Acta Phytopathologica et Entomologica Hungarica 38 (3–4), pp. 251–257 (2003)

Existence of Thaumatin-like Proteins (TLPs) in Seeds of Cereals

R. RADHAJEYALAKSHMI, K. YAMUNARANI,
K. SEETHARAMAN and R. VELAZHAHAN*

Department of Plant Pathology, Agricultural College and Research Institute,
Tamil Nadu Agricultural University, Madurai-625 104, Tamil Nadu, India

Seed extracts of pearl millet, sorghum, Japanese barnyard millet, foxtail millet, samai and proso millet were evaluated *in vitro* for their ability to inhibit the growth of *Rhizoctonia solani, Macrophomina phaseolina* and *Fusarium oxysporum.* Among them, seed extracts of pearl millet and sorghum were highly effective in inhibiting the growth of all three examined phytopathogenic fungi. The seed extracts were tested for the presence of thaumatin-like proteins (TLPs) by Western blot analysis using bean TLP antiserum. Results of Western blot analysis indicated the presence of a 23-kDa TLP in seeds of pearl millet, sorghum and Japanese barnyard millet. The 23-kDa TLP was more abundant in the seeds of pearl millet. The distribution of TLP in various parts of pearl millet was analyzed by Western blotting. The results indicated that the 23 kDa TLP was predominantly expressed in seeds and inflorescence of pearl millet.

Keywords: Antifungal protein, pathogenesis-related protein, pearl millet, *Pennisetum glaucum.*

Proteins with the ability to inhibit the growth of phytopathogenic fungi are abundantly present in plant seeds. These antifungal proteins include chitinases (Anuratha et al., 1992; Swegle et al., 1992; Shih et al., 2001b), ß-1,3-glucanases (Leah et al., 1991; Akiyama et al., 1996), thionins (Carmona et al., 1993; Terras et al., 1996), thaumatin-like proteins (Roberts and Selitrennikoff, 1990; Vigers et al., 1991; Shih et al., 2001a), ribosome-inactivating proteins (Leah et al., 1991), cysteine-rich proteins (Duvick et al., 1992; Terras et al., 1995), defensins (Terras et al., 1992; Osborn et al., 1995), 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). The presence of hard seed coat and the low water content of the seeds provide effective barrier against invasion by fungal pathogens. However, when plant seeds are sown in soil, these barriers are gradually disrupted during the imbibition phase of seed germination and then protection of germinating seeds from the invading soil-borne plant pathogens mainly depends on these antifungal proteins (Terras et al., 1992; Cammue et al., 1995). In the present study, the antifungal activities of seed extracts of pearl millet, sorghum, Japanese barnyard millet, foxtail millet, samai and proso millet against *Rhizoctonia solani, Macrophomina phaseolina* and *Fusarium oxysporum* were evaluated and the presence of thaumatin-like proteins (TLPs) in these seed extracts was analyzed.

*Corresponding author

0238–1249/2003/$ 20.00

Materials and Methods

Plant material

Seeds of pearl millet [*Pennisetum glaucum* (L.) R. Br.] cv. Co-7, sorghum [*Sorghum bicolor* (L.) Moench] cv. Co-26, kudiravali or Japanese barnyard millet (*Echinochloa frumentacea* L.) cv. Co-1, tenai or foxtail millet [*Setaria italica* (L) Beauv] cv. Co-5, samai (*Panicum miliare* L.) cv. Co-3 and panivaragu or proso millet (*Panicum miliaceum* L.) cv. Co-2 were obtained from the Millets Breeding Station, TNAU, Coimbatore, India.

Fungal culture

The following fungi were obtained from the culture collection of the Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai: *Rhizoctonia solani,* isolated from rice; *Macrophomina phaseolina,* isolated from groundnut and *Fusarium oxysporum* isolated from yam. The cultures were maintained on potato dextrose agar (PDA) medium.

Extraction of proteins from seeds

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 *g* for 15 min at 4 °C and the supernatant solutions were collected. These fractions were sterilized using 0.2-µm pore-size 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

A 9-mm-diameter disc containing 7-day-old fungal culture grown in PDA medium was transferred to the center of each Petri dish (90 mm in diameter) containing PDA medium. The plates were incubated for 1 day at 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 Petri dish and 20 µl of the supernatant fraction (50 µg of protein) was applied to each disc. The plates were further incubated at room temperature and monitored for inhibition of fungal growth.

