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Production of Stilbenoids and Phenolic Acids by the Peanut Plant at Early Stages of Growth

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The peanut plant (*Arachis hypogaea*) is known to produce stilbene phytoalexins as a defensive response to fungal invasion; however, the distribution of phytoalexins among different organs of the peanut plant at early stages of growth under axenic conditions has not been studied. Axenic plants produced a stilbenoid, resveratrol, as well as soluble bound and free phenolic acids, including 4-methoxycinnamic acid, which is reported in peanuts for the first time. Neither resveratrol nor phenolic acids were found in the root mucilage; the prenylated stilbenes were restricted to the mucilage and were not found in other organs of the peanut plant. These findings may lead to a better understanding of the defensive role of peanut stilbenes and phenolic acids.

KEYWORDS: Resveratrol; phytoalexins; mucilage; *p*-coumaric acid; ferulic acid; caffeic acid; 4-methoxycinnamic acid; phenolic acids; peanuts; groundnuts; *Arachis hypogaea*; HPLC-MS

INTRODUCTION

Stilbenoids are produced by the peanut plant as a defensive response to fungal invasion and other exogenous stimuli (*1*−*10*). Peanut stilbenoids are suggested to serve as phytoalexins (*5*, *6*, *8*, *9*, *11*, *12*) and possess antifungal activity against *Aspergillus flavus*, *A. parasiticus*, and other fungi (*2*, *7*, *11*, *13*) and play a key defensive role in peanuts when the water activity of the plant tissues is sufficiently high (*1*). Phenolic acids are aromatic secondary plant metabolites that are widespread throughout the plant kingdom (*14*). Phenolic acids have been connected with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, dormancy, and allelopathy; they are known to serve as structural components and are suggested to play a role as defensive compounds (*15*−*20*). At least seven basic phenolic acids have been detected in peanut kernels and are associated with peanut dormancy (*15*, *21*). The biosynthetic origin of phenolic acids as well as stilbenes is well-known (*22*−*24*); however, their particular role and sites of accumulation in many plants, including peanuts, are not known or understood. The purpose of this work was to characterize the distribution and levels of stilbenes and phenolic acids in peanut organs at early stages of growth under axenic conditions.

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EXPERIMENTAL PROCEDURES

Reagents, Materials, and Basic Apparatus. HPLC-grade solvents used in the sample extraction and preparation of the mobile phase were obtained from Fisher (Suwanee, GA). Celite 545, NaOH, and Na_2SO_4 were purchased from J. T. Baker (Phillipsburg, NJ). A 2% NaOCl solution was prepared by dilution of household bleach (Clorox) with distilled water (1:2, v/v). A high-speed blender (13000 rpm) with a glass jar (General Electric) was used in the research. Medium for peanut germination was composed of 1% agar (Difco) in sterile water; 25 mL was placed in each 100 mm × 15 mm Petri dish.

Reference Compounds. *trans*-Resveratrol (∼99%) was purchased from Sigma. *p*-Coumaric, ferulic, caffeic, and 4-methoxycinnamic acids were purchased from Aldrich (Milwaukee, WI). ESI-MS/MS (MS^2) and UV spectra were obtained for the above commercial compounds to serve as comparison standards for identifying *trans*-resveratrol and phenolic acids (**Figure 1**) extracted from peanuts. The values determined in this research are given in parentheses as $[M + H]^+$ values for *trans*-resveratrol and 4-methoxycinnamic acid, or as $[M - H]^-$ values for phenolic acids followed by UV absorption maxima: *trans*-resveratrol (*m*/*z* 229; 305 and 317 nm); *p*-coumaric acid (*m*/*z* 163; 293 sh, 300 sh, and 308 nm); caffeic acid (*m*/*z* 179; 299 and 323 nm); ferulic acid (*m*/*z* 193; 299 sh, and 322 nm); 4-methoxycinnamic acid (*m*/*z* 179; 293 sh, 300 sh, and 308 nm).

Plant Material and Processing. Seeds of two runner peanut cultivars, Georgia Green and Virugard, and a Valencia cultivar, GT-101, were used in the present study. Mature sound peanut pods were surface-sterilized with 2% NaOCl followed by drying at room temperature on paper towels overnight (*25*). The pods were manually cracked, the kernels were aseptically removed from the hulls, and blemish-free kernels were selected for the experiments. The peanut kernels (three per plate) were incubated on agar for 5 days at 30 °C

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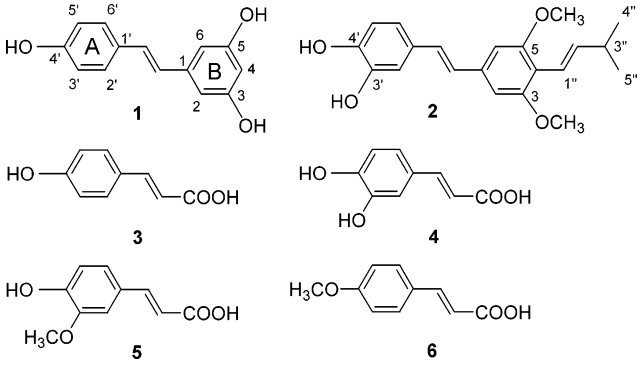

Figure 1. Structures of *trans*-resveratrol (**1**), mucilagin A (**2**), *p*-coumaric acid (**3**), caffeic acid (**4**), ferulic acid (**5**), and methoxycinnamic acid (**6**).

