Variation in Phytoalexin Production by Peanut Seed from Several Genotypes

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ABSTRACT

Evaluation of twenty peanut (*Arachis hypogaea* L.) genotypes for their phytoalexin producing ability showed wide variation in the amount and composition of phytoalexins produced. Some genotypes produced one major phytoalexin component while the other genotypes produced seven major phytoalexin components. In addition, high phytoalexin producing genotypes utilized more methionine-rich protein than the low phytoalexin producing genotypes suggesting that methionine-rich protein or its breakdown products may have a role in phytoalexin production.

Key Words: α-amino nitrogen, peanut genotypes, phytoalexins, methionine-rich protein.

Plants produce phytoalexins in response to injury and invasion by certain pathogens and appear to be involved in disease resistance (8, 10). Peanut seeds have been reported (2, 9, 11, 14) to produce phytoalexins when sliced and exposed to their native microflora at 25 C in the dark. These compounds have been implicated as a mechanism for natural resistance to fungal infection of peanuts (13). Peanut phytoalexins have been identified as isoprenylated stilbene derivatives closely related to 3,5,4' -trihydroxy-4-isopentenylstilbene (1, 11). Wooton and Strange (14) studied peanut stilbene phytoalexins elicited by slicing the seed and incubating the slices at 25 C in the dark. They found that these phytoalexins inhibited spore germination and hyphal extension of *Aspergillus flavus* with ED_{50} values in the range 4.9- 12.8 μg mL^{-1}. Their data also indicated that resistance of peanut seed to invasion by *A. flavus* was correlated with their capacity to synthesize phytoalexins as an early response to wounding. Conditions that promoted invasion of peanut by *A. flavus* inhibited phytoalexin production. Thus seeds of drought stressed plants, which are more susceptible to *A. flavus* than seeds of non-drought stressed plants, produced less phytoalexin in response to wounding by slicing than seeds from non-stressed plants (6, 7, 15).

In view of the evidence suggesting phytoalexin production as a mechanism for natural resistance to microbial invasion, this study was initiated to determine genotypic variation in the phytoalexin producing ability of the peanut seeds, and to determine the relationship between seed composition and the phytoalexin producing capacity of the seeds.

Materials and Methods

Materials

Twenty peanut (*Arachis hypogaea* L.) genotypes from the breeding program at the North Florida Research and Education Center, University of Florida, Marianna, Florida were used in this study. They represent a range of genotypes including cultivars and various breeding lines. Some of the lines have resistance to leaf spot (*Cercosporidium personatum*), rust, web blotch, white mold, and some insects.

Induction of Phytoalexins

Phytoalexins were elicited from peanut seeds as previously described by Aguamah *et al.* (1). About 6-8 g of dry peanut seeds were soaked in water at 4 C for 24 h, transferred into 9 cm petri dishes and cut into 1-2 mm thick slices. The petri dishes were then placed in a dessicator saturated with water and incubated for 4 days in dark at 25 C ± 1 C. All the incubations were conducted in three replications. After the 4 day incubation period the slices were freeze dried, ground into a meal and stored at -20 C until use.

Extraction and Fractionation of Phytoalexins

Phytoalexin extraction and fractionation was carried out as per the methods of Agumah *et al.* (1) and Dorner *et al.* (7). Peanut meal (2 g) was extracted with 20 mL of ethanol by homogenization for 1 min using a polytron homogenizer. The extracts were evaporated to dryness, redissolved in 5 mL benzene and passed through a silica Sep-Pak (Waters Assoc. Milford, MA) cartridge. The cartridge was eluted with 5 mL of benzene: ethylacetate (1:2, v/v) and an aliquot of the eluate was fractionated by HPLC. The HPLC system consisted of a Resolve silica column (3.9.mm x 15 cm; Waters Assoc. MA) and a UV/VIS detector set at 335 nm. The mobile phase consisted of cyclohexane methyl-t-butly ether:methanol (79:20:1, v/v/v) and the flow rate of the column was 1 mL/min. Since the chemical structure of individaual components have not been determined, comparative analysis of all the samples for phytoalexins was achieved by summing area counts for peaks corresponding to phytoalexins previously purified (7).

