Physical and Chemical Properties of Resistance Exhibited by Certain Genotypes of *Arachis hypogea* to Invasion by Aflatoxin Producing *Aspergillus* spp.

By

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1973

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Dr. J. A. Bartz, Chairman of the Supervisory Committee, for his guidance and advice as well as critical assistence during the preparation of this manuscript. Thanks also go to the other members of the Supervisory Committee: Dr. A. J. Norden, Dr. H. H. Luke, Dr. D. J. Mitchell, and Dr. T. A. Kucharek.

Acknowledgements are also extended to Thelma Carlysle for the scanning electron micrograph work, Dr. C. T. Edds for the aflatoxin determinations, Dr. A. C. Mixon and Dr. W. K. Bailey for counsel leading to the development of the screening technique.

Sincere appreciation is extended to his father, J. L. LaPrade, for his encouragement and to his wife, June, for her devotion, encouragement, and understanding throughout this study.

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Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Physical and Chemical Properties of Resistance Exhibited by Certain Genotypes of Arachis hypogea to Invasion by Aflatoxin Producing Aspergillus spp.

By

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December, 1973

Chairman: Dr. J. A. Bartz
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Data from inoculations of whole, shelled peanuts from 165 Florida breeding lines with conidia from three isolates of the Aspergillus flavus group (N.R.R.L. 3794, N.R.R.L. 2999, and one Florida isolate) revealed statistically significant differences in tolerance to colonization by the fungus. If seed coats were punctured with a needle or abraded with carborundum before inoculation there were no significant differences in tolerance among the lines. When intact seeds were soaked in an aqueous solution of 1.0 per cent 2, 3, 5-triphenyl-2H-tetrazolium chloride (T.Z.C.), a uniform reduction of T.Z.C. occurred on the cotyledons of the susceptible lines but not those of the tolerant lines. This suggested that the seed coats of the tolerant lines were not as permeable as those of the susceptible lines. Aqueous extracts of intact seeds of both tolerant and susceptible lines stimulated germination of

A. flavus conidia when compared to conidia incubated in distilled water. Diethyl ether extracts of intact seeds of tolerant peanut lines revealed no germination inhibition when compared with conidia incubated in distilled water. Thus an intact testa was required for tolerance and appeared to function as a mechanical barrier to penetration by the fungus.

Wax-like accumulations were noted in scanning electron micrographs on the testas of dried peanut seed. Seeds from breeding lines which were tolerant to colonization by Aspergillus flavus (N.R.R.L. 2999) appeared to possess more of the wax-like accumulations than did several which were highly susceptible. Extraction of waxes and lipids from intact seeds with chloroform: methanol, 2:1 (v/v), for up to 5 minutes increased the susceptibility of the extracted seeds. No reduction in germination percentage of the seed extracted for 5 minutes was noted. A suspension of A. flavus conidia was placed on the dried solvent residue from a 5 minute and a 2 hour extraction of intact tolerant peanut seed. Germination of A. flavus conidia was slightly stimulated by the residue, compared to distilled water. It appears that the wax-like accumulations help prevent A. flavus from penetrating the intact seed coat.

Several preconditioning factors that fluctuate under ordinary growing and storage conditions were shown to affect the tolerance expressed by two tolerant breeding lines and the moderately tolerant Florunner variety. Pod rot caused primarily by severe nematode infections on Florunner peanuts led to higher levels of aflatoxin contamination under field conditions compared with pod rot caused primarily by fungi.

Peanuts of a tolerant breeding line dug 5 weeks before or 1 week after inoculation were more susceptible than peanuts dug at optimum maturity. Tolerance was lost after 1 year of storage of shelled peanuts compared with non-shelled peanuts of the same tolerant genotype.

The term tolerance as used throughout this context refers to a variable level of infection by fungi of the A. flavus group on peanuts of breeding lines and cultivars. This term was used to differentiate the reaction observed in this study from a hypersensative reaction.

INTRODUCTION

In 1960 more than 100,000 young turkeys died in the course of a few months on poultry farms in England (9). This was one of the first diseases caused by a toxin produced by Aspergillus flavus Link ex Fries (77). The toxin was given the name "aflatoxin" in view of its fungal origin. Ducklings, partridges, and young pheasants were also found to be highly susceptible to this toxin. Heavy mortality due to aflatoxin has been experienced among those animals in England, Kenya, and Uganda (4).

Widespread mortality of farm animals in 1960 provided an unprecidented stimulus to researchers to gain information to combat the aflatoxin problem. Aflatoxin was shown to typically consist of a mixture of at least four different chemical compounds designated as B_1, B_2, G_1, and G_2, (3). The toxicity of each compound varies and sensitivity to aflatoxin varies with both animal age and species. The B_1 compound was shown to be more toxic than the other compounds and young ducklings were among the most sensitive animals to the ingestion of aflatoxin contaminated feed. In a comparison of the toxicity of aflatoxin compounds to one day old ducklings, Carnaghan (14) reported the following $L D_{50}$'s using a single injection: B_1 = 0.36 mg/kg, G_1 = 0.78 mg/kg, B_2 = 1.696 mg/kg, and G_2 = 2.45 mg/kg. Symptom development on a wide range of test animlas given various doses of aflatoxin showed that liver damage almost always occurred resulting in death if the concentration was sufficiently high (12). When

sub-lethal doses were administered, no effect was observed for some weeks, but after a period of approximately 6 months most test animals developed primary carcinoma of the liver (53). Thus, both acute toxicity and chronic toxicity are associated with aflatoxin ingestion depending on the dosage administered and the susceptibility of the test animal.

