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Roots of Sorghum Exude Hydrophobic Droplets Containing Biologically Active Components[1]

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ABSTRACT

Roots of *Sorghum* are known to exude materials that exhibit allelochemical activity, but the compounds identified do not completely account for the observed species-specific allelochemical activities. The purpose of this investigation was to characterize both water-soluble and water-insoluble exudates from roots of sorghum [*Sorghum bicolor* (L.) Moench] seedlings grown in petri dishes. Hydrophilic exudates included phenols, protein, and 3-deoxyanthocyanidin derivatives. Hydrophobic droplets, exuded from root hairs, tested positively for phenols and lipids. These hydrophobic exudates strongly inhibit (85%) root elongation in lettuce (*Lactuca sativa* cv. Great Lakes) seedlings, but do not affect that of corn (*Zea mays* L. cv. $B73_{Ht}$), nor the germination of either of these plants. Associated with these hydrophobic droplets are novel quinones, yet unidentified. The results indicate that these hydrophobic exudate droplets contain components that may have species-specific biological activities.

Additional index words: 3-Deoxyanthocyanidins, Root hairs, Root exudates, *Sorghum bicolor* (L.) Moench.

STUDIES on root exudates have generally employed systems in which plants are grown in either soil, sand, perlite, and/or vermiculite supports, and root exudates are collected from the watering system (Rice 1974). Based on these types of systems, biologically active compounds (i.e., *p*-hydroxybenzaldehyde, and chlorogenic, *p*-coumaric, ferulic, syringic, and vanillic acids) from aqueous washings of sorghum [*Sorghum bicolor* (L.) Moench] exudates have been identified (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1962 and 1966). However, these compounds are also found in exudates of many other plants (Putnam, 1985) and are known to be general plant growth inhibitors. Therefore, these compounds are not likely to be responsible for species-specific biological activities observed for sorghum (Abdul-Wahab and Rice, 1967; Brown and Edwards, 1944; Fletcher, 1912; Lehle and Putnam, 1983; Pope et al., 1985). Moreover, aqueous collection systems are limited to compounds that are either water-soluble or not readily bound to the support material. It has been reported that as much as 80% of the total C exuded from roots may be in the form of water-insoluble and/or volatile compounds (Rovira and Davey, 1974).

We have observed hydrophobic, pigmented droplets, not extractable in aqueous systems, that are exuded from root hairs of sorghum. We report here on the composition and biological activity of these droplets, as well as on water-soluble exudates.

MATERIALS AND METHODS

Plants, Growth Conditions, and Collection of Root Exudates

Sorghum cv. IS 8768 seeds were surface sterilized (Oberthur et al., 1983), germinated on water-saturated filter paper (Whatman no. 1) in petri dishes, and grown in the dark (28°C). Seven days after planting, 100 roots (about 3 g fresh wt) were excised from the caryopses and sequentially dipped (1 s) in each of the following three root wash solutions (20 mL each): distilled water (H_2O-A), chloroform ($CHCl_3$), and distilled water (H_2O-B). Root wash solutions were concentrated to near dryness under decreased pressure (35°C) and brought to 2 mL final volume of a water:methanol mixture (1:1).

Chemical Tests

The following reagents were added to either 200 μL of concentrated root wash solutions, with color changes detected visually, or to the exudate droplets present on excised roots which were mounted on slides, with changes in color observed using a microscope. Protein was detected by the appearance of a blue color after 100 μL of Coomassie Brilliant Blue G-250 reagent (100 mg L^{-1} H_2O) was added. Lipid

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was detected using Sudan IV (700 mL ethanol diluted to 1 L with H_2O saturated with Sudan IV) and Nile Blue (5 g L^{-1} H_2O) (Jensen, 1962). Ninhydrin (2 g L^{-1}) was used to test for the presence of free amino acids by the appearance of a purple color within 20 min. Phenols were detected by the appearance of a red color produced by aniline-iodate reagents (Mace, 1963) or a blue color produced by Prussian blue reagents (Price and Butler, 1977). The vanillin/H_2SO_4 test for proanthocyanidins and flavan-3-ols was performed as described by Swain and Hillis (1959).

Chromatography

Concentrated wash solutions (100 μL) were spotted on TLC plates (Anasil C Cellulose Analabs) along with known anthocyanidin standards, and developed in the following solvent systems (volume ratios in parentheses): (i) formic acid/H_2O/concentrated HCl (5:3:2), (ii) acetic acid/H_2O/concentrated HCl (10:87:3), and (iii) acetic acid/H_2O (30:70). For silica plates (EM Reagents, Keiselgel 60 F_{254}) solvent systems used were: (iv) ethyl acetate/formic acid/2 *N* HCl (85:9:6) and (v) hexane/ethyl ether/formic acid (40:10:1). Pigmented spots were scraped from developed plates, eluted with methanol/water (90:10), dried, dissolved in absolute methanol, and their absorbance spectra was recorded.