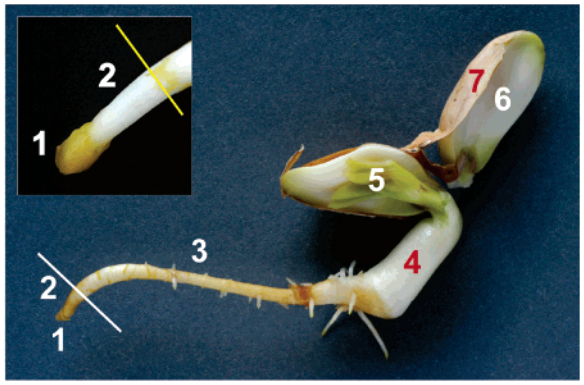

Figure 2. Germinated peanut seed after 5 days of incubation: 1, mucilage; 2, root tip (4–5 mm); 3, root; 4, hypocotyl; 5, epicotyl/plumule; 6, cotyledon; 7, testa.

without light and then examined under the stereomicroscope to ensure axenic conditions. Root mucilage was removed with a microspatula, and the germinated seed was manually dissected into the following parts: root tip (4−5 mm), root, hypocotyl, epicotyl, cotyledons, and testa (**Figure 2**).

To determine the production of phenolic compounds by the kernels in their transitional stage from dormancy to germination, peanut kernels were placed into a beaker with sterile distilled water and left at room temperature for 18 h to imbibe water (55% of the original kernel weight). The kernels then were removed from the beaker, blotted with a paper towel, and manually separated into the following parts: embryo, cotyledons, and testa. Dry peanut kernels were frozen for ease of separation into the same parts. In all experiments the plant parts were frozen before analysis to stop possible enzymatic reactions.

Extraction and Hydrolysis. Root mucilage (0.2 g) was mechanically removed from the root tips, mixed with an equal amount (w/w) of Celite 545, and ground in an agate mortar. The mixture was extracted with 1 mL of MeOH for 30 min with periodic shaking every 5 min. The extract was filtered through a glass-fiber filter. From 5 to 30 μL of the filtrate was analyzed by HPLC.

Soluble phenolic compounds were extracted from peanut organs with 80% MeOH in a high-speed blender for 1 min. The blender was flushed with N_2 before the extraction. The blended extract was then agitated by purging N_2 into the extract for 1 h at room temperature. A filtered aliquot of the extract was used for direct determination of free phenolic acids. Bound soluble phenolic acids were analyzed as free acids after basic hydrolysis with NaOH. For the hydrolysis, 1 mL of 2 or 4 N NaOH solution was added to 1 mL of the MeOH extract of each peanut organ, and the mixture was agitated by bubbling N_2 gas for 10−15 s to minimize the oxidation of phenolic acids. The mixture was allowed to stand for 2 or 4 h at room temperature. The sample was then adjusted to pH 2−3 with 6 N HCl, and 10 mL of H_2O was added. Phenolic compounds were extracted with EtOAc (4 × 10 mL). Combined EtOAc layers were dried over anhydrous Na_2SO_4, evaporated to dryness, and redissolved in 1 mL of MeOH. From 5 to 30 μL of the extract was analyzed by HPLC. Alternatively, the peanut organs were subjected to acidic hydrolysis (*20*) followed by extraction with EtOAc as described above.

Derivatization of *p*-Coumaric Acid. The methyl ester of *p*-coumaric acid was obtained by treatment of ~3 mg of the acid **3** (**Figure 1**) with 1.5 mL of BCl_3 in MeOH (10% w/v) (Alltech). The reaction mixture was heated for 5 min at 55 °C. Equal amounts of water and toluene (10 mL) were added to stop the reaction and to extract the methyl ester. The toluene layer was dried over Na_2SO_4, filtered, evaporated to dryness with a stream of N_2, redissolved in MeOH, and subjected to HPLC−diode array detection−MS analysis.

