In Vitro Activity of Sorgum Seed Antifungal Proteins against Grain Mold Pathogens

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Several sorghum seed antifungal proteins (AFP), including sormatin, chitinase, glucanase, and ribosome-inhibiting protein (RIP), were extracted, partially purified using sequential ammonium sulfate precipitation, and eluted from a CM-Sephadex column using a linear salt gradient. Eluted fractions were tested for bioactivity against *Fusarium moniliforme*, *Curvularia lunata*, and *Aspergillus flavus* using hyphal rupture, hyphal extension, and spore germination methods. A fraction containing several AFPs was most inhibitory against *F. moniliforme*, *C. lunata*, and *A. flavus*. Spore germination of all three species was markedly inhibited by 360 ppm of AFPs. The inhibitory effects were not observed when the protein fraction was boiled, suggesting the involvement of undenatured proteins. Hyphal elongation inhibition was the least sensitive assay to detect the inhibitory effects of AFPs on these fungi. *F. moniliforme* exhibited hyphal rupture at the growing tip at protein levels as low as 70 ppm. *C. lunata* required higher protein levels (70−360 ppm) and ruptured only at hyphal tips. *A. flavus* did not exhibit hyphal disruption when treated with AFPs.

Keywords: *Antifungal proteins; sormatin; chitinase; glucanase; sorghum; Fusarium moniliforme; Curvularia lunata; bioactivity*

INTRODUCTION

Antifungal proteins (AFPs) are components of the defense mechanisms of plants against pathogens. Numerous studies, in leaf and other nonstorage tissues, have shown that AFP levels change in plants in response to biotic and abiotic stress (Abeles et al., 1970; Pegg and Young, 1992; Mauch et al., 1984). However, changes in AFP levels are symptomatic in some instances (Ignatius et al., 1984) and causal in others (Mauch et al., 1988a). AFPs, individually or in combination, inhibit fungi *in vitro* (Mauch et al., 1988b; Leah et al., 1991). More recently, transgenic plants with one or more AFPs have been successfully created, and they limit the extent of disease and symptoms (Broglie et al., 1991; Logemann et al., 1992; Jach et al., 1995; Lin et al., 1995; Grison et al., 1996). Therefore, AFPs can be utilized to enhance disease resistance of plants.

Our long-term interest is to determine if AFPs can play a role in limiting infection of caryopses by fungi. Most studies pertaining to AFPs have been conducted in nonstorage tissues, and information pertaining to changes in AFPs in caryopses, i.e., their levels, and bioactivity on seed pathogens is limited (Mauch et al., 1988a; Bass et al., 1992; Cordero et al., 1994). Studies in our laboratory (Seetharaman et al., 1996) on sorghum caryopses have shown that the levels of three AFPs (sormatin, chitinase, and glucanase) peaked at physiological maturity [30 days after anthesis (DAA)], while ribosome-inactivating protein (RIP) levels peaked around midmaturity (15 DAA). Further, AFPs appeared to be mobile within the caryopses, moving outward from the endosperm upon imbibition. Results from these investigations suggest that understanding the interplay between AFP levels in caryopses, i.e., stage of maturity and environment (humidity/imbibition), and time of pathogen infection is important to maximize the bioactivity of AFPs *in vivo*.

After the presence of AFPs in caryopses had been established and the changes taking place during caryopses development and maturity had been documented, it was necessary to determine if these proteins were inhibitory against the sorghum grain molding pathogens. Sorghum grain molding is a serious problem affecting yield and quality of sorghum around the world. Grain mold infections result in poor seed quality, ranging from cosmetic deterioration of the pericarp to substantial deterioration in quality attributes (Rooney and Serna-Saldivar, 1991) such as smaller seed size, lower test weight, endosperm density, and decortication yield, increased amylase, protease, and lipase activities, and darker color of milled products. While several genera of fungi are associated with grain molds, *Fusarium moniliforme* Sheldon and *Curvularia lunata* (Wakker) Boedjin are the predominant causal agents worldwide (Castor and Frederiksen, 1980).

