Activity of Antifungal Proteins against Mold in Sorghum Caryopses in the Field

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Sorghums were stressed with pathogenic fungi and sprinkling to determine relationships between changes in chitinase and sormatin in caryopses and grain mold resistance. Panicles of 10 cultivars differing in mold resistance and accumulation of antifungal proteins (AFPs) were inoculated at anthesis with *Fusarium moniliforme* and *Curvularia lunata* spores. Panicles were sampled at 30 and 50 days after anthesis, and caryopses were evaluated for chitinase and sormatin using western blots. Sprinkling panicles (to mimic rainfall) decreased sormatin and chitinase in most cultivars. Inoculation decreased AFPs in susceptible cultivars, but resistant cultivars maintained or increased AFPs in caryopses. Grain mold resistance corresponded to induction of AFP synthesis in response to sprinkling, fungal stress, and/or adverse field conditions. Sormatin and chitinase appear to be an active part of the defense mechanism of the caryopsis against grain mold.

Keywords: *Chitinase; sormatin; antifungal proteins; grain mold; plant resistance*

INTRODUCTION

Grain molding by fungal species *Fusarium moniliforme* J. Sheldon and *Curvularia lunata* (Wakker) Boedjin is a serious biotic constraint in sorghum grain production worldwide. Like other crops, sorghum cultivars differ in their inherent mold resistance, which appears to be dependent on several factors. Menkir et al. (1996) found that mold resistance in sorghum was strongly associated with high concentrations of phenolic compounds, caryopsis hardness, and pericarp color. Likewise, Esele et al. (1993) suggested that a pigmented testa was the single most important trait conferring grain mold resistance in sorghum and, to a lesser extent, the red pericarp trait. Although sorghums with high phenol and tannin contents are resistant to molding, these compounds cause dark colors, astringency, and decreased nutritional value in foods or feeds (Earp et al., 1983; Hahn et al., 1984). Thus, recent studies have focused on hydrolytic enzymes and antifungal proteins (AFPs) in sorghum for the development of mold-resistant sorghums (Seetharaman et al., 1996; Rodriguez-Herrera et al., 1999).

The effectiveness of sorghum AFPs in vitro was demonstrated by Seetharaman et al. (1997) with a mixture of chitinase, sormatin, glucanase, and ribosome-inactivating protein that caused hyphal rupture and inhibited spore germination of *F. moniliforme*, *C. lunata*, and *Aspergillus flavus*. Caryopses resistant to grain molding showed greater response in vitro to physical damage and soaking with more chitinase and sormatin in endosperm and embryo tissues than did susceptible caryopses (R. D. Waniska, S. Clarke, K. Seetharaman, L. W. Rooney, and F. P. Bejosano, unpublished data, 1999). Barley AFPs were released from the caryopsis upon water imbibition in vitro, which was hypothesized to play a role in its natural defense against fungal infection (Swegle et al., 1992).

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AFPs are potentially important in fungal inhibition in the field; however, field data have been unclear about the relationships of AFP and grain mold resistance. Seetharaman et al. (1996) were the first to observe a significant inverse correlation coefficient between sormatin content in caryopses at 30 days after anthesis (DAA) with sorghum grain mold rating at harvest time. Field observations on how AFP levels in caryposes respond to fungal attack are needed to establish their role in grain mold resistance. Variations between resistant and susceptible lines in wet and dry field environments are needed. Thus, the objectives of this study were to determine the effects of inoculation with *F. moniliforme* and *C. lunata* at anthesis, periodic sprinkling, and the combination of stresses on AFP accumulation during caryopsis development.

MATERIALS AND METHODS

Plant Materials. Ten sorghum cultivars with different levels of AFPs and degrees of mold resistance (Seetharaman et al., 1996) were selected (Table 1). Characteristics related to grain molding, for example, maturity, pericarp color, pigmented testa, spreader gene, and endosperm hardness, were determined (Reichert et al., 1982; Rooney and Miller, 1982). Maturity was recorded the day when 50% of the plants of a plot were shedding pollen at least up to the middle of their panicles going downward from the tip to the bottom. Rachis branches containing caryopses 30 and 50 DAA were cut from the panicles, placed in a plastic bag, frozen, lyophilized, hand-threshed, and cleaned. Two replicated samples were achieved by compositing caryopses from three or more panicles.

Grain mold ratings were assessed in the field by visually estimating severity, based on a 1−5 scale: 1 = no mold; 2 = 1−10% molded grain; 3 = 11−25% molded grain; 4 = 26−50% molded grain; 5 = >50% molded grain (Castor et al., 1980).

