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Comparative studies on the changes of total soluble proteins and protease activity in Georgian commercial peanuts contaminated by *Aspergillus flavus*

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The presence of *Aspergillus* species is an indicator of storage conditions, which also suggests the possibility of several biochemical changes in grains. A comparative change in total soluble proteins and protease activity was determined in commercial peanut seeds collected from Georgia State. Protein contents of healthy peanuts, naturally contaminated peanuts and then artificially inoculated peanut seeds with *A. flavus* were estimated by Bradford method, and protease activity was also determined by using the Protease Detection Kits. Protein contents and the protease activity of the peanuts varied from sample to sample. The soluble protein content of seeds was significantly higher in healthy peanuts than in artificially inoculated or naturally infected peanuts with *A. flavus*. Protease activity was found to be higher in artificially inoculated seeds than in either naturally infected or healthy peanuts. Level of soluble proteins in buffer extracts of contaminated seeds decreased with incubation time, and protease activity increased with incubation time. These changes may be attributed to host response due to infection, contribution by *A. flavus* or due to biochemical alterations that occur naturally during the transition from endosperm to seedling during incubation period.

Keywords: *Aspergillus*; fungal contamination; peanuts; protease activity; soluble proteins

1. Introduction

Aspergillus flavus is the most commonly associated fungal species with peanut seeds (*Arachis hypogea* L.) and can invade in the field before harvest, during post-harvest drying and curing, and in storage and transportation. *A. flavus* is a predominantly aflatoxigenic fungi in the Southern United States, with >95% of isolates producing aflatoxins in the peanut-growing region of Southern Alabama and Georgia. Georgia produces 37% of the total peanuts produced in USA (Horn and Dorner 1999). These fungal species when grown on stored peanut seeds can reduce the seed protein qualitatively and quantitatively along with the loss of carbohydrate and oil content, can increase the free fatty acid and can also bring about several other biochemical changes (Cherry et al. 1972; Diener et al. 1981; Heath 1995; Sietsma et al. 1995; Wilson et al. 1995).

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It has been demonstrated that the seed components such as proteins and carbohydrates not only serve as a nutrient source for fungi during their invasion (Krupa and Branstorm 1974; Zscheile 1974) but also are involved in aflatoxin biosynthesis (Shih and Marth 1974; Buchanan and Lewis 1984). In addition to storage protein, changes in enzymes and isoenzymes patterns have also been reported in peanut seed due to infection by aflatoxigenic and non-aflatoxigenic forms (Cherry et al. 1978; Sulochana and Venkaiah 1990; Fajardo et al. 1994). Studies have shown that gel electrophoretic patterns of protein extracted from viable peanuts seeds are distinctly modified as a result of infection with the species of *Aspergillus* and other fungi (Cherry et al. 1974; Cherry 1975; Cherry and Beuchat 1976; Buchanan and Lewis 1984). Hammons (1972) expressed concerned over accumulation of evidence indicating that the genetic base or gene pool of cultivated peanut is becoming increasingly narrow, so that this crop is more vulnerable to invasion by fungi such as aflatoxin-producing species of *Aspergillus*. An initial survey of peanuts from selected commercial outlets in Georgia, showed the presence of *A. flavus* and production of aflatoxin after incubation of commercial peanuts (Achar and Sanchez 2006). Under favourable conditions of temperature and moisture *Aspergillus* species infestation causes a rapid change in seed composition and quality (Basha and Pancholy 1986). Stored peanuts in commercial outlets are exposed to varying environmental conditions, and this paper evaluates for the first time changes in soluble protein and protease in peanuts sold to consumers.

2. Materials and methods

2.1. Sample collection

A total of 10 different samples of peanuts (Raw Jumbo Virginia) were purchased from retail outlets in Kennesaw, Marietta and Cartesville in Georgia State. All the samples were brought to the laboratory in sterile plastic bags and kept at 4°C.