Protein Composition

The protein composition of various genotypes was determined by extracting the defatted peanut meal (25 mg) with 2 mL of 0.5 M NaCl, 0.01 M sodium phosphate (pH 7.0), 0.02% NaN_3 using a Polytron homogenizer. The homogenate was centrifuged and an aliquot of the supernatant was analyzed by HPLC (5). The HPLC system consisted of a model 510 pump, UV/VIS detector, Protein Pak SW 300 column and a 820 Data Station (Waters, Milford, MA).

α-amino Nitrogen, Soluble Sugars and Total Protein

A portion of the meal was defatted (4) using hexane and the defatted meal was extracted with methanol:chloroform:water (60:25:15, v/v/v) according to Young et al. (18), centrifuged and an aliquot of the supernatant was analyzed for α - amino nitrogen (16), and soluble sugars (17). Another portion of the defatted meal was homogenized with 1 M NaOH, centrifuged (4), and the protein content of the supernatant was determined according to the method of Lowry *et al.* (12), with bovine serum albumin as the standard.

Statistical Analysis

The significance of the data was tested by Duncan's Multiple Range Test using the SAS (1989) package.

Results and Discussion

Analysis of phytoalexin-induced peanut seed slices showed the presence of five to seven components not previously present in noninduced peanut seed slices. These components are considered as phytoalexins because they were produced by viable peanut seeds, only when wounded and incubated unsterilized. Thus, freeze-thawed peanut seeds (non-viable) or seeds sterilized with sodium hypochlorite (0.05%) prior to slicing, failed to produce these compounds. In previous studies, Dorner *et al.* (7) and Basha *et al.* (2) have reported the induction of seven phytoalexin components in Florunner peanut seeds following slicing and 4-day incubation. By definition, a phytoalexin is produced in response to microbial invasion and is not present in the absence of microbial invasion or simulated invasion. Using proton magnetic resonance analyses, Dorner *et al.* (unpublished data) have

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found some of these compounds to be stilbenes. Earlier, Keen (9) and Aguamah *et al.* (1) also observed that the seeds of peanuts accumulated phytoalexins when they were sliced and their natural microflora allowed to proliferate during a 3-5 day incubation period at 25 C in the dark.

These compounds have been identified as *cis*-and *trans*-isomers of 3, 5, 4' trihydroxy-4-isopentenylstilbene or Arachidin II (1, 8, 11), *cis*-and *trans*-3,5,4'-trihydroxystilbene or resveratrol, (8), 4-(3-methyl-but-l-enyl)-3, 5, 3', 4'-tetrahydroxystilbene (Arachidin I), and 4-(3-methyl-but-l-enyl)-3, 5, 4'-trihydroxystilbene or Arachidin III (1). These studies clearly demonstrated that peanut seeds accumulate stilbene phytoalexins when they were sliced and challenged with the native microflora.

Phytoalexin profiles of selected peanut genotypes used in this study are shown in Fig. 1. Compared to the Florunner cultivar (Fig. 1h), the phytoalexin composition differed widely among peanut genotypes. While some genotypes contained one major phytoalexin component, others contained more than one major component. For example, of the seven phytoalexin components, component VI was predominant in NC 6 (Fig. 1a) while in Southern Runner (Fig. 1f) all seven components were present in major proportions. In addition to qualitative differences, the peanut genotypes also showed quantiative differences in phytoalexin production. For example, component VI constituted 89% of the total phytoalexin produced in NC 6 (Fig. 1a), while it was only 7% in line 73x93-6-1-3-1-2-b3-B (Fig. 1b). Likewise, in line 79x4-6-2-1-1-b3-B 9 (Fig. 1c) component III constituted about 63% while in NC6 (Fig. 1a) it was only 5% of the total phytoalexins. In addition, component V constituted 13% in line 79x4-6-2-1-4-b3-B (Fig. 1g) while it was present in trace amounts in line 79x4-6-2-1-1-b3-B (Fig.1c). The data showed that phytoalexin composition differs widely among the peanut genotypes. To avoid confusion, the results of quantitation of various phytoalexin components (I through VII) are simplified by arranging peanut genotypes (1 through 20) in decreasing order based on the amount of individual phytoalexin component produced by each genotypes (Table I). For example, peanut genotype No. 6 (73x93-10-2-1-B-Pk-*b3*-B) produced the highest amount of component I while peanut genotype No. 17 (Florunner) produced the lowest amount of component I. Likewise, genotype No. 1 (Southern Runner) produced the highest amount of component VII while genotypes 15 through 20 produced only trace amounts of component II. As seen in the table, all twenty genotypes