Treatments. Ten sorghum cultivars were planted at two locations: College Station and Halfway, TX. Grain from Halfway served as a reference because Halfway has a history of dry weather and low mold incidence. Panicles were inoculated with a fungal solution (*F. moniliforme* and *C. lunata*) or just water in nurseries that did or did not receive overhead sprinkling (sprinkled) every 5 days. Spore suspensions of the

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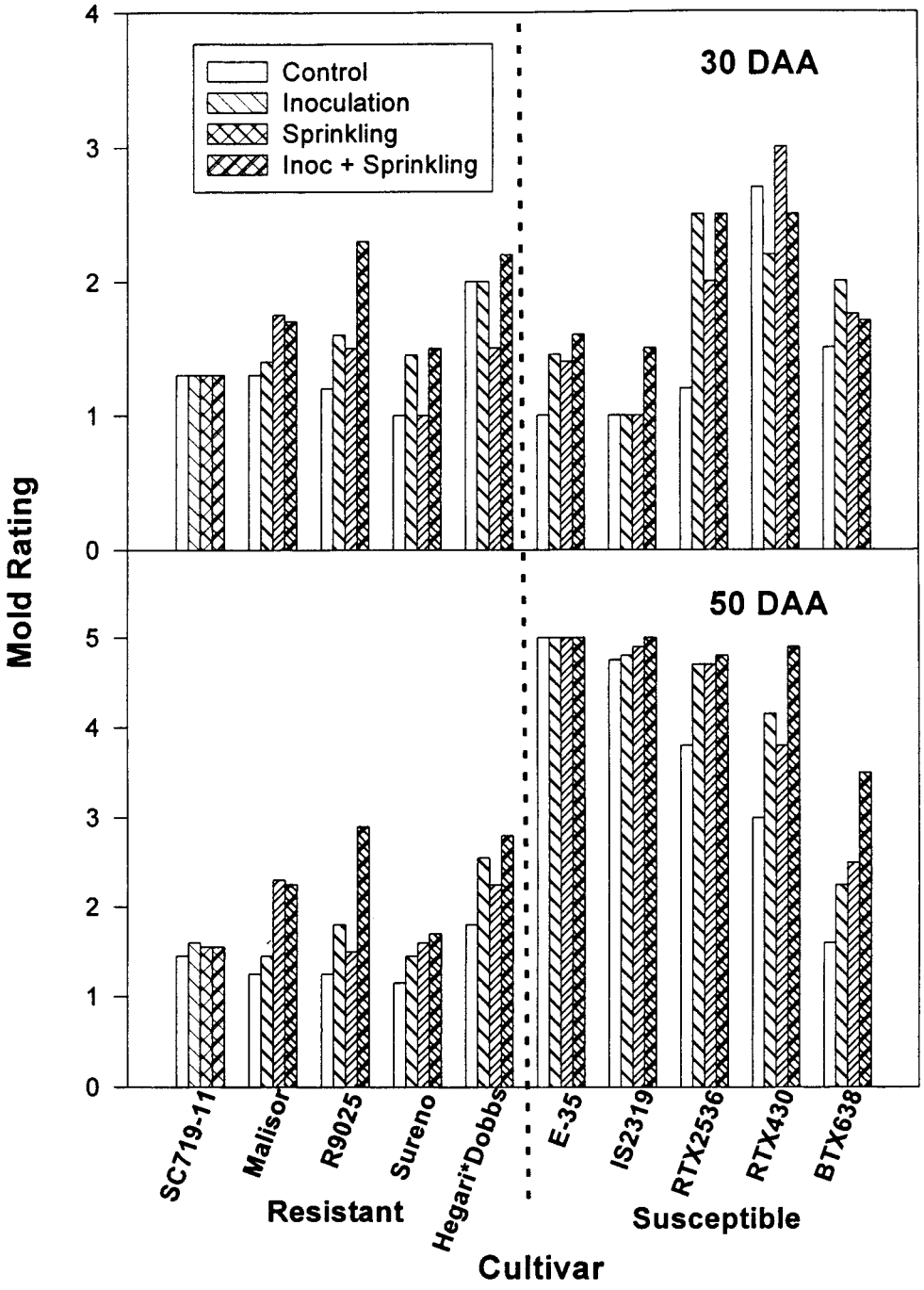

Figure 1. Effect of sprinkling and/or inoculation on mold ratings of sorghums at 30 and 50 DAA in College Station and of untreated sorghums in Halfway, TX.

two fungi were made separately. The fungal cultures were extracted from the agar medium by using a rubber policeman and distilled water. The suspension was filtered through a double layer of cheesecloth. Spore counting was done with the aid of a hemacytometer. The conidial suspension of *C. lunata* was diluted to a concentration of 5×10^4 spores/mL with distilled water, whereas *Fusarium* suspension was adjusted to 5×10^5 spores/mL. Equal quantities of both suspensions were mixed to form the inoculum. Treatments were fungal inoculation, sprinkling, and combined inoculation and sprinkling.