Several investigators have reported that fungi other than A. flavus produce at least some of the compounds collectively called aflatoxin; however, only A. flavus and Aspergillus parasiticus speare have been confirmed to produce aflatoxin (35). Thus, subsequent use of the name A. flavus herein will refer to the A. flavus group as described by Raper and Fennell (75).

The moisture or relative humidity surrounding a natural substrate has been recognized as the most important factor in growth and aflatoxin production by A. flavus (5). Aspergillus flavus has been classified as a mesophyte on the basis of having a minimum moisture requirement for growth between 80 and 90 per cent relative humidity (41,67,79,80). The actual minimum relative humidity required for growth was affected by temperature (83,87). Peanut seeds have been safely stored at substrate moisture in equilibrium with 70 per cent relative humidity (50). Both the rate of fungal growth and aflatoxin production have been influenced by the moisture content of the pods and temperature. Dickens and Pattee (24) reported that when the moisture content of freshly dug peanuts was between 15 and 30 per cent and the storage temperature was 90 F; aflatoxin was produced within 2 days. If the moisture level was 20 to 31 per cent with a 70 F storage temperature, aflatoxin developed

in four days.

Invasion of intact and undamaged peanut pods and kernels by A. flavus has been reported to occur during curing in the windrow (5,7,30,62). If the peanuts have been dried to safe storage levels within four or five days after digging, little opportunity for fungal invasion occurs (88). At digging, the moisture content of mature peanuts will normally range from 40 to 60 per cent (50). When harvesting is complete, the moisture content should have been reduced to 10 per cent or below. Either an interruption and retardation of the drying cycle by rainy weather, or an increase in moisture content of the peanuts after picking and storage has usually resulted in mold development with possible toxin formation (5,7,61,62,88).

Surveys have shown that the peanut industry spent over $12,000,000 between 1964 and 1966 on new inspection, sampling, drying, and storage facilities in order to ensure aflatoxin-free products (50). In 1964 the majority of the peanut shellers signed a contract (No. 1003) with the U. S. Dept. Agri. Commodity Credit Corporation whereby all lots of farm stock peanuts bought by the sheller or for his account were classified by grade factors into three segregations. These grade factors were soon modified to place increased emphasis on moldy kernels. Segregation I peanuts were to be allowed to move into food channels; Segregation II peanuts were to be held in reserve; and Segregation III peanuts were to be irreversibly diverted from food and feed channels. The provisions of this marketing agreement have been modified each year to further the objective of ensuring toxin-free products. In 1967 a modification stated that a sample of each lot of shelled peanuts would

have to be assayed for mycotoxins in an approved laboratory and that the results would have to be reported to the buyer (42). As a result of work by Dickens and Welty, (25) the agreement was amended in 1968 such that a sample of every lot of farmer stock peanuts was to be examined at the buying station by a Federal-State inspector, and that any lot containing visible A. flavus will be classified as Segregation III. By definition all Segregation III peanuts must be crushed and restricted to nonedible uses. Up until 1973 the peanut grower received a price support through the Commodity Credit Corporation for all peanuts that were placed in Segregation III. However, in 1973 growers were penalized $50.00 per ton for peanuts that were placed in Segregation III because of visible A. flavus growth or aflatoxin in excess of 20 ppb.

New techniques for reducing aflatoxin contamination during harvest, storage, and transport, have been developed. A digger-inverter was developed to promote rapid drying of the product in the field. Growers have been encouraged to use artificial drying facilities in order to reduce the period of time that peanuts are susceptible to fungal colonization (88). In addition, shellers and processors have been using low temperature and low humidity storage and transit facilities to minimize mold growth and aflatoxin accumulation.

A new approach to the problem was suggested through efforts to discover and/or develop a peanut variety which was resistant or tolerant to growth of A. flavus isolates capable of producing aflatoxin. The first concentrated effort was initiated at Auburn University in 1969 by Mixon (65) in cooperation with the United States Department of Agriculture,

Agricultural Research Service, (U.S.D.A., A.R.S.). In 1970 a similar program was initiated at the University of Florida through a cooperative grant also administered by the U.S.D.A., A.R.S. The objective of this work was to screen peanut varieties and breeding lines for resistance to colonization by Aspergillus flavus isolates capable of producing aflatoxin and to determine the inheritance of the resistance encountered. Twelve out of 398 lines screened proved to be statistically more tolerant by Duncan's New Multiple Range Test at the 5 per cent level.

The following study reports on the mechanism of tolerance encountered among the breeding lines screened. A knowledge of the chemical and physical nature of the tolerance mechanism is needed to properly evaluate and determine the usefulness and possible limitations of tolerant genotypes discovered.

REVIEW OF LITERATURE

The first report in the United States of the isolation of a strain of A. flavus capable of producing aflatoxin was made in 1963 by Diener, et al. (26). Day old ducklings were used to confirm the presence of aflatoxin, and thin layer chromatography was used to confirm the presence of the B_1 and G_1 components of the toxin. It was then pointed out that the aflatoxin problem was a fungus problem. From these findings, extensive research was stimulated in an attempt to better understand the fungi responsible and the conditions under which the production of aflatoxin takes place.

There have been numerous reports in the literature of fungi other than A. flavus producing aflatoxin in vitro. A list of such fungi, the investigator, and the aflatoxin component produced has been prepared by Diener and Davis (35), (Table 1).