Full methylation of *p*-coumaric acid to obtain methyl 4-methoxycinnamate was accomplished by dissolving ~1.5 mg of *p*-coumaric acid **3** (**Figure 1**) in 0.6 mL of acetone and combining the solution with 200 mg of K_2CO_3 and 80 μL of dimethyl sulfate in a 4-mL clear glass vial sealed with a Teflon-lined cap. The reaction mixture was agitated with a magnetic stirring bar for 1 h at room temperature. The vial was then charged with 2 mL of 10% NH_4OH and 1 mL of toluene and vigorously stirred for 20 min. The toluene layer was separated with a Pasteur pipet, dried with anhydrous Na_2SO_4, and evaporated to dryness under a stream of N_2. The residue was dissolved in methanol for HPLC analysis.

4-Methoxycinnamic acid (**6**; **Figure 1**) was obtained by hydrolysis of methyl 4-methoxycinnamate with 2 N NaOH for 35 min at room temperature as described above.

HPLC−Diode Array Detection−MS Analyses. Analyses were performed using an HPLC system equipped with an LC-10ATvp pump (Shimadzu), an SPD-M10Avp diode array detector covering the 200−500 nm range with Shimadzu Client/Server software, version 7.3, and a model 717 plus autosampler (Waters). The separation was performed on a 50 mm × 4.6 mm i.d., 2.5 μm XTerra MS C18 analytical column (Waters). H_2O (A), MeOH (B), and 1% HCOOH in H_2O (C) were combined in the following gradient: initial conditions, 95% A/0% B/5% C, increased linearly to 0% A/95% B/5% C in 15 min, held isocratic for 1 min, decreased to initial conditions in 0.01 min. The flow rate was 1.2 mL/min. The column was maintained at 35 °C in a model 105 column heater (Timberline Instruments, Boulder, CO). The eluate from the diode array detector was split with a T-unit (Upchurch Scientific, Oak Harbor, WA) for optimal MS performance. The flow rate through the ESI probe was set at 0.375 mL/min. MS analyses were performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with an ESI interface and operated with Xcalibur version 1.4 software (Thermo Electron Corp., San Jose, CA). The data were acquired in the full-scan mode (MS) from *m*/*z* 100 to 2000. Heated capillary temperature was 200 °C, sheath gas flow was 30 units, capillary voltage was 13 V, and source voltage was 4.5 kV. In MS^2 analyses, the $[M + H]^+$ and $[M - H]^-$ ions observed for each chromatographic peak in full-scan analyses were isolated and subjected to source collision-induced dissociation (CID) using He buffer gas. In all CID analyses, the isolation width, normalized collision energy, relative activation *Q*, and activation time were *m*/*z* 1.5, 25 or 30%, and 0.25 and 30 ms, respectively. The results of MS^2 experiments are represented throughout the text as follows: *m*/*z* aaa@bb → aaa, ccc, ddd, where aaa is parent ion, bb is normalized collision energy (%), and ccc and ddd are fragment ions. Concentrations of *trans*-resveratrol, *p*-coumaric (**3**), caffeic (**4**), ferulic (**5**), and 4-methoxycinnamic (**6**) acids (**Figure 1**) were determined by reference to peak areas of corresponding standards. Because extinction coefficients for new mucilage stilbenes could not be determined due to small quantities, we suggested them to be close to the extinction coefficient of a similar known compound, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (*7*). For our calculations we used an extinction coefficient of log 4.34 for each stilbene at the maximum absorption wavelength for that compound.

NMR Characterization. ^1H and ^{13}C NMR experiments were performed on a Bruker DRX-400.

RESULTS AND DISCUSSION

Structure Elucidation. In addition to known phenolic acids found in embryo, epicotyl/plumule, root, and hypocotyl extracts after basic hydrolysis (**Tables 1** and **2**), an unidentified

Table 1. Presence of Stilbenoids[a] and Major[b] Phenolic Acids in Parts of Peanut Seeds and Seedlings and Control Seeds of Tested Peanut Cultivars[c]

plant part	resveratrol (**1**)	mucilagin A (**2**)	minor prenylated stilbenes[d]	*p*-coumaric acid (**3**)[e]	caffeic acid (**4**)[e]	ferulic acid (**5**)[e]	methoxycinnamic acid (**6**)[e]
dry seed							
cotyledons	+						
embryo							
testa	+						
imbibed seed							
cotyledons	+			+			
embryo				+	+	+	+
testa	+						
seedling							
cotyledons	+			+		+	
epicotyl/plumule				+	+	+	+
hypocotyl	+			+	+		+
testa	+						
root	+			+	+		+
root tip (4–5 mm)							+
mucilage		+	+				

[a] Stilbenoids were determined before the hydrolysis. [b] Totaling ≥90% (area percent by HPLC). [c] Data from three replicates each of Georgia Green and Virugard runner cultivars and a Valencia cultivar; "+" indicates presence of the metabolite. [d] Sobolev et al. (*27*). [e] As free acids after basic hydrolysis.