Determination of AFP Content. Soluble proteins were extracted using the method described by Vigers (1992). Ground samples (0.1 g) were mixed with 1 mL of extraction buffer (25 mM sodium phosphate, 50 mM NaCl, and 5 mM EDTA, pH 7) with agitation for 1 h. After centrifugation at 10000 rpm for 20 min, the supernatant was collected. The supernatant was then mixed with BME buffer (1:1 v/v), 2-mercaptoethanol, and 0.002% bromophenol blue. Mixtures were boiled for 5 min, and 30 μL was loaded into 15% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane (Western blotting), rinsed, and blocked with 5% skim milk/TBS buffer for 1 h. Blocked membranes were soaked with the respective antibody [chitinase or sormatin raised using rabbits twice injected with purified sorghum proteins (Seetharaman et al., 1996)]. Upon >2 h of incubation, membranes were rinsed with TBS buffer and incubated in affinity-purified goat anti-rabbit IgG antibody for >1 h. Blots were then rinsed and proteins visualized. Blots were scanned and intensities of protein bands were quantified using NIH 1.59 software. Protein concentration was calculated on the basis of intensities of reference samples and standard curves. AFP content was reported as micrograms per caryopsis.

Table 1. Categorization of Cultivars, Plant Maturity, Caryopsis Properties, and Amounts of Sormatin and Chitinase in Sorghums Varying in Mold Resistance[a]

cultivar	AFP content[a]	mold resistance[a]	maturity[b]	pericarp color	pigmented testa	spreader gene[c]	endosperm hardness[d]	sormatin[e] (μg/caryopsis)	chitinase[e] (μg/caryopsis)	mold rating[f] at 50 DAA
SC719-11E	low	high	early	red	yes	yes	medium	13	101	1.4
Malisor 84-7	high	moderate	medium	white	no	no	high	111	400	1.2
R9025	high	high	medium	red	no	no	low	83	166	1.2
Sureno	high	moderate	medium	white	no	no	high	50	386	1.2
Hegari*Dobbs	high	moderate	medium	white	yes	no	medium	222	562	1.8
IS2319	low	low	late	white	yes	no	low	83	456	5.0
E35-1	low	low	late	white	no	no	medium	17	271	4.8
RTX2536	low	low	medium	white	no	no	low	32	246	3.8
RTX430	high	low	medium	white	no	no	low	153	528	3.0
BTX638	high	low	medium	red	no	no	medium	271	448	1.6

[a] Antifungal protein (AFP) content and grain mold resistance from Seetharaman et al. (1996). [b] Early maturity = <60 days after planting; medium = 61−80%; late = >81%. [c] When spreader (S) gene is dominant, tannins accumulate in pericarp and testa. [d] Hard = <30% abraded during decortication; medium = 30−50%; soft = >51%. [e] Sormatin and chitinase in control caryopses at 30 days after anthesis. Sormatin and chitinase had coefficient of variation of 11−23% for the different cultivars, locations, and age of caryopsis. [f] Mold rating (1 = no mold; 5 = >50% of surface molded) at 50 days after anthesis at College Station, TX.

RESULTS AND DISCUSSION

Mold Ratings of Sorghum Cultivars in the Field. Mold resistance (Table 1) was based on the history of each cultivar during the previous growing seasons. Mold ratings of each cultivar at 30 and 50 DAA reflect the variability of sorghums to mold infection in the field at College Station, TX (Figure 1). Mold ratings of sorghums grown at Halfway were 1.35 ± 0.15, evidence that the environment was not good for grain molding. Mold ratings between the resistant and susceptible cultivars exhibited no remarkable distinctions at 30 DAA even with fungal inoculation and sprinkling treatments. Accordingly, mold resistance and the mold rating scores at 30 DAA did not correlate significantly. This was expected because most susceptible sorghums do not have much deterioration before physiological maturity (30 DAA) (Bandyopadhyay et al., 1988). Nevertheless, after 30 DAA, mold growth was favored by warm/humid conditions in the field, and susceptible sorghums exhibited signs of deterioration. Hence, mold ratings increased considerably at 50 DAA and corresponded inversely with mold resistance.