Western blotting

Seeds were ground in a mixer and 0.5 g of the resulting ground seed was extracted in 1 ml of 25 mM sodium phosphate buffer (pH 7.0) for 3 hr at 4 ºC. The homogenate was then centrifuged at 10,000 *g* for 15 min and the supernatant solutions were collected. Protein concentrations were determined by Bradford assay (Bradford, 1976) using bovine serum albumin as a standard. Proteins (100 µg) were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mighty Small II gel electrophoresis unit

(Hoefer Scientific Instruments, San Francisco, CA, USA) with 12% acrylamide resolving gel and 4% acrylamide stacking gel according to the method of Laemmli (1970). The gels were electrophoresed for 2 h at a constant current of 20 mA. After electrophoresis, the proteins were electrotransferred to a polyvinylene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) for 30 min at 140 mA in a Bio-Rad semi-dry transblot apparatus (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturers instructions. The membrane was then blocked with Tris-buffered saline (10 mM Tris-HCl, pH 7.9, 140 mM NaCl) (TBS) containing 0.05% (v/v) Tween-20 (TBST) supplemented with 2.5% (w/v) gelatin for 2 h at 28 ± 2 °C, then washed (5 min) three times with TBST. The membrane was incubated in TBST containing primary antibody at 1:1000 dilution for overnight. Antiserum raised against a bean TLP (a gift of Dr. O. P. Sehgal, University of Missouri, Columbia, MO, USA) was used as primary antibody. The membrane was washed five times with TBST for 5 min. It was then incubated in TBST containing horse radish peroxidase conjugated goat-anti rabbit IgG (Bio-Rad, Hercules, CA, USA) at 1:1000 dilution for 3 h, washed (5 min) thrice with TBST and twice with TBS. Binding of the secondary antibody was detected by reaction of the antibody-HRP-conjugate with freshly prepared substrate solution consisting of 15 µl of 30% H_2O_2, 5 ml of 0.3% (w/v) 4-chloro-1-naphthol (Bio-Rad, Hercules, CA, USA) in methanol, and 25 ml of TBS for 3–5 min. Apparent molecular mass of proteins was determined by comparison with molecular weight standards (Rainbow markers, Amersham pharmacia, USA)

Results and Discussion

Antifungal proteins have been identified in seeds of several agronomically important monocot plants. Roberts and Selitrennikoff (1990) purified a 22-kDa antifungal protein (zeamatin) from *Zea mays* seeds. Vigers et al. (1991) reported the presence of a 22-kDa TLP in sorghum (sormatin), oats (avematin) and wheat (trimatin) seeds cross reacting with zeamatin antiserum. In the present study, seed extracts of pearl millet, sorghum, Japanese barnyard millet, foxtail millet, samai and proso millet were evaluated for their ability to inhibit the growth of three important phytopathogenic fungi viz., *R. solani, M. phaseolina* and *F. oxysporum.* Of the various seed extracts, the ones of pearl millet and sorghum exhibited high antifungal activity towards all three examined phytopathogenic fungi *(Table 1).* Seed extract of foxtail millet did not have antifungal effect on *M. phaseolina* and *F. oxysporum.*

Many plant seeds are known to possess TLPs (for review see, Velazhahan et al., 1999). TLPs are PR-5 group of pathogenesis-related (PR) proteins that show sequence homology to thaumatin, a sweet tasting protein isolated from a West African shrub *Thaumatococcus danielli.* TLPs have low molecular mass, i.e. 15 to 32 kDa, and are generally resistant to proteases and pH- or heat-induced denaturation. TLPs have been shown to exhibit antifungal activity against a wide range of fungi (Roberts and Selitrennikoff, 1990; Vigers et al., 1992; Ye et al., 1999). In the present study Western blot analysis of protein extracts of pearl millet, sorghum and Japanese millet seeds resulted in the detec-

Table 1

In vitro antifungal activity of seed extracts against *Rhizoctonia solani, Macrophomina phaseolina* and *Fusarium oxysporum*

Seed		Inhibition zone (mm)*		
		R. solani	*M. phaseolina*	*F. oxysporum*
Pearl millet	*Pennisetum glaucum*	14 b	19 a	20 b
Sorghum	*Sorghum bicolor*	21 a	18 b	23 a
Proso millet	*Panicum miliaceum*	10 e	18 b	10 e
Samai	*Panicum miliare*	11 d	10 d	15 c
Foxtail millet	*Setaria italica*	10 e	0 e	0 f
Japanese barnyard millet	*Echinochloa frumentacea*	13 c	11 c	13 d
Control		0 f	0 e	0 f

Mean within 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.

tion of a ca. 23-kDa TLP cross-reacting with bean TLP antiserum *(Fig. 1).* This 23-kDa TLP was abundantly expressed in the seeds of pearl millet, as a large, intense band that may be a doublet. This TLP could not be detected in seed extracts of foxtail millet, proso millet and samai. In order to study the distribution of TLP in various parts of pearl millet, protein extracts prepared from boot leaf, seeds, inflorescence, leaves, leaf sheaths and stem were analyzed in the above-mentioned manner. The results indicated that the 23-

