Ergosterol Concentration in Mold-Susceptible and Mold-Resistant Sorghum at Different Stages of Grain Development and Its Relationship to Flavan-4-ols†

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Sorghum [*Sorghum bicolor* (L.) Moench] germplasm and breeding lines exhibiting contrasting reactions to the grain mold complex were grown at Patancheru, India. Grains were harvested at different days after flowering (DAF) and analyzed for ergosterol. Methanol and acidified methanol extracts of grains were analyzed for flavan-4-ols. Ergosterol concentration increased with increasing DAF in the mold-susceptible accessions and was 10-fold higher in grains collected at 50 DAF than in the corresponding mold-resistant accessions. Ergosterol concentration could therefore be used to assess the magnitude of mold damage in sorghum grains. The correlation coefficient between ergosterol and flavan-4-ols concentrations was significant ($P < 0.01$) and negative in colored mold-susceptible and mold-resistant accessions that did not have testa, but no significant correlation was observed in white mold-resistant and mold-susceptible sorghum. This indicates that there must be another genetic trait or mechanism besides flavan-4-ols that is associated with mold resistance.

INTRODUCTION

Sorghum grain mold disease is caused by a complex range of fungi including *Fusarium moniliforme* (Sheld.), *Curvularia lunata* (Wakker) Boedijn, and *Phoma sorghina* (Sacc.) Boerma et al. Sorghum grain mold has been identified as an important disease in semiarid tropical areas where the crop matures under warm and humid conditions (Bandyopadhyay et al., 1988). Evaluation of sorghum germplasm accessions has led to the identification of many grain mold-resistant accessions (Bandyopadhyay et al., 1988). Chitin is a constituent of the cell walls of most fungi and has been used to measure fungal growth in stored corn, soybean (Donald and Mirocha 1977), and wheat (Golubchuk et al., 1960). However, this method is not as sensitive as some of the other methods, such as ergosterol assay, which is also a predominant sterol component in nearly all fungi (Seitz et al., 1977). Seitz et al. (1979) published a procedure to determine ergosterol and reported it to be a more sensitive, rapid, and convenient assay method than chitin in measuring *Alternaria alternata* and *Aspergillus flavus* growth on milled rice.

We have estimated the flavan-4-ols in mold-resistant and mold-susceptible sorghum grains and leaves and have established their positive association with grain mold resistance (Jambunathan et al., 1986, 1990; Jambunathan and Kherdekar, 1991). The objective of this study was to determine and compare the ergosterol concentration in developing grains of mold-susceptible and mold-resistant sorghum and to establish its relationship with flavan-4-ols concentration in grains.

EXPERIMENTAL PROCEDURES

Agronomy. Ten sorghum germplasm accessions and three breeding lines were grown during the 1989 rainy season on a Vertisol at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. They were screened for their resistance and susceptibility to grain mold at the ICRISAT Center (Bandyopadhyay et al., 1988). They were planted in a randomized complete block design with three replications. Other details of the planting plan and agronomy were as reported earlier (Jambunathan et al., 1990).

Grain Samples. Developing sorghum panicles were tagged at 50% flowering as described earlier (Jambunathan et al., 1990) and collected at 20, 30, 40, and 50 days after flowering (DAF). To closely monitor the fungal organism buildup, and hence the concentration of ergosterol in the grains, we collected the grains of mold-susceptible accessions IS 402 and IS 417 and the mold-resistant accession IS 14384 at shorter intervals. At each time of sampling, three randomly chosen panicles from each replication that best represented the plot were collected and freeze-dried soon thereafter. Grains from the freeze-dried panicles were removed and mixed thoroughly for subsequent analysis.

Ergosterol Extraction. A 25-g sample was ground in a Udy cyclone mill (U.D. Corp., Boulder, CO) to pass through a 0.4-mm screen. Ergosterol was determined according to the method of Seitz et al. (1977) with the following modifications. About 10 g of ground sample was accurately weighed in duplicate and extracted with 50 mL of methanol (MeOH) by vigorously shaking in a screw-capped bottle for 30 min. The mixture was allowed to settle, and 25 mL of clean extract was added into a screw-capped test tube containing 3 g of KOH. The mixture was vigorously agitated on a vortex mixer to dissolve KOH. Ten milliliters of *n*-hexane was added, the cap was replaced, and the mixture was incubated at 75 °C in a water bath for 30 min and then allowed to cool to room temperature. Distilled water (5 mL) was then added, the solution was mixed thoroughly and again cooled to room temperature, and the top hexane layer was carefully removed and transferred to a 50-mL beaker. To the remaining aliquot in the test tube, 10 mL of hexane was added and mixed thoroughly, and the hexane layer was carefully removed and pooled with the earlier aliquot. This procedure was repeated one more time. All the pooled hexane extracts in the beaker were evaporated to dryness on a hot-water bath. The residue was redissolved in 5 mL of methanol (HPLC grade) and filtered through a 0.45-μm filter (Millex, HV, Millipore Corp., Bedford, MA), and the filtrate was used for ergosterol analysis.