Physical Properties of Sorghum Caryopsis. The sorghum cultivars varied in maturity, pericarp color and composition, and endosperm hardness (Table 1), traits that have been associated with grain mold resistance (Bandyopadhyay et al., 1988; Esele et al., 1993; Menkir et al., 1996). Most of these traits, however, did not correlate with the established mold resistance (Table 1) and mold ratings at 30 DAA (Figure 1). Nevertheless, at 50 DAA, two of three sorghums with pigmented testa (i.e., condensed tannins) had less deterioration, but the third sorghum with pigmented testa molded (Figures 1 and 2). Low mold ratings were observed in two sorghums with red pericarp and no tannins and in two sorghums with white pericarp and no tannins. Three other sorghums with white pericarp and no tannins exhibited much mold damage. Therefore, red pericarp color (versus white) was related to less molding, even though the red pericarp sorghum, BTX638, exhibited more mold damage in previous years. Two sorghums with hard endosperm had the same low deterioration, as did sorghums with low and medium endosperm hardness, whereas the four sorghums with the most deterioration had low or medium endosperm hardness.

Endosperm hardness, as measured by decortication loss, correlated with grain mold resistance at 50 DAA (Table 2). Harder grain lost less during decortication and had lower mold ratings when stressed with sprinkling or inoculation with fungal pathogens. This trend was also observed in the control and the combined treatment but with $p \leq 0.10$.

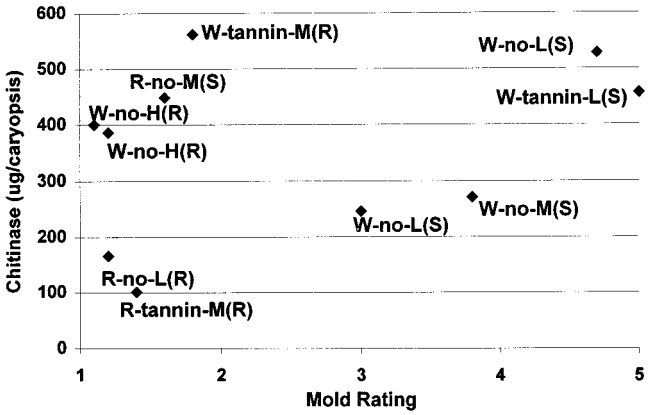

Figure 2. Scatter plot of chitinase in caryopsis at 30 DAA and grain mold rating of cultivars at 50 DAA. Labels are in the following format: pericarp color/presence of tannins/endosperm hardness/(grain mold resistance), where W = white pericarp, R = red pericarp, tannin = tannins present, no = no tannins, H = hard endosperm, M = medium endosperm hardness, L = low endosperm hardness, S = susceptible, and R = resistant.

Table 2. Correlation Coefficients between Mold Rating and Grain Hardness[a] at 50 DAA

treatment	correlation coefficient
control	0.57*
sprinkling	0.77**
inoculation	0.68**
inoculation + sprinkling	0.58*

[a] As determined by decortication (% removed). *, $p \leq 0.10$; **, $p \leq 0.05$.

Previous studies indicated that pigmented testa, red pericarp color, and harder endosperm confer mold resistance to sorghum (Glueck et al., 1980; Castor et al., 1980; Hahn and Rooney, 1984). The presence of proanthocyanidins but not flavon-4-ols and 3-deoxyanthocyanidins correlated with grain mold resistance (Melakeberhan et al., 1996). The mold rating at 50 DAA was lower when the sorghum had red pericarp and/or hard endosperm traits. However, hidden factors, such as the presence of AFPs in the caryopsis, might be involved. Seetharaman et al. (1996) noted differences in the mobility and extractability of chitinase and sormatin from sorghums of various tannin contents. They found that sorghums without tannins (type I) had variable levels of AFPs, type II (tannins present in pigmented layer) had increased sormatin and unchanged chitinase, and type III (tannins present in pericarp and pigmented layer) had decreased AFPs. Therefore, they speculated that several mechanisms could interact to achieve grain mold resistance.

