textural properties, yet they could not be distinguished on the basis of enzyme systems that were identified in extracts from seeds and seedlings. However, many other enzyme systems remain to be examined and evaluated. Thus, multiple enzyme expression in early stages of tomato plant development may still enable characterization of the plants for fruit firmness.

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Soluble Proteins and Enzymes as Indicators of Change in Peanuts Infected with *Aspergillus flavus*

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Buffered extracts of peanut (*Arachis hypogaea* Linn) seeds infected with four aflatoxigenic and five nonaflatoxigenic strains of *Aspergillus flavus* Link ex. Fries were examined electrophoretically for soluble proteins and selected enzymes. Quantitatively, soluble proteins in extracts of seeds infected 4 days were significantly lower than those in control seeds; however, the number of low molecular weight proteins in infected seeds increased. Enzyme patterns of extracts from seeds infected with aflatoxigenic *A. flavus* did not differ distinctively from patterns of seeds infected with nonaflatoxigenic strains. Esterase, leucine aminopeptidase, gluconate and alcohol dehydrogenase, and alkaline and acid phosphatase patterns in extracts could be distinguished between infected and control seeds.

Studies have shown that standard gel electrophoretic patterns of proteins extracted from viable peanut (*Arachis hypogaea* Linn) seeds are distinctly modified as a result of infection with species of *Aspergillus* and other fungi (Cherry et al., 1974, 1975, 1976; Cherry and Beuchat, 1976). Biochemical transformations include deletion of some proteins (including enzymes), intensification of others, and/or production of new components as evidenced by quantitative and qualitative changes in bands appearing in electrophoretic gels. These changes in band patterns indicate generally that biochemical mechanisms operative in saprophyte–seed interrelationships function efficiently and systematically in favor of the fungus. Studies suggested that leucine aminopeptidases, esterases, peroxidases, oxidases, and catalases, among other enzymes, were partially responsible for alterations in peanut proteins and for new bands appearing in gels. Many peanut enzymes remained active during invasion with Aspergilli. In some instances, intensification of activities was correlated with enzymes extracted from fungal mycelium collected from the infected seed surface. Quantitative and qualitative variations in gel patterns of certain enzymes also distinguished fungal mycelium grown on peanut seeds from that grown in a synthetic medium (Cherry et al., 1974).

Homologous protein fractions among various taxa can be useful in determining genetic relationships between organisms (Dessauer and Fox, 1964). Chang et al. (1962) demonstrated that protein complements of four *Neurospora* spp. were distinctive and recognizable. Shechter et al. (1966) reported that a number of homologous proteins present in *Microsporum* and *Trichophyton* were dissimilar to the protein complement of *Epidermophyton floccosum*, thus lending credence to the taxonomic placement of these species.

Protein homology does not always appear among taxonomically similar species of fungi, however. Kulik and Brooks (1970) examined three strains each of five members of the *Aspergillus flavus* group (*A. flavus*, *A. leporis*, *A. oryzae*, and *A. parasiticus*, and *A. tamarii*), *A. fumigatis*, and *A. ochraceus* using polyacrylamide gel electrophoresis. They concluded that although the seven taxa exhibited from 29 to 48 protein fractions, only two fractions were common to all taxa. Results did not support the contention that *A. flavus* and *A. parasiticus* may be more closely related to each other than to other members of the *A. flavus* group.

In a recent study, Schmidt et al. (1977) compared enzymes of aflatoxigenic and nonaflatoxigenic strains of *A. flavus* and *A. parasiticus* by polyacrylamide gel-slab

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electrophoresis. Enzymes were extracted from mycelium harvested from laboratory growth media. No distinct differences in enzyme patterns were detected between aflatoxigenic and nonaflatoxigenic strains. However, patterns of similarity suggested some degree of physiological relationships.

The study reported here was conducted to determine (1) if electrophoretic patterns of soluble proteins in general and certain enzymes extracted from peanut seeds infected with *A. flavus* could be distinguished from patterns developed from extracts of control seeds and (2) whether electrophoretic patterns of enzymes extracted from aflatoxigenic *A. flavus* infected peanut seeds were different from patterns of seeds infected with nonaflatoxigenic *A. flavus*.

