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Inhibition of Fungal Plant Pathogens by Seed Proteins of *Harpullia cupanioides* (Roxb.)

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Seed extracts of 50 plant species belonging to different families were evaluated for their ability to inhibit growth of *Trichoderma viride in vitro.* Of the various seed extracts, the seed extracts of *Harpullia cupanioides* (Roxb) belonging to *Sapindaceae* family exhibited very high antifungal activity. The seed extract of *H. cupanioides* strongly inhibited the growth of *Rhizoctonia solani, Curvularia lunata, Colletotrichum musae* and *Alternaria alternata.* Seed extract of *H. cupanioides* retained its antifungal activity even after heating at 100 °C for 10 min or autoclaving at 121 °C for 20 min. For partial purification of antifungal proteins, *H. cupanioides* seed extract was subjected to ammonium sulphate fractionation followed by gel filtration on Sephadex G-200 column. The fractions from sephadex G-200 were individually tested for their antifungal activity against *T. viride.* SDS-PAGE analysis of the fractions from Sephadex-G200 column indicated that the fractions with antifungal activity contained a 68-kDa band as well as other low molecular weight protein bands. The N-terminal amino acid sequence of the 68-kDa protein (13 residues) was determined by Edman degradation. However, comparison with sequences in the GenBank database (Swiss Prot) did not reveal any homology with known protein sequences.

Keywords: Antifungal protein, *Harpullia cupanioides, Trichoderma viride.*

The presence of antifungal proteins in seeds of many higher plants has long been recognized as an important factor in the protection of seeds from the invading soil-borne plant pathogens (Huynh et al., 1992). Many antifungal proteins have been purified and characterized from plant seeds including chitinases (Jacobsen et al., 1990; Huynh et al., 1992; Swegle et al., 1992), ß-1,3-glucanases (Leah et al., 1991), thaumatin-like proteins (Vigers et al., 1991; Garcia-Casado et al., 2000), thionins (Terras et al., 1996), ribosome-inactivating proteins (Leah et al., 1991), cysteine rich proteins (Duvick et al., 1992; Terras et al., 1995), defensins (Terras et al., 1992; Thevissen et al., 2000), cysteine protease inhibitor (Joshi et al., 1998), lipid transfer proteins (Cammue et al., 1995; Regente and Canal, 2000; Velazhahan et al., 2001) and 2S albumins (Wang et al., 2001). Increased knowledge about the structure and function of these antifungal proteins has implications for plant genetic engineering targeted towards development of disease resistant plants. Several studies have shown that transgenic crop plants constitutively overexpressing these antifungal proteins had enhanced resistance to pathogens than corresponding non-transformed plants (Lin et al., 1995; Chen et al., 1999; Datta et al., 2001). In the present study the antifungal activity of seed extracts of various plants belonging to different families against plant pathogenic fungi was determined. We report here for the first time the partial purification of an antifungal protein from *Harpullia cupanioides* (Roxb.) seeds.

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Materials and Methods

Fungal culture

The following fungi were obtained from the culture collection of the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore: *Trichoderma viride,* isolated from soil; *Rhizoctonia solani,* isolated from rice; *Fusarium oxysporum* f. sp. *cubense* and *Colletotrichum musae,* isolated from banana; *Alternaria alternata* and *Curvularia lunata,* isolated from tomato; *Diplodia natalensis* and *Diplocarpon rosae,* isolated from rose and *Pythium aphanidermatum,* isolated from turmeric. The cultures were maintained on potato dextrose agar (PDA) medium.

