Soluble Amino and Carbohydrate Compounds in the Testae of Six Experimental Peanut Lines with Various Degrees of *Aspergillus flavus* Resistance

Jaime Amaya-F., Clyde T. Young,*[1] Aubrey C. Mixon, and Allan J. Norden

The testae of three *Aspergillus flavus* resistant (PI 337394F, UF 734022, PI 337409) and three susceptible (UF 73515, PI 331326, PI 343419) lines of peanut (*Arachis hypogaea* L.) were analyzed for soluble amino compounds and carbohydrates. Resistant is defined as having a degree of resistance to testa penetration by the *A. flavus* fungus. Water-soluble nitrogenous compounds were found within testa of all resistant cultivars in significantly lower concentrations. Levels of total amino compounds in the hydrolyzed testae extracts were: 17.09, 22.36, 28.09, 33.94, 65.28, and 43.91 μmol/g, respectively. Most strongly correlated with susceptibility were concentrations of arginine, glycine, lysine, ammonia, methionine, and aspartic acid. The typical percent dry weight carbohydrate composition of the sulfuric acid hydrolysate of the testa was: arabinose (5.18), galactose (0.44), xylose (0.34), and glucose (0.14). The susceptible cultivar UF 73515 had an arabinose content significantly lower (2.92%) than the mean. These observations suggest that the unavailability of readily soluble, small molecular weight amino compounds on, or within, the testa matrix may play a role in the mechanism of *A. flavus* resistance exhibited by some peanut cultivars.

It has been suggested that the greater or lesser degree of resistance exhibited by certain peanut seeds against *Aspergillus flavus* colonization could be in part determined by the physical characteristics of the seed coat or testa. Resistant is defined as having a degree of resistance to testa penetration by the *A. flavus* fungus. Benedict et al. (1973) found that *A. parasiticus* grew well in both resistant and susceptible seeds after removal of the testa. Taber et al. (1973) detected morphological differences in the hila, the surface cuticular wax, and the palisade-like layers of the seed coats of resistant and susceptible varieties. They also showed differences between invasion of cotyledons before and after testa removal. Using the scanning electron microscope, LaPrade et al. (1973) noted that waxlike accumulations were more abundant on the testa of tolerant cultivars than on their susceptible counterparts. Glueck (1974), using light and scanning microscopes, found that the absence of cell composition in the New Mexico Valencia A variety, in combination with reduced cell wall thickness of inner epidermis, was responsible for improved integrity. He also found Starr and Florunner to have more splits after drying and related this to testae structure. The objective of this study was to examine the testae of several experimental peanut lines with various degrees of *A. flavus* resistance for amino and carbohydrate compounds that might be related to this resistance.

EXPERIMENTAL SECTION

Seeds and *A. flavus* Tests. Table I identifies the six peanut (*Arachis hypogaea* L.) cultivars selected for this study. In order to determine the degrees of susceptibility, 15- to 20-g samples of cured seeds were inoculated with *Aspergillus flavus* (NRRL A-13794 and NRRL 2999) spore suspensions as described by Mixon and Rogers (1973) and LaPrade et al. (1973). The degrees of susceptibility were made on the basis of a 2-year average of percent seed infection.

Preparation and Analysis of the Testae. About 50 seeds of each cultivar were lyophilized to approximately 4.0–4.5% moisture, and the testae were subsequently removed, avoiding pressure or rubbing between the surfaces of the testa and the cotyledon. Lowering the moisture of the seed to about 4.5% normally released the testa from the cotyledon. Frequently, however, it was necessary to begin lifting the testa with a small spatula.

Table I. Identification and Classification of the Six Peanut Lines with Various Degrees of Resistance or Susceptibility to *A. flavus* Colonization (Mean Values for Samples of Several Years)

Peanut identity	Seed source	Commercial type	% seed infection[b]
(I) PI 337394F[a]	Puerto Rico	Valencia	0
(II) UF 734022	Florida	Valencia	9
(III) PI 337409	Puerto Rico	Valencia	10
(IV) UF 73515	Florida	Spanish	60
(V) PI 331326	Maryland	Virginia	89
(VI) PI 343419	Georgia		91

[a] Those seeds with flesh-color testa from PI 337394. [b] Resistant lines had 16% infection or less.