Only a few studies have been reported that investigated the bioactivity of AFPs on these pathogens. Sorghum extracts were reported to be more inhibitory than maize or wheat extracts against 44 fungi tested. The extent of inhibition correlated with the level of chitinase detected in sorghum extracts (Darnetty, 1990). Hyphal elongation of *F. moniliforme* was not inhibited by any of the extracts. Three, as yet uncharacterized, sorghum endosperm proteins have been identified that inhibited the growth of *F. moniliforme* (Kumari et al., 1992). Hard endosperm caryopses had higher levels of these proteins and lower incidences of mold infection compared to soft endosperm caryopses. There have been no reports on the bioactivity of sorghum AFPs on

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C. lunata or *Aspergillus flavus*, an aflatoxin-producing species that infrequently contaminates sorghum grains.

Several studies have shown that a combination of AFPs is more inhibitory than an individual AFP (Mauch et al., 1988b; Leah et al., 1991). *F. solani* f. sp. *phaseoli* was inhibited by 10−30 μg/mL of chitinase and glucanase (Mauch et al., 1988b). Vigers (1992) reported that 5 μg of zeamatin or 10 μg of other permatins was inhibitory against *Neurospora crassa* or *Trichoderma reesei*. Barley RIP inhibited the growth of *T. reesei* at a concentration of 120 μg/mL (Roberts and Selitrennikoff, 1986), while some plant lectins are inhibitory at a higher concentration of 1 mg/mL (Brambl and Gade, 1980).

In an earlier paper we reported on the changes in APFs in caryopses during development and germination and suggested a role for AFPs in the defense of caryopses against fungi (Seetharaman et al., 1996). Our objectives, in this study, were to determine (a) if sorghum AFPs inhibited grain molding fungi and (b) if they shed light on the role of AFPs in defending caryopses against fungal invasion during development. In this paper, we demonstrate the inhibitory effects of sorghum extract containing sormatin, chitinase, glucanase, and RIP on *F. moniliforme*, *C. lunata* and *A. flavus* and that the inhibitory activity of these proteins varies with the stage of development of the pathogen.

MATERIALS AND METHODS

Biological Materials. Sorghum [*Sorghum bicolor* (L.) Moench; Malisor 84-7] was grown at the Texas A&M Agricultural Research Farm at College Station, TX. Seeds harvested 30 DAA were lyophilized and used for the experiments.

Antibodies against zeamatin (maize permatin) and bean chitinase were provided by Dr. Claude Selitrennikoff (University of Colorado, Denver, CO) and Dr. Karen Broglie (DuPont Co., Wilmington, DE), respectively. Antibodies against tobacco glucanase and maize RIP were provided by Dr. Michel Legrand (Université Louis Pastuer, Paris, France) and Dr. Rebecca Boston (North Carolina State University, Raleigh, NC), respectively.

A race of *F. moniliforme* (F+), which is specifically pathogenic against sorghum, was provided by Dr. John Leslie (Kansas State University, Manhattan, KS). Cultures of *C. lunata* and *A. flavus* papa 827 were provided by Drs. Richard Frederiksen and Nancy Keller (Texas A&M University, College Station, TX), respectively. Glycerol stock solutions of all cultures were maintained at −80 °C.

Preparation and Partial Purification of Sorghum AFPs. Sorghum (Malisor 84-7) AFPs were extracted and fractionated using the protocol described by Vigers (1992) with some modifications. Briefly, 225 g of sorghum flour was extracted for 1 h using 500 mL of cold extraction buffer (50 mM NaCl, 25 mM sodium phosphate, pH 7.2, and 5 mM EDTA). The slurry was centrifuged at 10000*g* for 20 min and the supernatant collected (S10 extract). The supernatant was sequentially fractionated with 30% and 55% ammonium sulfate (AS30 and AS55 fractions, respectively) and dialyzed against three changes of buffer. Dialyzed AS55 fraction was applied to a carboxylmethyl Sephadex (C-50−120, Pharmacia) column (1.5 in. × 12 in.) and eluted using a linear salt gradient (10−500 mM NaCl gradient). Column eluants (4 mL) were collected, and the absorbance (280 nm) was monitored. Eluants with absorbances higher than 0.05 were pooled into four fractions corresponding to four unresolved peaks (fractions 1−4). These fractions were concentrated by ultrafiltration and sterilized using a 0.2 μm filter. Proteins were fractionated under reducing conditions using 15% SDS−PAGE gels (Bio-Rad mini gel apparatus, Hercules, CA), and AFP bands were confirmed by Western blots (Seetharaman et al., 1996).

