Polyphenols in Groundnut Genotypes Resistant and Susceptible to Seed Colonization by *Aspergillus flavus*

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Abstract

Thirteen groundnut genotypes, eight resistant and five susceptible to in vitro seed colonization by Aspergillus flavus *were grown in replicated trials at three locations in Andhra Pradesh, India. Seed coats of these genotypes were analyzed for polyphenols using different methods. No significant correlation was observed between seed colonization and polyphenols content, which corroborates earlier observations on many genotypes using a single method for polyphenols estimation.*

Résumé

Les polyphénols dans les génotypes d'arachide résistants ou sensibles à la colonisation des graines par *Aspergillus flavus*: *Treize génotypes d'arachide, dont huit résistants et cinq sensibles à la colonisation in vitro des graines par* Aspergillus flavus *ont été étudiés dans le cadre d'essais répétés sur trois sites en Andhra Pradesh (Inde). Les téguments de ces génotypes ont été analysés par différentes méthodes pour la présence des polyphénols. On n'a observé aucune corrélation significative entre la colonisation et la teneur en polyphénols, ce qui corrobore une observation antérieure faite sur plusieurs génotypes en utilisant une seule méthode d'estimation des polyphénols.*

Resúmene

Los polifenoles presentes en los genotipos de cacahuate resistentes y susceptibles a la colonización de las semillas por *Aspergillus flavus*: *Trece genotipos de cacahuates, ocho resistentes y cinco susceptibles a la colonización de la semilla* in vitro *por* Aspergillus flavus, *se evaluaron en pruebas replicadas en tres localidades de Andhra Pradesh, India. Los tegumentos de las semillas de estos genotipos se analizaron para determinar su contenido de polifenoles, usando diferentes métodos. No se observó una correlación significativa entre la colonización de las semillas y su contenido de polifenoles, lo cual corrobora observaciones anteriores hechas en gran número de genotipos, usando un solo método de cuantificación de polifenoles.*

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Introduction

Tannins, which are polyphenols, may be defined as water-soluble phenolic metabolites of plants, with molecular weight of 500 or more, and with the ability to bind to and/or precipitate gelatin and/or other proteins from aqueous solutions (Butler In press). Tannins isolated from groundnut seed coat produce cyanidin during acid hydrolysis and therefore belong to the class of condensed procyanidins (Lansden 1982). Turner et al. (1975) isolated 5-7-dimethoxyisoflavone from groundnut and observed that it inhibited the growth of *Aspergillus flavus* Link ex Fries. Sanders and Mixen (1978) reported a significant and negative correlation between percentage seed colonization by *Aspergillus parasiticus* Speare and tannin content in groundnuts. Amaya et al. (1980) reported a possible relationship between resistance to *A. flavus* and total soluble amino compounds and arabinose content in groundnut cultivars. Lansden (1982) isolated three fractions of tannins from groundnut testae and observed that the fractions inhibited the growth of *A. parasiticus*. Karchesy and Hemingway (1986) have identified various compounds that are present in groundnut seed coats and determined the structure of some of the isolated polyphenolic compounds. We examined polyphenol content of seed coats in relation to seed colonization by *A. flavus* in several groundnut genotypes.

Materials and Methods

Fifty groundnut genotypes grown at ICRISAT Center in the 1982 rainy season, and 13 grown in replicated trials at three locations (ICRISAT Center, Tirupati, and Anantapur) in Andhra Pradesh State, India, in the 1985 rainy season were used in this study. The genotypes were tested for their reactions to in vitro seed colonization by *A. flavus* (Mehan and McDonald 1980).

Sample Extracts

Seed coats of groundnut genotypes were ground to a fine powder in a PICA® mill (Pitchford Industries, Pittsburg, USA), and defatted with n-hexane in a Soxhlet apparatus. The samples were air dried and then further dried at 37°C for 2 h. Ground seed coat (100 mg) was extracted with 5 mL methanol for 1 h in a test-tube mixer (Stuart Tube Rotator TR-2®, UK). After centrifuging, the supernatant was saved and the residue was reextracted with 5 mL methanol for 30 min and the methanol extracts were pooled together. The residue was extracted with 5 mL methanol containing 1% concentrated hydrochloric acid (vol/vol) and after centrifuging, this procedure was repeated and the extracts were pooled together. Both the methanol and acidified methanol extracts were used for the estimation of polyphenols. Analyses were carried out on individual replicate seed-coat samples from two locations as well as on pooled samples from all three locations. Results obtained on pooled samples are reported here as the agreement between the values obtained on individual replicate and pooled data was excellent.

