Weathered Grain Sorghum: Natural Occurrence of Alternariols and Storability of the Grain

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ABSTRACT

Sorghum grain of several commercial hybrids from seven locations in Kansas was studied to determine natural occurrence of *Alternaria* metabolites, alternariol monomethyl ether (AME) and alternariol (AOH), and to evaluate the storability of the grain. Amounts of AME and AOH found in the sorghums depended mainly on location and to a lesser extent on cultivar. Metabolite levels correlated with degree of grain discoloration and with number of rainy days during September and October. When sorghum was exposed to an adverse storage environment, grain which was weathered, discolored, heavily invaded by fungi, or even sprout-damaged, was no more susceptible to storage mold invasion than was clean, sound grain.

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Alternaria metabolites have been investigated by a number of workers because of their involvement in plant diseases and possibly in animal diseases (3, 4, 5, 6, 7, 9). While *Alternaria* is common in nature and a variety of metabolites are produced in culture, attempts to isolate metabolites in natural material (3, 4, 5, 6) had been successful only with tobacco (5) prior to our recent report of the identification of two *Alternaria* metabolites, alternariol monomethyl ether (AME) and alternariol (AOH), in weathered, discolored grain sorghum (8). We also noted that AME and AOH could be mistaken for zearalenone and/or aflatoxins if improper analytical tests were used.

We present in this paper results of a study of natural occurrence of AME and AOH in several commercial hybrid grain sorghums from seven locations in Kansas representing a range of weather conditions and on the extent of *Alternaria* invasion, discoloration, and sprout-damage. Much of the discolored grain also was badly sprout-damaged, leading to concern about its storability. By exposing grain of several hybrids from different locations to an adverse storage environment, we sought to find out if sprouting or weathering made the grain more susceptible to invasion by storage fungi.

MATERIALS AND METHODS.—*Sorghum samples.*—Grain sorghum samples were obtained from test plots managed by the Kansas Agricultural Experiment Station in seven different counties. At each location all hybrids were planted at the same time and harvested at the same time.

AME and AOH analysis.—Levels of AME and AOH were determined by an analysis procedure described previously (8). Whole grain (50 g) was homogenized with methanol in a blender for 2 minutes. Thirty milliliters of filtered methanol extract, 60 ml of 20% ammonium sulfate, and 30 ml hexane were shaken together in a separatory funnel for 30 seconds. After layers separated, the upper (hexane) layer was discarded. Then the aqueous ammonium sulfate-methanol layer was extracted twice with 5-ml portions of methylene chloride. The combined methylene chloride extracts were evaporated to dryness on a steam bath while flushing the container with a slow stream of nitrogen. The residue was dissolved in 0.5 ml benzene:acetonitrile (98:2, v/v) to produce a final extract ready for thin-layer chromatography (TLC). Final extracts (25 μliters each) were spotted on precoated SILG-HR-25 plates (Brinkman Instruments, Inc., Westbury, N.Y.) activated for 1 hour at 120 C. Also spotted on each plate was 10 μliters of a standard solution containing 16 ng/μliter AME and 17 ng/μliter AOH in benzene:acetonitrile (98:2, v/v). The standard solution was prepared from purified AME and AOH obtained

from R. W. Pero, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.

Relative fluorescence intensities of sample and standard AME and AOH spots were recorded using an Aminco fluorodensitometer (American Instrument Co., Silver Spring, Md.). To minimize effects from fluorescence fading, each plate was scanned during a 10- to 20-minute period after its removal from the developing tank. Also, all of the extracts of samples from one county were spotted on the same TLC plate along with the standard. There is greater fading of fluorescence with AME than AOH; hardly any fading was noted in the AOH spots.

The hexane extraction of the aqueous ammonium sulfate-methanol layer used to remove oil and pigments also removed 29% of the AME. No AOH was removed by the hexane. Thus, AME concentrations obtained by analyses of methylene chloride extracts were adjusted upward by a factor of 1.4. The average amount of AME lost was determined by extracting hexane washes from several samples with two 25-ml portions of 50% methanol in water. Methanol-water extracts were combined and partitioned with two separate 5-ml portions of methylene

TABLE 1. Levels (μg/g) of *Alternaria* metabolites (alternariol plus alternariol monomethyl ether) in sorghum hybrids from seven Kansas locations in 1973

Hybrid	County						
	Brown	Riley	Labette	Ford	Thomas	Greeley	Finney
RS 610	4.7	5.2	2.5	1.2	1.5	tr	0
RS 671	5.0	7.9	3.8	1.2	1.7	0	0
Asgrow Dorado	2.5	1.5	2.8	1.5	1.0	0	0
Pioneer 8442	1.2	0.7	1.3	0.5	0.2	0	0