Ergosterol Determination. Ergosterol was determined in a Shimadzu LC-6A high-performance liquid chromatograph with autoinjector SIL-6A. The extract was loaded on a reverse-phase column [3-μm particle size, 6 mm × 8 cm consisting of two 4-cm Zorbax Reliance cartridges (DuPont)]. The mobile phase was methanol–water (96:4 v/v) at a flow rate of 1.2 mL/min. The column temperature was maintained at 50 °C, and the absor-

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Table I. Description of Sorghum Germplasm and Breeding Lines, Grain Color, and Munsell Color Coding

group[a]	germplasm/ breeding line	grain color	Munsell color coding[b]
CRT$^+$ (colored, resistant, with testa)	IS 625	reddish brown	2.5 YR/3/3
	IS 9353	reddish brown	2.5 YR/4/4
	IS 18759	reddish brown	2.5 YR/3/4
CST$^-$ (colored, susceptible, without testa)	IS 402	reddish yellow	5 YR/6/8
	IS 417	reddish yellow	5 YR/6/8
CRT$^-$ (colored, resistant, without testa)	IS 14375	red	2.5 YR/4/8
	IS 14380	red	2.5 YR/5/6
	IS 14384	red	2.5 YR/4/8
WST$^+$ (white, susceptible, with testa)	IS 2433	white	10 YR/8/1
	IS 2516	white	10 YR/8/1
WRT$^-$ (white, resistant, without testa)	B 48826	yellowish white	7.5 Y/9/2
	B 48890	yellowish white	7.5 Y/8.5/2
	B 48971	yellowish white	5 Y/9/2

[a] Based on phenotypic grain color, reaction to mold, and presence or absence of testa. [b] Munsell color coding denotes the hue (first value), color (second value), and chroma (third value) (*Munsell Book of Color*, 1989).

bance of eluted ergosterol was detected at 282 nm. The standard ergosterol (Sigma) had a retention time of 8.3 min.

To determine the precision of our day to day analytical procedure, we used a susceptible check, Bulk Y, that was also used as a check by the pathologist for evaluating mold incidence in the field. Sufficient quantity of ground sample of Bulk Y was prepared and kept in a cold room (4 °C). A 10-g sample was used as a check every time along with the experimental samples. Twelve such determinations were made on Bulk Y during the period of our experiment. To determine the precision of detection over a range of ergosterol concentrations, we analyzed eight times each aliquots containing 1, 5, 10, and 25 μg of ergosterol. The recovery of ergosterol was also determined by spiking Bulk Y sample with 100 μg of standard ergosterol.

Flavan-4-ol Estimation. The concentration of flavan-4-ols in methanol (MeOH) and acidified methanol (H$^+$/Me) extracts of the grain samples was estimated in A_{550} g^{-1} as described earlier (Jambunathan et al., 1990).

All analyses were carried out at least in duplicate on each of the field replications. About 3 g of each sample was dried at 110 °C for 16 h to determine the moisture content. Mean values of observations are reported on a dry mass basis. Standard errors were calculated by one-way analysis, and the correlation coefficients were calculated as described by Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

The 13 germplasm and breeding lines were selected on the basis of their white (W) or colored (C) pericarp, resistance (R) or susceptibility (S) to grain mold, and presence (T$^+$) or absence (T$^-$) of testa (Table I). One additional group of breeding lines consisting of white, mold-resistant grains without testa (WRT$^-$) was included for the first time in our investigation.

