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Polyphenol Concentrations in Grain, Leaf, and Callus Tissues of Mold-Susceptible and Mold-Resistant Sorghum Cultivars

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Sorghum cultivars exhibiting both resistance and susceptibility to grain mold were subjected to a variety of assays for polyphenols including flavan-4-ols and proanthocyanidins. In methanol and acidified methanol extracts of grains of mold-resistant cultivars, the levels of flavan-4-ols were two- to threefold higher than in mold-susceptible cultivars. Similar results were noted when the leaves of resistant and susceptible cultivars grown under greenhouse conditions were harvested at different stages of growth and analyzed for flavan-4-ols.

INTRODUCTION

The role of plant phenolics including their protective effects against fungi and other pathogens has been reviewed (Friend, 1981). Harris and Burns (1973) reported that sorghum seed tannin content was strongly and negatively correlated with preharvest seed-molding indices. The condensed tannins (proanthocyanidins, oligomers of flavan-3-ols) are depolymerized in acid solution to form anthocyanidin pigments. Cyanidin is the anthocyanidin produced from sorghum tannins (Gupta and Haslam, 1978), which are therefore referred to as procyanidins. Certain monomeric flavanols such as flavan-3,4-diols and flavan-4-ols can also give rise to anthocyanidins and are therefore distinguished from the oligomeric flavan-3-ols by the name "leucoanthocyanidin" (Watterson and Butler, 1983). We observed mold resistance in sorghum cultivars that have relatively low levels of tannin and examined a representative group of susceptible and resistant cultivars with a variety of assays for polyphenols including flavan-4-ols and condensed tannin.

EXPERIMENTAL SECTION

Grain Samples. Sorghum cultivars were grown at the International Crops Research Institute for the Semi-Arid

Tropics, Patancheru, India, during the 1982 rainy season. Sorghum cultivars exhibiting both resistance and susceptibility to grain mold caused by a complex range of unspecialized fungi including *Fusarium moniliforme* and *Curvularia lunata* were selected according to established methods (ICRISAT, 1984). The seeds were exported to the U.S. and cleared through the Plant Protection and Quarantine Program of the U.S. Department of Agriculture. All analyses were carried out in the Department of Biochemistry, Purdue University.

All grain samples were ground in an Udy cyclone mill (U.D. Corp., Boulder, CO) to pass through a 0.4-mm screen. The color of the grain meal was measured in a Hunter Lab Colorimeter, Model D25-A2, using a yellow tile standard.

The sorghum meal was defatted by stirring with diethyl ether, 6 mL/g, for 30 min, and the mixture was filtered through a Whatman 541 filter paper. The residue was air-dried overnight and transferred to a screw-capped vial. The defatted sorghum meal was extracted with methanol, 6 mL/g, for 30 min in a screw-capped test tube. After centrifugation, the residue was reextracted with methanol, and the two methanol extracts were combined for analysis. The residue was further extracted twice with methanol containing 1% (v/v) concentrated HCl (H⁺/methanol), and these two extracts were pooled together for analysis.

Total phenols were measured by a modification of the Prussian blue assay as described by Butler (1982). Protein-precipitable phenols were estimated by using a modified procedure of Hagerman and Butler (1978), as follows:

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To 1.0 mL of the sample extract was added 2.0 mL of bovine serum albumin (BSA) solution (1.0 mg BSA/mL in 0.2 M acetate buffer, pH 4.8) and the resultant solution mixed. After 10 min, the contents were centrifuged in a clinical centrifuge for 10 min and the supernatant was discarded. Then, 2.0 mL of methanol was added without disturbing the pellet, the contents were centrifuged, and the supernatant was discarded. The pellet was dissolved in 4.0 mL of a solution containing 1% sodium dodecyl sulfate and 5% triethanolamine. Then, 1.0 mL of 0.01 M ferric chloride in 0.01 N HCl was added, and after 20 min the absorbance of the sample was read at 510 nm. A solvent blank and a sample blank (addition of ferric chloride was omitted) were also included. The vanillin assay was carried out with glacial acetic acid (Butler et al., 1982). With a solvent of 1-butanol and concentrated HCl, anthocyanidin formed from flavan-4-ols was measured at 550 nm (Butler, 1982). On heating, the unstable anthocyanidin pigment formed from flavan-4-ols is completely destroyed. However, under these conditions flavan-3-ol oligomers are converted to anthocyanidins; the resulting absorbance was measured at 550 nm. A solvent consisting of 50% concentrated HCl and 50% formic acid as reported by Dreyer et al. (1981) was used to estimate what they called "procyanidin", which we refer to a leucoanthocyanidins because true procyanidins are not present in sorghum leaf tissue (Watterson and Butler, 1983) and because this material has properties similar to flavan-4-ols (see Conclusions). To 7 mL of the solvent was added 0.25 mL of the sample extract, the resultant solution was mixed, and the rose-colored product (shades of pink and bluish purple were also given by some samples) was read at 550 nm.

All the results were expressed as absorbance per gram of sample to enable a comparison of the values obtained for various samples and represent the means of at least three determinations. The standard error for each estimation of the methods used was obtained by analyzing a particular sample 15 times. DeKalb BR 64, a sorghum hybrid from the 1982 harvest at Purdue University Agronomy Farm, was used as a check sample in all the analyses. In addition, condensed tannin purified from BR 64 (Hagerman and Butler, 1980) was included in all the polyphenol assays.

Leaf Tissue. Two sorghum cultivars (IS 14384, IS 14375) resistant to grain mold and another two cultivars (IS 402, IS 417) susceptible to grain mold were grown in the greenhouse at Purdue University. Four fully developed and the youngest leaves from each of the cultivars used were harvested and analyzed at least in duplicate for flavan-4-ols (Watterson and Butler, 1983). Moisture content was determined on another sample of leaf tissue. The leaf samples were taken from 6-week- and 8-week-old seedlings and thereafter at weekly intervals until such time that the flag leaves from each of the cultivars were harvested and analyzed for flavan-4-ol levels.

Tissue Culture. Callus tissues from the resistant cultivars IS 14384 and IS 14375 and susceptible cultivars IS 402 and IS 417 were established from seedling mesocotyl tissue by a method similar to that previously described (Oberthur et al., 1983). The tissues were transferred to fresh medium at approximately monthly intervals. Methanol extracts of callus tissues were analyzed in duplicate for flavan-4-ols and proanthocyanidins (Watterson and Butler, 1983) at the completion of their third transfer.

RESULTS AND DISCUSSION

The list of sorghum cultivars, their grain weight, and group descriptions are shown in Table I. For convenience,

Table I. Hundred-Grain Weight and Description of Sorghum Cultivars

gp ^a (based on grain color and reactn to mold)	cultivar	100-grain wt, ^b g
A	SPV 104	4.01
	M35 1	3.37
	IS 2327	2.73
B	IS 625	1.86
	IS 2821	2.55
	IS 18759	2.55
	IS 8763	2.77
C	IS 402	3.13
	IS 417	3.25
D	IS 14384	2.23
	IS 14375	2.07
	IS 14380	2.08
E	BR 64 (check sample)	2.64

^a Classification of groups: A, white, mold susceptible; B, brown/red brown, mold resistant; C, red/light red, mold susceptible; D, red/light red, mold resistant. ^b Means of two determinations.

Table II. Color of Sorghum Meal Measured in a Hunter Lab Colorimeter Using Yellow Standard

gp ^a	cultivar	color of ground meal ^b		
		<i>L</i>	<i>a</i>	<i>b</i>
A	SPV 104	78.2	-1.0	13.4
	M35 1	79.0	-1.2	12.7
	IS 2327	78.0	-1.4	12.8
B	IS 625	59.3	4.9	10.2
	IS 2821	61.6	3.4	11.1
	IS 18759	63.0	4.4	10.6
	IS 8763	62.3	3.1	11.0
C	IS 402	69.2	4.4	12.6
	IS 417	69.2	4.8	12.6
D	IS 14384	70.4	3.4	11.5
	IS 14375	70.6	3.1	11.6
	IS 14380	68.1	3.1	11.4
E	BR 64	64.9	2.8	9.2