2.2. Sample preparation for analysis

Dehulled and testa-free contaminated peanuts (200 seeds) from each sample were directly plated on potato dextrose agar (PDA) medium. For artificial inoculation, the inoculum was prepared by growing cultures of *Aspergillus* spores at 30°C for 7 days on PDA from which suspensions containing 10^5 spores/ml were made in sterile 0.05% Tween-20. Seeds were soaked in sterile water for 5–6 min, testa was carefully removed and seeds were inoculated by soaking them in conidial suspensions for 1 min. The control peanuts were similarly treated except for the omission of spores. Artificially inoculated, naturally infected and control seeds were plated on PDA and incubated at 30°C for 3 weeks. Experiments were replicated three times for all seeds. Changes in the protein content and protease activity were analysed at weekly interval of times for all the experimental design.

2.3. Protein estimation

After the desired incubation period, mycelial tissues were removed from contaminated seeds before analysis. Triplicates sets of uncontaminated, artificially inoculated and control seeds were collected. Two grams of whole peanuts were ground in a mortar and pestle containing sodium phosphate buffer pH 7.9 ($I = 0.01$)

and then centrifuged at 10,000 rpm for 30 min at 4°C (Cherry et al. 1974). Soluble protein concentration of the crude extracts was determined (modified Bradford) using the micro assay procedure of BIORAD Coomassie Blue-based dye (Wang et al. 1993). One millilitre of reaction mix volume comprised 200 μl of BIORAD dye and 800 μl of protein extract containing 1–20 μg of protein (Xia and Achar 2000). The reaction mix was incubated at room temperature for 5 min. The optical density value was recorded spectrophotometrically at 595 nm. Bovine serum albumin fraction IV was used for establishing a standard curve.

2.4. Protease assay

The total protease activity was determined from crude extracts obtained from peanut samples as above. Protease activity for both healthy and unhealthy peanuts was determined by Protease Detection Kits (Sigma-Aldrich), using highly labelled fluorescein isothiocyanate (FITC) casein as the substrate, modified procedure for protease detection (Twining 1984). The method is based on the proteolytic hydrolysis of a proprietary formulation of a FITC-labelled casein substrate. The method consisted of determining the proteolytic activity by measuring the fluorescence values obtained from the free fluorocrome released due to casein degradation. The final fluorescence values were measured using the fluorometer (OLIS DM45, TD-700 with standard PMT (P/N 7000-009). The trypsin control solution (20 μg/ml) form Sigma kit was used to generate a standard curve with serial dilution. Total protease activity was expressed as microgram per millilitre of dry weight. Twenty microlitre of incubation buffer (20 mM sodium phosphate with 150 mM sodium chloride, pH 7.6), 20 μl of FITC-casein substrate and 10 μl of the test sample were added to a microfuge tube. For control samples (trypsin control solution) were prepared by adding 20 μl of incubation buffer, 20 μl of FITC-casein substrate and 10 μl of the control sample. A blank sample was prepared by adding 20 μl of incubation buffer, 20 μl of FITC-casein substrate and 10 μl of millipore water. The tubes were mixed gently and incubated at 37°C in the dark for 60 min in a water bath with the samples covered in aluminium foil. After incubation 150 μl of 0.6 N trichloroacetic acid was added to each tube. The sample was gently mixed and incubated at 37°C in the dark for 30 min. After the incubation, the samples were centrifuged at 10,000 rpm for 10 min. Thirty microlitre of the supernatant was then added to 3 ml of the assay buffer in the fluorometer cuvette and mixed gently. The fluorescence intensity was recorded at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Total protease activity was expressed as microgram per millilitre of dry weight.

2.5. Statistical analysis

The Statistical Presentation System Software for Microsoft windows (version 10.2) was used to calculate the means, standard errors and standard deviations. One-way analysis of variance was applied to the data, and Tukey's multiple comparison test at 5% significance was applied.