Separation of hydrophobic exudates (chloroform wash) by high pressure liquid chromatography (Varian, Model 5000) was performed using a 10- × 0.4-cm Zorbax C_{18} reversed-phase column. Exudates, redissolved in methanol, were chromatographed using 60% solvent B (acetonitrile:acetic acid, 1000:1, v/v) in solvent A (methanol:water:acetic acid, 500:500:1, v/v/v) for 3 min followed by a 2 min linear gradient from 60% to 90% solvent B, then 7 min at 100% solvent B at a flow rate of 16.7 μL s^{-1}. Compounds were detected at 280 and 420 nm using a diode array detector (Hewlett Packard, Model 1040A).

Synthesis of 3-Deoxyanthocyanidin Standards

Naringenin (50 mg, Sigma) and eriodictyol (30 mg, Roth) were converted to the flavan-4β-ols (Attwood et al., 1983), (2,4-*cis*) apiforol and (2,4-*cis*) luteoforol, respectively, by reduction with $NaBH_4$ as described by Stafford and Lester (1984) for flavan-3,4-diols. After extraction of the products into ethyl acetate, the organic solvent was evaporated and the flavan-4-ols were dissolved in 2*M* HCl and converted to their respective 3-deoxyanthocyanidins, apigeninidin, and luteolinidin, by heating at 100°C for 1 h. After repeated extractions with ethyl acetate, the aqueous solution was used as the source of the purified 3-deoxyanthocyanidins.

Biological Assay

Components and standards dissolved in methanol (1 mg/mL) were added (1 mL) to paper disks and the methanol allowed to evaporate. Lettuce (*Lactuca sativa* L.) (50 seeds/disk) or corn (*Zea mays* L.) (6 seeds/disk) seeds were placed in the petri dishes and 2 mL of water added. After 6 days the root lengths and fresh weights were recorded. Each experiment ($n=2$) is an average of two replicates.

RESULTS AND DISCUSSION

Hydrophobic Droplets and Wash Solutions

Of the 25 different sorghum cultivars surveyed, all of them exuded hydrophobic, pigmented (yellow) droplets at the apex of nearly every root hair (Fig. 1). These droplets were present whether seedlings were grown in the light or dark, on distilled water with or without MS salts, or on agar medium. Droplets did not dissolve and were not extractable in aqueous media, but readily did so in the chloroform wash solution. Yellow material similar to that found in the exudates was not detected in chloroform extracts from ungerminated seeds or from shoots of light-grown seedlings, but was extracted from the filter paper on which seedlings were grown and from shoots of dark-grown seedlings (trace amounts compared to filter paper and roots). Chemical reagents did not show any qualitative differences in the droplets from six selected sorghum lines. Therefore, the remaining studies were conducted using sorghum cv. IS 8768.

In contrast to the exudates from sorghum, those of corn, pearl millet (*Pennisetum americanum* L. Leeke), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), or wheat (*Triticum aestivum* L.) did not exude hydrophobic, pigmented droplets. Clear, water-soluble globules similar to those previously reported for apple (Head, 1964) were present. Also, no yellow material was extracted from the roots or filter paper of these species.

Table 1 summarizes the results of the chemical reagent tests on both droplets and root wash solutions. Although both the Prussian blue and aniline-iodate

Table 1. Chemical tests of the hydrophobic droplets and wash solutions from roots of sorghum cv. IS 8768. See Materials and Methods for explanation of procedures.

Component reagent test	Droplets	Wash solutions		
		H_2O-A	$CHCl_3$	H_2O-B
Phenols				
Prussian Blue	+	++	+	++
Aniline-iodate	+++	–	+++	–
Tannins & Catechins				
Vanillin	–	–	–	–
Amino acids, free				
Ninhydrin	–	–	–	–
Protein				
Bradford	na†	+	–	+
Lipids				
Nile blue	+	na	na	na
Sudan IV	+	na	na	na
Anthocyanidins	na	+	–	+

† na = not applicable.