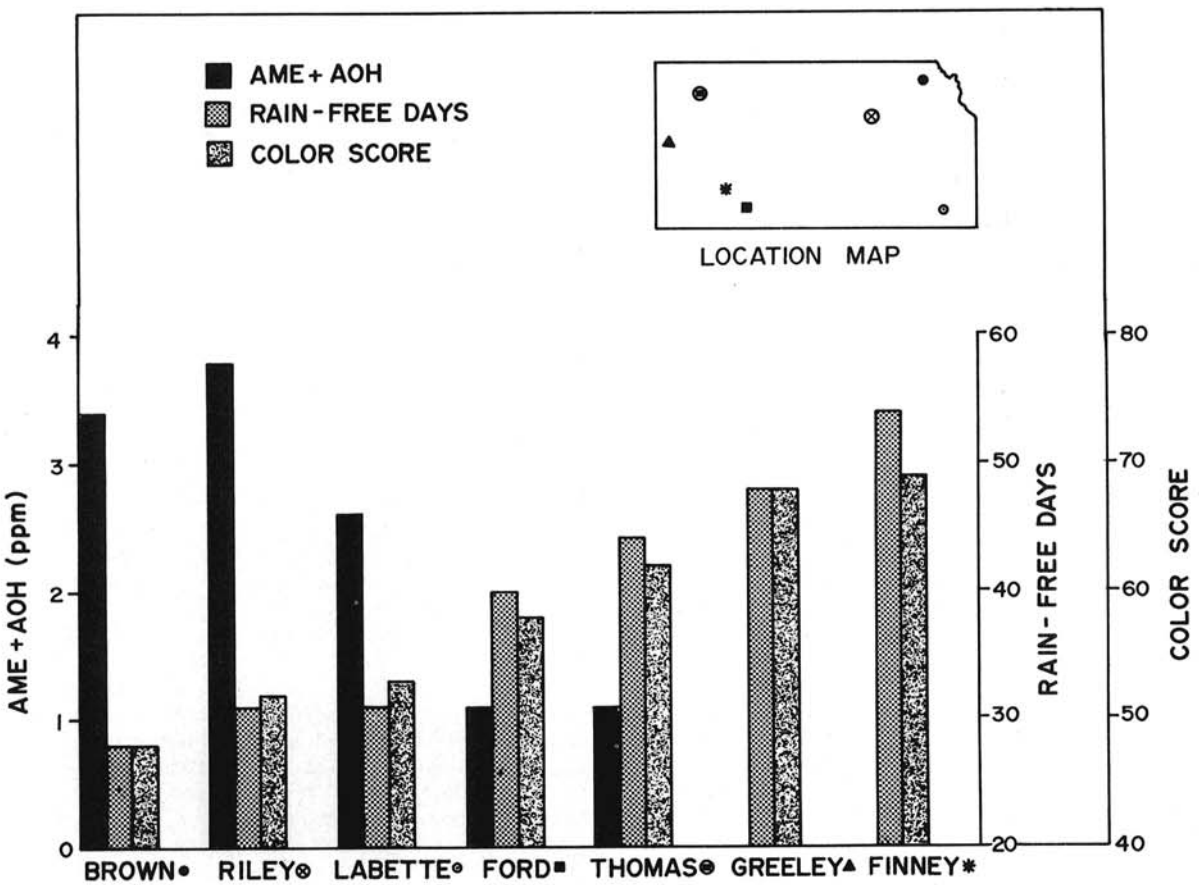

Fig. 1. Average *Alternaria* metabolite (AME + AOH) level, rain-free days, and Hunterlab color scores of four sorghum hybrids (RS 610, RS 671, Asgrow Dorado, and Pioneer 8442) from seven locations in Kansas in 1973. [Climatological data from U.S. Department of Commerce (10)]. Correlation coefficients (r) and standard errors (s.e.) for the three relationships are: rain-free days and alternariol level, $r = -0.91$, s.e. = 0.70; alternariols and color score, $r = -0.95$, s.e. = 2.8; rain-free days and color score, $r = 0.98$, s.e. = 1.6.

chloride. The methylene chloride extracts were combined and evaporated to dryness; the residue was redissolved in 0.5 ml benzene:acetonitrile (98:2, v/v). Amount of AME was determined by TLC as described above, and also by high-pressure liquid chromatography (HPLC).

Brightness and color measurements.—Reflectance and color characteristics of the sorghum samples were measured using a Hunterlab D25 color difference meter (Hunter Associates Lab, McLean, Va.).