^a As in Table I. ^b Means of two determinations: *L*, measures lightness and varies from 100 for perfect white to zero for black; *a*, measures redness when plus, gray when zero, and greenness when minus; *b*, measure yellowness when plus, gray when zero, and blueness when minus.

the sorghum cultivars were divided into five groups on the basis of their color and their reaction to grain mold infection (Table I). Sorghum cultivars in group A were white and susceptible to mold; those cultivars in group B were brown and resistant to mold. Cultivars in groups C and D were either light red or red; the cultivars in group C were susceptible while those cultivars in group D were resistant to mold. BR 64 was used as a check. The mean 100-grain weight was highest (3.19 g) for cultivars belonging to group C, and the mold-resistant cultivars in group D exhibited the lowest mean 100-grain weight of 2.13 g.

The color for sorghum meal as determined by the Hunter Lab Colorimeter showed that group A had the highest values, indicating the relative whiteness of the grains of the cultivars SPV 104, M35 1, and IS 2327 (Table II). The lowest mean values were obtained for cultivars IS 625, IS 2821, IS 18759, and IS 8763, indicating the relative influence of gray on the readings. The sorghum cultivars IS 402 and IS 417 exhibited more redness than the remaining cultivars as shown by their *a* values. The cultivars belonging to both groups B and D also showed some degree of redness. All the cultivars exhibited some yellowness as indicated by the *b*; in contrast BR 64 had the lowest value for this measurement.

With the exception of group B cultivars and the BR 64 check sample, all other cultivars had essentially negligible

Table III. Polyphenol Content in Sorghum Cultivars

gp ^a	cultivar	extractant	total phenols (A ₇₂₀ /g) ^b	vanillin assay (A ₅₁₀ /g) ^b	protein precip (A ₅₁₀ /g) ^b
A	SPV 104	methanol	4.70	0	0.04
		H ⁺ /methanol	3.30	0.18	0.03
	M35 1	methanol	3.36	0	0
		H ⁺ /methanol	2.73	0	0.09
	IS 2327	methanol	4.36	0	0.09
H ⁺ /methanol		4.40	0	0.02	
B	IS 625	methanol	102.97	76.09	8.26
		H ⁺ /methanol	42.29	23.46	1.33
	IS 2821	methanol	30.79	18.80	2.06
		H ⁺ /methanol	32.16	14.42	0.79
	IS 18759	methanol	35.91	19.67	2.34
		H ⁺ /methanol	35.77	17.05	0.81
	IS 8763	methanol	50.36	29.10	3.24
		H ⁺ /methanol	41.58	21.10	0.84
C	IS 402	methanol	7.37	0.75	0.01
		H ⁺ /methanol	5.93	0.69	0.04
	IS 417	methanol	5.92	0.75	0
D	IS 14384	H ⁺ /methanol	4.74	0.48	0.04
		methanol	8.55	1.65	0.05
	IS 14375	H ⁺ /methanol	7.44	0.84	0.04
		methanol	8.54	1.56	0.06
	IS 14380	H ⁺ /methanol	6.33	1.47	0.07
		methanol	6.24	1.41	0.03
E	BR 64	H ⁺ /methanol	6.59	0.94	0.07
		methanol	118.09	121.44	16.79
	tannin (isolated from BR 64, per 10 mg)	H ⁺ /methanol	34.09	20.53	0.75
		methanol	63.45	82.34	10.24

^a As in Table I. ^b Standard error of estimation of methods: total phenols, 1.47; vanillin assay, 1.71; protein precipitation, 0.40.

amounts of phenols extractable in methanol or acidified methanol. Similarly, the vanillin assay showed the absence of terminal units of flavan-3-ol oligomers in the group A cultivars, while the cultivars of both groups C and D showed negligible amounts. Furthermore, the values obtained for the cultivars in group B were also relatively low (about 30%) when compared to the values obtained for the BR 64 check. One method of identifying tannin is its capacity to precipitate protein. Therefore, the assay for protein-precipitable phenols gives an indication of the relative concentration of condensed tannin in these cultivars. The results clearly show (Table III) that cultivars belonging to groups A, C, and D had negligible levels of condensed tannins in the samples analysed while the group B cultivars had moderate concentration of tannins (about 24% of the level estimated in the check sample BR 64). Despite cultivars in both groups C and D having red or light red seed coats with negligible amounts of tannins, only the cultivars in group D were resistant to mold.