Extraction of proteins from seeds

Seeds from 50 plant species belonging to 19 different families were locally collected. Seeds were ground in a mixer and 0.5 g of the resulting ground seed was extracted in 1 ml of 5 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl and 1 mM EDTA for overnight at 4 °C (Vigers et al., 1991). The homogenates were centrifuged at 10,000 x *g* for 15 min at 4 °C and the supernatant solutions were collected. These fractions were sterilized using 0.2 µm filters (Millipore, MA, USA) and used for assay of antifungal activity. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Fungal growth inhibition assay

Growth inhibition of *T. viride* by seed extracts was determined as described by Radhajeyalakshmi et al. (2000). A spore suspension of *T. viride* was prepared in sterile distilled water and mixed with 20 ml of molten PDA medium to give final concentration of 5×10^3 conidia ml^{-1} and poured onto Petri dishes (90 mm in diameter). Sterile filter paper discs (6 mm in diameter) cut from Whatman No. 40 were laid on the agar surface one cm away from the periphery of the Petri dish and 20 µl of the supernatant fractions (50 µg protein) were applied to each disc. After the plates were incubated at room temperature for 48 h, inhibition of *T. viride* growth was measured.

Of the various seed extracts, the seed extract of *Harpullia cupanioides* (Roxb.) exhibited very high antifungal activity. Hence *H. cupanioides* seeds were chosen for further studies. The effect of different concentrations of *H. cupanioides* seed proteins (2, 5, 10, 15, 25, 40 and 50 µg) on inhibition of *Trichoderma viride* was also studied. The heat stability of the seed proteins of *H. cupanioides* was also determined by incubating protein samples for 10 min at 100 °C or by autoclaving (for 20 min at 121 °C).

For testing antifungal activity of the seed proteins of *H. cupanioides* against other fungi viz., *Alternaria alternata, Fusarium oxysporum* f. sp. *cubense, Colletotrichum musae, Diplodia natalensis, Diplocarpon rosae, Pythium aphanidermatum, Rhizoctonia solani* and *Curvularia lunata,* a 9-mm mycelial disc cut from a 5- to 7-day-old fungal culture was placed in the center of petridishes (90 mm in diameter) containing PDA medium. The plates were incubated for 24 h at room temperature (28 ± 2 ºC). At this time,

sterile filter paper discs (6 mm in diameter) were laid on the agar surface at 1 cm away from the periphery of the petridish and 20 µl of the supernatant fraction (50 µg of protein) was applied to each disc and the plates were further incubated at room temperature and monitored for inhibition of fungal growth.

Partial purification of antifungal proteins

Seeds (500 g) were homogenized with a blender by using 1200 ml of 0.1 M sodium phosphate buffer (pH 7.0). The homogenate was squeezed through a muslin cloth and then centrifuged for 20 min at 10,000 x *g*. Solid ammonium sulphate was added to the supernatant to yield 0–20, 20–40, 40–60 and 60–80% saturation and kept at 4 °C for overnight. Each fraction was obtained by centrifugation at 10,000 x *g* for 20 min at 4 °C. The precipitates were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) and dialyzed against the same buffer overnight at 4 °C. Each ammonium sulphate fraction was tested for its antifungal activity against *T. viride.* The major activity was in the 60–80 (i.e. 60–80% ammonium sulphate saturation) fraction. Hence the proteins from 60–80 fraction were separated by gel filtration using Sephadex G-200. Proteins were loaded onto a Sephadex G-200 column (30 x 1 cm) equilibrated with 1 mM Tris (pH 7.5) buffer and eluted with the same buffer. Fractions of 2 ml were collected using Bio-Rad automated Econo system (Bio-Rad, USA). Fractions containing proteins, as monitored by the optical density at 280 nm were subjected to 12% SDS-PAGE using a Mighty Small II unit (Hoefer, California, USA) according to the method of Laemmli (1970). Gels were run at 20 mA for 2 h and stained for protein with Coomassie Brilliant blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid. Apparent molecular mass of proteins was determined by comparison with molecular weight standards. Each fraction was tested for its ability to inhibit the growth of *T. viride in vitro* as described earlier. Analysis by SDS-PAGE of the active fractions showed that most of the active fractions contained a high molecular weight protein (68 kDa). The N-terminal sequence of the 68-kDa protein was determined.