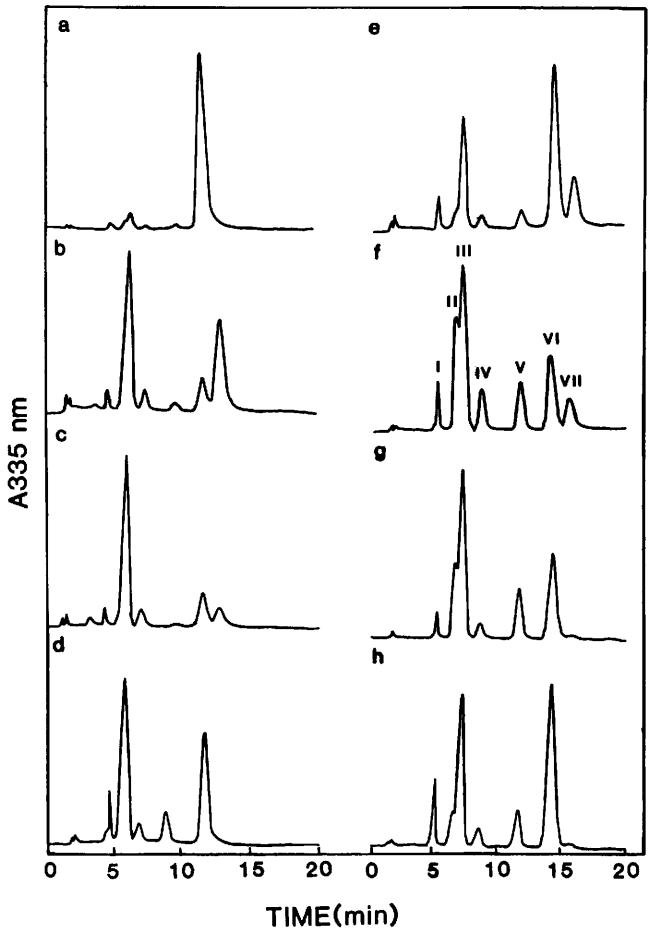

Fig. 1. Variation in the phytoalexin composition of selected peanut genotypes.

a=NC 6, b=73x93-6-1-3-1-2-b3-B, c-79x4-6-2-1-1-b3-B, d=79x6B-10-2-1-2-b3-B, e=73x93x10-2-1-B-PK-b3-B, f=Southern Runner, g=79x4-6-2-1-4-b3-B, h=Florunner. Peaks I through VII are numbered in the order of their elution.

Table 1. Rankings Among Twenty (1 through 20) Peanut Genotypes Based on their Phytoalexin Producing Ability.

Phytoalexin Components						
I	II	III	IV	V	VI	VII
a	a	a	a	a	a	a
9	4	4	1	1	8	1
ab	ab	ab	ab	ab	b	ab
8	7	1	9	8	10	9
abc	abc	abc	abc	abc	b	bc
1	1	18	2	4	9	7
abc	bc	abc	bcd	bcd	bc	bc
5	8	8	8	2	15	14
abc	bc	abc	bcd	bcd	bcd	bc
4	9	19	3	5	18	10
abc	bc	abc	bcd	bcde	bcde	bc
18	2	16	4	18	16	11
abc	bc	abc	bcd	bcde	bcdef	bc
2	5	5	5	3	13	3
abc	bc	abc	bcd	bcdef	bcdef	bc
19	3	9	14	9	14	12
abc	bc	abc	bcd	cdef	bcdef	bc
20	6	20	15	16	2	6
abc	bc	abc	bcd	cdef	bcdef	c
10	10	7	13	7	5	15
abc	bc	abc	bcd	cdef	bcdef	c
16	12	2	10	19	19	2
abc	bc	abc	cd	cdef	cdef	c
13	13	10	7	10	12	13
abc	c	abc	cd	cdef	cdef	c
15	11	3	12	20	3	5
abc	t	abc	d	def	cdef	c
6	14	13	11	15	6	4
abc	t	abc	d	def	cdef	t
3	15	17	20	14	1	8
abc	t	bc	d	ef	def	t
7	16	6	18	17	20	16
abc	t	bc	d	ef	def	t
14	17	14	16	13	7	17
abc	t	c	d	ef	ef	t
12	18	26	17	6	4	18
bc	t	c	d	ef	f	t
11	19	15	6	12	17	19
c	t	c	d	f	f	t
17	20	11	19	11	11	20