Table 2. Total Major[a] Free and Bound Soluble Phenolic Acids Content in Parts of Seeds and Seedlings of Different Peanut Varieties

	p-coumaric acid (**3**), caffeic acid (**4**), ferulic acid (**5**), and 4-methoxycinnamic acid (**6**) content[b] (mean ± SD, μg/g of wet wt; $n = 3$)		
organ/section of plant	Georgia Green	Valencia	Virugard
dry seed			
cotyledons	nd[c]	nd	nd
embryo	nd	nd	nd
testa	nd	nd	nd
imbibed seed			
cotyledons	(**3**) 79.0 ± 11.3	205.5 ± 46.9	34.6 ± 21.4
embryo	(**3**) 75.0 ± 8.4	73.9 ± 7.5	251.5 ± 48.8
	(**4**) 19.7 ± 6.6	22.8 ± 9.0	67.8 ± 20.7
testa	nd	nd	nd
seedling			
cotyledons	(**3**) 321.9 ± 46.5	212.3 ± 44.8	124.6 ± 19.1
	(**5**) 34.8 ± 6.2	18.6 ± 9.7	13.3 ± 7.4
hypocotyl	(**3**) 82.4 ± 17.9	83.5 ± 18.1	32.2 ± 21.9
	(**4**) 11.0 ± 2.3	13.8 ± 3.6	6.1 ± 3.0
	(**6**) 22.5 ± 7.7	20.4 ± 8.6	8.8 ± 7.4
root	(**3**) 136.8 ± 22.7	63.5 ± 37.8	89.0 ± 45.2
	(**4**) 56.7 ± 11.5	27.7 ± 8.8	63.6 ± 21.6
	(**6**) 109.6 ± 18.9	57.3 ± 9.5	81.2 ± 25.8
root tip (4–5 mm)	(**6**) 127.3 ± 88.9	no quantitative data	no quantitative data
epicotyl/plumule	(**3**) 708.3 ± 75.3	856.2 ±132.3	1305.8 ± 266.5
	(**4**) 202.3 ± 26.1	258.7 ± 37.5	415.2 ± 97.6
	(**5**) 72.1 ± 8.3	75.7 ± 16.6	103.2 ± 46.3
	(**6**) 50.5 ± 9.8	63.7 ± 12.9	97.0 ± 45.3
testa	nd	nd	nd
mucilage	nd	nd	nd

[a] Largest chromatographic peaks totaling ≥90% (area percent by HPLC). [b] Calculated as free phenolic acids after basic hydrolysis. [c] Not detected or a minor phenolic acid in the group of remaining compounds (≤10%, area percent by HPLC; see footnote *a*).

compound was detected in those same organs in concentrations comparable to concentrations of the major phenolic acids. The unknown compound was observed in root and hypocotyl extracts before hydrolysis only in extremely low concentrations (**Table 3**), which suggested its bound nature. The UV spectrum of the compound virtually matched that of *p*-coumaric acid (**3**), suggesting a characteristic *p*-coumaroyl moiety (*14*, *19*, *26*). The lower polarity of the unknown compound compared to other peanut phenolic acids suggested substitution of the carboxyl and/or phenolic hydroxy group of the *p*-coumaric acid with a hydrophobic moiety. Positive ESI-MS produced a complex spectrum (see Supporting Information); definite identification of the molecular ion was not assured. In contrast to other peanut phenolic acids, negative ESI-MS did not produce an informative spectrum. In addition, the unknown was not easily hydrolyzed, which suggested a phenol derivative rather than an ester. Negative ESI of the methyl ester of *p*-coumaric acid produced an informative spectrum (*m*/*z* 177 $[M - H]^-$), which did not match that of the unknown compound. On the other hand, basic hydrolysis of synthesized methyl 4-methoxycinnamate (positive ESI, *m*/*z* 193@30 → 161 $[M - OCH_3]^+$; no informative MS spectrum was produced by negative ESI) yielded a phenolic acid identical to the unknown compound. The retention time and UV and mass spectra of the acid matched those obtained

Table 3. Major[a] Free Phenolic Acids Content in Sections of the Seedlings of Georgia Green Cultivar

	mean ± SD, μg/g of wet weight; $n = 3$			
plant part	*p*-coumaric acid (**3**)	caffeic acid (**4**)	ferulic acid (**5**)	4-methoxycinnamic acid (**6**)
cotyledons	18.2 ± 2.7	nd[b]	nd	nd
epicotyl/plumule	384.7 ± 15.0	nd	nd	nd
hypocotyl	3.0 ± 0.4	nd	nd	1.6 ± 0.7
testa	nd	nd	nd	nd
root	3.3 ± 0.3	nd	nd	17.5 ± 3.7
mucilage	nd	nd	nd	nd

[a] Largest free phenolic acids chromatographic peaks totaling ≥90% (area percent by HPLC). [b] Not detected or a minor free phenolic acid in the group of remaining free phenolic acids (≤10%, area percent by HPLC; see footnote *a*).

for an authentic standard of 4-methoxycinnamic acid as well as 4-methoxycinnamic acid (**6**) isolated from peanuts (see Supporting Information).