AFP and Mold Resistance. Amounts of chitinase

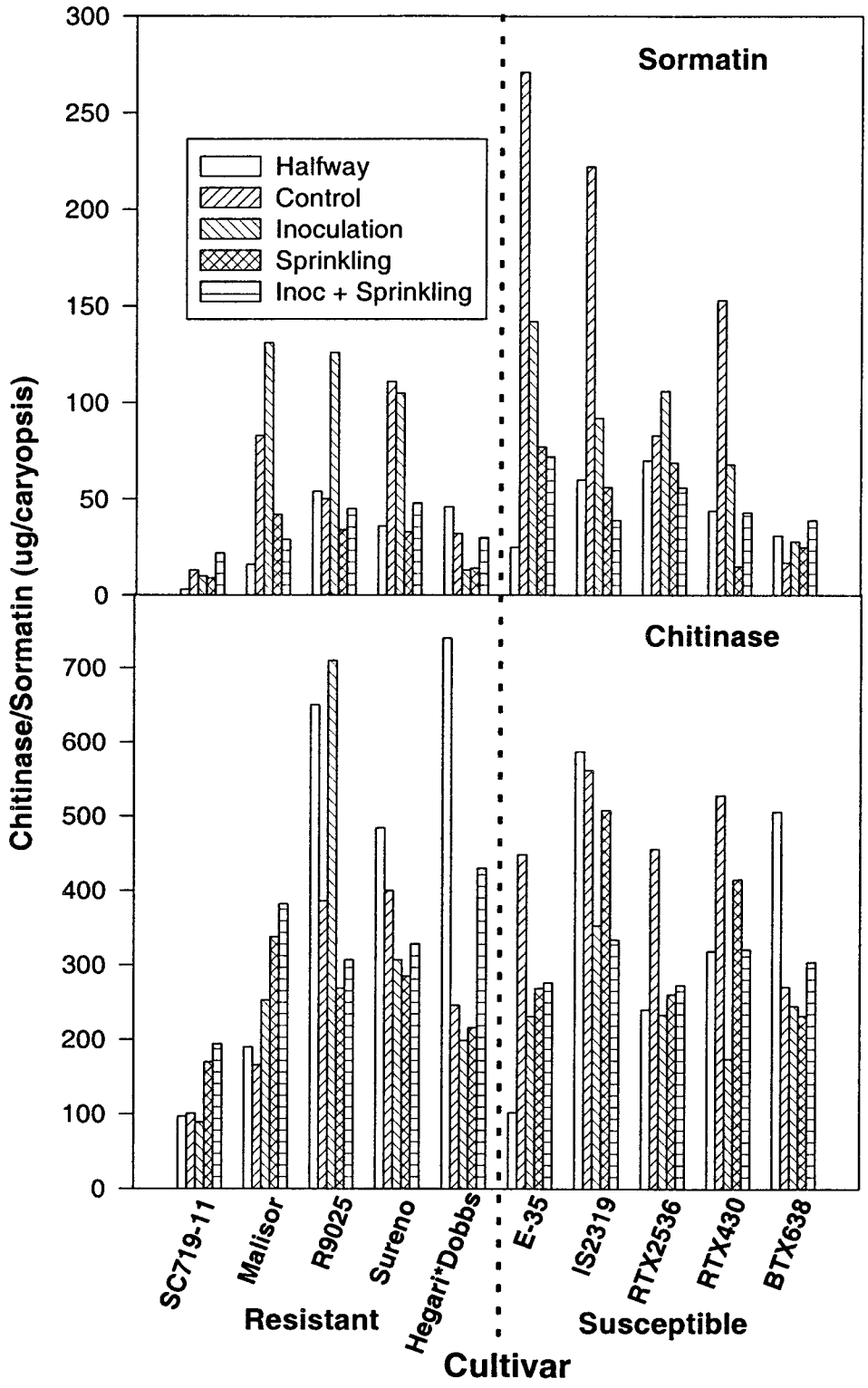

Figure 3. Effect of sprinkling and/or inoculation on AFPs in caryopses at 30 DAA in College Station and of untreated sorghums in Halfway, TX. Sormatin and chitinase had coefficients of variation between 11 and 23% for the different cultivars, locations, and age of caryopses.

and sormatin in caryopsis of control (untreated) sorghums at 30 DAA are shown in Table 1. Sormatin and chitinase had coefficients of variation between 11 and 23% for the different cultivars, locations, and age of caryopses. In the selection of cultivars, we intentionally chose those differing in mold resistance and AFP content. Hence, there were resistant/high-AFP, resistant/low-AFP, susceptible/high-AFP, and susceptible/low-AFP cultivars (Figure 2). Therefore, as we expected, AFP levels (both chitinase and sormatin) at physiological maturity did not significantly correlate with mold resistance. High levels of chitinase, however, were associated with resistant and susceptible tissues in vivo (Punja and Zhang, 1993); these authors speculated that expression of chitinase in combination with one or several different AFPs could have a greater effect on reducing disease development given the complexities of fungal−plant cell interactions and resistance responses in plants. Rodriguez-Herrera et al. (1999) observed increased levels of four AFPs in resistant sorghums.