The pooled testae were milled to pass a 40 mesh screen, and duplicate 100-mg portions were extracted twice in 4 mL of deionized water at 0 °C for 1 h. One aliquot of each extract was lyophilized, hydrolyzed under a nitrogen atmosphere in 6 N HCl at 145 °C for 2 hours, and neutralized with 12 N NaOH. In order to precipitate pigments, the pH was raised to about 12. After the color changed from gray to brown, HCl and citrate buffer were added to give a 2.2 pH, and the clear solution was analyzed in a Durrum D-500 amino acid analyzer.

Free monosaccharides in the aqueous extracts were determined in a low-pressure liquid chromatograph utilizing the tetrazolium blue reaction as a detection method (Mopper and Degens, 1972). Samples of milled testa (250 mg) were also hydrolyzed in 4 mL of 1 N H_2SO_4 in a boiling-water bath for 1 h; the hydrolysate was neutralized with 1 N $Ba(OH)_2$, centrifuged at 17 000 xg for 10 min, and analyzed for monosaccharides as described above.

RESULTS AND DISCUSSION

A highly significant correlation (r = 0.88) was found between the total amount of free, soluble amino compounds of the testa (Table II) and the degree of susceptibility to *A flavus* colonization (Table I). The amino acid content of the hydrolyzed soluble fraction is given in Table II. The concentration of 12 amino acids and that of

Department of Food Science (J.A.-F., C.T.Y.), University of Georgia, Experiment, Georgia 30212, Crops Research Unit (A.C.M.), U.S. Department of Agriculture, Agricultural Research Service, Southern Region, Tifton, Georgia 31794, Institute of Food and Agricultural Sciences, (A.J.N.), University of Florida, Gainesville, Florida 32611.

[1]Present address: Department of Food Science, North Carolina State University, Raleigh, N.C. 27607.

Table II. Amino Acid Composition (μmol/g) of the Water-Soluble Material in the Testa of Six Experimental Peanut Lines with Either Resistance or Susceptibility to *A. flavus* Colonization

Amino acid	Resistant			Susceptible			Correlation coefficient, r
	PI 337394F	UF 734022	PI 337409	UF 73515	PI 331326	PI 343419	
Asp	0.771	1.671	1.588	3.089	4.526	2.415	0.832
Thr	0.304	0.273	0.370	0.271	1.756	0.925	0.754
Ser	0.334	0.563	0.658	0.628	1.900	0.884	0.727
Glu	0.968	1.966	1.687	2.738	3.420	1.869	0.709
Pro	Tr[a]	Tr	Tr	Tr	1.886	Tr	
Gly	1.066	1.672	1.428	2.525	5.659	3.802	0.906
Ala	0.515	0.731	0.742	1.143	3.994	1.575	0.753
Cys	Tr	Tr	Tr	Tr	0.320	0.320	
Val	0.254	0.194	0.200	0.198	2.180	0.808	0.708
Met	0.320	0.430	0.532	1.257	1.036	1.005	0.875
Ile	0.484	0.568	0.522	0.536	1.754	0.818	0.674
Leu	0.486	0.504	0.593	0.492	2.842	0.900	0.627
Tyr	Tr	Tr	Tr	Tr	1.193	0.419	
Phe	0.713	0.692	0.844	0.340	2.368	1.075	0.559
His	Tr	Tr	Tr	Tr	0.739	0.891	
Lys	0.280	0.389	0.484	0.644	2.512	3.080	0.897
NH_3	10.600	12.704	18.445	19.281	25.690	22.737	0.898
Arg	Tr	Tr	Tr	0.883	1.508	1.988	0.977
Total	17.09 ± 1.41	22.35 ± 1.21	28.09 ± 1.49	34.02 ± 1.22	65.28 ± 0.84	43.91 ± 0.04	0.881

[a] Tr, 0.005 μmol/g or less.