Analysis of the ^{1}H NMR spectrum of the unknown compound revealed the presence of an oxygenated para-disubstituted aromatic ring, a *trans*-olefin, and a methoxy group. The ^{13}C NMR spectrum showed resonances consistent with these units, as well as a signal for an ester or acid carbonyl that must be linked to the olefin group based on δ_C values. Comparison of the ^{1}H and ^{13}C NMR spectra of the unknown compound with literature data allowed for the assignment of the compound as *trans*-4-methoxycinnamic acid.

***trans*-4-Methoxycinnamic acid:** ^{1}H NMR (CD_3OD, 400 MHz) δ 7.63 (1H, d, $J = 16$ Hz, H-7), 7.54 (2H, distorted d, $J = 8.8$ Hz, H-2,6), 6.96 (2H, distorted d, $J = 8.8$ Hz, H-3,5), 6.33 (1H, d, $J = 16$ Hz, H-8), 3.83 (3H, s, MeO-4); ^{13}C NMR (CD_3OD, 100 MHz) δ171.0 (C-9), 163.2 (C-4), 146.3 (C-7), 131.0 (C-2,6), 128.6 (C-1), 116.8 (C-8), 115.6 (C-3,5), 56.0 (MeO-4).

Phenolic acids obtained after hydrolysis were characterized by the following MS data: negative ESI-MS^2 *m/z* 163@30 → 163 $[M - H]^-$, 119 $[M - COOH]^-$ for *p*-coumaric acid; 179@30 → 179 $[M - H]^-$, 135 $[M - COOH]^-$ for caffeic acid; 193@25 → 193 $[M - H]^-$, 179 $[M - CH_3]^-$, 149 $[M - COOH]^-$ for ferulic acid. Positive ESI-MS^2 *m/z* 179@30 → 179 $[M + H]^+$, 161 $[M - H_2O + H]^+$, 133 $[M - H_2O - CO + H]^+$ were observed for 4-methoxycinnamic acid. The data for *trans*-resveratrol were as follows: positive ESI-MS^2 *m/z* 229@30 → 229 $[M + H]^+$, 211 $[M - H_2O + H]^+$, 135 $[M - C_6H_5OH + H]^+$. The above data matched data obtained for corresponding authentic standards.

Structure elucidation of the mucilage stilbenoids is described by Sobolev et al. (*27*).

Metabolite Distributions. Figure 2 shows the typical appearance of germinating peanut seed. Metabolism in dormant peanut seeds is extremely low at seed moisture levels of <10% but increases rapidly during seed water absorption and hydration of cell walls and protoplasm (*28*). **Tables 1**, **2**, and **4** show that the composition and concentrations of the metabolites changed from dry to imbibed to germinated seeds.

Stilbenoids. Resveratrol **1** (**Figure 1**) was detected in all peanut parts except embryo, epicotyl/plumule, root tip, and mucilage (**Tables 1** and **4**). The content of resveratrol in all parts was extremely low (**Table 4**); even in the testa, concentrations were <2 μg/g (fresh weight). There were no significant differences between the cultivars. Higher concentrations of resveratrol in the dry seed testa can be attributed to lower water content with a corresponding increase in resveratrol concentration. Fast-growing parts of the seedling, such as hypocotyl, root, root tip, and epicotyl/plumule, as well as fast-forming mucilage contained at most only traces of resveratrol (**Table 4**). None of the known stilbenes or phenolic acids was detected in the mucilage (**Tables 1**, **2**, and **4**); instead, several new prenylated stilbenes (*27*) were accumulated at extremely high concentrations. Concentrations of mucilagin A (**2**) and minor prenylated stilbenes (in parentheses) were as follows: Georgia Green, 251.2 ± 113.8 (127.5 ± 9.0); Valencia, 339.3 ± 127.5 (311.6 = 39.3); Virugard, 387.9 ± 31.6 (245.2 ± 44.1). In comparison, resveratrol content in other parts of the peanut plant (**Table 4**) did not exceed 1.93 μg/g (testa of Georgia Green seeds). None of the mucilage stilbenes was found in the root tip (**Table 1**). None of the known peanut stilbene phytoalexins, such as the arachidins and *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (*5*−*8*), were detected in any of the peanut organs.