t = trace

In each column, peanut lines 1 through 20 are arranged in decreasing order based on the amount of phytoalexin component produced by each line. Peanut lines 1-20 are as follows: 1 = Southern Runner, 2 = UF81206-1, 3 = UF81206-2, 4 = 79 x 4-6-2-1-4-b3-B, 5 = 79 x 4-6-2-1-1-b3-B, 6 = 73 x 93-10-2-1-B-PK-b3-B, 7 = 73 x 93-6-1-3-1-2-b3-B, 8 = NC 6, 9 = NC 15745, 10 = NC 10247, 11 = 72 x 41A-6-1-2-2-b3-B, 12 = 72 x 45-8-1-b3-B, 13 = 72 x 83B-7-1-1-B, 14 = UF79308-1, 15 = UF82107, 16 = Early Bunch, 17 = Florunner, 18 = 76 x 9-10-1-1-1-2-b2-B, 19 = 79 x 4-6-2-1-3-b3-B, 20 = 79 x 6B-10-2-1-2-b3-B

Genotypes (1-20) in columns followed by same letter are not significantly different according to Duncan's Multiple Range Test (P = 0.05).

produced varying amounts of phytoalexin components, I, III, IV, V and VI but components II and VII were produced only in trace amounts by cultivars 15 through 20. The quantitative and qualitative differences found in the phytoalexins indicate that peanut genotypes significantly differed in their ability to produce these components.

Relationship Between Seed Composition and Phytoalexin Production

In our previous study (2) we have found a decrease in Peak VI protein during phytoalexin induction. In view of this observation, seed protein profiles of the 20 genotypes used in this study were obtained using HPLC. Figure 2 shows protein profiles of selected peanut genotypes following a 4-day incubation period. As seen in Figure 2, the amount of protein in peak VI and Peak VII differed widely among the genotypes. In addition, the proportions of protein peaks I and II also varied among the genotypes. In view of the observed genetic variation in protein composition, an attempt was made to determine possible correlation between the amount of phytoalexin produced and a specific seed protein, and to identify a protein marker as an indicator of phytoalexin producing capacity of the seeds. Results showed that except for peak VI no correlation was found between the total phytoalexin amount and seed protein. In the case of peak VI a negative correlation was observed between the amount of phytoalexin produced by the seeds and the peak VI protein content of the seeds after incubation. The peanut seeds which produced higher amounts of phytoalexin contained lower amounts of peak VI after incubation while the seeds that produced less phytoalexin contained higher amounts of peak VI. For example, in the high phytoalexin producing genotype (NC6), phytoalexin and peak VI areas (microvolt-Sec) were 77 x 10^6 units and 150 x 10^3 units, respectively, while in the low phytoalexin producing genotype (73x93-6-1-3-1-2-*b3*-B) they were 11 x 10^6 units and 381 x 10^3 units. In our previous study (3) we reported peak VI protein as a 120,000 molecular weight methionine-rich protein, and is composed of six polypeptides with molecular weights between 16,000 and 21,000, and isoelectricpoints between 5.1 and 5.8.