TABLE 1. FUNGI PRODUCING AFLATOXIN IN VITRO (35)

Fungus	Investigator	Aflatoxin			
		B_1	B_2	G_1	G_2
Aspergillus flavus group					
A. flavus	Sargeant et al. (1961)	X	X	X	X
A. flavus var. columnaris	Van Walbeek et al. (1968)		X		
A. oryzae	Basappa et al. (1967)	X	X		
A. parasiticus	Codner et al. (1963)	X	X	X	X
A. parasiticus var. globosus	Murakami et al. (1966)	X	X	X	X
Other Species of Aspergillus, Penicillium, etc.					
A. niger	Kulik and Holaday (1967)	X			
A. wentii	Kulik and Holaday (1967)	X			
A. ruber	Kulik and Holaday (1967)	X			
A. ostianus	Scott et al. (1967)	X		X	
A. ochraceus	Van Walbeek et al. (1968)	X			
Penicillium puberulum	Hodges et al. (1964)	X		X	
	Kulik and Holaday (1967)	X	X	X	X
P. variable	Kulik and Holaday (1967)	X			
P. frequentans	Kulik and Holaday (1967)	X			
P. citrinum	Kulik and Holaday (1967)	X			
Rhizopus sp.	Van Walbeek et al. (1968)	X		X	

Many investigators have failed to corroborate findings that fungi other than A. flavus or A. parasiticus produce aflatoxin. A screening study of 121 fungus isolates representing 29 species, revealed aflatoxin formation only in the A. flavus group (91). Eight of the negative organisms were subcultures of isolates reported to produce aflatoxin by other investigators. The authors discussed possible reasons for false positive findings, and gave precautionary measures and useful differential analytical procedures for future aflatoxin screening studies. The most obvious source of error in false positive findings was shown to be the utilization of a purely chemical or physical test to detect aflatoxin without also using a bioassay. The most widely used physical test for toxin detection is based on the fact that aflatoxins fluoresce when irradiated with U.V. light. A similar fluorescent behavior has been noted not only for a number of natural biochemical plant constituents but also for chemical compounds produced by many fungi. Unless those compounds were properly removed in the analytical procedure, the presence of aflatoxin could have been wrongly attributed to many samples (13,17,78,82). Results of bioassays and thin layer chromatography must, therefore, be correlated and carefully evaluated before concluding that aflatoxin is present.

Isolates generally classified as A. parasiticus have been among the most active aflatoxin producers (16,46). Kulik and Holaday (51) noted that the members of the A. flavus group, which include A. parasiticus, have had the greatest capacity for aflatoxin production. Members of the A. flavus group appeared to be the primary and perhaps the only source of aflatoxins. However, not all isolates of a particular species within

this group produce toxin. From a survey of isolates of the A. flavus group found on peanuts in the United States, Ambrecht et al. (1) found that seven out of 10 isolates produced aflatoxin. Diener and Davis (32) reported that 35 out of 44 isolates produced detectable aflatoxin, and Taber and Schroeder (85) found that only 107 out of 213 isolates from Spanish peanuts were capable of producing detectable aflatoxin. Aspergillus flavus isolates from peanuts grown in Alabama, Texas, and Virginia all produced aflatoxin when cultured on peanuts or in nutrient solution (31).

In order to determine the ideal, as well as the limiting environmental conditions for which the production of aflatoxin takes place, Diener and Davis (29) studied aflatoxin production by A. flavus on heat treated, sterile peanuts. Relative humidity was more closely correlated with aflatoxin content than kernel moisture content. Aspergillus flavus grew well at a wide range of temperatures (14-43 C) if held in 97-99 per cent relative humidity, but grew well only on a comparatively narrow range of relative humidity (86-99 per cent at 30 C). Aflatoxin was found in peanut kernels from storage in high humidities at 14 C and 40 C, but not at 12 C and 43 C. Optimum and cardinal temperatures for aflatoxin production were found to vary slightly with the fungal isolate and species of Aspergillus, as well as the peanut variety used as substrate (28,31,32,69). One isolate of A. flavus found to produce only aflatoxin B_1 had an optimal temperature near 25 C (28). The optimal temperature range for production of aflatoxin B_1 and G_1 by A. parasiticus was 25 to 30 C. The ratio of one aflatoxin compound to another was shown to be influenced by temperature and

composition of the substrate (19,21,22,28,73).

Davis et al. (21) studied the factors influencing the production of aflatoxin by A. flavus growing on laboratory media. Chemically defined media such as Czapek-Dox broth supported growth of the fungus, but very little aflatoxin was produced unless 1 per cent yeast extract had been added (22). The carbon source present in the substrate was shown to affect total aflatoxin production with glucose, ribose, xylose, or glycerol necessary for production of detectable quantities of aflatoxin (19).

The presence or absence of minor elements in the nutrient medium has also been shown to affect aflatoxin production (20). When the 15 elements generally required by plants were added to the fungus substrate, increased yields of aflatoxin were obtained. Sporulation and reproductive growth were not necessary for aflatoxin synthesis. Very little aflatoxin was found in the mycelium, but was readily demonstrated in the medium, indicating that diffusion of the toxin took place at about the same rate as it was formed.

The relationship between concentration of atmospheric gases and aflatoxin production in peanuts was investigated by several researchers (8,54,76). Aflatoxin synthesis was highly aerobic and maximum aeriation of cultures resulted in highest toxin yields (54). These results supported those of Diener and Davis (36) who reported that an increase in CO_2 concentration resulted in decreased levels of aflatoxin production by all strains of A. flavus tested.

The physiological condition of the peanut has also been shown to affect its susceptibility to colonization by A. flavus. Overmaturity

of the peanut at harvest was shown to be correlated with a high incidence of A. flavus infection, and toxin accumulation (24,27,30,64). From a study on the growth of A. flavus and production of aflatoxin in peanuts, McDonald and Harkness (62) determined the following relationships: (a) A. flavus grew much more successfully on dead peanut tissue than on living tissue; (b) There was virtually no aflatoxin in the crop at harvest with the exception of broken pods; (c) Contamination of kernels by A. flavus and the appearance of toxin did not occur until at least 5 days after harvest; (d) Kernels from broken pods were more likely to be contaminated with A. flavus and to contain toxin than were kernels from undamaged pods; (e) Aflatoxin content of the crop varied from year to year at specific localities.

