Short communication

Effect of chitinase and thaumatin on mycelial growth of five sorghum [*Sorghum bicolor* (L.) Moench] grain molding fungi under *in vitro* conditions

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Abstract

The effects of various concentrations of chitinase, thaumatin, and their mixtures on radial mycelial growth of five grain molding fungi were studied. When compared with the control, chitinase and chitinase+thaumatin mixtures markedly reduced mycelial growth of the fungal species studied, except for *Fusarium semitectum*. Reductions in fungal radial mycelial growth of 65.4% for *Bipolaris* sp. and 61.7% for *Colletotrichum graminicola* were noted when these fungi were grown on half-strength-potato dextrose agar amended with chitinase. A mixture of chitinase at 50 ml L^{-1}+thaumatin at 37.5 mg L^{-1} reduced mycelial growth of *Bipolaris* sp. by 61.7%, *C. graminicola* by 56.2%, and *Curvularia lunata* by 44.9% when compared with the control. The levels of thaumatin used in this study were less effective in inhibiting mycelial growth, except for *F. semitectum*. This study for the first time showed the suppression of mycelial growth *in vitro* of *F. thapsinum*, *Bipolaris* sp., and *C. graminicola* isolated from sorghum grain.

Keywords: Antifungal proteins, Fungal pathogens, Grain mold.

Fungi like *Fusarium thapsinum* Klittick, Leslie, Nelson and Marasas, *Fusarium semitectum* Berk. & Ravenel, *Curvularia lunata* (Wakk.) Boedijin, *Colletotrichum graminicola* (Ces.) G.W. Wilson (syn. *C. sublineolum*), and *Alternaria alternata* (Fr.:Fr.) Keisel have been associated with grain mold of sorghum [*Sorghum bicolor* (L.) Moench] (Singh and Bandyopadhyay, 2000). The use of resistant cultivars offers the best means for controlling grain mold (Esele et al., 1995; Singh and Bandyopadhyay, 2000). However, grain mold resistance is complex and it is enhanced by factors such as hardness of the kernel, presence of red pericarp, endosperm texture, high tannins, high concentrations of flavan-4-ol, tan plant color, and the occurrence of pericarp intensifier (*I*) gene (Esele et al., 1995). Over the years, studies have shown that antifungal proteins (AFP) such as chitinases, glucanases, sormatin, thaumatin-like proteins, and ribosome-inhibiting protein (RIP) isolated and characterized from sorghum and other plants may play a role in protecting these plants from fungal invasion (Seetharaman et al., 1996; 1997; Selitrennikoff, 2001). Chitinase has been shown to weaken fungal cell walls by cleaving the cell wall chitin polymers *in situ*. β-glucanase also weakens the cell walls, resulting in lysis and cell death, whereas thaumatin-like proteins have been shown to cause changes in the permeability of fungal cell walls (Selitrennikoff, 2001). Seetharaman et al. (1996) noted increased levels of chitinase (28 kDa), sormatin (22 kDa), β-glucanase, and reduced levels of RIP in mature sorghum caryoses. During *in vitro* assays, the use of a combination of these antifungal proteins extracted from sorghum seeds were found to inhibit spore germination but were less effective in inhibiting hyphal elongation of *Fusarium moniliforme* Sheldon, *C. lunata*, and *Aspergillus flavus* Link (Seetharaman et al., 1997). This study evaluates the influence of various concentrations of chitinase, thaumatin, and mixtures of these compounds on mycelial growth of five grain mold fungi.

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The study was conducted at the USDA-Southern Plains Agricultural Research Center, College Station, Texas. Single-spore cultures of *F. thapsinum*, *F. semitectum*, *C. lunata*, *C. graminicola* (syn. *C. sublineolum*), and *Bipolaris* sp. were isolated from grain mold-infected sorghum kernels and maintained on dried colonized Whatman No. 2 filter papers stored in a freezer at –7 ºC. The AFPs comprised of a sugar beet-derived chitinase in liquid formulation, and thaumatin from *Thaumatococcus daniellii* as a wettable powder (Sigma-Aldrich Co., St. Louis, MO).

