COMPARATIVE STUDIES OF PROTEIN AND AMINO ACID CHANGES IN PEANUTS INFECTED WITH *NEUROSPORA SITOPHILA* AND *RHIZOPUS OLIGOSPORUS*

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Proteins and total and free amino acids of noninfected 'Florunner' peanut (*Arachis hypogaea* L.) seeds were characterized and compared to seeds infected for intervals up to 7 days with the ontjom fungi, *Neurospora sitophila* and *Rhizopus oligosporus*. Amounts (based on evaluations taken as mg/ml or percentage) of proteins in dilute buffer (sodium phosphate; I = 0.01; pH 7.9)-soluble extracts of seeds infected with either fungus declined similarly during most of the test period and, simultaneously, percentages of these components increased in buffer-insoluble preparations. Amounts of protein in whole seeds and soluble and insoluble fractions of uninoculated peanuts showed only minor quantitative changes during the test period. The percentage of protein in whole seeds infected with either fungus increased slightly at 2 days after inoculation and thereafter remained constant. Polyacrylamide gel electrophoresis showed that shortly after the start of the test period, most of the proteins that were soluble in buffer fractions of *R. oligosporus*- and *N. sitophila*-infected peanut seeds were converted to small molecular-weight components. Quantities of some free amino acids in infected whole seeds increased to levels above those of uninoculated seeds, while others decreased during the 7-day test period. Growth of either *R. oligosporus* or *N. sitophila* on seeds altered the percentages of total amino acids in the different fractions analyzed when compared to those of uninoculated seeds. These changes corresponded with the qualitative changes in proteins shown by gel electrophoresis. Changes in protein and total and free amino acid composition of peanut seeds associated with fungal catabolism and anabolism may contribute to nutritional quality alterations associated with Oriental peanut fermentation processes.

Host-parasite and host-saprophyte relations between plant tissues or seeds and invading fungi have been studied extensively. Efforts have been concentrated mainly on research to determine plant disease susceptibility characteristics. On the other hand, destructive effects associated with field and storage fungi can also have beneficial and desirable attributes. For example, fermented oilseeds and grains are popular in Asia, Africa, and parts of Latin America (1). The Indonesians prepare a product called "ontjom" from peanut press cake by fermenting the cake with the reddish-orange mold, *Neurospora sitophila*. Less often, *Rhizopus oligosporus* is used to produce white ontjom (2). The latter organism is also used to manufacture tempeh from soybeans. Nearly all of the research data reported on biochemical changes in peanut seed substrates as a result of growth of either *N. sitophila* or *R. oligosporus* have been in the form of comparisons of proximate composition of finished products with that of non-fermented substrates (2–5). Without exception, these studies were conducted using peanut meals.

Enhanced nutritive quality and digestibility of fermented oilseeds have been partially attributed to proteolytic activities of fungi (6–8). Both *N. sitophila* and

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R. oligosporus are reported to exhibit such activities (1,5–12). Research data describing stepwise processes of chemical breakdown in viable peanut seeds infected with *N. sitophila* and *R. oligosporus* are lacking. A series of studies was therefore designed to examine this complex host-fungus interrelationship from a macro-molecular viewpoint. This paper reports on changes in proteins and free and total amino acids in whole peanut seeds and in soluble and insoluble extracts made from seeds infected with either *N. sitophila* or *R. oligosporus* for various time intervals.