A study of fungal penetration characteristics on dried peanuts revealed that A. flavus penetrated the peanut kernel directly (57). Inner kernel tissues were ramified by mycelium and toxin was distributed throughout the kernel. Lindsey (59) reported that when peanuts grown under gnotobiotic conditions were inoculated with A. flavus there was no evidence of colonization of plants and no pod rot symptoms. Aspergillus flavus penetrated the shell tissue consistently but was limited in its seed invasion to the testa. Wells and Kreutzer (89) showed that under gnotobiotic conditions, A. flavus colonized peanut flowers nonpathogenically during the blossom period, and remained associated with the apparently healthy developing pod tissue.

Certain common soil inhabiting fungi have been shown to have an antagonistic effect on the colonization of peanuts by A. flavus in the soil. Wells, et al. (90) found that colonization of immature and mature

peanut pericarps by A. flavus was reduced in the presence of Trichoderma viride Person ex Fries, under gnotobiotic conditions; however, the presence of Penicillium funiculosum Thom appeared to nulify the antagonistic effect of T. viride and stimulated colonization of mature peanut pericarps and testae by A. flavus. Burnett and Rambo (11) reported a 12 per cent reduction in toxin formation by A. flavus if Aspergillus niger van Tieghem had been previously grown on the peanut substrate or if culture filtrates from A. niger were used as a soak on peanuts before the latter were inoculated with A. flavus. Griffin (44) found that A. flavus conidia placed in the peg geoscarposphere did not germinate readily and only trace germination was noted in the fruit geoscarposphere of intact undamaged pods and fruit. If, however, pods were superficially injured, A. flavus conidia germinated readily. It can be postulated that A. flavus conidia in the soil do not readily germinate, although soil temperature and moisture may favor germination.

There have been numerous attempts to control or reduce the incidence of aflatoxin in peanut products. Some of the first attempts were directed toward developing faster drying and more efficient curing techniques immediately following digging (62). Such techniques did reduce but not eliminate the incidence of aflatoxin (10). Controlled atmosphere storage was investigated as a possible means of preventing growth of A. flavus and/or aflatoxin production after harvest. Sanders et al. (76) showed that growth of A. flavus and aflatoxin production could be inhibited at CO_2 concentrations as low as 20 per cent in combination with reduced temperatures and low relative humidities. However, peanuts stored for 2 weeks under high carbon dioxide, high temperature, and

high humidity conditions led to a product with poor edible quality (54).

Jackson (48) conducted a preliminary investigation on the use of five fungicides to control peanut pod surface fungi, including Aspergillus spp. in the windrow during the dehydration period. Difolatan and Du-Ter gave the best control under test conditions. Jackson (49) further evaluated fungicides under laboratory conditions and showed that it took only 5 micro-grams of Difolatan or Daconil 2787 to kill spores and mycelial fragments of A. flavus or A. parasiticus. Although those results seemed encouraging, a fungicide study conducted under field conditions by Pettit et al. (70) did not show a statistical difference in degree of fungal infestation, level of toxin, or grade factors between any fungicide treatment. Difolatan was included in that test but Du-Ter and Daconil 2787 were not. Fungicides are not currently applied to peanuts to control storage fungi primarily due to a decided lack of efficiency.

Investigations were directed toward detoxification since toxin formation could not be prevented. Detoxification of 78 per cent of the aflatoxins in contaminated peanut meal was accomplished by a 1 hour ozone treatment at high temperatures and high meal moistures (40). However, it was subsequently determined that the treated meal had a reduced protein efficiency ratio of approximately 40 per cent over non-treated high quality peanut meal (37). Dollear further reported that complete elimination of aflatoxins from contaminated peanut meal was achieved by extraction with a 9:1 (v/v) acetone: water solvent system, while maintaining an 85 per cent protein efficiency ratio compared to that of high quality non-contaminated peanut meal. Rambo and Bean

(74) found that treating peanut seed with dimethyl sulfoxide (D.M.S.O.) solution at concentrations of 2.5 per cent or higher prior to inoculating with A. flavus caused approximately 62-64 per cent inhibition in aflatoxin production. Dimethyl sulfoxide could possibly be applied to peanuts in the windrow or prior to processing to inhibit aflatoxin formation, provided that the chemical was found to be non-toxic to animals and that market quality of the treated peanuts was not impaired.

Attempts to remove moldy contaminated kernels from healthy, clean kernels during the shelling and sorting process have proven partially successful in reducing levels of alfatoxin (50). Manual and electronic sorters have been used in the peanut industry since McDonald and Harkness (62,64) reported that overmature, broken and damaged kernels were more likely to contain aflatoxin.

An average of 80 per cent B_1 and 60 per cent B_2 aflatoxin in peanut seed was removed by roasting for 30 minutes at 150 C (58). However, if the toxin level was originally high in the raw peanut, it would continue to be high even after roasting.