N-terminal sequencing

Partially purified proteins were subjected to 12% SDS-PAGE and then they were electroblotted onto a PVDF (Immobilon-P, Millipore, Bedford, MA) membrane using a Bio-Rad semi-dry transblot unit (Bio-Rad, USA). The membrane was stained with Coomassie blue and the 68-kDa band was excised and then subjected to automated Edman degradation using an Applied Biosystems Sequencer at the Biotechnology Microchemical Core Facility, Kansas State University, Manhattan, Kansas, USA. Database search was performed with the BLAST 2.0 service from the National Centre for Biotechnology Information World Wide Web server.

Results and Discussion

In recent years, several proteins with antifungal properties have been detected in plant seeds (Joshi et al., 1998; Son et al., 1998; Lucca et al., 1999; Oliveira et al., 1999;

Regente and Canal, 2000; Velazhahan et al., 2001). In the present study, seed extracts of fifty plant species belonging to 19 different families were evaluated for their ability to inhibit the growth of *T. viride in vitro.* Of the various seed extracts, the seed extracts of *Harpullia cupanioides* (Roxb.), *Pennisetum glaucum* (L.) R. Br.) and *Sorghum bicolor* (L.) Moench alone showed antifungal activity against *T. viride* and recorded inhibition zones of 42 mm, 17 mm and 15 mm, respectively. The seed extract of *H. cupanioides* exhibited antifungal activity against *T. viride* even at 2 µg level *(Fig. 1).* The seed extract of *H. cupanioides* also exhibited strong antifungal activity against other fungal pathogens

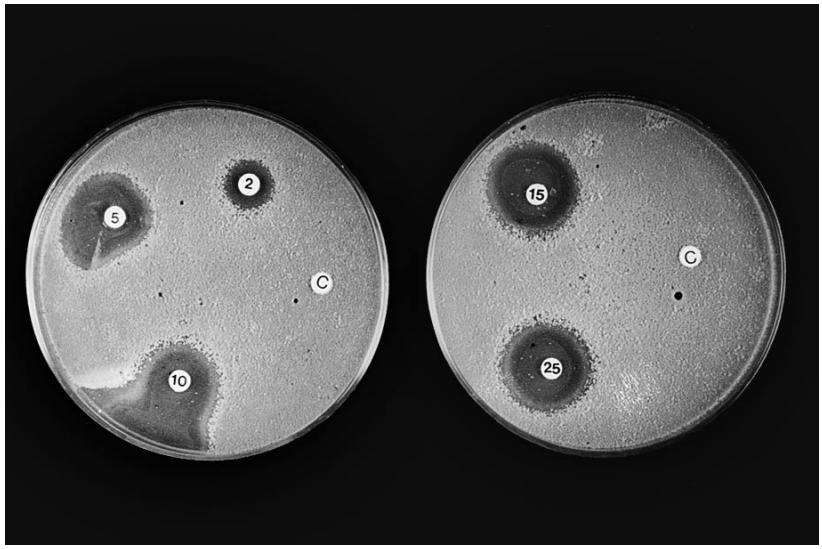

Fig. 1. Antifungal activity of various concentrations (µg) of seed proteins of *Harpullia cupanioides* against *Trichoderma viride.* C – control

viz., *Rhizoctonia solani, Curvularia lunata, Colletotrichum musae* and *Alternaria alternata* but not against *Pythium aphanidermatum, Diplodia natalensis, Fusarium oxysporum* f. sp. *cubense* and *Diplocarpon rosae (Table 1).* Seed proteins of *H. cupanioides* heated for 10 min at 100 °C or autoclaved for 20 min at 121 °C retained antifungal activity (data not shown). For partial purification of antifungal proteins, *H. cupanioides* seeds were extracted with phosphate buffer and the proteins were fractionated using ammonium sulphate. *In vitro* antifungal activity of different ammonium sulphate fractions was assessed against *T. viride*. The maximum antifungal activity was observed in the 60–80% fraction (i.e. 60–80% ammonium sulphate saturation) *(Table 2).* The 60–80% ammonium sulphate fraction was subjected to gel filtration on Sephadex-G200. The fractions from sephadex-G200 were individually tested for their antifungal activity against *T. viride*. Antifungal activity was found within the range of fractions 16–26 and corresponded to UV absorption peak *(Fig. 2).* SDS-PAGE of the fractions from