Blots were scanned using a HP DeskScan IIIC. Images were analyzed using NIH image (v. 1.04) to obtain pixel density for each band. Relative amounts of AFPs were calculated by measuring the band intensities of known amounts of pure AFPs in each immunoblot.

Antifungal Assays. All manipulations were carried out under sterile conditions. Fungi were grown on potato dextrose agar (PDA) plates for 5 days, after which time spores were harvested from sporulating colonies and suspended in sterile Tween 20 (1%) water. The concentrations of spores in suspension were determined using a hemacytometer and adjusted to 1.0×10^6 spores/mL. *F. moniliforme* and *C. lunata* cultures were maintained and tested at 25 °C, and *A. flavus* cultures were maintained and tested at 30 °C.

Hyphal Elongation Inhibition. Freshly prepared inoculum (10 μL) was placed in the center of a 9 cm Petri dish containing 20 mL of PDA and incubated for 24 h. To test for antifungal activity, test samples (50 or 100 μL) were applied to sterile filter disks (0.5 in. diameter; Difco Labs, Detroit, MI) placed equidistant around the periphery of the growing colony. The plates were incubated for an additional 12−18 h in the dark and examined for zones of inhibition around each disk. Control included filter disks immersed in buffer alone.

Spore Germination Inhibition Assay. Fresh inoculum (10 μL) was transferred into each well of a 96-well tissue culture plate. The wells also contained 100 μL of 0.5× PDA broth. Different volumes of extracts (0, 2, 10, 20, 50, or 100 μL) made up to 100 μL with water were added to wells to give a final volume of 210 μL in each well. The plates were then incubated in the dark for 18−24 h. Spore germination was rated according to the length of the germ tube after incubation: − = inhibited, no visible germ tube after incubation; + = slight protrusion of germ tube from spore; . . . to ++++ = normal germination. Wells were photographed using a microscope (Model BH-2, Olympus).

Hyphal Rupture Assay. Hyphal rupture assays were performed using a modification of the method described in Roberts and Selitrennikoff (1986). Spores were germinated in a 96-well tissue culture plate containing 100 μL 0.5× PDA broth and incubated for 24 h. Mycelium was removed from the well and placed on a microscope slide in 100 μL of protein extract. Methylene blue (4 μL) was added to the slide, mixed gently, and viewed under a microscope. Hyphae were either ruptured or unchanged when observed several minutes after addition to protein extracts. Ruptured hyphae were seen as "clouds" of blue-stained cytoplasm emanating from hyphal tips.

RESULTS

Confirmation of AFPs in Column Eluants. Positively charged water soluble AFPs eluted from the Sephadex column and were detected in three of four fractions (Figure 1). Fraction 1 did not exhibit antifungal activity or substantial immuno-cross-reactions to any AFP. Chitinase, glucanase, and RIP were detected in fraction 2, where they constituted about 22% of the proteins in that fraction. Two cross-reactive proteins against RIP were detected (Figure 1B); this could be due to proteolytic activity. Fractions 3 and 4 contained chitinase, glucanase, sormatin, and RIP. AFPs accounted for 25% of the proteins in fraction 3 and 75% of the proteins in fraction 4. The antifungal activity of proteins in the concentrated, sterilized column fractions, especially fraction 4, were determined using three fungal pathogens in three bioassays.