In order to prevent peanuts containing high levels of aflatoxin from being processed, a rapid technique was needed for determining A. flavus contamination or toxin content in a given sample. A correlation between free fatty acid production by A. flavus isolates capable of producing aflatoxin, and growth of the fungus was observed by Pattee and Sessoms (68). It was suggested that a rapid method of determining free fatty acids might be used to screen peanut samples for the possible presence of aflatoxins. However, the presence of free fatty acid was found to be closely correlated with growth of many fungi,

and not just those capable of aflatoxin production (33,34). Dickens and Welty (25) developed a technique for detecting aflatoxin contamination in farmers' stock peanuts by examination of the kernels for visible growth of A. flavus. This technique is currently used in determination of the grade or quality level placed on farmers' stock peanuts. Several rapid chemical techniques for detecting aflatoxin have also been developed and are currently used with considerable success (2,18,43,45,47,71,72).

Reports concerning varietal differences in peanuts as to the level of toxin production stimulated hope that resistance to toxin production might be achieved with these varieties. One variety, designated as U.S. 26, was reported to be resistant to toxin production (84). Another report which stated that a Tanganiyaka peanut variety, "Asiriya Mwitunde", was "tolerant" to aflatoxin production stimulated further interest in varieties resistant to toxin accumulation (52). However, when these two varieties were inoculated with A. flavus, aflatoxin was found to be produced at substantial levels (38). Doupnik further reported no inhibition of aflatoxin accumulation from a screening study of 20 additional breeding lines maintained by R. O. Hammons at Tifton, Georgia. These results agree with earlier work by McDonald and Harkness (62) who reported that out of four peanut varieties assayed for toxin formation under natural conditions, no variety was more resistant than another.

Observations indicated that heavy colonization of breeding lines by toxin producing fungi always led to high levels of toxin accumulation (65). Therefore, large numbers of breeding lines were screened

in an effort to determine if resistance or tolerance to colonization might exist among them. LaPrade and Bartz (55) reported finding several breeding lines among those maintained by A. J. Norden that were statistically more tolerant to colonization than were other breeding lines. Mixon and Rogers (66) reported finding two peanut accessions which averaged less than 5 per cent seeds colonized by toxin-producing strains of A. flavus using laboratory inoculation procedures which favored heavy colonization of susceptible accessions.

There have been several reports in the literature concerning the reasons why certain peanut samples appeared to be more tolerant to colonization by A. flavus than were others. Early workers first observed that excessive damage to the peanut pods at the time of harvest led to increased toxin levels over undamaged peanuts (61,62,63). Bampton (7) observed that damage to the peanut testa, especially before drying to a safe moisture level, increased fungal invasion and possibilities of toxin development in the kernel. Diener and Davis (35) speculated that damage to the shell or kernel afforded increased opportunities for direct invasion of the kernel by A. flavus and increased nutrient availability. Thus, the extent of fungus growth and toxin formation at minimal temperature and relative humidity would be determined by time, nutrient availability, and damage.

Carter (15) reported on the possible factors leading to greater tolerance of a red seed coated peanut variety versus that of a white seed coated variety. Tannens, derived from seed coat pigments were implicated as being the major barrier to fungal invasion, while only minor significance was given to the seed coat as a physical barrier to

fungal colonization. LaPrade and Bartz (55), Taber et al. (86), Deickert et al. (23), and LaPrade et al. (56), have suggested a purely mechanical tolerance mechanism for dried, shelled peanuts. LaPrade and Bartz demonstrated that the testa functions as a physical barrier to colonization for peanuts from breeding lines that proved to be tolerant to A. flavus colonization. It was suggested in this work that differential seed coat thickness or permeability could play a part in such tolerance. Taber et al. observed differences in the seed coat of peanuts from Mixon's tolerant accessions compared to peanuts from highly susceptible accessions. Such differences included the size and shape of the hila, abount of wax secretion, thickness of the palisade-like layers, and size and arrangement of cells within these layers. Deickert et al. (23) showed that seed coat structure played a part in the tolerance mechanism by providing a physical barrier to complete fungus hyphal-peg penetration. LaPrade et al. (56) reported that heavier cuticular wax accumulations occurred on peanuts of tolerant breeding lines and that this wax accumulation was capable of significantly reducing the colonization level by means of mechanical protection to fungal penetration.

Part II - Section I

Mechanical Tolerance of Selected Genotypes of Dried Peanuts to Colonization by Strains of Aflatoxin Producing Aspergillus spp.

INTRODUCTION

Preventing the occurrence of aflatoxin in peanuts or peanut products has been a major concern since finding mycotoxin responsible for Turkey "X" disease in England in 1960 (9). Peanut breeders tried to prevent the occurrence of aflatoxin by developing varieties that could inhibit toxin formation. The peanut varieties, U. S. 26 (84) and Asiriya Mwitunde (52) were first reported to inhibit the production of aflatoxin, but were later shown to be contaminated with substantial levels when colonized with a toxin producing strain of Aspergillus flavus Link ex Fries (38). Mixon (65) reported that colonization percentage of peanuts of a given breeding line by a toxin producing isolate of A. flavus was correlated with toxin level accumulation by that line. Percentage of colonization was further shown to vary widely among peanuts of different genotypes (56). Four breeding lines out of 165 maintained by A. J. Norden at the University of Florida were found to be more tolerant to colonization by three isolates of A. flavus during laboratory tests.

Carter (15) observed consistent differences in the amount of A. flavus colonization of peanuts with red versus white seed coats. The tolerance to colonization by the peanuts with red seed coats was

attributed to the presence of tannic acid, not found in the white seed coats. However, peanuts of several breeding lines with red seed coats were as susceptible as shite seed-coated lines. The following is a study of the mechanism of tolerance found among peanuts with red seed coats. The susceptible lines used as controls also had red seed coats.