The concentration of flavan-4-ols in the MeOH and H$^+$/Me extracts of grains of four sorghum groups at different DAF is shown in Table II. The flavan-4-ol concentration (A_{550} g^{-1}) in MeOH extract of the CRT$^+$ group was around 7.8, both at 20 and 50 DAF, and it showed little variation in the case of the CRT$^-$ group also. The concentration of flavan-4-ols in the CST$^-$ group decreased from 10.7 at 20 DAF to 3.2 at 50 DAF; i.e., the mold-susceptible group had a higher concentration of flavan-4-ols than the mold-resistant group in the early stages of grain formation. A similar observation was made earlier (Jambunathan et al., 1990). Flavan-4-ols were not detected in the MeOH extracts of grains of the WRT$^-$ group. This observation is unique because our observation so far has indicated an association of flavan-4-ols with grain mold resistance in the CRT$^+$ and CRT$^-$ groups. The concentration of flavan-4-ols in the H$^+$/Me extracts of the CST$^-$ group at 20 DAF decreased to 28% of its value at 50 DAF; its decrease in the CRT$^-$ group was not of the same magnitude as that in the CST$^-$ group. The CRT$^-$ group had a much higher (about 70%) concentration of flavan-4-ols at 50 DAF than the CRT$^+$ group; i.e., sorghum accessions without testa had higher flavan-4-ol concentration in the H$^+$/Me extract than the sorghum accessions with testa. The presence of testa is associated with tannin in sorghum (Butler, 1988). This observation indicates that the concentration of flavan-4-ols need not be associated with tannin content.

We determined the precision of the ergosterol method by extracting and analyzing the ergosterol concentration from one susceptible check sample, Bulk Y, along with our experimental samples. The mean ergosterol concentration of Bulk Y based on 12 determinations was 97.8 μg g^{-1}, range 92.6–102.6 μg g^{-1}, and the coefficient of variation was 4.0%. The coefficients of variation for the various concentrations of ergosterol that were used to check the precision of detection by HPLC were as follows: 1 μg, 8.1%; 5 μg, 3.9%; 10 μg, 2.6%; and 25 μg, 2.7%. The recovery of spiked ergosterol (100 μg) added to Bulk Y varied between 89.0 and 101.0 μg with a mean of 96.0 μg based on 10 determinations. The coefficient of variation was 3.6%.

The grains of mold-susceptible accession IS 402 were collected at 18, 21, 23, 25, 27, 30, 33, 36, 39, 41, 43, 48, 50, 52, and 54 DAF. The concentration of ergosterol and total flavan-4-ols (sum of flavan-4-ols in MeOH and H$^+$/Me extracts) of these grains is shown in Figure 1. Ergosterol was not detected at 18 DAF when the first sampling was done, but it increased rapidly from 1.6 μg g^{-1} at 21 DAF to 164.1 μg g^{-1} at 54 DAF. In contrast, flavan-4-ols in MeOH and H$^+$/Me extracts showed a continuous decrease from 18 DAF. Flavan-4-ol concentration (A_{550} g^{-1}) in MeOH extract at 18 DAF was 13.2, decreasing to 3 at 54 DAF. The concentration of flavan-4-ols in the H$^+$/Me extract was 9.3 at 18 DAF, decreasing to 3 at 54 DAF. The grains of the second mold-susceptible accession, IS 417, were collected at 18, 21, 25, 30, 36, 41, 50, and 54 DAF. The buildup of ergosterol was similar to that of IS 402 initially, but the concentration of ergosterol was much lower at later stages (Figure 1). At 50 DAF, ergosterol concentration in IS 417 was the highest (89.1 μg g^{-1}), which was about 57% of the value obtained with IS 402. However, the concentrations of flavan-4-ols both at 18 and 54 DAF were similar to those of IS 402. This indicated a difference in the response of these two sorghum accessions to grain mold, although they both had similar concentrations of flavan-4-ols.

The data in Figure 2 show the concentration of ergosterol and total flavan-4-ols in a mold-resistant accession, IS 14384. The grains were collected at 20, 25, 30, 35, 40, 45, 50, and 55 DAF. The difference between the mold-susceptible and mold-resistant accessions in the buildup of ergosterol can be seen by comparing its concentration at different DAF (Figures 1 and 2). Up to 25 DAF, the ergosterol could not be detected in IS 14384. Between 35 and 55 DAF, the ergosterol concentration varied from 6.8 to 11.2 μg g^{-1} (Figure 2). It may be noted that the ergosterol concentration scale in Figure 2 is 10 times lower than the concentration scale used in Figure 1 for the mold-susceptible accessions, while the flavan-4-ol scales for the two are identical. The flavan-4-ol concentration (A_{550} g^{-1})