Polyphenol Estimation

Total phenols were estimated using the modified Prussian blue method (Butler 1982). Total phenols were also extracted independently from each seed coat sample using acidified methanol extract and then estimated by the Folin-Denis method (AOAC 1984). Protein-precipitable

phenols were estimated by using a modified procedure of Hagerman and Butler (1978) as described by Jambunathan et al (1986). The vanillin assay was carried out with glacial acetic acid (Butler et al. 1982). Leucoanthocyanidin and proanthocyanidin were estimated according to Walterson and Butler (1983). All the results are expressed as absorbance units g^{-1} of defatted seed coat except in the case of the Folin-Denis method where the results are expressed as mg g^{-1} of tannic acid equivalents.

Results and Discussion

The ranges and means of seed colonization and polyphenols contents in 50 genotypes are shown in Table 1. A significant and negative correlation ($r = 0.33$, $P < 0.05$) was found between seed colonization by *A. flavus* and polyphenols content estimated by the Folin-Denis method. However, when the groundnut genotype OG 43-4-1, which showed 97% seed colonization and had the lowest polyphenols content (2 mg g^{-1}), was excluded from the analysis, the correlation coefficient ($r = 0.06$) became insignificant.

Table 1. Seed colonization (%) by *Aspergillus flavus* and polyphenol content in groundnut genotypes[1].

Number of genotypes		Seed colonization (%)	Polyphenols[2], ($\mu g\ g^{-1}$)	Correlation coefficient (r)
50	Range	25.6-97.0	2-388	-0.33*
	Mean	40.4	267	
	SE	±1.7	±10.1	
49	Range	25.6-71.6	149-388	0.06
	Mean	39.2	272	
	SE	±1.3	±8.6	

1. Rainy season 1982.
2. Estimated by the Folin-Denis method on acidified methanol extract.
* Significant at $P < 0.05$.

The reactions of 13 groundnut genotypes to in vitro seed colonization by *A. flavus* are shown in Table 2. All the resistant lines had less than 15% seed colonized, except ICGS 58 at Tirupati (19.8%) and ICGS 78 (39.3%), ICGS 58 (36.8%), and PI 337394 F (15.8%) at Anantapur. All the five susceptible genotypes had more than 30% of their seed colonized across all three locations. The ranges and mean values of polyphenols estimated by the Folin-Denis method in the acidified methanol extract of seed coats and by Prussian blue method in the methanol extract are shown in Table 3. Variation between resistant and susceptible cultivars at any of the locations was not apparent from these values. The vanillin assay method has been used as one of the standard methods for the estimation of degree of polymerization of proanthocyanidin. The ranges and mean values obtained did not show a large variation between the resistant and susceptible genotypes at any location though the absorbance values g^{-1} of the seed coat were high for both the resistant and susceptible genotypes (Table 4). In contrast, protein-precipitable

Table 2. Reaction of groundnut genotypes from three Indian locations to in vitro seed colonization by *Aspergillus flavus*.

Genotypes	Reaction[1]	Seed colonized (%)		
		ICRISAT Center	Tirupati	Anantapur
Ah 7223	R	8.9	8.2	10.7
J 11	R	10.5	10.8	10.3
U 4-47-7	R	14.8	10.0	13.8
UF 71513	R	11.1	11.1	11.1
PI 337394F	R	11.5	14.0	15.8
Var 27	R	13.8	8.1	10.0
ICGS 58	R	13.2	19.8	36.8
ICGS 78	R	12.5	11.5	39.3
TMV 2	S	32.4	32.0	33.3
Faizpur 1-5 × NC Ac 17090	S	33.9	38.0	44.2
Gangapuri	S	37.4	34.0	34.6
NC Ac 17090	S	36.5	45.2	46.3
EC 76446(292)	S	38.0	44.9	41.1
SD		±12.1	±14.5	±14.7

1. R = Resistant; S = Susceptible.

Table 3. Polyphenol content in seed coats of groundnut genotypes grown at three Indian locations[1].