Table 1

Inhibitory effects of seed proteins of *Harpullia cupanioides* on hyphal growth of various fungi

Fungi	Inhibition zone (mm)
Rhizoctonia solani	40 b
Alternaria alternata	33 e
Curvularia lunata	37 c
Fusarium oxysporum f. sp. *cubense*	0 f
Pythium aphanidermatum	0 f
Colletotrichum musae	35 d
Diplodia natalensis	0 f
Diplocarpon rosae	0 f
Trichoderma viride	42 a

Means with in a column followed by a common letter are not significantly different (P = 0.05) by Duncan's multiple range test.
The data are mean of three replications.

Table 2

In vitro antifungal activity of ammonium sulphate fractions obtained from seed extracts of *Harpullia cupanioides* against *Trichoderma viride*

Fraction	Inhibition zone (mm)
0–20	25 c
20–40	30 b
40–60	31 b
60–80	40 a

Means with in a column followed by a common letter are not significantly different (P = 0.05) by Duncan's multiple range test.
The data are mean of six replications.

Sephadex-G200 column indicated that the fractions with antifungal activity contained a 68-kDa band as well as other low molecular weight protein bands *(Fig. 3).* Therefore, it is most likely that the 68-kDa protein observed in the active fractions plays an important role in the antifungal activity of the seed extract of *H. cupanioides*. The N-terminal amino acid sequence of the 68-kDa protein was determined by Edman degradation. The sequence is as follows: GVFEELYFDDGYK. A comparison with sequences in the GenBank database (Swiss Prot) did not reveal any homology with known protein sequences. Voutquenne et al. (1998) isolated five saponins from the bark of *H. cupanioides* and elucidated the structures by analysis of 2D NMR spectra and mass spectra. The results of the present study provide evidence for the existence of antifungal proteins in seeds of *H. cupanioides*. The seed proteins of *H. cupanioides* strongly inhibited the growth of other agronomically important fungal pathogens like *Rhizoctonia*

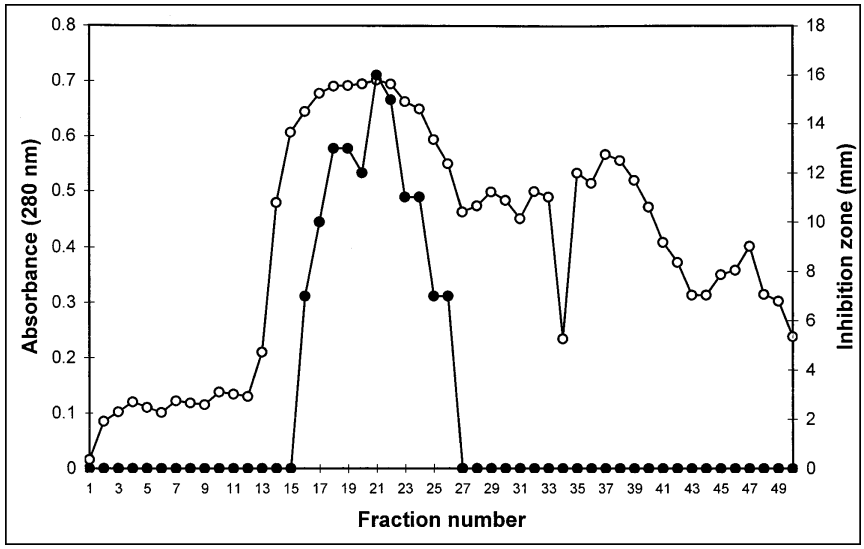

Fig. 2. Fractionation of seed proteins of *Harpullia cupanioides* on Sephadex G-200. – ❍ –, absorbance at 280 nm; – ● –, antifungal activity against *T. viride* (inhibition zone in mm)