EXPERIMENTAL PROCEDURES

Four aflatoxigenic and five nonaflatoxigenic strains of *Aspergillus flavus* were cultured on potato dextrose agar (pH 5.6) slants at 29 °C for 16 days. Conidia were harvested by flooding the culture surfaces with sterile 0.005% Span 20 and then gently rubbing with a glass rod. Suspensions were diluted in water to contain 700 to 1000 colony-forming units/mL; these suspensions served as inocula for all experiments.

Peanut (*Arachis hypogaea* L. cv. Florunner) seeds were rinsed twice in sterile water and then soaked for 5 to 6 min. Skins were carefully removed and the seeds were inoculated by submerging them in conidial suspensions for 1 min. After excess water was removed, seeds were deposited in petri dishes and placed in ventilated containers lined with water-saturated cotton to maintain an environment with high humidity. Control peanut seeds were similarly treated except not inoculated with conidia. Seeds were incubated at 29 °C for 4 days. Two independent experiments were conducted.

At day 4, duplicate sets of inoculated and control seeds were collected; 2.0 g were combined with 7 mL of pH 7.9 (I = 0.01) sodium phosphate buffer, ground with a pestle in a mortar, and centrifuged at 43 500g. The supernatant liquid was carefully removed from the centrifuge tube and analyzed for protein content using the method of Lowry et al. (1951); bovine serum albumin was used for establishing a standard curve.

Soluble proteins and enzymes in the supernatant fractions collected from infected and control peanut seeds were characterized using gel electrophoretic techniques. Disc gel electrophoresis of proteins was performed on polyacrylamide gels according to procedures of Canalco (1973) and Cherry et al. (1970). Methods for detecting enzyme activity on polyacrylamide gels included those of Cherry and Katterman (1971) for esterase, Scandalios (1969) for leucine aminopeptidase, and Nielson and Scandalios (1974) for α-N-benzoyl-DL-arginine-β-naphthylamide (BANA) peptidase.

A horizontal starch gel electrophoresis apparatus was constructed according to the procedures described by Brewbaker et al. (1968). Methods for preparing the starch gel and the analyses of samples were essentially those developed by these authors. Alcohol and malate dehydrogenases, acid and alkaline phosphatases, and catalase were determined on starch gels using the methods of Scandalios (1969). Glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities were analyzed according to procedures described by Bakay and Nyhan (1969).

Control and infected seeds were analyzed for cumulative aflatoxin B_1, B_2, G_1, and G_2 content at the end of the 4-day incubation period. A volume of acetonitrile–water (8:2, v/v) five times the weight of the seeds was added to the sample and the mixture was blended in a Sorvall blender for 3 min at high speed. After filtering through Whatman No. 1 filter paper, a 20-mL aliquot of filtrate was transferred to a separatory funnel and extracted with 25 mL of 5% NaCl and 3 mL of benzene. The benzene fraction was extracted with an additional 25 mL of 5% NaCl and then transferred to a vial containing 0.1 g of Na_2SO_4. Samples were spotted on Absorbosil-1 (Applied Science Laboratories, State College, Pa.) thin-layer chromatography plates and developed in chloroform–acetone (90:10); aflatoxins were quantitated against standards using a Photovolt Photodensitometer (Photovolt Corp., New York, N.Y.).

Table I. Soluble Protein and Aflatoxin Content in Control and Infected Peanut Seeds

Treatment	Protein,[a] mg/mL	Aflatoxin, ppm[b]
Control 1 (initial)	52.4 a	ND[c]
Control 2 (4 days)	51.5 a	ND
Strain 1 (4 days)	20.0 cd	ND
2	22.8 cd	ND
3	24.2 cd	ND
4	24.5 cd	ND
5	32.6 b	ND
6	25.5 c	0.5
7	19.4 d	5.7
8	22.4 cd	8.6
9	24.7 cd	4.5

[a] Values followed by the same letter are not significantly different at the 95% level of significance. [b] Cumulative of aflatoxins B_1, B_2, G_1, and G_2. [c] None detected.