The levels of flavan-4-ols and proanthocyanidins in these sorghum cultivars showed very interesting trends (Table IV). Group A showed the absence of flavan-4-ols and proanthocyanidins. The methanol extracts of groups B and D showed similar mean values for flavan-4-ols while the mean value of group C, representing susceptible cultivars, was only about 33% of groups B and D values. In all the cultivars, the values obtained for the acidified methanol extracts were lower than those obtained for the corresponding methanol extracts. However, the acidified methanol extracts of group D cultivars showed the highest values for flavan-4-ols of all the cultivars. Cultivars of groups C and D contained very little, if any, proanthocyanidins, while group B showed a considerable amount of proanthocyanidins, but lower levels than for the BR 64 check. The results obtained from the assay for leucoanthocyanidins showed a similar trend to the conventional flavan-4-ols assay although the distribution was marginally different. The mean value of leucoanthocyanidins for the methanol extract of group C sorghum cultivars was about

half that of the cultivars in groups B and D. However, the acidified methanol extract of group D samples again showed the highest values among all the cultivars. A correlation coefficient (*r*) of 0.823 was observed between the results obtained for flavan-4-ols and leucoanthocyanidins.

Levels of Flavan-4-ols in Leaf Tissues of Sorghum Plants at Different Stages of Development. The leaf tissues of mold-resistant and -susceptible cultivars were also examined for flavan-4-ols at different stages of their development. Detectable levels of flavan-4-ols were not found in plants younger than 6 weeks. Leaves from 6-week-old plants of the cultivars IS 402, IS 417, IS 14384, and IS 14375 failed to show significant differences in their levels of flavan-4-ols. However, the amounts of flavan-4-ols from acidified methanol and methanol extracts of leaves of 8 weeks or older plants showed a striking difference between the mold-susceptible and mold-resistant sorghum cultivars (Table V).

Levels of Flavan-4-ols in Callus Tissue of Sorghum. The flavan-4-ols values were slightly higher in the methanol extracts of callus tissues of mold-resistant cultivars (Table VI). However, there does not appear to be any marked trend in the values obtained for flavan-4-ols and proanthocyanidins in the callus tissues obtained from the mold-resistant and mold-susceptible sorghum cultivars.

CONCLUSIONS

It is evident from the data presented on a limited number of cultivars that the concentrations of flavan-4-ols in the methanol and acidified methanol extracts of the grains of mold-resistant cultivars were much higher than those of the mold-susceptible cultivars. Similar trends were observed although of a lower magnitude, in the leucoanthocyanidin assay. The data indicate that, in addition to the protective role played by proanthocyanidins, other compounds in plants may also play a role in their defense mechanism. Although the leucoanthocyanidin assay is more sensitive than the flavan-4-ols assay, we do not know