MATERIALS AND METHODS

N. sitophila NRRL 2884 and *R. oligosporus* NRRL 2710 were cultured on potato dextrose agar slants for 9 to 12 days at 24° C. Conidia of *N. sitophila* and spores of *R. oligosporus* were harvested by washing the culture surface growth with sterile deionized water containing 0.005% Span 20. Testa-free 'Florunner' peanut seeds uniform in size and weight (0.73 ± 0.04 g/dry seed plus skin) and assumed to be viable upon initiating these experiments were soaked in these solutions of diluted conidial or spore suspensions for 1 min and then placed in petri dishes set in a ventilated container lined with water-saturated absorbent cotton to maintain an environment of high humidity. Average weight of each seed after soaking in water 1 min and removing skin was 0.75 ± 0.04 g. Uninoculated peanuts were similarly treated, omitting the fungi in the inoculation step. The experimental substrates were incubated at 29° C for time intervals of 2, 4, and 7 days. After each test period, duplicate samples of three control and three each of *N. sitophila*- and *R. oligosporus*-infected peanuts were collected. Control seeds showed no visible evidence of mold growth. Only seeds free of visible fungal growth or showing *N. sitophila* or *R. oligosporus* mycelia (and sometimes conidia or spores) were selected as control or infected material, respectively, for use in these experiments. After careful removal of mycelia/conidia or mycelia/spores from the surface of the test seeds, seeds were individually ground in 7 ml of sodium phosphate buffer (pH 7.9; I = 0.01) in a mortar with a pestle. The mycelia/conidia and mycelia/spore samples collected from the seeds were similarly ground in 2–3 ml of buffer. Peanut and fungal preparations were centrifuged at 43,500 × *g* for 30 min to separate soluble (supernatant) and insoluble (pellet plus fat pad) fractions.

The method of Lowry *et al.* (13) was used to determine protein quantities in the soluble fractions of peanut and fungal samples. The soluble proteins were further characterized by gel electrophoresis on 10% polyacrylamide disc gels according to previously published procedures (14,15).

Uninoculated and inoculated whole seeds, soluble and insoluble fractions made from similarly treated seeds, and fungal tissue from the seed surface were lyophilized, ground into their respective meals and/or protein concentrates, and defatted with diethyl ether. These products were then analyzed for protein content (percentage) using the macro-Kjeldahl technique (N-to-protein conversion values of 5.46 for peanut preparations and 6.25 for fungal samples were used). Free and/or total amino acids of these fat-free preparations were determined by ion-exchange chromatography using a Durrum Model D-500 amino acid analyzer as previously described (16–18).

Data presented in this paper represent means from two independent trials or replicates run in duplicate.

RESULTS AND DISCUSSION

Quantities of Protein in Various Fractions

Protein (Lowry *et al.* (13)) content of soluble fractions prepared from uninoculated seeds varied between 51.0 and 63.5 mg/ml during the 7-day test period (Fig. 1). On the other hand, quantities of proteins in extracts of *R. oligosporus*-infected seeds dropped to 33.0 mg/ml by 2 days after inoculation, and continued to decline to levels of 14.0–15.0 mg/ml between 4 and 7 days. At day 2, quantities of protein in soluble fractions of *N. sitophila*-infected seeds did