MATERIALS AND METHODS

Two of the A. flavus isolates used in the screening procedure were obtained from the National Regional Research Laboratory (N.R.R.L.) designated as (N.R.R.L. 2999 and N.R.R.L. 3794). Both isolates were found to produce high levels of aflatoxin when growing on peanuts (81). The "Fla. isolate" was isolated from an infected peanut found on the University of Florida Agronomy farm. This isolate produced moderate levels of aflatoxin in peanuts. The amount of toxin was measured by the millicolumn assay technique developed by Holaday (47).

Only hand-shelled, dried, peanut seeds that had been stored at room temperature for at least 60 days were used in the screening procedure. These seeds were carefully examined prior to inoculation and all seeds with damaged seed coats were discarded.

Inoculum was prepared from an aqueous conidial suspension of 5 per cent Tween 20 (v/v) in sterile distilled water with ca. 8.0×10^6 conidia/ml. One-half ml of this conidial suspension was added to each of three 15 g replications of seed from each breeding line in a 200 X 20 mm

petri plate. Seeds of each replicate had been adjusted to 20 per cent water content before inoculation. The percentage of colonized peanuts was determined after 1 week incubation at 25 C.

Two 15 g replicates of hand-shelled seed of UF70101 and UF71206 (tolerant to A. flavus colonization) and UF711441 (susceptible to A. flavus colonization) were used in the following treatments: (a) All seed coats were punctured with a brass pin at random locations on each seed. Each puncture location was marked with black indelible ink. (b) All seed coats were punctured with a fine glass needle ca. one-fourth the diameter of the pin, in the same manner as treatment one. (c) A 4 to 5 mm^2 section of the seed coat of each seed was removed with a sterile scalpel exposing the cotyledon. (d) Seeds were abraded with sterile no. 600 carborundum grit by swirling each seed for ca. 5 seconds in a 600 ml beaker containing the carborundum. Comparisons of colonization of peanuts with wounded seed coats versus peanuts of the same breeding lines with non-wounded seed coats were made 3 days after inoculation as described above.

Three 10 seed replicates of UF71316 (tolerant to A. flavus colonization) and UF711441 (susceptible to A. flavus colonization) were stored at 4 C for ca. 24 hours. Each replicate of seed was then introduced to a separate container of 1 per cent aqueous 2, 3, 5-triphenyl-2H-tetrazolium chloride (T.Z.C.) solution. After 15 minutes, the seeds of each replicate were removed from the T.Z.C. solution and rinsed thoroughly in distilled water ca. 24 C. Each replicate was then placed separately on filter paper in a 200 X 20 mm petri plate and incubated at 4 C for 24 hours. After the 24 hours, the seed coats were cut with a scalpel around the

circumference of the seed and removed with forceps. An immediate observation was made of the concentration of reduced T.Z.C. (carmine red in color) on the cotyledon.

Aqueous extracts of peanuts from the tolerant and susceptible lines UF71206 and UF711441 respectively, were obtained by soaking seed of two 15 g replicates in 20 ml of sterile distilled water for 24 hours at 5 C. One-half ml of each of the extracts was filtered through a 0.2 micron millipore filter and diluted in sterile distilled water. The final dilutions ranged from one-half to one-sixteenth times the original concentration of the extracts. An aqueous conidial suspension of 5 per cent Tween 20 in sterile distilled water (v/v) was prepared with ca. 1.0×10^6 conidia/ml. One-tenth ml of this A. flavus conidia suspension (N.R.R.L. 2999) was mixed with equal volumes of each concentration of the peanut extracts, czapeks broth, and sterile distilled water. The aqueous peanut extracts and check solutions containing A. flavus conidia were transferred to individual wells of depression plates that were kept in a petri plate containing moist filter paper. The percentages of germinated conidia were determined from 10 microscope fields taken at random for each treatment after 20 hours of incubation at 25 C.

Diethyl ether extracts of peanuts from UF71206 and UF711441 with the same replication size as used above were obtained by soaking seed of each replicate in 25 ml of diethyl ether for 1/2 hour at room temperature. The remaining diethyl ether extract was then placed in watch glasses and allowed to evaporate to ca. 1 ml volume. Dilutions with diethyl ether were prepared at relative concentrations from one-half to one-sixteenth times the original concentration. Diethyl ether in the final

dilutions was evaporated in a vacuum oven at (25 P.S.I. vacuum) 35 C for 4 hours prior to adding aqueous conidial suspensions prepared as described previously. After 20 hours incubation at 25 C, percentage germination of conidia was determined as described above.

RESULTS

The tolerance of different peanut genotypes to A. flavus colonization was measured by comparing the percentage of uninjured peanut seeds which were visibly colonized by the fungus. The tolerance of UF711441 and UF71510, respectively, to colonization by all three isolates of A. flavus was significantly higher than for UF71206, UF71104, UF71316, or UF70101 (Tables 1-3). Average colonization ranged from 13.2 to 39.7 per cent of the kernels for the tolerant breeding lines compared to 87.4 to 100 per cent for the susceptible breeding lines.

The effect of injury on tolerance was studied since it has been shown that injured seeds are more susceptible to colonization (63). Seed coats of tolerant and susceptible seed shown in Tables 1-3 were wounded as described earlier and subsequently inoculated with A. flavus conidia. All treatments involving mechanical damage to the seed coats of tolerant peanuts rendered those seed susceptible. Colonization was apparent only 3 days after inoculation and was initially confined to the wound area on tolerant seed (Figs. 1 and 2) but not on susceptible seed (Fig. 3). The exposed cotyledon of either tolerant or susceptible peanuts was equally susceptible.

An intact undamaged seed coat seemed to be required for tolerance, therefore, the possibility that natural or artificial microscopic breaks in the seed coat was responsible for the susceptibility of certain peanut lines was studied. Peanut seeds were immersed in a T.Z.C. solution for 15 minutes which was a modification of a test for cracks in soybean seed coats (39).