Table IV. Flavan-4-ols and Proanthocyanidin Content in Sorghum Cultivars

gp ^a	cultivar	extractant	flavan-4-ols (A ₅₅₀ /g) ^b	proanthocyanidin (A ₅₅₀ /g) ^b	formic acid-HCl assay leucoanthocyanidin (A ₅₅₀ /g)
A	SPV 104	methanol	0	0	0
		H ⁺ /methanol	0	0	0
	M35 1	methanol	0	0	0
		H ⁺ /methanol	0	0	0
IS 2327	methanol	0	0	0	
	H ⁺ /methanol	0	0	0	
B	IS 625	methanol	24.67	56.68	45.96
		H ⁺ /methanol	5.24	26.01	10.71
	IS 2821	methanol	15.16	12.29	26.82
		H ⁺ /methanol	5.28	16.90	8.74
	IS 18759	methanol	11.67	17.97	19.79
		H ⁺ /methanol	5.25	14.31	9.54
IS 8763	methanol	14.74	23.41	23.74	
	H ⁺ /methanol	5.40	16.82	9.83	
C	IS 402	methanol	6.14	0.36	18.28
		H ⁺ /methanol	3.53	0.36	12.10
	IS 417	methanol	4.36	0.32	13.45
D	IS 14384	H ⁺ /methanol	3.02	0.08	8.04
		methanol	17.25	1.26	31.60
	IS 14375	H ⁺ /methanol	11.93	0	20.87
		methanol	19.19	1.22	35.37
IS 14380	H ⁺ /methanol	11.87	0	22.79	
	methanol	9.25	0.58	21.05	
E	BR 64	H ⁺ /methanol	8.25	0.18	14.60
		methanol	7.29	73.94	36.17
	tannin (isolated from BR 64, per 10 mg)	H ⁺ /methanol	1.89	19.93	9.06
		methanol	2.06	31.21	14.54

^aAs in Table I. ^bStandard error of estimation of methods: flavan-4-ols, 0.16; proanthocyanidin, 1.00.

Table V. Levels of Flavan-4-ols in Sorghum Leaves at Different Stages of Plant Development

age of plant after planting, weeks	extractant	flavan-4-ols (A ₅₅₀ /g dry wt)				A/B
		mold resistant (A)		mold susceptible (B)		
		IS 14375	IS 14384	IS 402	IS 417	
8	methanol	13.66	8.92	1.76	2.51	5.56
		(80.67) ^a	(79.70)	(80.10)	(81.66)	
9	methanol	36.82	23.56	5.16	10.46	3.87
		(77.08)	(77.55)	(79.07)	(79.35)	
10	H ⁺ /methanol	19.15	10.16	3.20	5.13	3.52
	methanol	336.50	300.08	61.42	47.45	
			(74.41)	(74.42)	(76.44)	
11 (flag leaves)	H ⁺ /methanol	128.45	113.33	30.05	23.32	4.53
	methanol	321.30	292.39	160.79	81.06	
			(65.87)	(67.03)	(70.90)	
	H ⁺ /methanol	118.75	106.31	67.42	36.81	2.14

^aFigures in parentheses show leaf moisture (%).

Table VI. Analysis of Methanol Extracts of Callus Tissues for Flavan-4-ols and Proanthocyanidins

cultivar	flavan-4-ols (A ₅₅₀ /g of dry wt)	proantho- cyanidins (A ₅₅₀ /g dry wt)	moisture, %
IS 402	0	4.38	92.0
IS 417	0.99	2.25	92.9
IS 14375	1.32	1.43	90.9
IS 14384	1.24	2.48	91.1

the types of compounds that are measured by this assay. However, when the colored products of the leucoanthocyanidin assay obtained with group D samples were heated in a boiling water bath for 1 h, the absorbance values were considerably reduced, to less than 20% of the original values. This indicated that, similar to flavan-4-ols, these compounds were also unstable and therefore were destroyed. Leaves of mold-resistant sorghum cultivars, 8 weeks or older in age contained significantly higher concentrations of flavan-4-ols than those of mold-susceptible cultivars. The differences however, appeared to diminish with increasing age of the plants. Callus tissues from the

mold-resistant and -susceptible cultivars did not reveal any particular trend in their tissue concentrations of flavan-4-ols.

It is yet to be determined whether flavan-4-ols play any direct role in preventing the attack by mold-causing organisms or they are released or accumulated as a byproduct of a defense mechanism by the host plant. Experiments to analyze grains from several mold-resistant lines and their hybrids in crosses with susceptible lines are being carried out. Furthermore, experiments are being conducted on the role of flavan-4-ols in grain mold resistance.