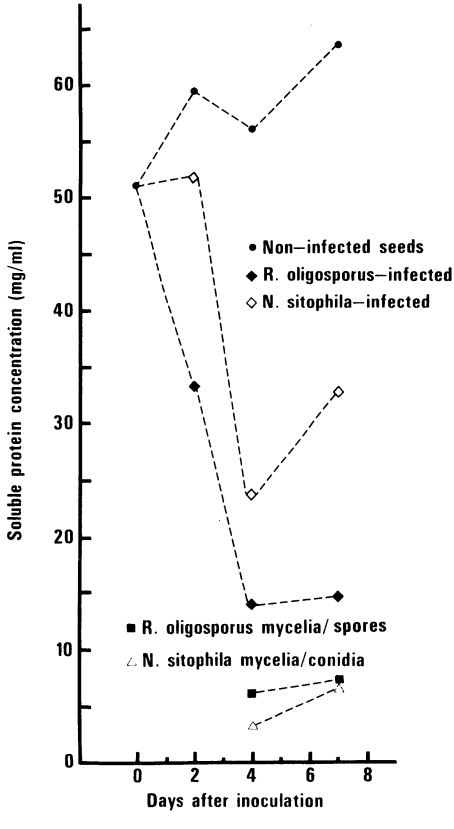


Fig. 1. Quantitative changes in soluble proteins (Lowry *et al.* (13)) of noninfected and *R. oligosporus*- and *N. sitophila*-infected seeds during the test period of 0 to 7 days. Included are values for soluble protein from *R. oligosporus* mycelia/spores and *N. sitophila* mycelia/conidia collected from the seed surface during the test period. Duplicate control and infected (surface fungal growth removed) samples of three seeds uniform in size and weight (0.75 ± 0.04 g/testa-free seed at day 0) from each test interval were ground in 7 ml of buffer, centrifuged, and the soluble fractions analyzed for protein content (mg/ml) according to the procedure of Lowry *et al.* (13). The mycelia/conidia and mycelia/spore samples from the surfaces of three seeds collected at each test interval were pooled and similarly prepared in 2–3 ml buffer for protein analysis.

not decline. However, between days 4 and 7, levels of soluble protein in the soluble extracts declined to 24.0 mg/ml, then increased to 34.0 mg/ml, respectively. *N. sitophila* grew somewhat slower than did *R. oligosporus,* which accounted in part for the delayed change in level of soluble protein noted at 2 days after inoculation. The 29°C incubation temperature was evidently more favorable for growth of *R. oligosporus* than for *N. sitophila* on peanut seeds.

Quantities of soluble proteins in extracts of *R. oligosporus* mycelia/spores and *N. sitophila* mycelia/conidia were similar, raising slightly from 4.5 to 7.0 mg/ml between 4 and 7 days of infection (Fig. 1).

Mean percentages of crude protein (macro-Kjeldahl) in lyophilized and defatted samples of whole seeds (total protein) and soluble and insoluble fractions of noninfected seeds were approximately 43.0, 60.0, and 34.0%, respectively, during the test period (Fig. 2). Lyophilized soluble extracts of

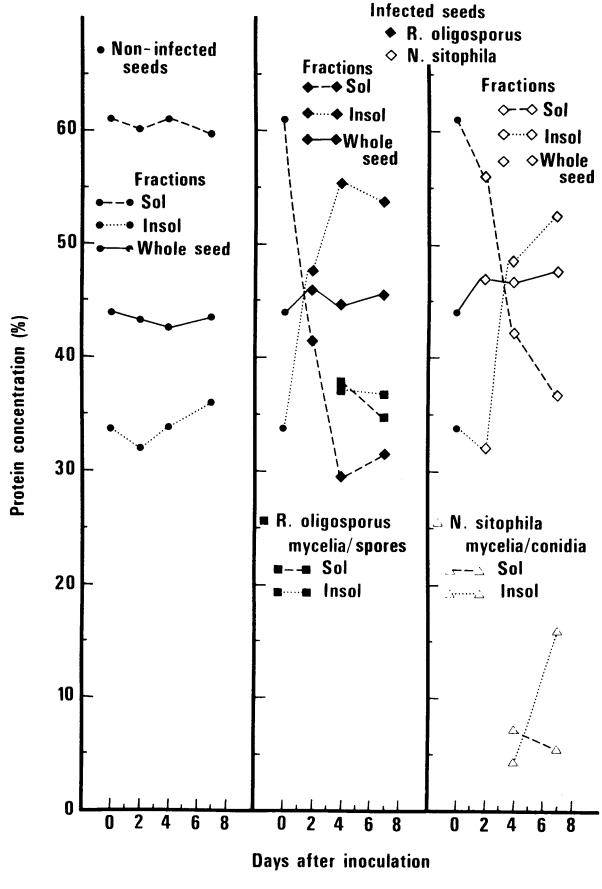


Fig. 2. Percentage protein (macro-Kjeldahl) in fat-free meals and/or concentrates of whole seed and soluble (sol) and insoluble (insol) fractions of noninfected and *R. oligosporus*- and *N. sitophila*-infected seeds during the test period of 0 to 7 days. Included are protein values of fat-free concentrates of soluble and insoluble fractions of the fungi.