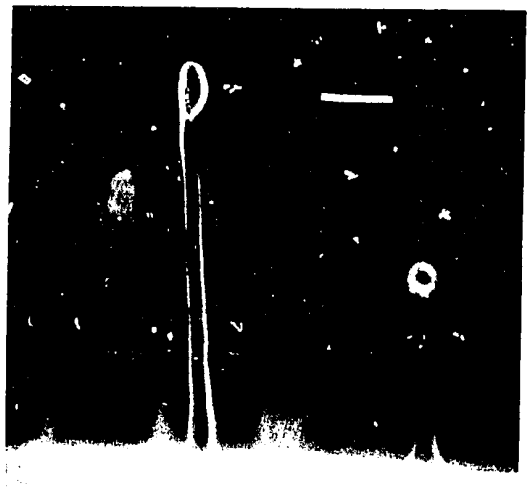

Fig. 1. Pigmented, hydrophobic droplet exuded from a root hair of *Sorghum bicolor* (L.) Moench cv. IS 8768. Bar = 35 μm.

tests detect phenolic compounds, aqueous and chloroform wash solutions tested differently with these reagents. The Prussian blue assay is a measure of the color produced from Fe^{2+} by the oxidation of phenols. In the aniline-iodate assay, phenols are determined by the colored products formed from the addition of aniline to quinones derived from phenols (Mace, 1963). Moreover, exudates from the chloroform wash that were dissolved in an ethanol solution (2 mL) and treated with $NaBH_4$ (20 mg) changed immediately from an intense yellow to a clear solution, a standard test for the reduction of quinones (Thomson, 1971). Quenching the $NaBH_4$ with 100 μL of 4*M* HCl restored the yellow color immediately. This result indicates that the chloroform wash solution contains preformed quinones.

Hydrophobic Exudates

Development by TLC of the chloroform wash solution revealed only one major compound (yellow pigment). The R_f values for this pigment in five solvent systems (see Materials and Methods) were as follows: 0.0 for (i), (ii), and (iii); 0.98 for (iv); and 0.19 for (v). However, chromatography by reversed-phase HPLC revealed one major (Y-1, 75% of the total absorbance at 280 nm) and five minor components (Y-2, Y-3, Y-4, Y-5, and Y-6) all with UV-visible spectra (286 nm major peak, 420 nm inflection) identical to that of the yellow pigment detected by TLC. These compounds exhibit a reversible pH-dependent color change (yellow to purple-red) characteristic of quinones, which was also observed in chloroform wash solutions and exudate droplets. Chemical and physical properties of these compounds do not correspond with any previously reported flavonoid or anthocyanidin of sorghum (Nip and Burns, 1969 and 1971; Stafford, 1966 and 1969; Yasumatsu et al., 1965). We are presently attempting to identify these novel quinones.

Hydrophilic Exudates

The aqueous wash solutions (Table 1) had qualitatively identical TLC patterns. Four of the eight compounds detected after separation by TLC were pigmented. A yellow-orange spot with an absorbance spectrum (280, 475 nm) and chemical properties like that of apigeninidin, but having different R_f values in several solvent systems indicated it was an apigeninidin derivative. Based on the absorbance spectra (280, 495 nm) and chromatographic properties of three orange spots detected, one was identified as luteolinidin and the other two as luteolinidin derivatives (Stafford, 1966, 1969). The remaining non-pigmented compounds were not characterized further.

Table 2. Effects of sorghum exudate components on lettuce and corn root elongation.

Component	Root length	
	Lettuce	Corn
	mm	
Standards		
Control	14a*	69a
Apiforol	14a	60a
Luteoforol	13a	82a
Water-soluble		
Apigeninidin	12b	61a
Luteolinidin	11b	61a
Water-insoluble		
Chloroform wash	2b	62a

* Means in each column followed by a different letter are significantly different ($P < 0.05$) from the control as determined by F and t tests ($n = 2$).

Biological Activity (Table 2)

Hydrophobic exudates inhibited root elongation of lettuce by 85%, while 3-deoxyanthocyanidins had only a slight effect. None of these components had any significant effect on corn roots or on the germination of lettuce or corn seeds.

CONCLUSIONS

The identification of allelochemicals from sorghum, as well as those from other plants is highly desirable for possible implementation as species-specific herbicides or growth regulators (Putnam and DeFrank, 1983). Hydrophobic droplets exuded from sorghum roots contain novel quinones, possibly unique to the genus, and materials of selective biological activity. Further work is necessary to determine if the quinones are themselves the active components. As far as the authors are aware, these hydrophobic, pigmented droplets of sorghum roots have not been previously reported, although a reference to yellow material from sorghum extracts (Yasumatsu et al., 1965) may possibly refer to the compounds reported here.

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