Table 4. Resveratrol (**1**) Content in Parts of Seeds and Seedlings in Different Peanut Varieties

	resveratrol content (mean ± SD, μg/g; $n = 3$)		
plant part	Georgia Green	Valencia	Virugard
dry seed			
cotyledons	0.02 ± 0.01	<0.01	<0.01
embryo	nd[a]	nd	nd
testa	1.93 ± 0.78	1.26 ± 0.27	1.02 ± 0.14
imbibed seed			
cotyledons	<0.01	0.01 ± 0.06	<0.01
embryo	nd	nd	nd
testa	0.58 ± 0.08	0.69 ± 0.31	0.38 ± 0.02
seedling			
cotyledons	0.17 ± 0.05	0.03 ± 0.02	0.02 ± 0.02
hypocotyl	nd–<0.01	nd–<0.01	nd–0.01
root	0.06 ± 0.1	nd–<0.01	nd–<0.01
root tip (4–5 mm)	nd	nd	nd
epicotyl/plumule	nd	nd	nd
testa	1.27 ± 0.27	0.58 ± 0.02	0.75 ± 0.25
mucilage	nd	nd	nd

[a] Not detected.

Phenolic Acids. Phenolic acids were detected at higher levels compared to resveratrol. Phenolic acids in free form were observed in the extracts of cotyledons, hypocotyl, and root at very low concentrations except in embryos (~0.4 mg/g) (**Table 3**). *p*-Coumaric acid was detected in cotyledons, epicotyl/plumule, hypocotyl, and root, whereas 4-methoxycinnamic acid was found only in hypocotyl and root (**Table 3**). None of the free acids was found in testa and root mucilage. Free caffeic and ferulic acids were not detected in any of the organs.

Much higher concentrations of phenolic acids were observed in the extracts of organs after acidic or basic hydrolysis (**Table 2**), indicating the bound nature of the phenolic acids. The major peanut phenolic acids in tested peanuts were identified as *p*-coumaric (**3**), caffeic (**4**), ferulic (**5**), and 4-methoxycinnamic (**6**) acids (**Figure 1**). Compared to dry seeds, embryos of the imbibed seed produced *p*-coumaric and caffeic acids as major acids and ferulic and 4-methoxycinnamic acids as minor components. The epicotyl/plumule part of the seedlings produced all four phenolic acids as major acids (**Tables 1** and **2**). The epicotyl/plumule in seedlings produced significantly higher concentrations of ferulic and 4-methoxycinnamic acid compared to imbibed seeds (**Table 2**). The most significant qualitative and quantitative changes were observed in the seedlings (**Tables 1**, **2**, and **4**). Developing parts of the seedling such as hypocotyl, root, and root tip produced 4-methoxycinnamic acid in addition to *p*-coumaric and caffeic acids. Interestingly, the metabolite profile of the 4−5-mm-long root tips did not match that of the

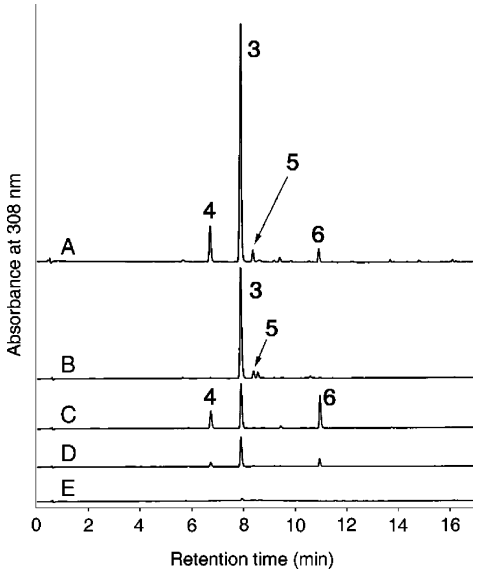

Figure 3. HPLC of peanut organ extracts after basic hydrolysis: **A**, embryos; **B**, cotyledons; **C**, roots; **D**, hypocotyls; **E**, testae. Peaks: coumaric acid (**3**); caffeic acid (**4**); ferulic acid (**5**); 4-methoxycinnamic acid (**6**). The chromatograms are scaled for direct comparison of concentrations of phenolic acids.