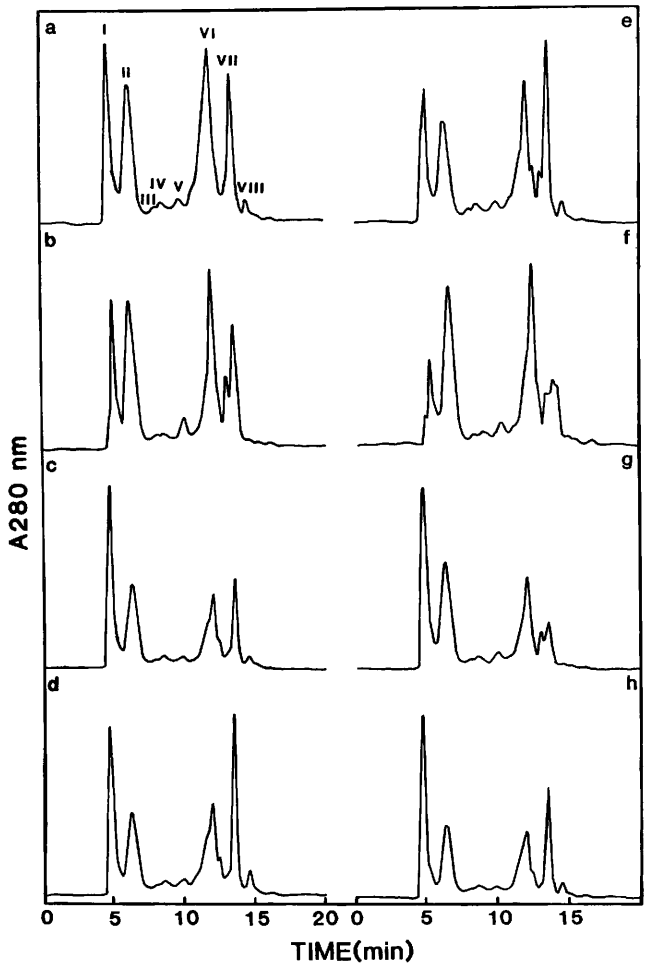

Fig. 2. Protein profiles of selected peanut genotypes resolved by HPLC.

a=NC6, b=73x93-6-1-3-1-2-b3-B, c=79x4-6-2-1-1-b3-B, d=79x6B-10-2-1-2-b3-B, e=73x93x10-2-1-B-PK-b3-B, f=Southern Runner, g=79x4-6-2-1-4-b3-B, h=Florunner. Peaks I through VIII are numbered in the order of their elution and decreasing molecular weight..

Seed Composition

Analyses of unicubated and incubate seed slices for protein, α-amino nitrogen and sugar showed that the incubated seeds contained 0.4% to 17% less protein than the unicubated seeds (Table 2). Likewise free sugar content of the seeds also decreased between 0.9% to 4.5% during the incubation. Unlike the protein and sugar, the α-amino nitrogen (total free amino acids) content of the seeds increased after incubation. The increase in the α-amino nitrogen content of the seeds may be due to protein breakdown. Increase in α-amino nitrogen content of the seeds varied among the genotypes and ranged between 0.17% to 1.45% (Table 2).

In summary, quantitative and qualitative differences

Table 2. Differences in the seed composition between unincubated and incubated peanut seed slices during phytoalexin induction.

Genotype[a]	Unincubated			Incubated		
	Protein	α-NH_2 Nitrogen	Sugars	Protein	α-NH_2 Nitrogen	Sugars
	g/100 g deatted meal					
8	47.01	1.45	7.07	35.24	2.17	5.31
1	54.00	0.87	5.64	50.03	1.86	4.48
9	54.21	1.41	5.91	37.76	2.20	4.55
2	46.27	1.63	5.97	45.87	2.52	2.78
19	49.44	1.38	8.45	43.31	2.17	6.18
5	54.83	1.27	8.60	42.02	2.86	4.58
12	52.72	1.00	6.66	35.24	2.45	5.89
14	58.35	1.08	6.52	49.10	2.33	4.98
4	51.93	1.58	8.96	44.63	1.33	4.46
6	52.80	1.61	8.55	48.12	1.73	7.57
7	55.11	0.80	6.39	49.51	1.37	5.38

[a]The peanut lines are listed in the decreasing order (P =0.05) of their ability to produce phytoalexins(total). To avoid duplication, only few genotypes representing high, medium and low phytoalexin producing genotypes are shown in the table.

1 = Southern runner, 2 = UF 81206-1, 4 = 79x4-6-2-4-b3-B,

5 = 79x4-6-2-1-1-b3-B, 6 = 73x93-10-2-1-B-PK-b3-B,

7 = 73x93-6-1-3-1-2-b3-B, 8 = NC6, 9 = NC 15745, 12 = 72x45-8-1-b3-B,

14 = UF 79301-1, 19 = 79x4-6-2-1-3-b3-B

observed in the phytoalexin components among the genotypes suggest that peanut seeds vary in their response to induction of these components following injury by slicing and incubation under non-sterile conditions. In addition, seed components such as methionine-rich protein and amino acids may have a role in phytoalexin production. From these observations it can be attributed that inherent differences among the peanut genotypes to produce these components may have a role in their ability to resist microbial infestation. Additional studies are in progress involving chemical identification and biological activity of these components against *A. flavus*.

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