Table II. Flavan-4-ol Concentration [A_{550} (g^{-1})] in Methanol (MeOH) and Acidified Methanol (H^+/Me) Extracts, Rainy Season, 1989

	days after flowering							
	20		30		40		50	
group[a]	MeOH	H^+/Me	MeOH	H^+/Me	MeOH	H^+/Me	MeOH	H^+/Me
CRT^+	7.8 ± 2.17	7.8 ± 0.40	5.8 ± 0.24	4.4 ± 0.20	7.6 ± 0.24	3.8 ± 0.23	7.8 ± 0.80	3.3 ± 0.21
CST^-	10.7 ± 0.50	9.7 ± 0.51	7.7 ± 0.90	7.4 ± 0.48	4.4 ± 0.18	3.8 ± 0.71	3.2 ± 0.72	2.8 ± 0.35
CRT^-	7.7 ± 0.37	10.9 ± 0.19	6.1 ± 0.52	8.5 ± 0.20	6.8 ± 0.11	6.1 ± 0.26	7.1 ± 1.20	5.6 ± 0.27
WST^+	0.3 ± 0.01	0.7 ± 0.04	0.4 ± 0.20	1.0 ± 0.16	0.5 ± 0.30	1.2 ± 0.11	0.5 ± 0.22	0.7 ± 0.13

[a] As in Table I. Flavan-4-ols were not detected in breeding lines belong to the WRT^- group.

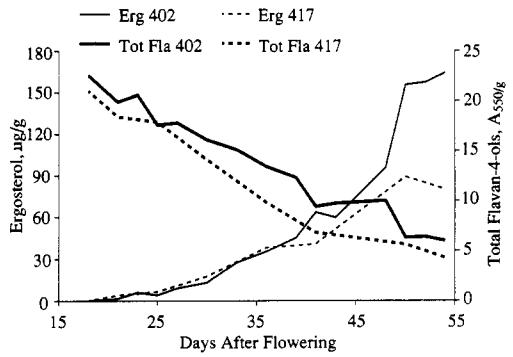

Figure 1. Concentration of ergosterol and total flavan-4-ols in developing grains of mold-susceptible sorghum accessions IS 402 and IS 417, collected at frequent intervals. Each value represents the mean of three field replicates. Standard error for ergosterol ranged from 0 to 38.22 with a mean of 9.52 and for total flavan-4-ols from 0.06 to 0.99 with a mean of 0.51.

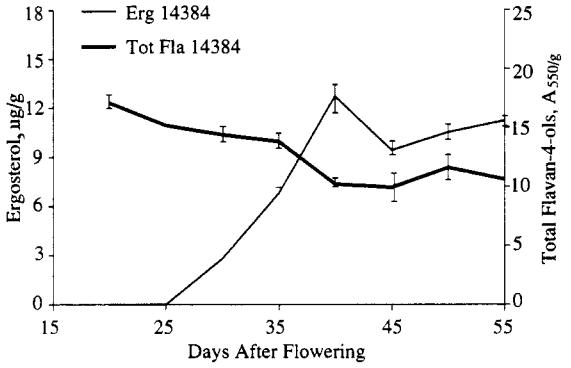

Figure 2. Concentration of ergosterol and total flavan-4-ols in developing grains of mold-resistant sorghum accession IS 14384, collected at frequent intervals. Each value represents the mean of three field replicates, and standard errors representing means of groups are shown by vertical lines.

in the MeOH extract of IS 14384 was only 6.3 at 20 DAF, while in IS 417 it was 9.5 at 21 DAF, and in IS 402 it was 11.8 at 21 DAF. In the resistant accession, flavan-4-ols showed a small variation in concentration at different stages of grain maturity, while in the mold-susceptible accessions, the values showed a gradual decrease between 21 and 55 DAF (Figures 1 and 2). The concentration of flavan-4-ols (A_{550} g^{-1}) in the mold-resistant accession was at least 2-fold higher than the mold-susceptible accessions at 55 DAF, which confirmed our earlier observations (Jambunathan et al., 1986, 1990).