Storage tests.—Grain was placed in shallow layers on trays in a controlled environment chamber at 24 C and approximately 87% relative humidity and held for 6 weeks. Grain was tested weekly for moisture content and percentage invasion by storage fungi. Moisture content (wet-weight basis) was determined by drying 10 g of grain in a forced draft oven for 17 hours at 120 C. Fungal invasion was determined by surface sterilizing the grain in 2% NaOCl for 1 minute, rinsing in sterile water and plating 100 kernels on Difco (Difco Laboratories, Detroit, Mich.) malt agar containing 4% NaCl and 200 μg/g Tergitol NPX (Union Carbide Corp., New York, N.Y.). Plates were incubated at 25 C until fungi could be identified and counted. Germination percentage of the sorghum was determined initially and after 4 weeks of storage; 200 kernels were placed between moist paper towels in an aluminum foil folder and held at room temperature for 6 days.

RESULTS.—*Occurrence of Alternaria metabolites.*—Levels of alternariols (AME + AOH) in four grain sorghum hybrids (RS 610, RS 671, Asgrow Dorado, and Pioneer 8442) from seven locations in Kansas are shown in Table 1. Ten additional hybrids were tested from any of the seven locations from which they were available. The average number of hybrids analyzed from each location was nine; no location was represented by fewer than six. AME-AOH values were comparable to those shown in Table 1. Pioneer 8442 had the lowest average value (0.6 μg/g) and RS 671 the highest (2.8 μg/g) of the 14 tested. Levels of alternariols were not related to difference in maturity times for the various hybrids. The ratios of AME to AOH in sorghum samples were variable, although AOH was generally the more abundant of the two metabolites. AOH concentration was frequently twice, and sometimes three times as high as AME, although in a few samples AME levels were twice as high as AOH.

There was a definite relationship between location and levels of alternariols. The three eastern counties (Brown, Riley, Labette) had more wet weather during September and October than the western counties (Ford, Thomas, Greeley, Finney). Using the number of rain-free days during September and October as an indicator of the kind of weather that occurred in each county during and after maturation of the sorghum, one can see from Fig. 1 that, in general, the AME-AOH level decreased as the number of rain-free days increased. Greeley County experienced nearly as much rain as Thomas County; however, the planting date in Greeley County was 14 days later than that of Thomas County. Consequently, Greeley County sorghum may have arrived at the critical stage of maturity, as far as *Alternaria* is concerned, just after the wet period. Finney County was driest of the seven counties.

Eastern Kansas sorghum samples were visibly discolored and darkened by the growth of fungi in contrast to bright-looking grain from western counties. High readings for the three Hunter values (total reflectance "L", redness "a", and yellowness "b") indicated bright, clean-looking grain, so the values were totaled for each sample to obtain a single score. Averages of Hunterlab color difference meter scores for the four hybrids grown at the seven locations are included in Fig. 1. The data indicate that locations with high AME-AOH values tended to have low Hunter scores as well as a low number of rain-free days.

Weather conditions at Finney and Ford County stations, separated by only about 88 km, differed considerably during September. Finney County had rain on 7 days for a total of 5.8 cm, whereas Ford County had rain on 21 days for a total of 17.3 cm. Station observers in Ford County noted that frequent precipitation during the maturation period slowed drying and caused deterioration. On the other hand, conditions at the Finney County station were considered excellent for normal grain maturation.

AME and AOH, and probably most of the discoloration, resulted from growth of *Alternaria;* so we tried to find a satisfactory way to measure the amount of *Alternaria* in a sample. *Alternaria* grew from nearly 100% of surface sterilized kernels plated on agar media whether they were discolored or not. Various attempts to make dilution cultures from ground or chopped samples also failed to show differences between heavily invaded and lightly invaded lots. Dilution cultures produced very low numbers of *Alternaria* colonies per gram of grain, even in samples that appeared heavily invaded.

Alternaria appeared to grow out faster and heavier from surface sterilized kernels of discolored samples than from bright grain. We tried washing spores from seeds after 2 or 3 days of incubation on plates and then counting the spores in a hemacytometer, but this also failed to show large or consistent differences between discolored and normal grain.

Using techniques similar to those of Hyde and Galleymore (2), kernels were soaked in water to loosen and remove the pericarps which were then stained, cleared, and examined under a microscope. More fungal mycelium could be seen in the pericarps of discolored than in bright kernels, but mycelium of different species of fungi could not be distinguished. The rather tedious technique did not seem to be a practical way to measure degree of invasion by *Alternaria.*

Storage tests.—Table 2 shows typical storage data for one hybrid (RS 671) from three of the seven counties. Similar data were obtained for 38 lots of grain representing seven hybrids from the seven locations. Not all hybrids were available from all locations. Sorghum from the three counties included in Table 2 varied widely in initial condition including level of alternariols. Moisture contents remained constant during 4 weeks of storage, then declined slightly because of a drop in relative humidity in the chamber. The main storage fungi which grew in the sorghum were the *Aspergillus glaucus* group and to a lesser extent *Penicillium* species. The principal field fungi, *Alternaria* (not shown in Table 2), and in eastern Kansas, *Fusarium,* remained static or declined during storage. Storage and the resultant mold