Number of genotypes	Folin-Denis (mg g^{-1})[2]			Prussian blue (A_{720} g^{-1})[3]		
	ICRISAT Center	Tirupati	Anantapur	ICRISAT Center	Tirupati	Anantapur
8 Resistant	224	221	215	262	265	343
	(171-271)	(124-255)	(167-236)	(146-422)	(96-438)	(232-440)
SD	±29.2	±41.3	±22.6	±81.8	±95.6	±68.5
5 Susceptible	220	238	223	279	302	343
	(161-286)	(163-285)	(175-272)	(149-453)	(100-467)	(246-507)
SD	±46.2	±45.9	±34.6	±110.0	±131.7	±105.3

1. Means and (ranges) are given.
2. Acidified methanol extract.
3. Methanol extract.

phenols estimated using bovine serum albumin showed much lower values in the resistant and susceptible genotypes though the susceptible genotypes had slightly higher values than resistant cultivars (Table 4). However, the differences were not large enough to distinguish between susceptible and resistant types. We observed that leucoanthocyanidin concentration in susceptible genotypes was higher at each of the locations than the resistant genotypes and the leucoanthocyanidin values were the lowest of all the polyphenols that were estimated in these samples (Table 5). However, when they were heated to measure proanthocyanidin, dramatic increases in the values were observed (Table 5).

Table 4. Polyphenol content (A510 g^{-1})[1] in seed coats of groundnut genotypes grown at three Indian locations[2].

Number of genotypes	Vanillin assay			Protein-precipitable phenols		
	ICRISAT Center	Tirupati	Anantapur	ICRISAT Center	Tirupati	Anantapur
8 Resistant	645	664	716	59.6	68.1	73.0
	(330-1020)	(196-1046)	(408-830)	(22.4-95.8)	(18.6-92.0)	(36.4-95.0)
SD	±199.4	±242.7	±141.0	±20.7	±22.9	±18.7
5 Susceptible	665	624	664	68.5	70.9	76.0
	(295-1102)	(190-984)	(388-1028)	(20.2-100.6)	(24.4-94.4)	(35.4-106.6)
SD	±288.2	±303.4	±248.2	±33.1	±26.8	±27.1

1. Absorbance at 510 nm calculated as units g^{-1}, methanol extract.
2. Means and ranges are given.

able 5. Polyphenol content (A550 g^{-1})[1] in seed coats of groundnut genotypes grown at three Indian ıcations.

Number of genotypes	Leucoanthocyanidin			Proanthocyanidin		
	ICRISAT Center	Tirupati	Anantapur	ICRISAT Center	Tirupati	Anantapur
8 Resistant	15.1	9.8	11.0	659	585	742
	(11-19)	(4-16)	(9-12)	(230-1130)	(140-884)	(308-996)
SD	±3.1	±3.4	±0.8	±253.4	±216.7	±207.3
5 Susceptible	24.0	14.6	19.4	559	594	734
	(10-63)	(3-48)	(9-54)	(220-1185)	(112-926)	(324-1164)
SD	±22.8	±18.8	±19.5	±391.8	±296.7	±318.9

1. Absorbance at 550 nm calculated as units g^{-1}, methanol extract.

Although only the results obtained with the methanol extract have been discussed, the results obtained with the acidified methanol extract also did not show any large differences in the ranges or in the mean values between resistant and susceptible genotypes. Table 6 shows the correlation coefficients between the results obtained with polyphenol methods and the seed colonization data for groundnut genotypes grown at ICRISAT Center, Tirupati, and Anantapur. There was no significant correlation between any of the methods and seed colonization data. Also, no significant correlation was obtained between seed colonization data and the results of polyphenols obtained with acidified methanol extract of 8 resistant and 5 susceptible genotypes (Table 6). When the data for total polyphenols (polyphenols measured in methanol plus acidified methanol extracts) was compared with the seed colonization data from each of the locations, no significant correlation was obtained (Table 6).

Analysis of variance was carried out to examine the differences in resistant versus susceptible groups, genotypes within the groups, and their interaction with locations for seed colonization and various polyphenols estimations. Results showed (Table 7) significant interactions between the groups, and also between genotypes within the groups with locations for most of the

Table 6. Correlation coefficients between seed colonization by *Aspergillus flavus* and polyphenols content of seed coats of groundnut genotypes grown at three Indian locations.