peanuts that had been inoculated with *R. oligosporus* showed a decline in percentage of protein to levels of 29.5 and 31.5% between days 4 and 7. A continuous decline from 61.0 to 36.5% was noted in lyophilized soluble extracts of *N. sitophila*-infected seeds during this same test period. The decline in proteins noted in these soluble fractions as determined by the macro-Kjeldahl technique is in accord with data derived using the method of Lowry *et al.* (13). Simultaneously, percentage of protein in lyophilized insoluble fractions of peanuts infected with the test fungi increased to levels between 52.5 and 55.0%. The percentage of protein in inoculated whole seeds was similar to that of uninoculated peanuts, varying between 44.0 and 47.5% during the test period. Thus, these results show that infection of peanut seeds with either *N. sitophila* or *R. oligosporus* produces a decline in buffer-soluble proteins. Moreover, these data suggest that during the infection period soluble proteins are mainly converted to insoluble forms rather than being transformed into other chemical constituents or volatile components, since the total protein content of infected whole seeds changed only slightly during the test period.

Lyophilized soluble and insoluble extracts of *R. oligosporus* mycelia/spores contained higher percentages of protein than did those of *N. sitophila* mycelia/conidia. For *R. oligosporus,* the percentage of protein in the insoluble material remained at approximately 37.0%, while that of the soluble fraction decreased slightly from 38.0 to 35.0%. The insoluble material of *N. sitophila* mycelia/conidia increased in percentage of protein from 4.0 to 16.0%, while a decrease from 7.0 to 5.5% was noted in the soluble fraction. Proportionately higher calculated values for soluble and insoluble protein in lyophilized fungal samples analyzed by the macro-Kjeldahl technique as compared to values obtained using the technique of Lowry *et al.* (13) (*cf.* Figs. 1 and 2) probably reflect high levels of nucleic acid synthesis associated with fungal growth in the seeds.

Gel Electrophoresis of Soluble Proteins

Comparison of the gel pattern representing seeds inoculated with *R. oligosporus* for 2 days to that of the uninoculated seeds showed: a) three new bands in region 0–0.7 cm; b) a slight increase in mobility of the two large molecular-weight storage components of arachin (two bands, (19)) from region 0.7–1.7 to 0.9–2.0 cm; c) a disappearance of two major dark staining bands in region 1.7–7.5 cm; and d) a new group of polypeptides in region 3.0–5.0 cm (Fig. 3). The gel pattern representing seeds inoculated with *N. sitophila* for 2 days showed only a broadening of the arachin bands (region 0.7–1.7 to 0.7–2.1 cm) and disappearance of the protein in region 1.7–2.3 cm when compared to the "standard" gel profile of uninoculated seeds. Between 4 and 7 days after inoculation with either test fungi, the number of small molecular-weight protein components increased. In both cases, the arachin bands increased in mobility and became more diffuse, while the number of components in the lower half of the gels (region 3.0–7.0 cm) increased. By day 7, after inoculation of seeds with either fungus, many of the proteins (especially in region 2.0–7.0 cm) became difficult to distinguish in the gel patterns. Evidently, substantial hydrolysis to yet smaller polypeptides, free amino acids, and/or insoluble forms which are not detected on electrophoretic gels was occurring.

The gel patterns of mycelia/conidia and mycelia/spores of both test fungi

differed at days 4 and 7 (Fig. 3). The gel patterns showed that each fungus synthesized more of certain proteins and formed a new group of components which were soluble in dilute buffer solution. Moreover, the position in the gels of many of these bands coincided in mobility with those in patterns of soluble fractions from infected peanuts. These observations suggested that certain bands appearing in gels prepared from infected seeds were of fungal origin or vice versa.