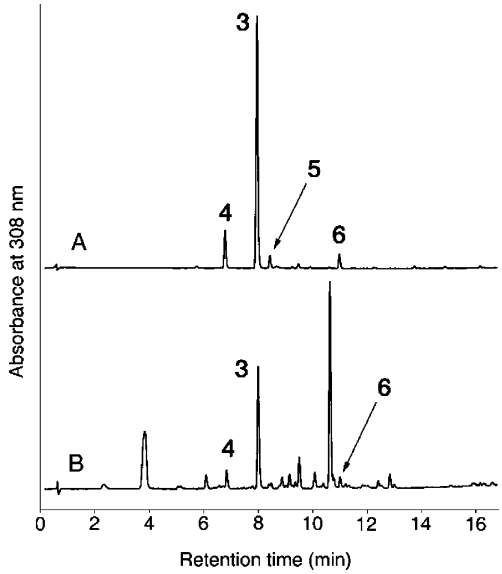

Figure 4. HPLC of embryo extract (Georgia Green cultivar) after basic hydrolysis (**A**) and acidic hydrolysis (**B**). Peaks: *p*-coumaric acid (**3**); caffeic acid (**4**); ferulic acid (**5**); 4-methoxycinnamic acid (**6**). Chromatogram **B** is up-scaled compared to chromatogram **A** for a better visualization of minor extract components.

whole root (**Tables 1** and **2**). The tips produced 4-methoxycinnamic acid and did not produce other phenolic acids.

The concentrations of major phenolic acids for three different peanut genotypes are given in **Table 2**. Dry seed parts did not accumulate detectable amounts of phenolic acids, whereas cotyledons and particularly embryos of imbibed seeds produced phenolic acids up to 0.25 mg/g (*p*-coumaric acid in the Virugard cultivar). None of the acids was detected in the testa. Of the seven parts of the seedling, four produced phenolic acids (in descending order): epicotyl/plumule, cotyledons, root, and hypocotyl. The epicotyl/plumule part accumulated high concentrations of *p*-coumaric acid (up to 1.3 mg/g in the Virugard cultivar). Ferulic acid was produced only by the epicotyl/plumule and cotyledons only in the seedling. Testa and mucilage of experimental and control peanuts did not accumulate any of the phenolic acids (**Tables 1** and **2**). In all of the experiments phenolic acids were represented only by the cinnamates **3**−**6** (**Figure 1**). Concomitant distribution of phenolic acids and resveratrol was observed in embryo, epicotyl/plumule, and testa (**Tables 1**−**4**).

Experimental Rationale. The experiments were performed with axenic seedlings grown under controlled conditions of constant temperature and minimal light and nutrients. The rationale for this experimental setup was dictated by the need to exclude the complexities prevailing in the field (*29*, *30*). For example, peanut plants can be systemically invaded by *A. flavus* and *A. parasiticus* from soil during peanut seedling emergence from the soil (*31*). Our research concentrated on the determination of two important groups of phenolic metabolites that may play a role in young peanut plants as defensive compounds: stilbenoids and phenolic acids.

Eighty percent MeOH was chosen as an effective solvent both for free phenolic acids and for soluble bound acids (*32*). To study the distribution of major phenolic metabolites in different organs of the peanut plant, a gradient HPLC method was developed. The method allowed for a baseline separation of all major soluble metabolites of different polarities (**Figure 3**). Organ extracts were analyzed by HPLC without any purification to avoid possible loss of stilbenoids and phenolic acids. Peanut stilbenoids are labile and light-sensitive, and phenolic acids, particularly ferulic acid, can be easily oxidized during preparation and analysis (*4*, *19*). The acidic pH was chosen to keep the stilbenes and phenolic acids in their neutral form, which would provide predictable elution order and better separation with improved peak shape. Recoveries of *trans*-resveratrol were similar to those previously described (*4*) and exceeded 95% with spike levels of >100 ng/g. The detection limit of *trans*-resveratrol was 10 ng/g, and that for *p*-coumaric (**3**), caffeic (**4**), ferulic (**5**), and 4-methoxycinnamic (**6**) acids ~15 ng/g (**Figure 1**), which was adequate for the quantitation of trace amounts of the metabolites.

Peanut organs were subjected to acidic and basic hydrolysis (**Figure 4**) to determine bound phenolic acids because only a minor fraction of phenolic acids exists in plants as free acids (*14*, *16*, *19*, *33*, *34*). Acidic hydrolysis of Georgia Green embryos yielded 133.1 ± 15.3 μg/g of *p*-coumaric acid and 20.3 ± 3.5 μg/g of caffeic acid. These values were significantly lower than the values obtained after basic hydrolysis (708.3 ± 75.3 and 202.3 ± 26.1 μg/g, respectively) (**Table 2**). Phenolic acids obtained by basic hydrolysis demonstrated more than satisfactory stability under nitrogen at room temperature (*19*); therefore, basic hydrolysis was chosen in this research.