The T.Z.C. is reduced upon contact of cotyledons of viable seeds changing from colorless to carmine red (60). A distinct carmine red spot or narrow band on the cotyledon of a susceptible treated seed would indicate that a microscopic puncture or crack had been present in the seed coat of that seed. There were no such spots or bands observed on any of the susceptible seed. Instead, the cotyledons of susceptible treated seed appeared uniformly light pink while the cotyledons of tolerant seed remained white (Figs. 4 and 5).

Peanut seed of the tolerant lines might possess a conidial germination inhibitor. Thus seeds of tolerant and susceptible lines were soaked in a polar and non-polar solvent and the effects of each extract on conidial germination was tested. No germination inhibition was noted among the extracts tested (Table 4). In fact a stimulation in conidial germination occurred for all extract dilutions as compared to distilled water. There were no significant differences in the per cent germination of conidia in similar or different dilutions of the residues from polar or non-polar extractions of tolerant or susceptible breeding lines.

Table 1. Percentages of seed from different peanut lines colonized by *Aspergillus flavus* (N.R.R.L. 2999) seven days after inoculation with ca. 4.0×10^6 conidia

Breeding line	Rep.1	Rep.2	Rep.3	%mean	Arcsin mean 1/
UF711441	100	83.3	87.5	90.3	75.1 a
UF71510	100	89.4	78.9	89.4	74.4 a
UF71206	18.1	9.5	19.0	15.5	23.0 b
UF71104	16.0	12.5	11.1	13.2	21.3 b
UF71316	20.0	10.0	9.5	13.2	21.3 b
UF70101	17.6	12.1	11.8	13.8	21.8 b

1/ All mean arcsin values followed by the same letter are not significantly different at the 5.0% level by Duncan's new multiple-range test.

Table 2. Percentages of seed from different peanut lines colonized by Aspergillus flavus (N.R.R.L. 3794) seven days after inoculation with ca. 4.0 X 10^6 conidia

Breeding line	Rep.1	Rep.2	Rep.3	%mean	Arcsin mean 1/
UF711441	84.0	89.3	88.9	87.4	69.3 a
UF71510	84.2	94.4	94.7	91.1	73.2 a
UF71206	25.0	33.0	36.4	31.6	34.1 b
UF71104	31.1	29.6	40.7	33.8	35.5 b
UF71316	23.8	30.4	31.8	28.7	32.3 b
UF70101	18.3	21.8	29.4	23.2	28.8 b

1/ All mean arcsin values followed by the same letter are not significantly different at the 5.0% level by Duncan's new multiple-range test.

Table 3. Percentages of seed from different peanut lines colonized by *Aspergillus flavus* (Florida isolate) seven days after inoculation with ca. 4.0 X 10^6 conidia

Breeding line	Rep.1	Rep.2	Rep.3	%mean	Arcsin mean 1/
UF711441	100	100	100	100	90.0 a
UF71510	94.7	88.9	100	94.5	82.1 a
UF71206	33.3	41.7	44.0	39.7	39.0 b
UF71104	31.1	29.6	40.7	33.8	35.5 b
UF71316	23.8	30.4	31.8	28.7	32.3 b
UF70101	29.4	33.6	37.9	34.0	35.7 b

1/ All mean arcsin values followed by the same letter are not significantly different at the 5.0% level by Duncan's new multiple-range test.

Table 4. Mean germination of A. flavus conidia (N.R.R.L. 2999) in four dilutions of aqueous and diethyl ether residues of the tolerant line UF71206 versus the susceptible line UF711441

	Mean germination of conidia (arcsin degrees) 1/			
	Aqueous extracts		Diethyl ether residues	
Dilutions	UF71206	UF711441	UF71206	UF711441
1/2	77	81	69	65
1/4	70	73	63	66
1/8	62	60	56	57
1/16	63	60	55	58

Check Solutions	Mean germination (arcsin degrees)
Czapeks	73
Sterile distilled water	54

1/ The lsd between paired observations for any dilution is 7.2 arcsin degrees.

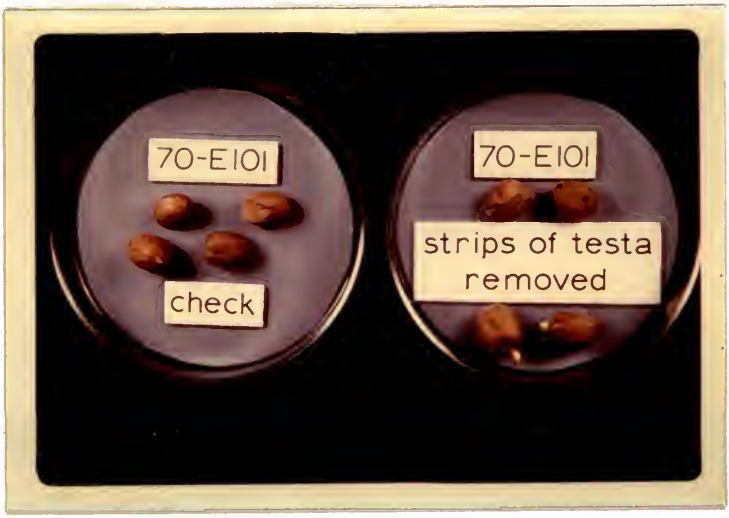

Figure 1. Seeds of the tolerant line UF70101 inoculated with A. flavus (N.R.R.L. 2999) and incubated at 25 C for three days. Treatments were performed as indicated.