These studies showed that gel electrophoretic profiles of soluble proteins from noninoculated seeds are distinctly modified by inoculation with either *R. oligosporus* or *N. sitophila*. The biochemical transformations, differing

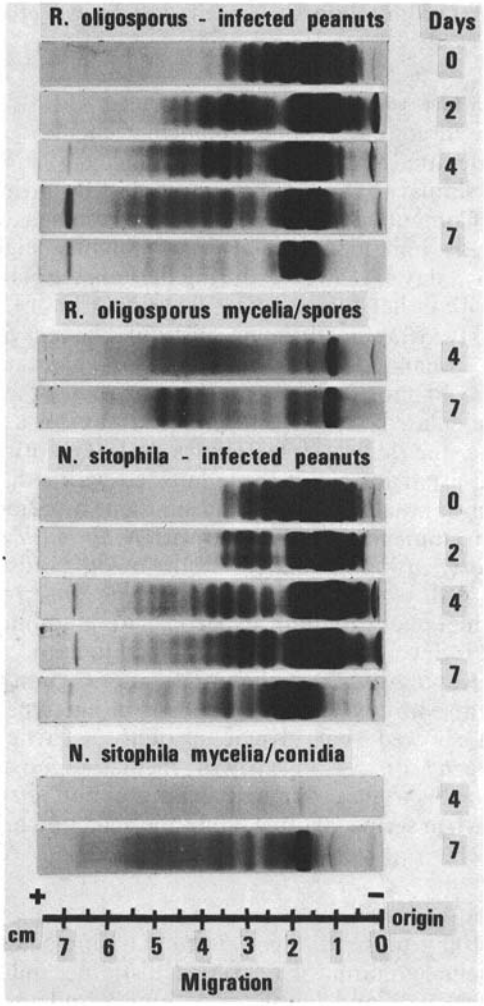


Fig. 3. Polyacrylamide disc-gel electrophoretic patterns of soluble proteins of noninfected (0 day) and *R. oligosporus*- and *N. sitophila*-infected (2–7 days) seeds. Data from soluble mycelia/spores and mycelia/conidia-fractions from the seed surfaces are presented for days 4 and 7.

somewhat in the rate at which they occur, are similar for both fungi and basically include a breakdown or alteration of storage proteins to other small molecular-weight components. Extending the test period caused continued decreases in soluble proteins. Simultaneous increases in other constituents including, possibly, insoluble forms and/or free amino acids and polypeptides not detectable on electrophoretic gels are evidently occurring. The observed proteolytic activities of *N. sitophila* and *R. oligosporus* on peanut seeds are in agreement with data reported by others on soybeans (6–8, 10–12), peanut press cake (2), and wheat (7,20). Although *R. oligosporus* may possess stronger proteolytic capabilities than *N. sitophila,* both organisms are capable of causing substantial protein modification in oilseeds (*cf.* fungal effects on proteins of peanut seeds in Figs. 1, 2, and 3).