Role of Metabolites. The most interesting results in this research were obtained from the mucilage, which produced extremely high concentrations of stilbenoids. The generally accepted definition of phytoanticipins, antimicrobial compounds that are present in plants before the plants are challenged by microorganisms (*35*), suggests that mucilage stilbenoids represent phytoanticipins. In some instances, the same compound may serve in the same plant as a phytoalexin and as a phytoanticipin (*35*). Data on accumulation of preformed peanut stilbenoids in axenic plants are lacking in the literature. Uncertainty of the roles that stilbenoids play in the resistance of plants to microbial challenge is due in part to insufficient data on the antimicrobial properties of this group of compounds. It is not clear why high concentrations of mucilagin A **2** (**Figure 1**) are produced by the mucilage under unchallenged conditions, whereas other parts of the plant do not produce any measurable

amounts of stilbenes. The embryo/epicotyl, which is protected by cotyledons, testa, and peanut shell, does not need to produce any defensive chemicals at the expense of internal nutrients, whereas the mucilaginous external layer produces such chemicals in response to challenge by soil pathogenic microorganisms. Extremely high concentrations of mucilage stilbenes suggest that they may play a defensive role in the peanut plant because several of the peanut stilbenes are known to be biologically active (*2*, *6*, *7*, *11*). Stilbenes likely possess high antimicrobial activity because of the presence of lipophilic isoprenyl groups. Isoprenylation increased the fungitoxicity of corresponding nonprenylated stilbenes that had higher polarity and lower activity (*8*). The interface between root and soil could be not only a region of high metabolite activity as a result of plant−microflora associations (*36*, *37*) but also a region of antagonistic plant−microflora interactions. Root mucilage of Virugard and Valencia cultivars produced significantly higher levels of stilbenoids than Georgia Green, which may indicate higher resistance of Virugard and Valencia root systems to exogenous infection.

Resveratrol **1** (**Figure 1**) has been found at low levels in sound peanuts (*38*, *39*); however, a lack of potential fungal infection was not assured. In the present study all experiments were performed under axenic conditions. Resveratrol does not seem to serve as a phytoanticipin in axenic peanuts due to its low concentrations in peanut organs. Damaged parts of the peanut plant challenged by a fungus produced resveratrol and other stilbenes in much higher concentrations (*1*−*8*). Whether the above parts of an intact peanut seed and seedling in a fungal environment are able to synthesize higher levels of resveratrol was not addressed. Lack of known stilbenoids (*5*−*8*) in axenic peanuts and their accumulation in challenged peanuts indicate that they should be regarded as phytoalexins.

Occurrence of phenolic acids in different peanut organs and their concentrations varied considerably (**Table 2**). The presence of these acids in peanut organs in imbibed seeds and seedlings, in which metabolism increased with increasing water activity levels, but not in dry seed (**Table 2**), suggests that those acids are associated with peanut dormancy or growth (*28*). Challenged peanuts have been used to study phenolic acid elicitation and phenolic acid antifungal activity (*16*, *21*, *40*). Ferulic acid, for instance, possesses a wide spectrum of antiviral and antimicrobial activity (*40*). As an antioxidant, caffeic acid was a potent anti-aflatoxigenic agent that completely inhibited production of aflatoxins by a toxigenic *A. flavus* strain at a concentration of 0.1% (*18*). At the same time caffeic acid did not have a visible effect on fungal development at this concentration (*18*). Other phenolic acids and stilbenoids that also possess antioxidative properties may share a similar protective mechanism suggested to be fungal "oxidative stress" (*18*). Ferulic and *p*-coumaric acids at concentrations of 0.1 mM completely inhibited aflatoxin B_1 biosynthesis by *A. flavus* on day 4 of incubation (*16*). However, a "phytoalexin-like" elicitation of *p*-coumaric and ferulic acids in peanuts challenged by *A. flavus* cannot be explained merely by their antifungal properties (*16*). More likely, hydrophobic phenolic acids, such as 4-methoxycinnamic acid **6** (**Figure 1**), and an unknown low-polarity phenolic acid in peanuts (*16*) may serve as antimicrobial agents. Hydrophobic moieties may increase the antimicrobial activity of peanut preformed or elicited cinnamates on the basis of their similarity to peanut stilbenoids (*8*).

This research demonstrated that seven parts of the peanut plant had different abilities to produce stilbene phytoalexins and phenolic acids under axenic conditions. Three different peanut genotypes produced the same set of stilbene phytoalexins and phenolic acids. Root mucilage contained several new low-polarity stilbene compounds and none of the known peanut stilbenes or phenolic acids; the prenylated stilbenes were restricted to the mucilage and were not found in other organs of the peanut plant. Mucilagin A **2** (**Figure 1**) and other root mucilage stilbenoids (*27*) may play a role as antimicrobial compounds. A new low-polarity phenolic acid, 4-methoxycinnamic acid, may possess properties of a phytoanticipin/phytoalexin on the basis of its production by the peanut root as one of the major phenolic acids.

ABBREVIATIONS USED

ESI, electrospray ionization; HPLC, high-performance liquid chromatography.

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Supporting Information Available: Positive ESI-MS spectrum of **6** and positive ESI-MS^3 spectrum of **6**. This information is available free of charge via the Internet at http://pubs.asc.org.

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