Bioactivity Assays. *Hyphal Elongation Inhibition*. Initial trials to test inhibition of hyphal elongation of *F. moniliforme* using the S10 extract from sorghum were unsuccessful. A clear zone of inhibition was not achieved, although there were indications of inhibition before the disk was overgrown with mycelium. Inhibition zones were observed when concentrated fractions, i.e., AS30, AS55, and fractions 3 and 4, were evaluated after 24 h (Figure 2). Protein levels in these fractions

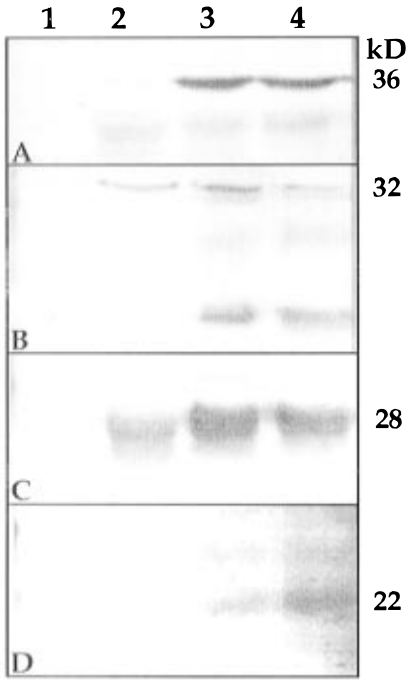

Figure 1. Detection of glucanase (A), RIP (B), chitinase (C), and sormatin (D) in different fractions of proteins eluted from a Sephadex column using a linear salt gradient (10−500 mM). Lanes: 1, fraction 1; 2, fraction 2; 3, fraction 3; 4, fraction 4.

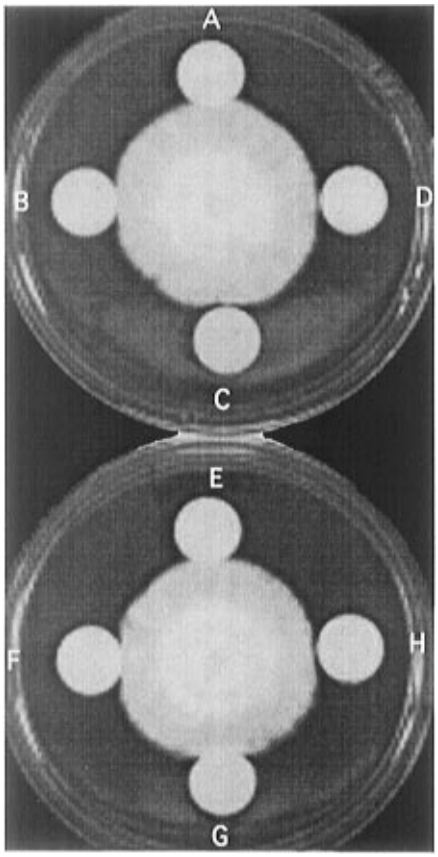

Figure 2. Inhibition of hyphal elongation of *F. moniliforme* by different protein fractions (100 μL) eluted from a Sephadex column using a linear salt gradient (10−500 mM): A, E, control; B, 30% ammonium sulfate insoluble proteins (AS 30); C, AS 55; D, fraction 2; F, fraction 3; G, fraction 1; H, fraction 4.

were 425, 466, 208, and 220 μg per disk, respectively. AFP levels were 52 and 165 μg per disk for fractions 3 and 4, respectively. The fungi, however, overcame the inhibitory effects of these proteins by 36 h. Using *C. lunata*, only fraction 4 with 165 μg of AFP per disk had an inhibitory effect after 24 h (Figure 3). Even with relatively high levels of protein loading, the inhibitory effect on hyphal elongation was detectable but not effective in stopping hyphal elongation.