The fungi were cultured in 100 x 15 mm petri-dishes containing 10 ml of sterile half-strength potato dextrose agar (PDA) into which the AFP were added. AFP treatments comprising of 25 and 50 ml L^{-1}chitinase 18.75 and 37.5 mg L^{-1} thaumatin, and a mixture of the AFPs at their low and high concentrations (i.e., mixtures of 1: 25 ml L^{-1}chitinase + 18.75 mg L^{-1} thaumatin and 2: 50 ml L^{-1}chitinase + 37.5 mg L^{-1} thaumatin) were evaluated. Agar plates without AFP served as control. The center of each petri-dish was subsequently inoculated with 0.4 mm diameter fungal plugs obtained from 7 day-old PDA cultures of *F. thapsinum*, *F. semitectum*, *C. lunata*, *C.graminicola*, and *Bipolaris* spp. and incubated at 25±1ºC. Each petri-dish was considered a replicate. All seven treatments were replicated three times for each fungal species, and the experiment was repeated twice in February and March, 2009. The response of mycelial growth of each fungal species to AFP type and concentration was determined by daily measurement of colony diameter. These daily measurements started four days after inoculation, were terminated eight days post-inoculation. Percent reduction of mycelial growth for each fungal species was determined using the formula

$$\textit{Percent reduction of radial growth} = \frac{C - T}{C} \times 100,$$

where C = rate of growth in petri-dish without AFP eight days after inoculation, and T = rate of growth in petri-dish with AFP's, eight days after inoculation. The data were subjected to analysis of variance using the command PROC GLM (Version 9.2, SAS Institute Inc., Cary, NC.) to determine the effect of AFP type, AFP concentration, fungal pathogen, and their interactions. Due to heterogeneity of the error variances, data for the two experiments were analyzed separately. Mean comparisons were conducted using Tukey-Kramer at 5% probability level.

Radial growth of the fungal cultures was significantly (p<0.01) affected by AFP concentrations, fungal pathogen, and their interactions (Table 1). Significant fungal pathogen x AFP interaction indicates that the fungal species respond differently to chitinase, thaumatin, and their various mixtures. In this study, there was no complete inhibition of mycelial growth of the different grain mold fungi when treated with different concentrations of the AFPs or their mixtures. Except for *F. semitectum*, chitinase and mixtures of chitinase+ thaumatin significantly suppressed the mycelial growth of all other fungal species tested. *F. semitectum* was more sensitive to thaumatin than to chitinase and chitinase+ thaumatin mixtures in experiment 1 (Table 1).

In experiment 1, chitinase applied at 50 ml L^{-1} and a mixture of chitinase+thaumatin at higher concentrations (Mix 2) caused the highest reduction in mycelial growth of *Bipolaris* spp., *C. graminicola*, *C. lunata*, and *F. thapsinum*. Compared to the control plates, mycelial growth of *Bipolaris* sp. was reduced by 61.7%, *C. graminicola* by 56.2%, and *C. lunata* by 44.9% in agar plates amended with Mix 2. Chitinase applied at 50 ml L^{-1} reduced mycelial growth of *Bipolaris* sp. by 65.4%, *C. graminicola* by 61.7%, and *F. thapsinum* by 45%.

In experiment 2, mixtures of chitinase+thaumatin at their higher concentrations caused the highest reduction in mycelial growth of *C. lunata* (39.8%) and *C. graminicola* (34.9%), whereas 29.5% reduction in mycelial growth of *Bipolaris*sp. was observed when treated with a mixture of chitinase and thaumatin at lower concentrations. There were variations in the results of the two experiments. The possible cause of these variations may be the lack of thorough mixing of different AFPs and agar or more frequent opening of the incubator in experiment 2, thereby allowing for fluctuating temperatures and less heat for plates placed closest to the incubator door.

This result is in agreement with the findings of Kumari

Table 1. Percent suppression of mycelial growth of five sorghum grain molding fungi grown on half-strength potato dextrose agar amended with different concentrations of chitinase and thaumatin and their mixtures.