Quantitative Changes in Free Amino Acids

Quantities of free threonine, alanine, isoleucine, leucine, tyrosine, methionine, lysine, and NH_4 in defatted whole peanut seeds infected with *R. oligosporus* or *N. sitophila* remained similar to those of noninfected seeds during the first 2 days of infection (Fig. 4). Contents of isoleucine, leucine, tyrosine, and methionine in infected whole seeds remained similar to or slightly higher than those of uninoculated seeds at day 4. However, during the balance of the infection period, seeds inoculated with either fungus contained quantities of threonine, alanine, isoleucine, leucine, tyrosine, methionine, histidine, lysine, others (unidentified amino acids or small peptides), and NH_4 greater than those in uninoculated seeds. In most cases, quantities of these free amino acids were greater in seeds inoculated with *N. sitophila* than in those inoculated with *R. oligosporus* during the longer infection period. Quantities of free valine, phenylalanine, and aspartic acid in seeds infected with either fungus remained lower than those of uninoculated seeds during the test period. Serine, glutamic acid, proline, and arginine in seeds infected with *N. sitophila* remained greater in quantity than those in uninoculated seeds or in seeds infected with *R. oligosporus*. After 7 days of infection with *R. oligosporus,* quantities of free serine, glycine, valine, phenylalanine, aspartic acid, glutamic acid, proline, and arginine decreased to levels below those contained in uninoculated seeds. These data indicate that *R. oligosporus* preferentially uses certain free amino acids as sources of nitrogen. Moreover, these data are in agreement with a previous report (21) which showed that glycine, proline, aspartic acid, and leucine supplied as nitrogen sources in growth media supported growth of *R. oligosporus* comparable to that obtained on ammonium nitrogen. Good growth was also obtained when serine, alanine, glutamic acid, and arginine were used as nitrogen sources (21).

Quantitative Changes in Total Amino Acids

Percentages (g/100 g protein) of certain total amino acids including glycine, alanine, valine, leucine, glutamic acid, cystine, histidine, and lysine in proteins of uninoculated whole seeds and lyophilized soluble and insoluble fractions of uninoculated seeds were distinctly different throughout the 7-day test period (Fig. 5A, B). The remaining total amino acids were either similar in content at specific test intervals or fluctuated to levels above and below each other. This made comparisons among the three noninfected fractions of seeds difficult.

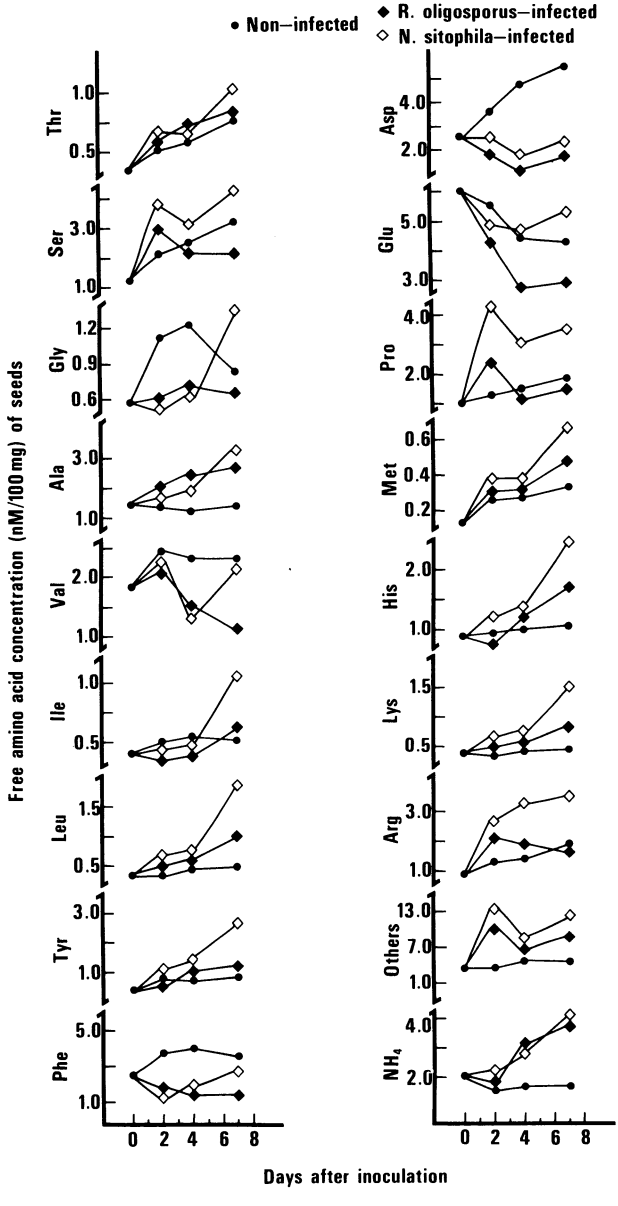


Fig. 4. Quantitative changes in free amino acid content of fat-free meals from noninfected and *R. oligosporus*- and *N. sitophila*-infected whole seeds during the test period of 0 to 7 days.