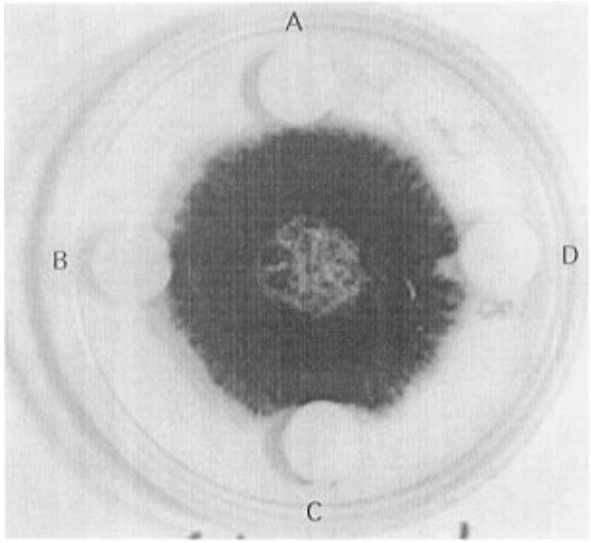

Figure 3. Hyphal elongation inhibition of *C. lunata* by different protein fractions eluted from a Sephadex column using a linear salt gradient (10−500 mM): A, 50 μL of fraction 4 (83 μg of AFPs); B, 50 μL of fraction 3 (26 μg of AFPs); C, 100 μL of fraction 4 (165 μg of AFPs); D, 100 μL of fraction 3 (52 μg of AFPs).

Table 1. Effect of Protein Fraction Eluted from a Sephadex Column Using a Linear Salt Gradient (10−500 mM) on Spore Germination[a]

fraction	antifungal protein (ppm)	*F. moniliforme*	*C. lunata*	*A. flavus*
1	0	++++	++++	++++
2	240	++++	++++	++++
3	260	++	++	+++
4	160	+	+	+

[a] Scale: − = inhibited, no germination, through ++++ = normal germination

Table 2. Effect of Concentration of Proteins from Fraction 4 Eluted from a Sephadex Column Using a Linear Salt Gradient (10−500 mM) on Spore Germination of *C. lunata* and *F. moniliforme*[a]

antifungal protein (ppm)	*F. moniliforme*	*C. lunata*
0	+++++	+++++
15	+++	+++
80	+	++
160	+	+
390	−	−
790	−	−
790, boiled	++++	++++

[a] Scale: − = no germination, through ++++ = normal germination.

Spore Germination Inhibition. The inhibitory activity of AFPs in each column fraction was evaluated using the spore germination method. Fraction 4 was most inhibitory to the germination of spores of each fungus tested (Table 1). This fraction also contained significant amounts of AFPs, while fraction 3, which contained lower levels of sormatin, was less inhibitory. Spore germination of *C. lunata* and *F. moniliforme* were markedly inhibited by 160 μg of AFPs (Table 2). Spores of *A. flavus* were completely inhibited from germinating

