Presence of Polyphenolic Materials, Including Condensed Tannins, in Sorghum Callus

With age, callus tissue of sorghum forms relatively large amounts of phenolic materials. Among those present are polyphenols which strongly bind and precipitate proteins and which respond to tests for condensed tannins (proanthocyanidins). Condensed tannins have not previously been found in vegetative tissue of sorghum, although their presence in seed has been well established. Like the condensed tannins from seed, those from callus tissue of the same cultivar cannot be extracted with methanol but are readily extracted by acidic methanol.

Among the cereals, sorghum is remarkable for the quantity and variety of phenolic materials it produces (Hulse et al., 1980). The condensed tannins (proanthocyanidins, oligomers of flavan-3-ols) are phenolics of considerable agronomic and nutritional significance, which may be present in tissues of sorghum seed in amounts up to 3% of the dry weight (Butler et al., 1980). Sorghum tannins bind certain proteins very strongly (Hagerman and Butler, 1981) and can interfere with digestion of the grain, diminishing its nutritional quality (Schaffert et al., 1974). Tannins are also thought to be responsible for desirable agronomic traits such as resistance to bird depredation (McMillian et al., 1972) and to infestation of the grain by microorganisms (Harris and Burns, 1973).

We have sought means to minimize the disparate effects of sorghum tannins in production (beneficial) and utilization (harmful) by techniques such as chemical detoxification of tannins in the grain (Price et al., 1979). A more satisfactory solution might involve biochemical manipulation of polyphenol metabolism; however, this requires a thorough understanding of the biosynthetic reactions and their regulation. We have explored the possibility that tissue culture might provide a convenient system for investigating polyphenol biosynthesis in sorghum. Tannins are apparently not present in vegetative tissues of normally grown plants (Watterson and Butler, 1983). However, sorghum callus has been reported to produce pigments (Masteller and Holden, 1970; Brar et al., 1979), and these may include flavanoids and condensed tannins.

MATERIALS AND METHODS

Tissue Culture. Seed of *Sorghum bicolor* (L.) Moench, variety IS 2319, with white pericarp and brown testa (Rooney and Miller, 1982), was provided by Dr. John Axtell, Department of Agronomy, Purdue University. Culture techniques were as described by Masteller and Holden (1970). Seeds were surface-sterilized 30–40 min with a commercial sodium hypochlorite solution (1 part of bleach to 9 parts of water) to which Alconox detergent (approximately 12 g/L) had been added. Seeds were then rinsed 3 times with sterile distilled water and allowed to germinate for 5–7 days in the dark at 28 °C. The shoots were excised below the first node and plated on the revised basal medium of Murashige and Skoog (1962) containing indoleacetic acid at 1 mg/L and kinetin at 0.04 mg/L. The medium was modified to contain 5.5% (w/v) sucrose, 10% (v/v) coconut milk, and (2,4-dichlorophenoxy)acetic acid, 1 mg/L (Masteller and Holden, 1970). Before being autoclaved, the medium was adjusted to pH 5.6–5.8 with NaOH or HCl.

Four weeks after placing shoots on the agar medium the callus which had formed was excised and transferred to fresh medium. Callus pieces were then routinely divided and transferred to fresh medium at 4-week intervals. Callus was induced and maintained at 24 °C under constant fluorescent light (30 μEinstein·m^{-2}·s^{-1} across the range 400–700 nm). Callus analyzed for polyphenols had been divided 1 or more times.

Tissue Extraction and Polyphenol Analysis. Tissue was extracted with methanol twice and the residue was then extracted twice with 1% HCl in methanol as described by Butler (1982a) except that extraction with ether was omitted and the amount of extractant used was 10–20 mL/g (fresh weight) of tissue. A separate portion of callus was weighed and dried to constant weight in order to present the results of polyphenol analyses on a dry weight basis.

The Prussian blue assay for total phenols was as described by Price and Butler (1977). Protein precipitable phenols were assayed as described by Hagerman and Butler (1978) except that the precipitated phenols were quantitated by the Prussian blue reaction. The centrifuged pellet of precipitated protein and tannin was dissolved in 30 mL of 1% sodium dodecyl sulfate in water, and the color was developed as in the conventional Prussian blue assay (Price and Butler, 1977). Protein precipitation using ^{14}C-labeled bovine serum albumin, prepared by the method of Jentoft and Dearborn (1979), was carried out as described by Hagerman and Butler (1980). Assays of flavan-4-ols and proanthocyanidins, by their conversion to anthocyanidins while absorbed on insoluble poly(vinylpyrrolidone), were as described by Watterson and Butler (1983).

RESULTS AND DISCUSSION

Sorghum lines differ greatly in their adaptability to growth as callus in culture (Brar et al., 1979). Most of our observations have been made with the variety IS 2319, which is the most readily cultured and rapid growing of the 12 lines we have tested.