RESULTS

Soluble Proteins and Aflatoxins. Listed in Table I are data showing relative amounts of soluble protein in control peanut seeds and in seeds on which *A. flavus* had grown for 4 days. Levels of soluble proteins in buffer extracts of infected seeds were significantly lower than those in controls. There appears to be no correlation between the soluble protein and aflatoxin contents of infected seeds.

Gel electrophoretic patterns of proteins in soluble fractions of peanuts revealed that *A. flavus* caused changes in these components during the 4-day infection period (Figure 1). No changes were noted in gel patterns of soluble proteins in control seeds. Changes in infected peanuts were evidenced mainly by increased numbers of small molecular weight components with increased mobility rates. With the exception of slight variations in the band pattern for strain 6 in the 2.5–3.5 cm migration region, differences in soluble protein patterns from seeds infected with nonaflatoxigenic strains (1–5) and aflatoxigenic strains (6–9) of *A. flavus* were essentially the same.

Esterase. Figure 2 shows esterase zymograms for control seeds and for seeds infected with *A. flavus*. No changes in esterase bands were noted for controls. Seeds infected with *A. flavus*, however, exhibited quantitative and qualitative changes in esterase activity. Enzyme bands in the 3.0–4.0 and 5.0–6.5 cm regions of gels prepared from extracts of infected seeds were either absent or less distinct as compared to bands from control samples. Patterns from infected seeds varied among strains.

Leucine Aminopeptidase. Analysis for leucine aminopeptidase activity in extracts from control peanut seeds yielded five bands in the 4.0–7.5 cm region of polyacrylamide gels (Figure 3). Four days after inoculation, extracts from each of the peanuts infected with the nine *A. flavus* strains produced a zymogram differing from the controls

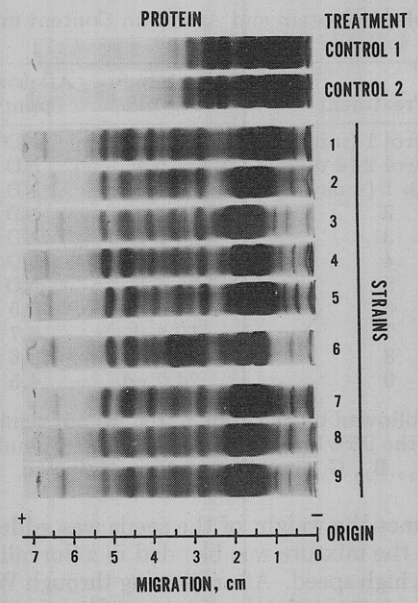

Figure 1. Polyacrylamide disc gel electrophoretic patterns of soluble proteins from control peanut seeds and from seeds infected with nonaflatoxigenic (strains 1–5) and aflatoxigenic (strains 6–9) *A. flavus*.

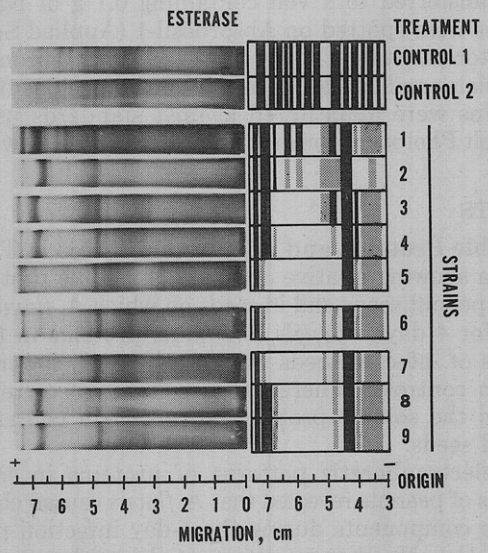

Figure 2. Polyacrylamide disc gel electrophoretic patterns of esterase from control peanut seeds and from seeds infected with nonaflatoxigenic (strains 1–5) and aflatoxigenic (strains 6–9) *A. flavus*.

and from each other. The numbers of bands in the 3–8 cm region of gels which were developed using extracts from infected peanuts ranged between 6 and 8 as compared to 4 or 5 in gels from control extracts.