The development and concentration of ergosterol and its relationship to total flavan-4-ols in the CRT^+, CRT^-, WRT^-, CST^-, and WST^+ groups are shown in Figures 3 and 4. In the CRT^+ group, the data shown at 20 DAF represent the value obtained from only one accession, IS 9353, and at 30 DAF represent the values obtained from two accessions, IS 9353 and IS 18759. Total flavan-4-ols (A_{550} g^{-1}) decreased from 15.6 at 20 DAF to 11.1 at 50 DAF, and during the same period, ergosterol concentration

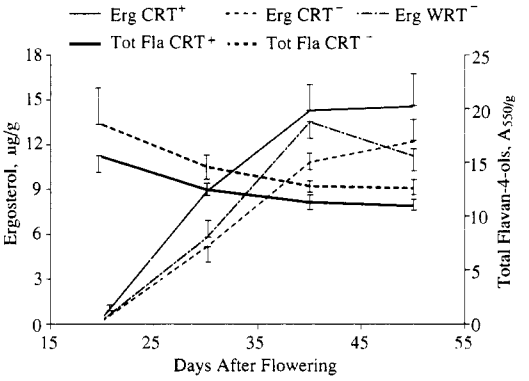

Figure 3. Concentration of ergosterol and total flavan-4-ols in developing grains of mold-resistant sorghum accessions and breeding lines (CRT^-, CRT^+, and WRT^-). Each value represents the group mean. Standard errors representing means of groups are shown by vertical lines.

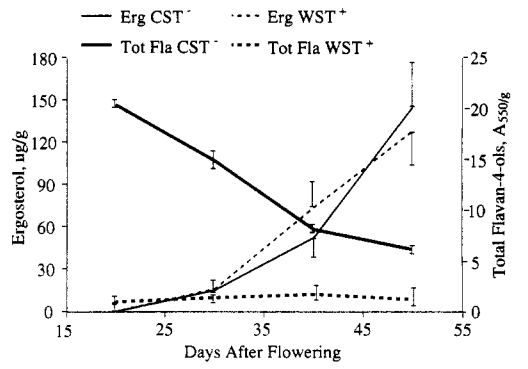

Figure 4. Concentration of ergosterol and total flavan-4-ols in developing grains of mold-susceptible sorghum accessions (CST^- and WST^+). Each value represents the group mean. Standard errors representing means of groups are shown by vertical lines.

(µg g^{-1}) increased from 0.7 to 14.6. In the CRT^- group, total flavan-4-ols (A_{550} g^{-1}) decreased from 18.6 at 20 DAF to 12.7 at 50 DAF (Figure 3). During the same period, ergosterol concentration (µg g^{-1}) increased from 0.4 to 12.3. In white resistant breeding lines without testa (WRT^-), ergosterol concentration (µg g^{-1}) varied from 0.4 at 20 DAF to 11.3 at 50 DAF (Figure 3). Total flavan-4-ols in this group were negligible and ranged from 0.0 to 0.6 (A_{550} g^{-1}) and hence are not shown in the figure.

In the mold-susceptible accessions (CST^-), total flavan-4-ols (A_{550} g^{-1}) at 20 DAF were 20.4 and decreased to 6.0 at 50 DAF (Figure 4). During the same period, ergosterol concentration (µg g^{-1}) increased from 0.1 to 144.8. In white, susceptible accessions, the grains of which have testa (WST^+), total flavan-4-ols (A_{550} g^{-1}) were low throughout and ranged from 1.0 at 20 DAF to 1.2 at 50 DAF (Figure 4) while the ergosterol concentration (µg g^{-1}) increased from 0.2 at 18 DAF to 128 at 50 DAF.

The variability in the ergosterol concentrations among the five groups of sorghum accessions can be compared by expressing the values as a ratio of CST^- in comparison

with other groups. The CST^-/CRT^+ ratios at different DAF varied from 0.2 at 20 DAF to almost 10 at 50 DAF. A similar result was obtained in the case of CST^-/CRT^- and CST^-/WRT^-, indicating that the mold-susceptible germplasm accessions had almost 10 times the ergosterol concentration in the mature seeds than the mold-resistant accessions. The ratios of CST^-/WST^+ at different DAF varied only from 0.5 at 20 DAF to 1.1 at 50 DAF. This indicated that the ergosterol values were similar in both susceptible groups, although the accessions in one of them had testa (WST^+) and in the other they did not (CST^-).