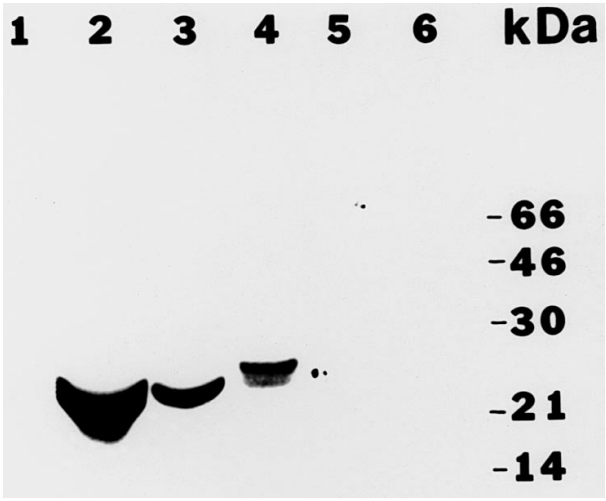

Fig. 1. Western blot showing constitutive expression of thaumatin-like proteins in various seed extracts. Each lane contains 100 µg of proteins from seeds of foxtail millet (lane 1), pearl millet (lane 2), sorghum (lane 3), Japanese millet (lane 4), proso millet (lane 5) and Samai (lane 6). Molecular mass markers are shown on the right

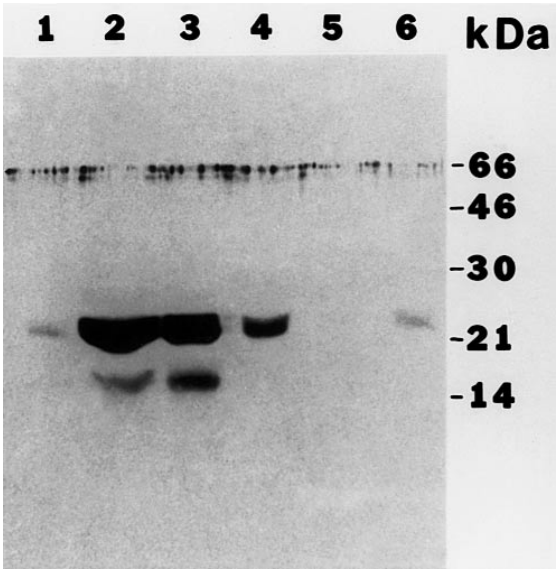

Fig. 2. Immuno blot showing constitutive expression of thaumatin-like proteins in various parts of pearl millet. Each lane contains 100 µg of proteins from boot leaf (lane 1), seeds (lane 2), inflorescence (lane 3), leaf (lane 4), leaf sheath (lane 5) and stem (lane 6). Molecular mass markers are shown on the right

kDa TLP was predominantly expressed in seeds and inflorescence *(Fig. 2).* In addition to the 23-kDa TLP, extracts of pearl millet seeds and inflorescence showed another protein with a molecular mass of 17 kDa cross-reacting with bean TLP antiserum. At a lower intensity leaves also constitutively expressed the 23-kDa TLP *(Fig. 2).*

Many antifungal proteins have been detected in pearl millet (Joshi et al., 1998; Kini et al., 2000; Radhajeyalakshmi et al., 2000; Velazhahan et al., 2001). Joshi et al. (1998) purified a cysteine protease inhibitor with a molecular mass of 24 kDa and an isoelectric point (pI) of 9.8. The purified protein exhibited antifungal activity against *Trichoderma reesei* as well as against a number of phytopathogenic fungi. Kini et al. (2000) purified a major isoform of ß-1,3-glucanase from *Sclerotinia graminicola*-inoculated pearl millet seedlings. The purified protein had a molecular mass of 20.5 kDa with a pI of 9.6. Radhajeyalakshmi et al. (2000) reported constitutive expression of a 30-kDa chitinase in the seeds and a 45-kDa chitinase in the inflorescence of pearl millet. They further purified the 45-kDa chitinase from the pearl millet inflorescence and demonstrated its antifungal activity against *Trichoderma viride* and *Rhizoctonia solani.* Velazhahan et al. (2001) purified an antifungal 23-kDa lipid transfer protein (LTP) from the seeds of the same species. In the present study we report the existence of TLP in pearl millet for the first time. Further purification and characterization of the ca. 23-kDa TLP from pearl millet seeds are in progress.

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