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Epicuticular Waxes of Glaucous and Nonglaucous Durum Wheat Lines

Giorgio Bianchi* and Maria L. Figini

Surface chemical constituents of glaucous and nonglaucous wheat lines were obtained by dipping the aerial plant organs into chloroform. Surface chemicals comprised alkanes, aldehydes, alcohols, acids, esters, β -diketones, and hydroxy β -diketones. These classes of compounds were quantitatively separated by column chromatography and the homologues analyzed by glass capillary gas chromatography. The relationships between glaucous and nonglaucous appearance and the presence or absence of β -dicarbonyl components in two Durum wheat cultivars and six derived lines are presented and discussed.

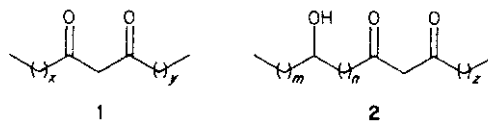
The epicuticular wax layer is the area of contact of plant aerial organs with the surrounding atmosphere. As such it is important in studies on water and gas exchange, retention, and penetration of air-borne substances such as herbicides and plant growth regulators. Epicuticular wax can also be of further agricultural relevance, representing the habitat for parasitic and saprophytic organisms, the barrier to fungal pathogens (Hamilton and Hamilton, 1972).

Surface wax is responsible for the appearance and development of the aerial organs of plants that can appear "green" (i.e., nonglaucous) or blue, gray, or even white according to the nature of the epicuticular lipid layer. Glaucousness, due to a superficial deposit of light-scattering crystallites of wax, is generally thought to be due to the amount and chemical composition of the epicuticular material on the aerial organs of plants (Baker, 1982).

Bloom and bloomless sorghum lines have been defined also on the basis of different wax composition (Wilkinson and Cummings, 1981). Furthermore, it has been shown that mutants of *Brassica oleracea* (Holloway et al., 1977), *Pisum sativum* (Holloway et al., 1977), and *Zea mays* (Bianchi et al., 1985), when nonglaucous, have wax of a different chemical composition to that of the normal glaucous plants.

The ubiquitous wax components of the cereals most studied so far, namely wheat, barley, maize, sorghum, oat, rye, triticale, and rice, are alkanes, aldehydes, alcohols, acids, and esters (Tulloch et al., 1980; von Wettstein-Knowles, 1982; Bianchi et al., 1982; Dalton and Mitchell, 1959; Neucere and Sumrell, 1980; Avato et al., 1984; Tulloch and Hoffman, 1973; Streibl et al., 1974; Bianchi et al., 1979).

In addition to these classes of compounds, the waxes of barley, wheat, oat, rye, and triticale comprise relevant amounts of β -diketones and hydroxy β -diketones (henceforth called β -diketones or β -dicarbonyl compounds) of the types 1 and 2.



The glaucous or less glaucous appearance of the plant organs of the latter cereals has been usually correlated with the presence and percent contents of β -diketones in the surface waxes (von Wettstein-Knowles, 1972; Barber and Netting, 1968; Tulloch and Hoffman, 1974; Streibl et al., 1974; Bengston et al., 1978; Bianchi et al. 1980, 1982).

In particular, a number of studies on tetraploid and hexaploid *Triticum* species and cultivars have been carried out by several investigators during the last two decades to examine the appearance of the plant organ surfaces visually or as seen in the electron microscope and, more importantly because of the compositional data obtainable, the relation between visual appearance and wax chemistry. An extensive review on the waxes of numerous genera of the tribe *Triticeae* has been made by Tulloch et al. (1980).

Examination of the data on this subject available in the literature as well as our own results gained from a chemical genetics study on wheat varieties and mutants has evidenced some major points regarding wheat epicuticular wax: (i) The so-called glaucous wheats are characterized with wax structures in which are present long, thin tubes (also called rods and spicules). (ii) Nonglaucous (green, glossy "waxless") lines are associated with plate-type wax structures. (iii) Wax chemical composition studies have disclosed that both glaucous and nonglaucous wheats have waxes comprising the same compounds, that is the ubiquitous classes and β -diketones. In a recent report (Plant

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