The majority of the callus tissue was hard and compact rather than friable and resembled that previously described (Masteller and Holden, 1970). With age, large segments of callus appeared red-brown to black (Figure 1, 1) and pigment of the same color was also dispersed into the surrounding agar medium. This pigment development has been referred to as blackening (Masteller and Holden, 1970; Brar et al., 1979). Masteller and Holden (1970) also reported the presence of pigmented inclusion bodies in cells of sorghum callus. Such inclusion bodies were also present in our callus preparations (Figure 1, 2). In living cells inclusion bodies varied in pigmentation from colorless to intense red-brown to orange-brown, whereas only red-brown to orange-brown bodies were associated with dead cells. Inclusion bodies in cells which first appeared following callus transfer were typically colorless. Thus, color development appeared to occur as cells aged. The red-brown to orange-brown pigment was sometimes associated

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Table I. Polyphenol Analyses[a]

sample	times divided	weeks since last divided	extractant	total phenols, A_{720}/g	protein precipitable phenols, A_{720}/g	protein precipitation, mg of BSA/g	proanthocyanidins, A_{550}/g	flavan-4-ols, A_{550}/g
callus tissue	1	1	methanol	1.25	0	0	0.012	0
			H^+/methanol	0.613	0.533	6.2	0.035	0.047
callus tissue	4	6-9	methanol	2.57	0	1.83	0.164	0.094
			H^+/methanol	2.88	0.681	11.3	0.219	0.152
seed			methanol	0.154	0	1.86	0.062	0.050
			H^+/methanol	0.362	0.304	23.8	0.362	0.050
medium from no. 2			methanol	0.30	0	0.15	0	0
			H^+/methanol	0.167	0.073	0.48	0.007	0.005
medium (control, no callus)			methanol	0.02	0	0	0	0
			H^+/methanol	0.007	0	0	0	0

[a] All values are the average of duplicate analyses and are presented on a dry weight basis.

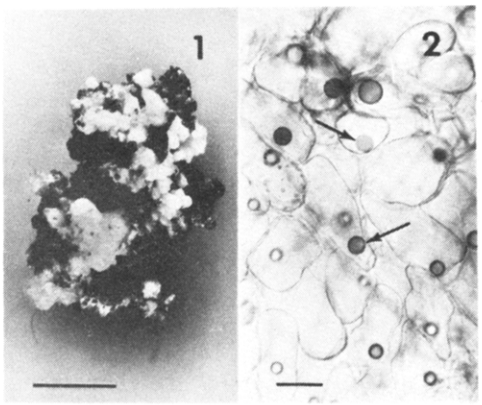

Figure 1. (1) Callus tissue of the sorghum cultivar IS 2319. Note that the callus is composed of intensely pigmented as well as unpigmented lobed segments. Magnification bar represents 0.5 cm. (2) Inclusion bodies (arrows) within cells of callus of the sorghum cultivar IS 2319. Magnification bar represents 20 μm.

with cell walls, especially walls of cells which had died. This type of pigmentation was never observed in vacuoles. Pigments in vacuoles were rare and when present the vacuolar pigment was pink, was uniformly dispersed throughout the vacuoles of living cells, and was never associated with inclusion bodies or cell walls.

As cultures aged pigmentation associated with inclusion bodies and cell walls became more intense. Therefore, polyphenol analyses were carried out on both older, heavily pigmented callus and the younger, less pigmented greener callus. Analyses were also done on the culture medium "blackened" by callus growth, on unused medium, and on seeds, for comparison. Extracts of callus were intensely reddish brown, fading to pink on dilution.

The assays indicate that large amounts of polyphenols are produced and the amounts are greater for older callus (Table I). However, even young callus contained more phenols on a dry weight basis than are found in seeds. As in immature seeds (Butler, 1982b), the high moisture content of callus tissue may interfere with polyphenol extraction, so these differences between callus tissue and seeds are to be regarded as minimal values. Significant amounts of polyphenols can be extracted from the agar medium immediately surrounding where callus had been grown. Microscopic examination suggested that the pigments that diffuse into the agar originate from ruptured cells of the callus tissue.

The identity of these phenolic materials is not yet established, but the protein binding properties and the formation of anthocyanidins on heating in acidic butanol strongly indicate the presence of condensed tannins similar to those of seeds (Table I). Moreover, the flavan-4-ols reported to be present in leaf tissue of some sorghum lines (Watterson and Butler, 1983) were also present in callus tissue. The amount of tannin present appears to be a few milligrams per gram dry weight of callus.