Treatments[1]	*Bipolaris* sp.		*Colletotrichum graminicola*		*Curvularia lunata*		*Fusarium semitectum*		*Fusarium thapsinum*	
	Expt. 1	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 1	Expt. 2
25 ml L^{-1}	12.2±7.5^{efgh}	22.3±5.4^{bcd}	3.5±1.7^{h}	13.2±2.7^{defg}	10.5±2.2^{fgh}	21.6±5.2^{bcd}	0.01±0.01^{h}	0.01±0.01^{g}	0.01±0.01^{h}	4.8±2.5^{fg}
50 ml L	65.4±2.7^{a}	17.6±4.3^{cdef}	61.7±1.0^{ab}	0.8±0.5^{g}	30.3±2.3^{cde}	11.4±3.5^{defg}	3.3±2.1^{h}	0.01±0.01^{g}	45.0±4.9^{bc}	0.3±0.3^{g}
18.75 mg L^{-1}	0.01±0.01^{h}	2.5±1.1^{fg}	6.2±1.1^{h}	4.5±1.4^{fg}	0.01±0.01^{h}	0.01±0.01^{g}	28.8±3.4^{cdef}	0.01±0.01^{g}	0.5±0.4^{h}	3.0±1.3^{fg}
37.5 mg L^{-1}	0.01±0.01^{h}	5.7±1.9^{efg}	2.1±2.1^{h}	0.01±0.01^{g}	0.5±0.5^{h}	2.1±0.9^{g}	26.4±3.2^{cdefg}	3.0±1.9^{fg}	7.1±5.3^{gh}	9.6±4.9^{defg}
MIX 1	11.6±3.0^{efgh}	29.5±6.1^{abc}	13.8±10.0^{defgh}	11.7±2.7^{defg}	5.7±1.8^{h}	20.4±4.8^{bcde}	0.01±0.01^{h}	0.01±0.01^{g}	1.1±0.9^{h}	5.1±2.7^{fg}
MIX 2	61.7±7.8^{ab}	23.0±5.4^{bcd}	56.2±1.5^{ab}	34.9±4.0^{ab}	44.9±3.9^{bc}	39.8±6.5^{a}	0.01±0.01^{h}	0.01±0.01^{g}	31.8±7.4^{cd}	2.6±2.6^{fg}

[1]25 and 50 ml L^{-1}chitinase; 18.75 and 37.5 mg L^{-1}thaumatin; MIX 1 = Mixture of 25 ml L^{-1}chitinase + 18.75 mg L^{-1}thaumatin; MIX 2 = Mixture of 50 ml L^{-1}chitinase + 37.5 mg L^{-1}thaumatin; Means (with standard error) within an experiment followed by the same letter(s) are not significantly different (p=0.05) based on Tukey-Kramer adjustment for multiple comparisons.

et al. (1994) who showed that reduction in mycelial growth of *F. moniliforme*, *F. oxysporum*, *F. semitectum*, *A. alternate*, and *C. lunata* when exposed to three AFPs of approximate sizes of 18, 26, and 30 kDa extracted from sorghum endosperm, albeit the AFPs were not identified as chitinase and thaumatin. Seetharaman et al. (1997) reported that higher levels of mixtures of chitinase, glucanase, sormatin, and RIPs in protein fractions obtained from sorghum caryopses exhibited more inhibition to spore germination of *F. moniliforme*, *C. lunata*, and *A. flavus* than protein fractions with lower levels of these AFPs**.** In this study, chitinase +thaumatin mixtures exhibited greater suppression of mycelial growth of the grain mold fungi, indicating that the AFPs acted synergistically (Table 1). In conclusion, this study indicates that AFPs, especially in combination may play a significant role in reducing fungal colonization in the grain mold disease complex. It also for the first time demonstrated the ability of chitinase and thaumatin, either added singly or in combination, for suppressing mycelial growth *in vitro* of *F. thapsinum*, *Bipolaris* sp. and *C. graminicola* isolated from sorghum grain.

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