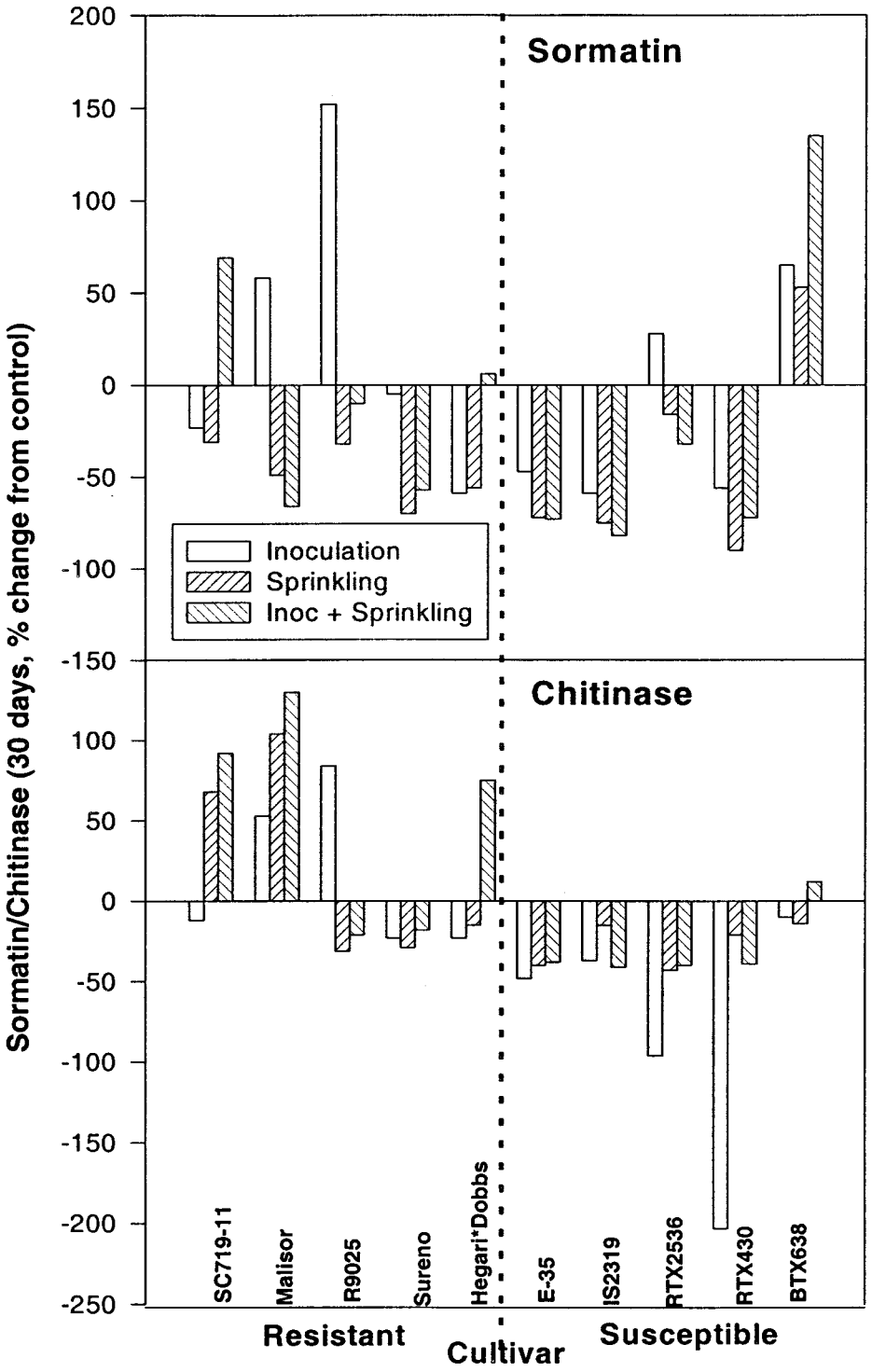

Figure 4. Changes in AFPs as effected by sprinkling and/or inoculation in caryopses at 30 DAA. All values are compared to the control at 30 DAA in College Station, TX.

However, the importance of AFP in grain mold resistance was not discernible in these studies.