BANA Peptidase. Polyacrylamide gels developed from extracts of control and infected seeds showed no major band differences in BANA peptidase activity; all gels contained one major (dark-staining) band in the 4.0–4.3 cm region. A light-staining band was noted in control gels at about 4.5 cm migration which was not observed in gels developed from infected seeds.

Phosphogluconate and Alcohol Dehydrogenases. Starch gels developed from extracts of seeds and then analyzed for phosphogluconate and alcohol dehydrogenases revealed that the two enzymes were generally less easily discerned in infected samples (Figure 4). Activity of alcohol dehydrogenase in seeds infected with 7 of 9 *A. flavus* test strains was essentially lost. A phosphogluconate dehydrogenase band at 3.5 cm migration in gels developed

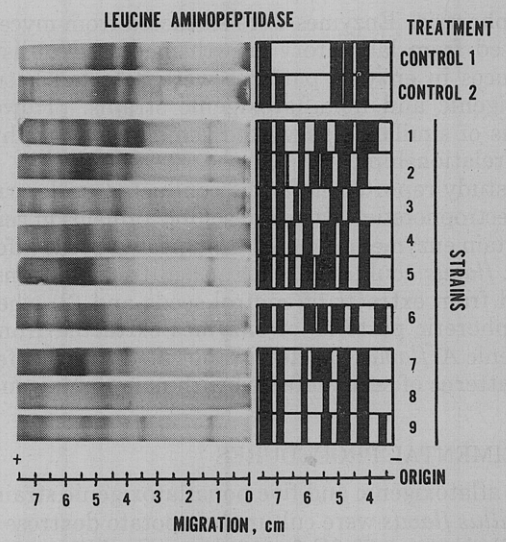

Figure 3. Polyacrylamide disc gel electrophoretic patterns of leucine aminopeptidase from control peanut seeds and from seeds infected with nonaflatoxigenic (strains 1–5) and aflatoxigenic (strains 6–9) *A. flavus*.

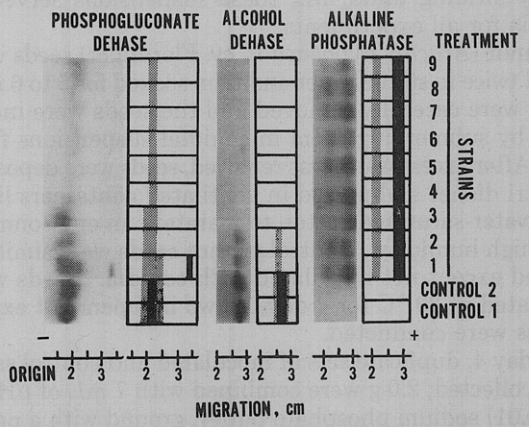

Figure 4. Starch gel electrophoretic patterns of phosphogluconate dehydrogenase, alcohol dehydrogenase, and alkaline phosphatase from control peanut seeds and from seeds infected with nonaflatoxigenic (strains 1–5) and aflatoxigenic (strains 6–9) *A. flavus*.

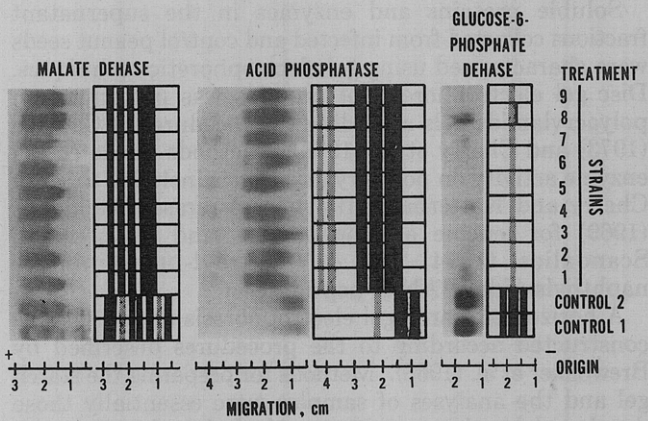

Figure 5. Starch gel electrophoretic patterns of malate dehydrogenase, acid phosphatase, and glucose-6-phosphate dehydrogenase from control peanut seeds and from seeds infected with nonaflatoxigenic (strains 1–5) and aflatoxigenic (strains 6–9) *A. flavus*.

from peanuts infected with one strain of *A. flavus* was not noted in other gels.