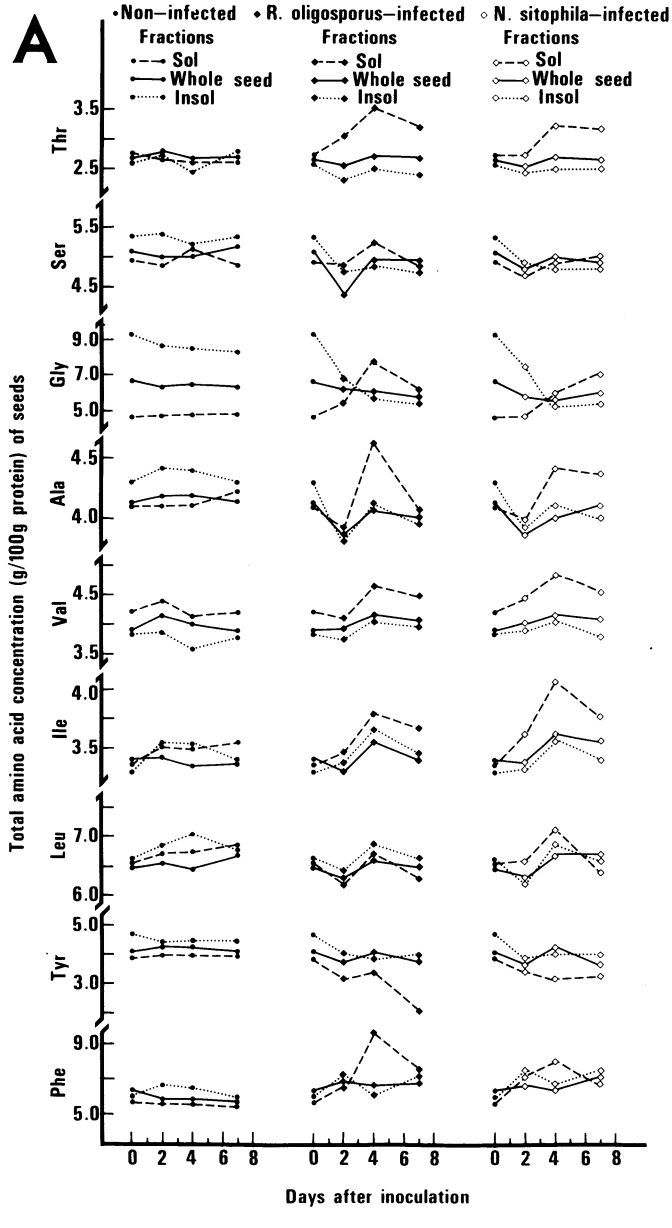
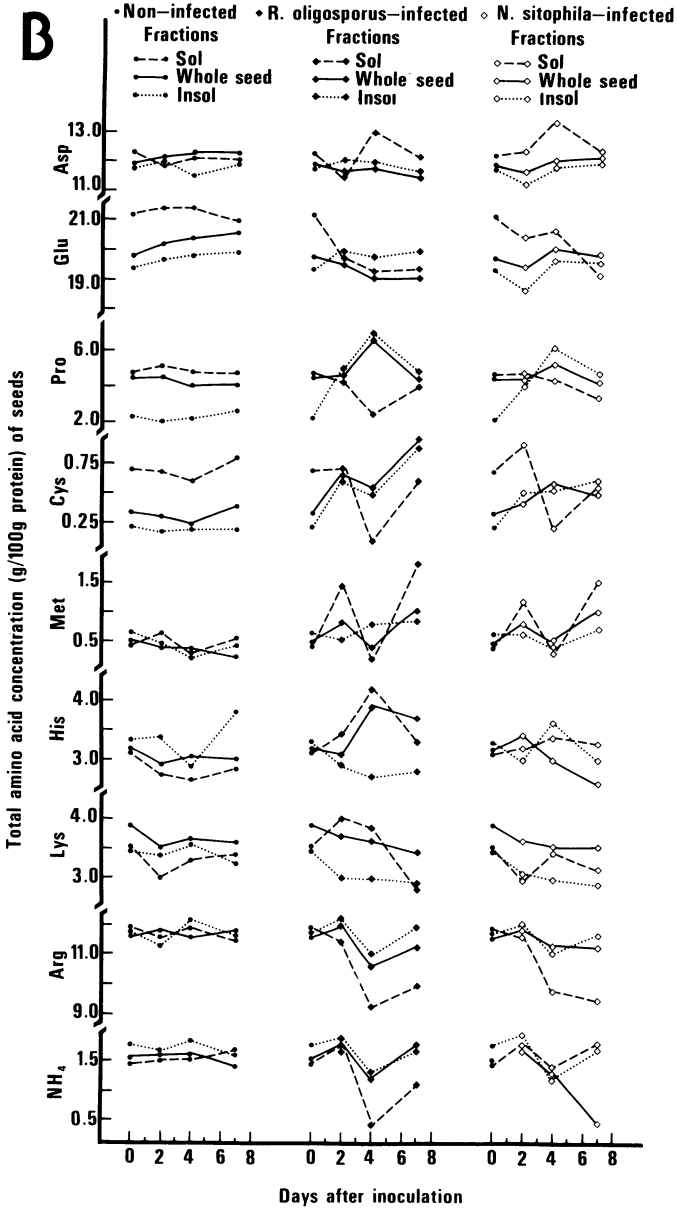