Response of AFPs to Field Conditions and Stress. Field conditions of College Station and Halfway, TX, affected AFPs at 30 DAA (Figures 3 and 4). Sormatin levels in caryopses were either higher (five) or unchanged (five) for sorghums grown in College Station compared to Halfway, TX. Chitinase levels in resistant sorghums were either decreased (three) or unchanged (two), whereas chitinase levels in susceptible sorghums increased in three, decreased in one and was unchanged in another cultivar when grown in College Station compared to Halfway, TX. This suggests that less ideal field conditions promote higher levels of sormatin and chitinase in susceptible cultivars and higher levels of sormatin but lower levels of chitinase in resistant cultivars.

Field conditions at College Station affected AFPs in caryopses between 30 and 50 DAA (Figures 3 and 5). Sormatin increased in four resistant cultivars, whereas chitinase increased in only one resistant cultivar. Sormatin and chitinase decreased in one resistant and all susceptible cultivars. It appears that elevated humidity induced AFP synthesis in some resistant cultivars but

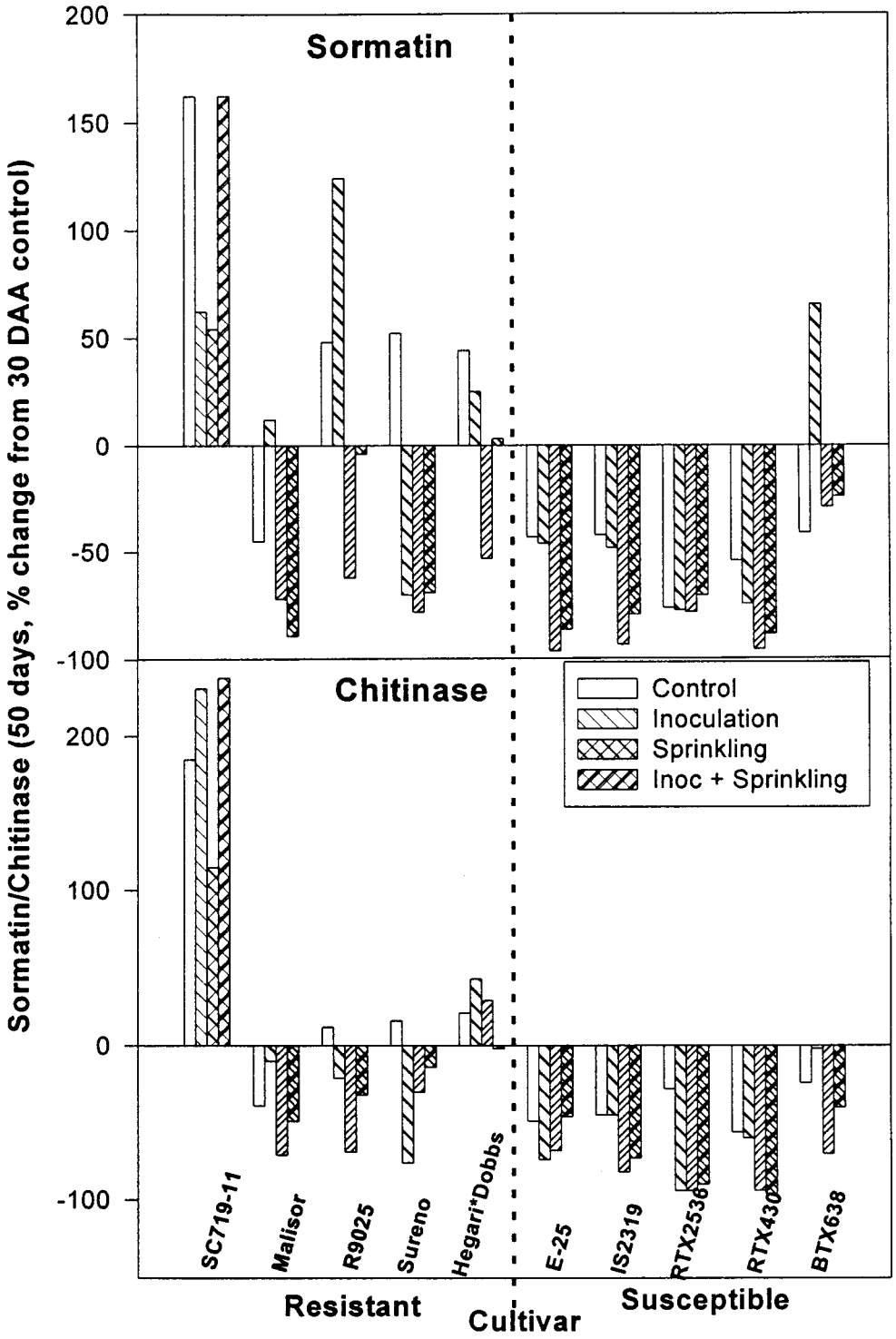

Figure 5. Changes in AFPs as effected by sprinkling and/or inoculation in caryopses at 50 DAA. All values are compared to the control at 30 DAA in College Station, TX.

not in any susceptible cultivars. This partially contradicts the findings of Seetharaman et al. (1996), who reported that AFPs decreased in caryopses after 30 DAA; however, the results could be evidence of an active defense response of AFPs in the caryopsis.