It is of considerable interest that extraction with acidified methanol is required to remove the majority of assayable phenolic components from seed, callus tissue, and also the pigment-impregnated culture medium (Table I). The effect of acid on extraction is especially evident in the assays involving protein precipitation. This requirement for acid extraction is characteristic of the tannin of the seed of sorghum lines which have been designated as group II types (Price et al., 1978). We are currently investigating the basis for this unique characteristic.

As far as we are aware, this is the first report of a condensed tannin found in vegetative tissue of sorghum. We have recently shown that condensed tannins are not present in detectable amounts in fully developed leaves of 43 lines of sorghum (Watterson and Butler, 1983). We speculate that synthesis of condensed tannins in callus but not in the actively growing vegetative tissues of the sorghum plant may result from stresses recognized only by the less differentiated cells and morphologically undeveloped tissues of callus. Identification of these stresses and their subsequent manipulation should allow for the study of tannin biosynthesis and its regulation in sorghum. Such augmented phenolic synthesis would be similar to that which has made the investigation of flavonoid synthesis possible through the use of parsley suspension cultures (Hahlbrock, 1981).

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Vertical Transport of Pesticides into Soil When Adsorbed on Suspended Particles

It is generally assumed that pesticides with very high K_D values are virtually immobile in the soil, though they may be transported laterally by erosion. Data presented in this paper show that vertical transport of [^{14}C]DDT and [^{14}C]paraquat adsorbed on suspended material can occur. Under favorable conditions for transport, 18% of the applied [^{14}C]DDT was transported on solids in sewage effluent to a depth greater than 9 cm in a sandy loam soil. Dispersed Li-montmorillonite suspension transported over 50% of applied [^{14}C]paraquat to a depth of 12 cm. The conditions required for such transport to occur are described.

It is observed in the literature that pesticides with high K_D values such as DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] and paraquat (1,1′-dimethyl-4,4′-dipyridylium chloride) are so strongly adsorbed by soils as to be virtually immobile (Guenzi and Beard, 1967). Such pesticides may, however, be transported laterally in sediments removed by erosion [see Wauchope (1978) for a review] and in runoff water. Rao and Davidson (1982) point out that unless the K_D (partition coefficient) is greater than about 100 or the sediment load is high (>0.1%) most of the pesticide transported during lateral flow is in the aqueous phase because of the low concentration of solids in suspension. K_D is defined by the equation $X_e/n = K_D S_e$ where K_D = partition coefficient (mL/g), n = suspension concentration (g/mL), X_e = concentration of adsorbate on the adsorbed phase (g/mL), and S_e = concentration of adsorbate in the solution phase (g/mL). By contrast, during the flow of water through soil the contribution of pesticides in solution to vertical transport is always low if K_D is large and if the pesticide is rapidly transferred to adsorbing surfaces. Instantaneous equilibration is a good approximation of pesticide adsorption on the soil solid phase unless the flow rate is high (Davidson and Chang, 1972). Thus, only if the pesticide is complexed or adsorbed on mobile colloids will any significant vertical transport occur. Ballard (1971) had demonstrated the role of humic substances, dispersed by addition of urea to a forest soil, in complexing and transport of DDT. Guenzi and Beard (1967) postulated transport of DDT adsorbed on soil colloids as a mechanism of downward transport. The experiments reported here were part of two studies on the transport of suspended solids through soil. [^{14}C]Paraquat adsorbed on Li-montmorillonite in suspension was used in one study (Vinten, 1981) and [^{14}C]DDT adsorbed on the organic suspended solids in sewage effluent was used in the other (Vinten et al., 1983). As a side shoot of these investigations these data show that under favorable conditions transport of pesticides with high K_D values on mobile colloids is feasible.

Table I. Characteristics of Soils

soils	% clay	% organic matter	% carbonates	pH	packing density, g/cm^3
		[^{14}C]DDT Experiments			
Bet Dagan, sandy loam	13.7	0.68	2.3	7.9	1.30
Gilat, silty loam	23.1	0.95	12.9	7.8	1.59
Bene Darom, coarse sand	1.2	0.21	2.8	7.8	1.30
		[^{14}C]Paraquat Experiments			
Begbroke, sandy loam	18	2.5	n.a.[a]	6.1	1.4

[a] Not available.

EXPERIMENTAL SECTION

Materials. [^{14}C]Paraquat and [^{14}C]DDT were obtained from Amersham International, Ltd., United Kingdom. They had specific activities of 425 and 80.8 mCi/g, respectively. Sewage effluent was obtained from Kibbutz Givat Brenner, Israel. It had a pH of 7.6, EC of 2.0 mmho/cm, COD of 316 mg/L, and a suspended solids concentration of 98 mg/L. Unaltered effluent (A) and effluent after filtration through a Whatman No. 92 filter (B) were used. Details of soils used in the experiments are given in Table I. Counting of samples was done on

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