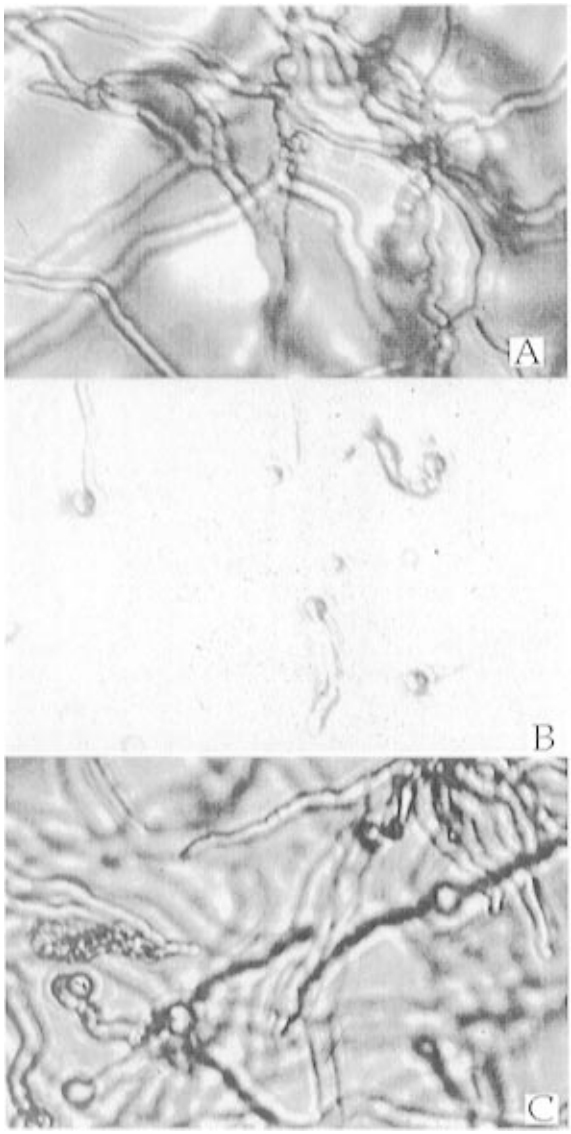

Figure 4. Spore germination inhibition of *A. flavus* by fraction 4 eluted from a Sephadex column using a linear salt gradient (10−500 mM): A, control; B, fraction 4 (165 μg of AFPs); C, boiled fraction 4 (165 μg of AFPs).

at 390 μg of AFPs (Figure 4B). Spore germination was not inhibited when the protein fraction was boiled (Table 2; Figure 4C), suggesting the involvement of denaturable proteins.

Hyphal Rupture. F. moniliforme exhibited hyphal rupture at the growing tip and other regions of mycelium at protein levels as low as 160 ppm of AFPs of fraction 4 (Figure 5). Hyphae of *C. lunata* ruptured only at hyphal tips at protein levels of 360 ppm of AFPs of fraction 4. Hyphal rupture was not detected in *A. flavus* even at 1650 ppm of AFPs of fraction 4.

DISCUSSION

Hitherto, evidence that sorghum seed AFPs inhibit grain molding pathogens has been unclear (Darnetty, 1990; Kumari et al., 1992). We have demonstrated that a combination of AFPs from sorghum is inhibitory against grain molding pathogens *in vitro.* In this study we demonstrate that fungal pathogens at different stages of growth and development exhibit different levels of tolerance to AFPs; that is, spore germlings are more susceptible to inhibition by AFPs than are fungal

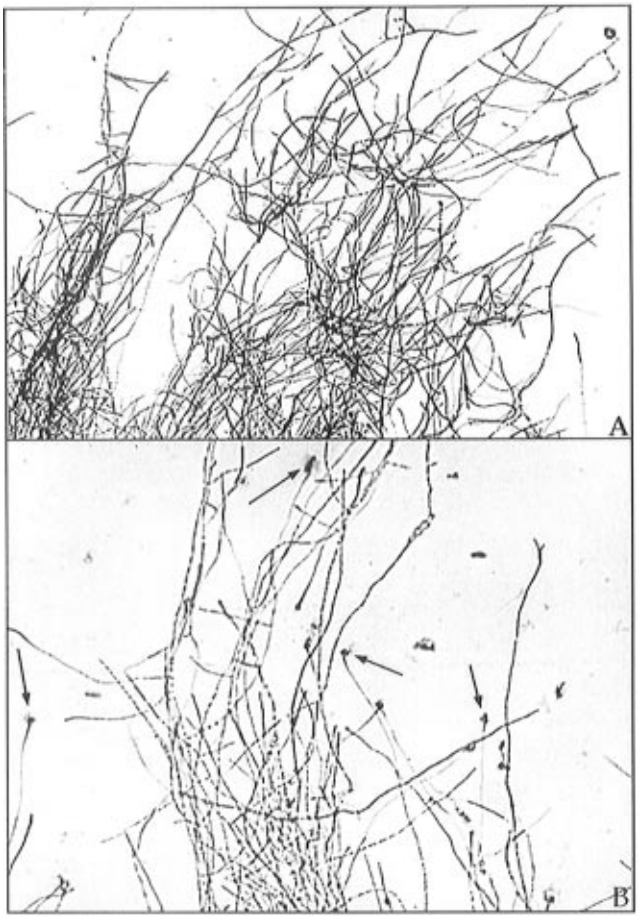

Figure 5. Hyphal rupture of *F. moniliforme* by proteins in fraction 4 eluted from a Sephadex column using a linear salt gradient (10−500 mM): A, control; B, fraction 4 (33 μg of AFPs). Arrows indicate ruptured hyphae.

hyphae. Thus, bioactivity of AFPs depends upon the stage of development of the pathogen, since the presence of AFPs at the time of fungal infection (spore germination assay) is more inhibitory than during fungal colonization (hyphal elongation and hyphal rupture assays).