The sprinkling treatment (intended to mimic brief showers) was conducted to determine mobility of AFPs in vivo. Sprinkling decreased sormatin in most cultivars at 30 and 50 DAA (Figures 4 and 5). Sprinkling increased sormatin in one susceptible cultivar, BTX638, at 30 DAA and one resistant cultivar, SC719-11E, at 50 DAA. Likewise, sprinkling decreased or did not change chitinase in most cultivars; however, sprinkling increased chitinase in two resistant cultivars, SC719-11E and Hegari*Dobbs, at 30 and 50 DAA. Sprinkling caused mobility or a loss of AFPs in the caryopses of most susceptible and several resistant cultivars.

Panicles were inoculated with fungal pathogens to determine whether AFPs were accumulated actively or passively. AFP levels decreased in most susceptible cultivars at 30 and 50 DAA (Figures 3−5). Inoculation and fungal stress increased sormatin and chitinase at 30 DAA in two resistant cultivars (Malisor and R9025) and sormatin in two susceptible cultivars (IS2319 and

Table 3. Correlation Coefficients between Mold Rating at 30 and 50 DAA and Percent Change in Sormatin and Chitinase in Sorghum Caryopses[a]

	30 DAA		50 DAA	
treatment	sormatin	chitinase	sormatin	chitinase
inoculation	0.09	−0.51	−0.63**	−0.50
sprinkling	−0.02	−0.05	−0.58*	−0.56*
inoculation + sprinkling	−0.16	−0.31	−0.58*	−0.66**

[a] Values for control at 30 DAA were used as reference to calculate change due to treatment. *, $p \leq 0.10$; **, $p \leq 0.05$.

BTX638). Sormatin increased at 50 DAA in one susceptible and three resistant cultivars, and chitinase increased in two resistant cultivars. Sormatin levels at 50 DAA increased more consistently than chitinase levels in resistant cultivars. Fungal stress induced AFP synthesis more in resistant than in susceptible cultivars. Mold resistance appears to be related to the degree of AFP induction and not on the relative amount of AFP in the unstressed sorghum caryopsis.

Changes of AFPs in caryopses due to inoculation with fungal pathogens and sprinkling to mimic brief showers are also shown in Figures 4 and 5. Most cultivars had unchanged or decreased AFP levels at 30 and 50 DAA. The resistant cultivar, SC719-11E, had increased sormatin and chitinase at 30 and 50 DAA. Susceptible BTX638 had increased sormatin at 30 DAA, whereas two resistant cultivars, Malisor and Hegari*Dobbs, had increased chitinase at 30 DAA. The resistant-like behavior of BTX683, a susceptible cultivar, may be an anomaly of the growing season, misclassification of resistance, or other factors. The combined treatment of fungal inoculation and sprinkling resulted in AFP changes more similar to the sprinkling than to the inoculation treatment. Sprinkling generally caused reduced AFP in all cultivars, whereas inoculation increased AFP levels in some of the resistant cultivars.

The resistant cultivar that initially had low levels of AFP (SC719-11E) was induced by the treatments to accumulate more sormatin and chitinase at 30 and 50 DAA. The other resistant cultivars, except Sureno, responded to one or more of the stresses with higher levels of sormatin or chitinase during caryopsis development. The susceptible cultivars, regardless of AFP level, responded with lower levels of AFP with only one exception. This implies that the amount of AFP in caryopses is less important than induction of AFP accumulation in the caryopsis by stresses leading to grain molding.

Mold Rating and AFP. Mold rating of sorghum caryopses did not correlate to AFPs (discussed earlier), but mold rating did correlate with percent change in AFP (Table 3). Significant correlation coefficients were obtained at 50 DAA but not at 30 DAA. Grain molding increased during 30−50 DAA. Mold rating at 50 DAA was negatively correlated to percent change in sormatin after inoculation and in chitinase after inoculation and sprinkling. This means that less fungal infection occurred when AFP levels increased. The imposed stresses either diminished AFP content in some cultivars and/or induced AFP synthesis in other cultivars. Hence, inherent grain mold resistance was more clearly expressed in less ideal field conditions. Conversely, grain mold resistance was associated with the amount of AFP synthesis that was induced by the stress.

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