The relationship between ergosterol and total flavan-4-ols in the five groups was studied. A significant and negative correlation coefficient ($r = -0.73$, $P < 0.01$) was obtained between the concentration of ergosterol and flavan-4-ols in the CST^- group. A significant and negative correlation ($r = -0.60$, $P < 0.01$) was also obtained between the concentration of ergosterol and flavan-4-ols in the CRT^- group. The correlation coefficient obtained between the concentration of ergosterol and flavan-4-ols for the CRT^+ group was negative and significant ($r = -0.60$, $P < 0.01$), and for WST^+ it was positive but not significant ($r = 0.28$). The correlation coefficient between ergosterol and flavan-4-ols for accessions belonging to all of the groups CRT^+, CST^-, CRT^-, and WST^+ was negative and significant ($r = -0.50$, $P < 0.01$). The correlation coefficient between ergosterol and flavan-4-ols for accessions having testa (CRT^+ and WST^+) was negative and significant ($r = -0.49$, $P < 0.01$), and for accessions without testa (CRT^- and CST^-) it was also negative and significant ($r = -0.65$, $P < 0.01$).

Seitz et al. (1983) studied the buildup of ergosterol concentration in sorghum hybrids and lines that were grown in Kansas and Texas and harvested at 12 weekly intervals. They investigated the relationships among grain maturity, harvest date, kernel discoloration, hybrid type, and fungal invasion. They observed a varying degree in the susceptibility of hybrids and lines. The ergosterol concentration increased due to wet weather in all of them. Visual ratings did not adequately indicate the extent of fungal invasion, although it was highly correlated with ergosterol concentration. Although grain mold resistance or susceptibility was not the objective of this study, ergosterol concentration varied greatly in sorghum lines, roughly from 15 to 120 ppm of ergosterol, in College Station, TX (Sietz et al., 1983), and we report a similar range in our investigation. They concluded that it is important to know the relationship between harvest date and physiological maturity to relate these observations. Forbes et al. (1989) studied the ergosterol concentratioon, kernel weight and density, percentage germination, etc. of grains of six sorghum cultivars that were inoculated with *F. moniliforme* in the field and compared them with noninoculated controls. Ergosterol concentration varied from 25 to 353 ppm in inoculated cultivars; it varied from 5.2 to 21.4 ppm in controls. The ergosterol concentration in our mold-susceptible and mold-resistant sorghum accessions and breeding materials grown under semiarid tropical conditions ranged from 10 (B 48890) to 168 $\mu g\ g^{-1}$ (IS 402), indicating the importance of environmental influence in these measurements.

CONCLUSIONS

Data obtained on developing grains of sorghum having contrasting reactions to sorghum grain mold showed that in mold-susceptible sorghum accessions there was a steep increase in ergosterol concentration during the grain developmental stage compared with the mold-resistant accessions. In the mold-resistant accessions, ergosterol concentration was only about 10% of the value of mold-susceptible accessions in the mature grains. From our limited data, it may be said that ergosterol concentration of 30 $\mu g\ g^{-1}$ or below in mature sorghum grains that are grown under conducive conditions for the development of mold could be an indicator of the mold-resistant nature of the sorghum lines. Pathologists and breeders have usually relied on visual examination of sorghum grains to give ratings for mold damage and to select the desired entries. However, ratings can sometimes be erroneous depending on the extent to which mold damage can be seen visually by the naked eye and also will be difficult to judge in the case of close similarity between two samples. Ergosterol concentration, a highly sensitive indicator of total fungal biomass, gives an accurate method of choice for confirming the ratings given by the pathologists and breeders and could be an important tool to supplement the selection criteria.

We have shown that in the case of colored sorghum accessions with or without testa there is a negative and significant relationship between ergosterol and flavan-4-ols, and the concentration of flavan-4-ols in the acidified methanol extract need not be associated with tannin content. The significance of this finding and the role that flavan-4-ols play in mold resistance still need further elucidation. A new group, WRT^-, mold-resistant sorghum without testa having white grains, showed a negligible concentration of flavan-4-ols which needs further investigation. This indicates that there must be at least another genetic trait besides flavan-4-ol that is associated with mold resistance. This group would be of considerable interest as white grains are preferred than colored grains by traders and consumers in several countries. There is also a possibility that other colored genotypes may behave as the white ones have concerning the correlation between flavan-4-ol and ergosterol concentration because the polyphenolic diversity and effects thereof in sorghum are enormous.

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