	ICRISAT Center			Tirupati			Anantapur		
	M[1]	AM	MAM	M	AM	MAM	M	AM	MAM
Total phenols (Prussian blue)	0.11	-0.08	0.11	0.28	0.32	0.40	0.40	0.31	0.49
Protein-precipitable phenols	0.19	-0.29	0.15	0.23	-0.07	0.25	0.45	-0.35	0.44
Vanillin Assay	0.04	-0.25	0.01	0.03	-0.40	0.01	0.12	0.28	0.16
Leucoanthocyanidin	0.38	-0.09	0.38	0.34	0.11	0.30	0.33	0.29	0.32
Proanthocyanidin	-0.19	-0.19	-0.21	0.17	-0.06	0.15	0.34	-0.27	0.27
Folin Denis	-	-0.05	-0.05	-	0.32	0.32	-	0.46	0.46

1. M = Methanol extract; AM = Acidified methanol extract; MAM = Methanol plus acidified methanol extract.

Table 7. Analysis of variance (mean squares and probability levels).

		Variables						
Source	d.f.	SC[1]	PB	PP	VA	L	FP	FD
Location	1	17.9	13389.5	8204.1	615.0	3.2	41961.0	1295.1
Replicate/ Location	6	6.3	1718.7	833.8	10317.0	21.3	19202.0	22.9
Genotype	12	1376.4	67234.3	4658.3	403630.3	1298.3	540923.0	9574.8
Resistant (R) vs Susceptible (S)	1	15776.9	1380.0	4166.0	7939.0	796.3	28613.0	232.3
Resistant	7	42.1	60533.9	3410.0	337743.0	23.8	409652.0	7009.2
Susceptible	4	111.3	95423.6	6964.6	617855.0	3655.2	898725.0	16400.3
Genotype × location	12	39.0	4471.6	329.7	41741.3	83.2	31494.0	681.3
(R vs S) × location	1	71.5	2700.9	354.6	16089.0	447.2	7920.0	61.3
Resistant × location	7	31.6	5195.8	195.0	49639.0	61.1	17054.0	467.2
Susceptible × location	4	46.2	3646.9	559.0	34333.0	30.8	62656.0	1210.8
Error	72	5.1[2]	360.0	73.8	4263.0	4.04	5065.0	73.8

1. SC : Seed colonization; PB : Prussian blue; PP : Protein-precipitable phenols; VA : Vanillin acetic acid; L : Leucoanthocyanidin; FP : Proanthocyanidin; and FD : Folin Denis.
2. Error d.f. for SC is 64 due to 8 missing observations.

** $P < 0.01$

variables (as denoted by asterisks). Sanders and Mixon (1978), using the Folin-Denis method, reported a negative and significant correlation (r = 0.76) between seed color and tannin content in 16 groundnut genotypes. The tannin content in seed coats of these samples ranged from 33.5 mg g^{-1} in the highly susceptible to 344 mg g^{-1} in the resistant genotypes. In our 13 genotypes, the lowest value was 161 mg g^{-1} obtained in the susceptible genotypes grown at ICRISAT Center, and the highest value was again obtained in the susceptible genotype grown at ICRISAT Center that had 286 mg g^{-1}. In the 49 genotypes that were grown in the 1982 rainy season, the lowest value was 149 mg g^{-1} and the highest value was 388 mg g^{-1}. Thus our values in the susceptible cultivars were much higher than those reported by Sanders and Mixon (1978). However, the seed colonization data of Sanders and Mixon (1978) showed a range between 10.3 and 91.3%

while our seed colonization data on 13 genotypes ranged from 8.1 to 46.3%. In our data obtained on 50 genotypes, if we eliminated one genotype that showed 97.0% seed colonization, the correlation was not significant. It is true that the absolute quantity of polyphenols reported from laboratories varies widely because of the nature of the compound measured, the methodologies involved, and the influence of such other factors as environment and laboratory variations. However, we observed no significant correlation between seed colonization data and polyphenols content in methanol and acidified methanol extract using a variety of methods.

We made an attempt to find out if any of the various methods of polyphenols estimation could be useful to aid pathologists and plant breeders in evaluating and screening resistant and susceptible groundnut genotypes. Although we observed no significant correlation between seed colonization data and polyphenols, it is necessary to determine whether any of the various individual polyphenolic compounds could be involved in resistant characteristics of groundnut genotypes. However, from the data obtained so far, we can conclude that total polyphenols estimation in groundnut seed coat is not a reliable indicator when screening resistant genotypes for seed colonization by *Aspergillus flavus*.

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Discussion

R.E. Pettit. I understand that what you are reporting here are total phenols, if you look at the individual phenol compounds you might get a better correlation.

R. Jambunathan. I agree; and we plan to do this when we have found suitable methods.