M NaCl in the same buffer. F1 fraction was pooled and submitted to one cycle of gel filtration chromatography in Sephadex G-50 (column of 1.5×50 cm) equilibrated and developed in 20 mM Tris-HCl buffer (pH 8.0). The column was calibrated with several proteins of known molecular masses (bovine serum albumin, ovalbumin, soybean trypsin inhibitor and ribonuclease). The peak containing antifungal activity was collected and diluted in 0.1 % (v/v) TFA and injected onto an HPLC Vydac C4 reverse phase column (50×5 mm). The chromatography was developed at a flow rate of 0.7 mL·min^{-1} with 100 % solvent A (0.1 % TFA in water) for 10 min, 80 % solvent B (80 % acetonitrile containing 0.1 % TFA) over 55 min and finally 100 % solvent B over 10 min. Proteins were monitored by on-line measurement of the absorbance at 280 nm.

4.4. Gel electrophoresis

SDS-tricine-gel electrophoresis was performed according to the method of Schagger and Von Jagow [21].

4.5. Western blotting

Extraction of proteins from the different legume seeds was performed basically as described by Terras et al. [27]. After extraction, the material was extensively dialysed against distilled water and utilised for western blotting analysis. Antisera against purified LTP (from Epace-10 seeds) were prepared by immunization of white rabbits. Pre-immune sera were collected before immunization. After primary immunization, boosts were done and rabbit blood was obtained by puncture of the marginal ear vein of animals on each subsequent week. Total sera used for western blotting was the one taken at the fourth week after immunization [18]. Western blotting was done by transferring proteins to nitrocellulose membranes after tricine polyacrylamide gel electrophoresis, according to the method described by Towbin et al. [29].

4.6. Amino acid sequence analysis

N-Terminal amino acid sequencing was performed on a Shimadzu PPSQ-10 Automated Protein Sequencer performing Edman degradation. Sequences were determined for purified peptides blotted on PVDF membranes after tricine-gel electrophoresis. PTH-amino acids were detected at 269 nm after separation on a reverse-phase C4 column (4.6×2.5 mm) under isocratic conditions, according to the manufacturer's instructions. The sequences were compared to sequences reported in amino acid data banks and submitted to automatic alignment by using the NCBI-BLAST search system.

4.7. Effect of peptides on fungi growth

To monitor the effect of peptide rich fractions on the growth of fungi, cells and spores (20 000 in 1 mL Sabouraud broth) were incubated at 28 °C in 200-μL microplates followed by the addition of several peptides solutions ($20\ \mu g \cdot mL^{-1}$). Optical readings at 630 nm were taken at the zero time and at each 6 h for the following 44 h. A general control without addition of peptides was also used. Readings were taken against a blank containing only the culture medium. After the 44-h growth period, cells were separated from the growth medium by centrifugation, washed in 0.1 M Tris-HCl (pH 8.0) and plated for observation in an optical microscope at 400× magnification. All the experiments were run in triplicate and the reading averages, the standard errors and coefficients of variation were calculated.

4.8. Organ-specific expression of antifungal peptides for indirect immunofluorescence assay

For organ-specific localization analysis of antifungal peptides (nsLTP), cowpea (Epace-10) sections (cotyledon and embryonic axes) were first submitted to fixation for 1 h at room temperature in a solution containing 0.1 % glutaraldehyde, 2.0 % formaldehyde in 0.1 M phosphate buffer (pH 7.3), rinsed three times with 0.1 M phosphate buffer (pH 7.3), dehydrated in solutions of increasing concentrations of methanol (30–90 %) and processed for LR Gold embedding. Sections (0.8–1.0 μm thick) were laid on slides pretreated with 0.1 % poly-L-lysine (p/v) and submitted to the immunofluorescence assay. The sections were immunolabelled by treating the slides as follows: (a) PBS (phosphate saline buffer) + BSA (bovine serum albumin) buffer (10 mM phosphate buffer, 0.15 M NaCl, pH 7.5 with 1 % BSA), 30 min; (b) pre-immune serum in PBS + BSA, 20 min; (c) anti-nsLTP serum (1:50) in PBS + BSA, 2 h at room temperature; (d) ten changes of PBS + BSA, 10 min each; (e) goat anti-rabbit IgG antibody conjugated with FITC (Sigma) (1:100) in PBS + BSA, 2 h at room temperature; (f) ten changes of PBS + BSA, 5 min each; (g) PBS + BSA, 20 min; (h) deionized water, 20 min. The slides were viewed by epi-fluorescence, using a light microscope (ZEISS). Control sections were prepared by replacing the primary antiserum with pre-immune serum.

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