Fig. 5. A, B. Quantitative changes in total amino acids of fat-free meals and/or concentrates of noninfected and *R. oligosporus*- and *N. sitophila*-infected whole seeds and amino acids in soluble (sol) and insoluble (insol) fractions during the test period of 0



to 7 days. Data were quantitated based on protein content of individual fat-free samples (whole seed, and soluble and insoluble fractions).

Growth of either *R. oligosporus* or *N. sitophila* on seeds altered the percentages of total amino acids in the various protein fractions analyzed (Fig. 5A, B). For example, percentages of threonine, alanine, valine, isoleucine, phenylalanine, aspartic acid, and histidine in the soluble fraction increased at 4 days after inoculation to values exceeding those observed for uninoculated seeds. In most cases, the same amino acids in insoluble fractions from infected seeds decreased or remained lower than those of noninfected seeds during the test period. Proline in insoluble fractions of seeds infected with either fungus increased at day 4 to levels greater than those in the other preparations of both infected and noninfected whole seeds. Methionine and cystine in soluble fractions of seeds infected with either fungus changed similarly, increasing at day 2 to levels above those of whole seed and insoluble fractions, then decreased at day 4, and finally increased at day 7. In both *R. oligosporus* and *N. sitophila*-infected seeds, arginine decreased in soluble fractions made from seeds infected for 2 to 7 days to levels substantially lower than those in whole seeds and insoluble fractions.

Relative levels of total amino acids in various peanut seed fractions analyzed in this study changed substantially as a result of *R. oligosporus* and *N. sitophila* infection. Whether the nutritional value of partially hydrolyzed proteins was altered as a result of fungal proteolysis remains to be demonstrated. It seems plausible that increased levels of low-molecular-weight peptides resulting from fungal proteolysis could affect the digestibility and nutritional quality of fermented peanuts. Thus, contentions put forth in previous reports (3,5,6) regarding possible increases in nutritional value of fermented peanut seeds may be founded in part as a consequence of fungal proteolytic activity.

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