3. Results

The total soluble protein content in extracts of seeds was significantly higher in healthy peanuts than in artificially inoculated or naturally infected peanuts with

A. flavus (Figure 1). A maximum protein content 19.67 ± 0.4 mg/ml, 20.39 ± 0.58 mg/ml and 22.8 ± 0.10 mg/ml was recorded from the samples collected from Kennesaw, Marietta and Cartersville, respectively, and not much variation was recorded among the healthy or artificially inoculated or naturally infected peanuts seeds at first day of incubation. A minimum protein content 6.31 ± 0.05 mg/ml, 6.92 ± 0.01 mg/ml and 5.1 ± 0.05 mg/ml was recorded from the naturally infected samples collected from Kennesaw, Marietta and Cartersville, respectively, at 2 weeks after incubation. Moreover, protein content increased with incubation time until the third week in healthy peanuts, whereas it significantly decreased in unhealthy peanuts. From the third week of incubation, when seeds were about to germinate, there was a slight increase in protein content in both artificially and contaminated seeds. Soluble protein content in both contaminated and uncontaminated peanut seeds varied from one commercial outlet to another. While peanut seeds from Cartersville showed the highest protein content, those from Kennesaw showed the least.

Analysis for total protease activity in crude extracts showed significantly higher enzyme activity in contaminated than in healthy peanut seeds. Moreover, protease activity was significantly higher in artificially inoculated peanut seeds than in either naturally infected or healthy peanut seeds. Enzyme activity increased with incubation period in healthy samples until the second week of incubation; thereafter, a slight decrease was recorded in the third week. Protease activity varied in both contaminated and uncontaminated peanuts from one commercial outlet to another. A minimum enzyme activity of 72.61 ± 0.15 μg/ml was recorded at first day of incubation and maximum of 79.22 ± 0.01 μg/ml at the second week in healthy samples from Kennesaw; thereafter, a slight decrease was noted. A similar enzyme

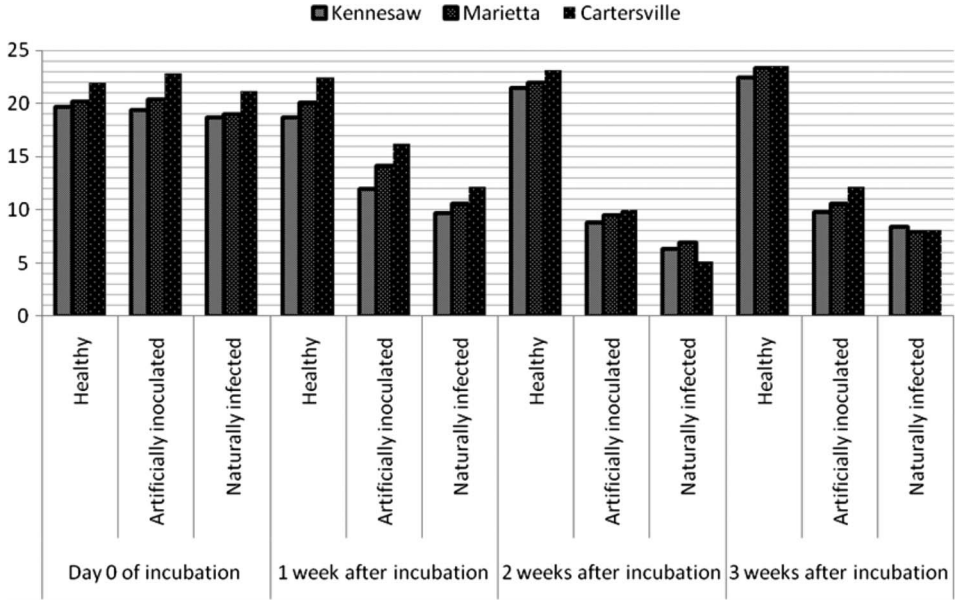

Figure 1. Total protein content (mg/ml) of peanut seeds in naturally and artificially inoculated with *A. flavus* and healthy seeds collected from Georgia state.

activity pattern was noted in healthy samples from Marietta and Cartesville. While a minimum of 81.2 $\pm$ 0.01 μg/ml was recorded in artificially inoculated peanuts on the first day of inoculation, a maximum of 88.65 $\pm$ 0.69 μg/ml was recorded in the third week of inoculation in peanut samples from the same commercial outlet. A similar pattern was noted in artificially inoculated samples from Marietta and Cartesville. Maximum enzyme activity was recorded for both naturally infected and artificially inoculated peanut samples from Cartersville, while peanuts from Kennesaw showed the least enzyme activity (Figure 2).