Table III. Major Water-Soluble Monosaccharides in the Testa (mg/g, Dry Weight Basis)

Sugar[a]	Resistant			Susceptible		
	PI 337-394F	UF 734-022	PI 337-409	UF 73515	PI 331-326	PI 343-419
Xylose	0.42	0.99	0.25	0.77	1.48	0.99
Galactose	ND[b]	ND	ND	ND	0.24	0.15
Glucose	0.34	0.96	0.32	0.43	1.15	0.92

[a] Two minor unknowns were often detected early in the chromatograms. [b] ND, not detected.

ammonia were positively correlated ($0.55 \leq r \leq 0.97$) with the susceptibility of the seed. Most highly correlated were the levels of arginine ($r = 0.97$), glycine ($r = 0.90$), lysine ($r = 0.89$), ammonia ($r = 0.89$), methionine ($r = 0.87$), and aspartic acid ($r = 0.83$). Since ammonia was by far the largest contributor of the nitrogenous compounds, its presence was considered highly significant to the overall relationship. The high correlation coefficient of most amino acids, however, suggested the susceptible character of a seed was a function of the total amount of soluble amino compounds readily available to germinating spores within the testa.

The amino acid pattern given in Table II appeared to be unique compared with the typical hydrolysate of the whole peanut (mostly cotyledon) or the free amino acid pattern of cured raw peanuts (Young et al., 1974a,b). These observations support the possibility that soluble amino compounds are characteristically associated with the testa rather than being a contaminant from the cotyledon.

Although the extracted amino compounds were able to diffuse through a dialysis membrane, the material was hydrolyzed in 6 N HCl in order to (a) remove pigments which interfered with the ninhydrin reaction and (b) enhance the free amino acid yield, implying that small peptides were probably present. No unusually high proportions of asparagine could be inferred from the data in Table II.

Whether the soluble amino compounds are located on the inner surface or throughout the matrix of the testa has not yet been determined. Whatever the case, it would appear that minute amounts of diffusable amino compounds are available to germinating fungal spores, under high-moisture conditions, on the testa of certain peanut lines.

In view of the structural differences detected under the scanning electron microscope by LaPrade et al. (1973), it was of importance to analyze both the soluble and the remaining hydrolyzable (hemicellulose) carbohydrate fractions in the testa. Variable levels of xylose, galactose, and glucose were commonly found in the water extracts of all the testae (Table III). Hydrolysis of the ground testa in 1 N H_2SO_4 released mostly arabinose, xylose, galactose, and glucose (Table IV). The concentration of arabinose, the major monosaccharide in the testa, was found to be fairly constant for all the lines tested except the susceptible UF 73515, which contained 57% of the mean value of the remaining lines. Since UF 73515 also contained somewhat lower levels of soluble amino compounds than the other

Table IV. Monosaccharides in Testa Hydrolysates (mg/g of Dry Testa[a]) of Six Experimental Peanut Lines Considered Either Resistant or Susceptible to *A. flavus* Colonization

Sugar	Resistant			Susceptible		
	PI 337394F	UF 734022	PI 337409	UF 73515	PI 331326	PI 343419
Unk_1	0.90 ± 0.29	1.28 ± 0.61	0.91 ± 0.09	0.98[b]	1.03 ± 0.10	1.05 ± 0.09
Unk_2	0.76 ± 0.09	1.00 ± 0.23	0.77 ± 0.09	0.62 ± 0.10	0.62 ± 0.07	1.17[b]
Unk_3	0.84 ± 0.19	ND[c]	ND	1.84[b]	0.96 ± 0.58	1.33[b]
Arabinose	52.70 ± 3.25	49.37 ± 1.94	55.11 ± 1.90	29.28 ± 1.02	47.20 ± 1.13	54.64 ± 1.64
Xylose	2.80 ± 1.12	3.74 ± 0.20	3.60	2.85 ± 0.00	3.55 ± 0.40	3.87 ± 0.29
Galactose	3.26 ± 1.16	5.01 ± 0.55	4.39 ± 1.93	2.80 ± 0.10	4.42 ± 0.48	5.36 ± 0.89
Glucose	0.42 ± 0.19	2.01 ± .122	1.00[b]	1.58[b]	1.14 ± 0.47	2.76 ± 0.51