Alkaline and Acid Phosphatases. Shown in Figures 4 and 5, respectively, are starch gels exhibiting activities

of alkaline and acid phosphatases. Although no alkaline phosphatase activity was detected in control peanuts, upon invasion by *A. flavus* two similar bands evolved. Acid phosphatase from infected peanuts was detectable as three major and two minor staining bands. These bands were at different positions in the starch gels than were those detected in extracts from control seeds; however, like the alkaline phosphatase zymograms, acid phosphatase bands were not observed to differ in gels prepared from extracts of peanuts infected with nonaflatoxigenic or aflatoxigenic *A. flavus*.

Malate and Glucose-6-Phosphatase Dehydrogenases. The four major malate dehydrogenase bands on starch gels developed using extracts from infected peanuts had mobilities similar to the seven bands detected in extracts from control seeds (Figure 5). Patterns for the nine *A. flavus* infected seeds were essentially the same. The intensity of glucose-6-phosphate dehydrogenase bands observed in gels developed from extracts of control seeds was greatly deminished as a result of *A. flavus* infection. No new bands were noted in infected seeds.

Catalase. Starch gels of catalase showed no major band differences between extracts from control and infected seeds; all gels contained two closely migrating bands in region 2.0–3.5 cm.

DISCUSSION

Observations that soluble proteins decreased markedly in peanuts infected with *A. flavus* are in agreement with data reported on *A. oryzae* (Cherry et al., 1975) and *A. parasiticus* (Cherry et al., 1976). Concurrent with this change was a decomposition of high-molecular-weight peanut proteins such as arachin to yield small-molecular-weight proteins and polypeptides as evidenced by a larger number of fast-moving bands in electrophoretic gels developed using extracts from infected seeds. These protein components are undoubtedly further hydrolyzed to yield carbon and nitrogen sources for fungal growth. Others have demonstrated that plant tissue proteins serve as substantial sources of nutrients for fungi during infection (Krupa and Branstrom, 1974; Zscheile, 1974).

Although soluble protein gel patterns from infected seeds were readily distinguishable from patterns of control seeds, no distinct differences were noted between patterns from seeds infected with aflatoxigenic and nonaflatoxigenic *A. flavus*. Slight variations appeared among the nine test strains. Furthermore, patterns from *A. flavus* infected seeds had many similarities to those noted for seeds infected with other *Aspergillus* spp. (Cherry et al., 1975, 1976) and with *Neurospora sitophila* and *Rhizopus oligosporus* (Cherry and Beuchat, 1976). Thus, although electrophoretic gel patterns of soluble proteins can be used to give some indication of fungal infestation in peanut seeds, it does not appear that such patterns give an indication of the fungal species or even genera responsible for deterioration.

Differences in zymograms of esterase, leucine aminopeptidase, phosphogluconate, and alcohol dehydrogenase, and alkaline and acid phosphatase activity distinguished infected seeds from controls. Since alkaline phosphatase is not normally present in peanuts, this enzyme may be a more valuable indicator of fungal degradation than some of the other enzymes tested.

Our inability to differentiate between enzyme patterns from aflatoxigenic and nonaflatoxigenic *A. flavus* grown on peanut seeds is consistent with a report by Schmidt et al. (1977) who examined mycelial enzymes from aflatoxigenic and nonaflatoxigenic strains of *A. flavus* and *A. parasiticus* cultured in laboratory media. They found that there was no relationship between the production of different aflatoxins, such as B_1 and G_2, and a specific enzyme-banding pattern. Sorenson et al. (1971) suggested that a similarity index of protein patterns from *Aspergillus* spp. might be useful in assessing relatedness between species of the same genus but that patterns were not useful in identification of different species. From data presented here, it does not appear feasible to ascertain the aflatoxin-producing ability of *A. flavus* by examining enzyme patterns from peanut seeds infected with the organism.

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