4. Discussion

Fungi causing grain deterioration have always been a major concern for food experts because of their ability to produce mycotoxins. Fungi, especially those belonging to the genera *Aspergillus, Penicillium* and *Fusarium* have been reported to occur with high per cent incidence on cereals (Diener et al. 1981; Abdel-Hafez et al. 1990; Pitt et al. 1994; Gassen 1999). Peanut seeds are indeed susceptible to infection by several species of *Aspergillus*. The field *s*urveys conducted worldwide also revealed that the occurrence of *A. flavus* is known to contaminate frequently the peanuts with concomitant production of aflatoxins (Ellis et al. 1991).

In the present study, *A. flavus* in contaminated and when inoculated in commercial peanuts from time intervals ranging from 0 to week 3, altered protein content when compared to control, healthy samples. Our observation that soluble proteins decreased in peanuts infected with *A. flavus* is similar to those made previously in peanuts with *A. oryza* and with *A. parasiticus* (Cherry et al. 1978). The soluble protein content of the peanut seeds decreased during the infestation period of 21 days in this study. This decrease may be due to the action of endogenous

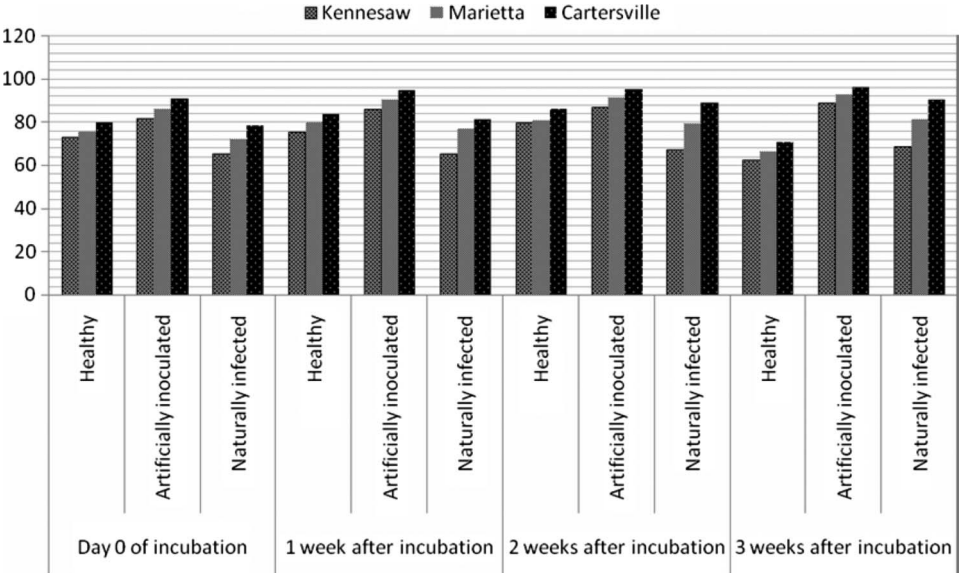

Figure 2. Protease activity (μg/ml) of peanut seeds in naturally and artificially inoculated with *A. flavus* and healthy seeds collected from Georgia state.

peptidases and/or proteolytic enzymes of the peanut seed that may have been activated or *de novo* synthesised (Basha and Cherry 1978) during the longer incubation periods. Reports indicate that major storage proteins extracted from viable peanuts seeds are distinctly modified and converted to numerous low-molecular-weight components following fungal invasion (Cherry 1975; Cherry and Beuchat 1976; Cherry et al. 1978). It has also been demonstrated that plant tissue proteins serve as a substantial sources of nutrients for fungi during infection (Krupa and Branstorm 1974; Zscheile 1974).