[a] Not corrected for losses. Arabinose recovery was estimated 70.95 ± 1.48%. [b] Peak measurable in one analysis only. [c] ND, not detectable.

two susceptible lines, it was conceivable that seed coats of this particular line had a weakened polysaccharide network which facilitated the diffusion of soluble material to the testa surface. The monosaccharide patterns found in six experimental peanut lines also appeared to be unique to the testa. Large amounts of glucose and fructose over those of arabinose and galactose, which is characteristic of the cotyledon (Amaya-F. and Young, 1976), contrasted with the sugars present in the testa.

The amino acid pattern of hydrolyzed water extracts from the peanut testa was unique to this organ and was characterized by a large proportion of ammonia. Most of these diffusable nitrogenous compounds seemed to be implicated in the growth of germinating fungal (*A. flavus*) spores assumed to be randomly distributed over the seed surface. Soluble monosaccharides found in testae did not appear to be correlated with susceptibility or resistance of the seed. Lower contents of arabinose in the acid hydrolysate of a susceptible line, however, was an indication of debilitated polysaccharide structures which could be important in maintaining the physical barrier characteristics of the testa.

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A Comparison of Various Mass Spectrometric and a Chemiluminescent Method for the Estimation of Volatile Nitrosamines

Terry A. Gough,* Kenneth S. Webb, Martin A. Pringuer, and Beryl J. Wood

Extracts of a variety of commodities have been analyzed for the presence of volatile nitrosamines using high- and low-resolution mass spectrometry and chemiluminescent detection. The quantitative results obtained by these methods have been compared to assess their reliability. The high-resolution peak matching and chemiluminescent data are in agreement with each other for all the analyses, but inconsistencies were observed using the other mass spectrometric procedures.

It has been established that many nitrosamines are carcinogenic (Magee and Barnes, 1967; Wolff and Wasserman, 1972), and their formation is possible between secondary or tertiary amines and nitrite (Mirvish, 1970; Fan and Tannenbaum, 1971). The need to detect trace amounts of nitrosamines in foodstuffs, biological fluids, vegetation, and other matter has been met by using a variety of analytical procedures. Volatile nitrosamines, after gas chromatographic (GC) separation, have been detected down to mg/L amounts, using nitrogen selective detectors such as the flame thermionic and the Coulson electrolytic conductivity detector. Extracts of biological origin are still complex mixtures even after extensive cleanup and frequently contain nitrogen-containing compounds. Some of these will have identical retention characteristics to nitrosamines, so that nitrogen selective detectors can give rise to false positive results (Goodhead and Gough, 1975). Some means of confirming the presence of nitrosamines tentatively observed using GC detectors is necessary, and mass spectrometry (MS) offers the most reliable means of achieving this. Several workers have developed methods based on mass spectrometry (Fazio et al., 1971; Gough and Webb, 1972; Telling et al., 1971), all of which involve a prior separation of the nitrosamines from each other and from extraneous material, using combined gas chromatography and mass spectrometry (GC–MS). The degree of sophistication of the GC apparatus varies from single isothermal packed columns to systems incorporating solvent-venting pressure programming (Gough and Webb, 1973) and high-efficiency narrow bore columns (Essigmann and Issenberg, 1972; Gough and Sugden, 1975). The identification of a nitrosamine is based both on its GC retention time and some characteristic of its mass spectral fragmentation. Quantitation is normally based on the intensity of selected ions in the spectrum, after calibration using standard nitrosamine solutions. Using GC–MS several groups have reported the presence of nitrosamines in food such as cured meat, fish, and cheese (Crosby et al., 1972; Fazio et al., 1971, 1973; Fong and Chan, 1976; Gough et al., 1976; Sen et al., 1973; Wasserman et al., 1972).

The only other technique available at the present time which is likely to offer the specificity necessary for the unequivocal detection of nitrosamines is that based on chemiluminescence, and such a system has been described by Fine et al. (1975). Nitrosamines are catalytically cleaved

Department of Industry, Laboratory of the Government Chemist, London SE1 9NQ, England.