Darnetty (1990) tested the bioactivity of sorghum protein extract on *F. moniliforme* along with 44 other fungi. Sorghum extract (138 μg of protein/25 μL) only weakly inhibited hyphal elongation of *F. moniliforme.* Similar results were obtained when hyphal elongation was assayed in this study. However, Kumari et al. (1992) reported a significant inhibition of hyphal elongation of *F. moniliforme* by sorghum extract containing $<$50 μg of protein/100 μL.

In this study *F. moniliforme* was markedly inhibited at protein concentrations as low as 160 ppm of AFPs using spore germination inhibition or hyphal rupture evaluations. *C. lunata* and *A. flavus* required higher AFP concentrations for inhibition in these assays when compared to *F. moniliforme.* Further, *A. flavus* did not exhibit hyphal rupture or hyphal elongation inhibition when treated with AFPs. *A. flavus* was inhibited by maize seed proteins and polysaccharides using the spore germination method (Neucere and Zeringue, 1987; Neucere and Godshall, 1991).

While the levels of AFPs in mold-resistant and mold-susceptible sorghums differ significantly (Vigers, 1992; Seetharaman et al., 1996), their role in protecting seeds, *in vivo,* during caryopses development is not clear. AFPs increase during caryopsis development (Seethara-

man et al., 1996); only trace levels of AFPs are detectable 10 DAA in most caryopses. Further, AFPs leach out of immature caryopses (10 DAA) upon imbibition, while they are bound to the pericarp in mature caryopses (Seetharaman et al., 1996). Hence, AFPs may not be a significant factor in defending initial developing caryopses against pathogens since AFP levels are lowest in caryopses 5−10 DAA when fungal spores more easily infect sorghum caryopses (Castor and Fredericksen, 1980).

The amount of AFPs in a caryopsis (33 mg) of Malisor 84-7 at physiological maturity was estimated to be 260 ppm of AFPs. Seetharaman et al. (1997) reported that AFPs in the caryopsis are mobile when wetted, e.g., by rain, and are "bound" to the components in the pericarp. The concentration of AFPs in the pericarp could be increased 2−10-fold during this mobilization process, thereby strengthening the caroypsis' AFP fungal defense mechanism near the exterior of the caryopsis. Therefore, sorghum caryopses may contain sufficient amounts of AFPs to inhibit grain molding fungi *in vitro* and, with some speculation, *in vivo*.

Seetharaman et al. (1997) observed increased levels of AFPs during seed germination. It is possible that AFPs are deposited in developing caryopses primarily for the protection of the next generation, i.e., the protection of seeds during storage and germination. Research is in progress to elaborate the nature of seed−pathogen interactions as it pertains to AFPs.

Enhancing the levels of AFPs at the earlier stages of caryopses development could help minimize caryopsis infection. Advances in genetic techniques to create transgenic plants offer promising alternatives to enhance seed AFP levels. Successful transformations of tobacco (Broglie et al., 1991; Logemann et al., 1992; Jach et al., 1995), rice (Lin et al., 1995), and rape seed (Grison et al., 1996) with genes encoding RIP, chitinase, and/or glucanase have been reported. It has been shown that a "multi-transgene", i.e., incorporating gene for more than one AFP, can be created and is more resistant to disease than transgenes containing only one AFP (Jach et al., 1995). However, the success of similar strategies against seed diseases may prove to be more difficult, although research is in progress presently to address this question. Results from our investigations indicate that any strategy undertaken should consider changes in these proteins during caryopses development and the stage of development of the pathogen during infection to have maximum inhibitory effect on pathogens.

ABBREVIATIONS USED

AFPs, antifungal proteins; AS30/55, ammonium sulfate 30%/55%; DAA, days after anthesis; PDA, potato dextrose agar; RIP, ribosome-inhibiting protein; S10, supernatant extract.

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