Interestingly in our study, after the third week of incubation, when seeds were about to germinate, there was a slight increase in soluble protein content in both artificially and naturally contaminated seeds. This increase is probably due to inclusion of proteins from *A. flavus* as reported previously. A similar suggestion was made by Cherry et al. (1974) in peanuts infected by *A. parasiticus*. Possibly, this increase may be because of the higher solubility of the seed proteins resulting due to their breakdown into low-molecular-weight polypeptides by the fungal proteases (Basha and Pancholy 1986). Decomposition of high-molecular-weight protein to low-molecular-weight proteins are further hydrolysed to yield carbon and nitrogen sources for fungal growth (Cherry et al. 1978). Within 2–3 days of fungal inoculation with *Aspergillus* species, arachin and conarachin begin to hydrolyse and form new low-molecular-weight polypeptides (Cherry et al. 1974), and free amino acids varied after 5 days indicating a variable hydrolysis/utilisation of proteins by fungus (Cherry 1975; Desphande and Pancholy 1979). The breakdown of arachin, the main seed protein (Basha and Pancholy 1986), into low-molecular-weight components was attributed to the proteolytic action of fungal enzyme (Fajardo et al. 1994). Enzymatic hydrolysis by invading fungi, synthesis of novel proteins of microbial origin, and *de novo* synthesis of protein of host origin were reported to occur during *A. flavus* colonisation of peanuts (Szerszen and Petit 1990). Gel electrophoresis of soluble proteins showed that during fungal proteolysis, there was a decomposition of high-molecular-weight peanut globulins, arachin, to low-molecular-weight protein components (Cherry et al. 1978).

In our study, transformations such as the catabolism of soluble proteins coincided with changes in protease activities in the commercial peanuts. In addition to proteins, enzymes such as esterase, leucine amino peptidase, catalase, alcohol dehydrogenase, alkaline phosphatase, glucose 6-phosphate dehydrogenase, mannitol dehydrogenase and malate dehydrogenase are also affected by fungal infestation (Cherry et al. 1972; Cherry et al. 1978; Buchanan and Lewis 1984). Analysis of protease activity in crude extracts of all commercial peanuts in our study showed significantly higher enzyme activity in contaminated than in healthy ones. Moreover, maximum enzyme activity was recorded in both naturally infected and artificially inoculated peanut samples from Cartersville, while peanuts from Kennesaw showed the least enzyme activity. This could be attributed to variation in environmental conditions at storage in these commercial outlets. Our observation corroborates with previous reports of alteration in enzyme activity in the host due to fungal invasion (Fajardo et al. 1994). Cherry et al. (1974) suggested that the changes in protein and enzyme patterns were probably related to a premature senescence of contaminated peanuts caused by active utilisation of peanut components by the fungus. Alkaline and neutral proteinases of *A. oryzae* were shown to dissolve and hydrolyse soybean proteins to peptides (Nakadai et al. 1972). On the other hand, protease activity in our study was lower in healthy than in contaminated samples; moreover, activity

increased with incubation period until the second week of incubation, and thereafter, a slight decrease was recorded in the third week. The latter may be attributed to the utilisation of protein by the host at the onset of germination.

Even though there are several reports on changes in seed protein and enzyme activities due to infestation by *A. flavus*, ours is the first report in this aspect of biochemical changes in commercial peanuts available to consumers in Georgia. As indicated above infestation by *A. flavus* is correlated to biochemical transformation that may include deletion of some proteins (including enzyme), intensification of others, and/or production of new components (Cherry et al. 1978). It has been well documented that biochemical transformation in peanut seeds due to infestation by *A. flavus* leads to aflatoxin production. This study stresses the importance of screening for other commercial cultivars on shelf in this region in view of consumer's health. An effective, economical and promising control of aflatoxin-producing fungi is possible by manipulating specific enzyme activities.

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