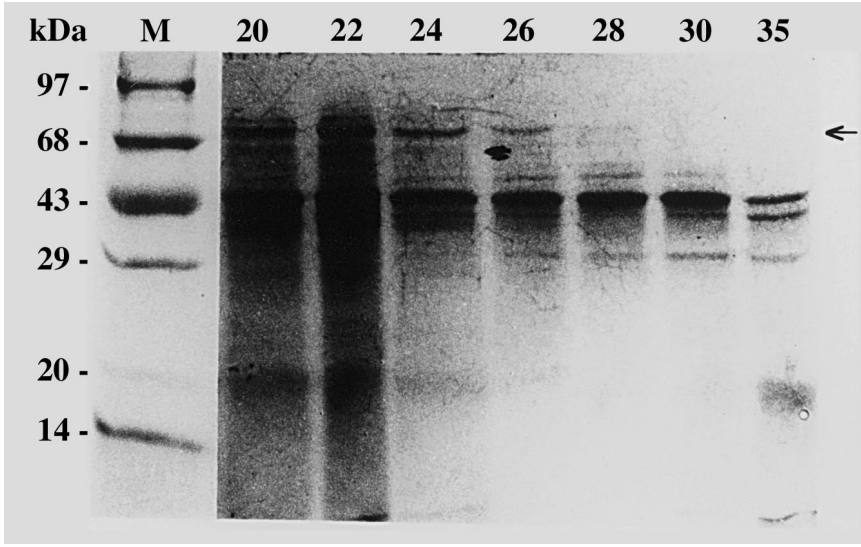

Fig. 3. Coomassie blue-stained SDS-PAGE of fractions from Sephadex G-200. Lane 1, Molecular weight marker; Lanes 2–8, fractions from Sephadex G-200 column

solani, Curvularia lunata, Colletotrichum musae and *Alternaria alternata* indicating that the genes encoding for these antifungal protein(s) may be the promising candidates for genetic engineering of fungal resistant crops.

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Literature

Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cammue, B. P. A., Thevissen, K., Hendriks, M., Eggermont, K., Goderis, I. J., Proost, P., Van Damme, J., Osborn, R. W., Guerbette, F., Kader, J. C. and Broekaert, W. F. (1995): A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. Plant Physiol. 109, 445–455.

Chen, W. P., Chen, P. D., Liu, D. J., Kynast, R., Friebe, B., Velazhahan, R., Muthukrishnan, S. and Gill, B. S. (1999): Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene. Theor. Appl. Genet. 99, 755–760.

Datta, K., Tu, J., Oliva, N., Ona, I., Velazhahan, R., Mew, T. W., Muthukrishnan, S. and Datta, S. K. (2001): Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars. Plant Sci. 160, 405–414.

Duvick, J. P., Rood, T., Rao, A. G. and Marshak, D. R. (1992): Purification and characterization of a novel antimicrobial peptide from maize (*Zea mays* L.) kernels. J. Biol. Chem., 267, 18814–18820.

Garcia-Casado, G., Collada, C., Allona, I., Soto, A., Casado, R., Rodriguez-Cerezo, E., Gomez, A. and Aragoncillo, C. (2000): Characterization of an apoplastic basic thaumatin-like protein from recalcitrant chestnut seeds. Physiol. Plant. 110, 172–180.

Huynh, Q. K., Borgmeyer, J. R. and Zobel, J. F. (1992): Isolation and characterization of a 22 kDa protein with antifungal properties from maize seeds. Biochem. Biophys. Res. Commun. 182, 1–5.

Jacobsen, S., Mikkelsen, J. D. and Hejgaard, J. (1990): Characterization of two antifungal endochitinases from barley grain. Physiol. Plant. 79, 554–562.

Joshi, B. N., Sainani, M. N., Bastawade, K. B., Gupta, V. S. and Ranjekar, P. K. (1998): Cysteine protease inhibitor from pearl millet: a new class of antifungal protein. Biochem. Biophys. Res. Commun. 246, 382–387.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277, 680–684.

Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. (1991): Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. 266, 1564–1573.

Lin, W., Anuratha, C. S., Datta, K., Potrykus, I., Muthukrishnan, S. and Datta, S. K. (1995): Genetic engineering of rice for resistance to sheath blight. Bio/Technology 13, 686–691.

Lucca, A. J. De., Jacks, T. J. and Broekaert, W. J. (1999): Fungicidal and binding properties of three plant peptides. Mycopathologia 144, 87–91.

Oliveira, A. E. A., Gomes, V. M., Sales, M. P., Fernandes, K. V. S., Carlini, C. R. and Xavier-Filho, J. (1999): The toxicity of jack bean [*Canavalia ensiformis)* (L.) DC] canatoxin to plant pathogenic fungi. Revista Brasileira de Biologica 59, 59–62.

Radhajeyalakshmi, R., Meena, B., Thangavelu, R., Deborah, S. D., Vidhyasekaran, P. and Velazhahan, R. (2000): A 45-kDa chitinase purified from pearl millet [*Pennisetum glaucum* (L.) R. Br.] shows antifungal activity. J. Plant Dis. Protect. 107, 605–616.

Regente, M. C. and Canal, L. De La. (2000): Purification, characterization and antifungal properties of a lipid-transfer protein from sunflower *(Helianthus annuus)* seeds. Physiol. Plant. 110, 158–163.

Son, D. Y., Shin, B. C., Yun, D. J., Seong, K. Y. and Chung, Y. R. (1998): Purification and characterization of an antifungal peptide from the seeds of *Phytolacca americana.* Korean J. Plant Pathol. 14, 203–208.

Swegle, M., Kramer, K. J. and Muthukrishnan, S. (1992): Properties of barley seed chitinases and release of embryo-associated isoforms during early stages of imbibition. Plant Physiol. 99, 1009–1014.

Terras, F. R. G., Eggermont, K., Kovaleva, V., Raikhel, N. V., Osborn, R. W., Kester, A., Rees, S. B., Vanderleyden, J., Cammue, B. P. A. and Broekaert, W. F. (1995): Small cysteine-rich antifungal proteins from radish: their role in host defence. Plant Cell 7, 573–588.

Terras, F. R. G., Schoofs, H., De Bolle, M. F. C., Van Leuven, F., Rees, S. B., Vanderleyden, J., Cammue, B. P. A. and Broekaert, W. F. (1992): Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J. Biol. Chem. 267, 15301–15309.

Terras, F. R. G., Torrekens, S., Van Leuven, F. and Broekaert, W. F. (1996): A six-cysteine type thionin from the radish storage organ displays weak *in vitro* antifungal activity against *Fusarium culmorum.* Plant Physiol. Biochem. 34, 599–603.

Thevissen, K., Osborn, R. W., Acland, D. P. and Broekaert, W. F. (2000): Specific binding sites for an antifungal plant defensin from dahlia *(Dahlia merckii)* on fungal cells are required for antifungal activity. Mol. Plant-Microbe Interact. 13, 54–61.

Velazhahan, R., Radhajeyalakshmi, R., Thangavelu, R. and Muthukrishnan, S. (2001): An antifungal protein purified from pearl millet seeds shows sequence homology to lipid transfer proteins. Biol. Plant. 44, 417–421.

Vigers, A. J., Roberts, W. K. and Selitrennikoff, C. P. (1991): A new family of plant antifungal proteins. Mol. Plant Microbe Interact. 4, 315–323.

Voutquenne, L., Lavaud, C., Massiot, G. and Delaude, C. (1998): Saponins from *Harpullia cupanioides*. Phytochemistry 49, 2081–2085.

Wang, X., Bunkers, G. J., Walters, M. R. and Thoma, R. S. (2001): Purification and characterization of three antifungal proteins from cheeseweed *(Malva parviflora).* Biochem. Biophys. Res. Commun. 282, 1224–1228.