TABLE 2. Fungal invasion, moisture content, and germinability of hybrid RS 671 sorghum grain from three locations during 6 weeks storage at 24 C

Location and storage time (weeks)	Moisture content (%)	Germination (%)	Percentage of kernels invaded by:		
			Aspergillus glaucus	*Penicillium* sp.	*Fusarium* sp.
Riley County					
0	14.5	32(67)[a]	0	0	56
1	17.8	...	0	0	54
2	17.7	...	2	2	59
3	17.8	...	11	3	43
4	17.7	7	51	5	38
6	17.3	...	40	21	42
Thomas County					
0	14.5	76(29)	...	...	16
1	18.2	...	0	0	18
2	18.1	...	15	2	2
3	18.1	...	32	6	0
4	18.2	26	29	13	0
6	17.7	...	39	28	4
Finney County					
0	...	93(4)	0	0	18
1	17.9	...	0	0	26
2	17.8	...	36	0	1
3	17.8	...	58	1	0
4	17.8	34	53	7	3
6	17.5	...	46	19	1

[a]Percentage of kernels with sprout-damage or cracks over germ.

TABLE 3. Characteristics of sorghum grain from seven Kansas locations and extent of mold invasion during storage at 24 C[a]

Location (county)	Before storage							During storage		
	Sprouted or cracked pericarp over germ (%)	Germination (%)	1000-kernel weight (g)	Initial field fungi (%)				Average moisture content (%)	Storage mold[b]	Germination after 4 weeks (%)
				Alternaria	*Fusarium*	*Cladosporium*	*Helminthosporium*			
Brown	64	40	25	98	49	9	6	17.6	97	30
Riley	56	46	25	98	62	2	3	17.7	105	14
Labette	43	81	23	93	58	1	10	17.6	38	55
Ford	18	93	21	99	8	17	1	17.6	36	79
Thomas[c]	19	85	24	95	18	2	1	17.9	120	36
Greeley[c]	9	84	21	75	...	9	1	18.4	201	32
Finney[c]	4	95	28	86	15	16	1	17.6	141	45

[a]Averages for hybrids RS 610, RS 671, Asgrow Dorado, and Pioneer 8442.
[b]Sum of percentages of kernels invaded by *Aspergillus* and by *Penicillium* after 1, 2, 3, and 4 weeks.
[c]Irrigated plots.

invasion did not alter the concentration of AME and AOH.

A summary of the relationships between various characteristics of four hybrids from the seven locations and the extent to which they were invaded by storage fungi during the first 4 weeks of storage is shown in Table 3. Equilibrium moisture contents varied slightly with hybrids, but more noticeably among counties. Greeley County samples, judged by appearance and low 1000-kernel weights, were somewhat immature which may have affected equilibrium moisture contents and susceptibility to invasion by storage fungi.

DISCUSSION.—Date of harvest was not a factor influencing the AME-AOH level produced. Sorghum hybrids from Finney and Greeley Counties, which did not contain AME or AOH, were harvested much later than sorghum from the other five counties which did contain alternariols. Although average daily temperatures were higher in the eastern counties than in the western counties, it was not apparent what role, if any, temperature played in determining AME-AOH level or degree of discoloration. It appears that most of the weathering occurred well before harvest, probably during September when most of the rainfall occurred.

The specific stage of maturity at which the grain is most susceptible to *Alternaria* invasion during wet weather cannot be determined from the information available. However, grain that remained green during the wet period and matured later was not discolored. In October shortly after the wet weather, we collected green and

mature heads from a Riley County field. Some of the mature heads were discolored; others were normal and bright. Only discolored grain contained AME and AOH.

Germination tests on sorghums prior to storage showed a definite relationship between germinability and cracked seeds (Tables 2 and 3). In most cases the percent germination plus the percent cracked seeds totaled approximately 100. If we assume that cracked, sprouted, dead seeds are more easily invaded by storage fungi than sound seeds (1), then some other factor which accompanies sprout-damage, such as heavy invasion by field fungi, must offset the vulnerability of the sorghums to storage fungi.

Nothing in the data suggests that sorghum that is weathered, discolored, heavily invaded by field fungi, or even sprout-damaged is more susceptible to storage mold invasion than is clean, sound grain. In fact, the best quality grain (Finney County) became moldy in storage faster than did the worst grain from the three eastern counties. Perhaps the heavy invasion by field fungi made the weathered grain more difficult for other fungi to attack. Such "resistance" could result from inhibitory fungal metabolites or other biological activity of the fungi already present in the seeds. We observed a similar phenomenon in southern corn leaf blight-infected corn heavily invaded by field fungi. The grain was less susceptible to storage mold invasion than was similar corn free of blight (Sauer and Burroughs, *unpublished*).

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