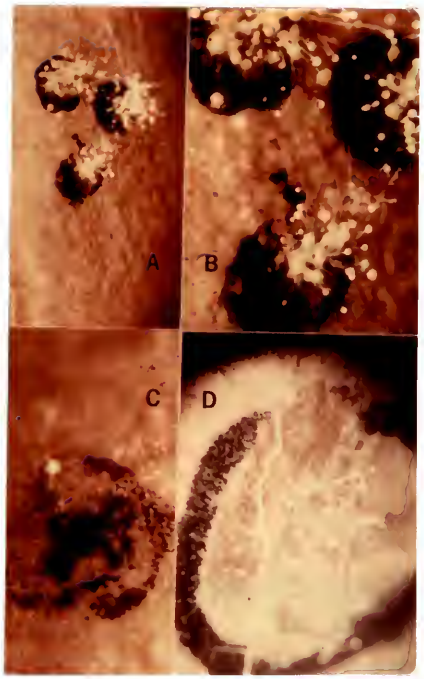

Figure 2. Seed of the tolerant line UF71104 inoculated with A. flavus (N.R.R.L. 2999) and incubated at 25 C for three days. The seeds in Fig. A-C were punctured in the areas marked; A and B with a pin, C with a glass needle. The seed coat in the area within the circle of Fig. D was mechanically abraided with carborundum.

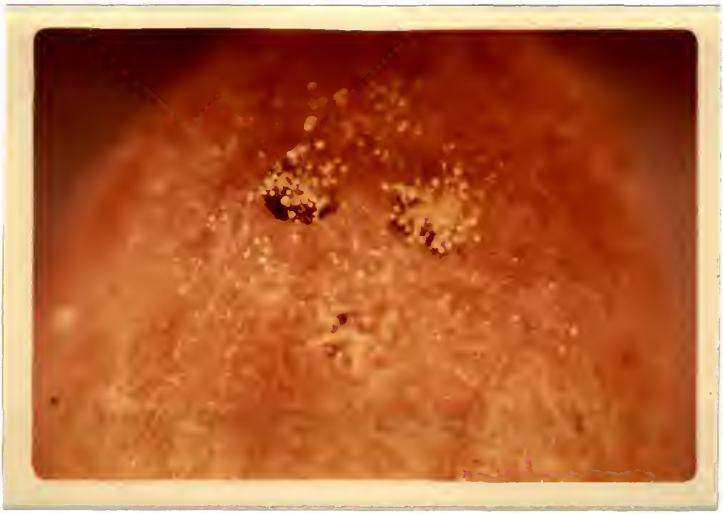

Figure 3. A seed of the susceptible line UF711441; the seed coat was punctured with a pin, inoculated with A. flavus (N.R.R.L. 2999) and incubated for three days.

Figure 4. Differential reduction of 1.0% aqueous T.Z.C. solution (v/v) on the cotyledons of seeds from the tolerant line UF71316 (lab. no. 52) versus the susceptible line UF711441 (lab. no. 200).

Figure 5. Differential reduction of aqueous 1.0% T.Z.C. solution (v/v) on the cotyledons of seeds from the tolerant line UF71104 (lab. no. 4) versus the susceptible line UF71510 (lab. no. 82).

DISCUSSION

Peanuts from four breeding lines out of 165 screened were found to be significantly less colonized by three isolates from the A. flavus group capable of producing aflatoxin. The tolerance mechanism of these breeding lines was studied.

Mechanical damage of the seed coat or testa of tolerant seed rendered them susceptible to colonization, but fungal growth was initially confined to the damaged areas. Similarly damaged susceptible seeds were colonized rather indiscriminately.

Three theories were formulated from the above observations: (a) Natural microscopic breaks occurred in the susceptible seed coats that allowed indiscriminate colonization. (b) Tolerant seed coats possessed an inhibitor that prevented or retarded germination of conidia. (c) Tolerant seed coats served as a physical barrier to conidia germ tube penetration.

There was no visible or microscopic evidence of breaks in the susceptible seed coats. However, the cotyledons of susceptible seed exhibited a uniformly increased quantity of reduced T.Z.C. Either the seed coats of susceptible seed were more permeable to this solution or those seed were more viable. Since it has been shown that non-viable seed are more susceptible to A. flavus colonization (63), permeability differences were believed to exist. Seed coat thickness could also affect the amount of reduced T.Z.C. observed on the cotyledon since a thin seed coat would allow more T.Z.C. to pass through in a given time

interval if permeability rates of both seed coats are equal.

Aspergillus flavus conidia in aqueous and diethyl ether residue extracts from tolerant seed germinated at approximately the same rate as similar extracts from susceptible seed. No conidium germination inhibition was found from diethyl ether or water soluble peanut extracts from tolerant lines compared to conidia germinated in sterile distilled water. Conidia germ tubes were observed to be more robust in the higher concentrations of aqueous peanut extracts from tolerant and susceptible seed and pure Czapeks solutions than were germ tubes developing in the less concentrated extracts or distilled water. This was believed to be a response by A. flavus conidia to various levels of nutrient present in the different extract concentrations.

The tolerance mechanism appears to reside in the seed coat which is thought to function as a mechanical barrier to conidial germ tube penetration. The maximum benefit that will be derived from peanut varieties incorporating this tolerance mechanism will occur before the seed coat is damaged by cultivation, harvest, or shelling. Peanut cultivars with tolerance of this nature should, therefore, be more beneficial to the grower who handles his crop to minimize damage. Toxin accumulation in the windrow of undamaged peanuts should be reduced. However, toxin accumulation in storage of shelled peanuts would not be expected to be reduced by varieties with this tolerance mechanism since the seed coat is often damaged by mechanical shelling. Storage of unshelled peanuts should be beneficial using these tolerant genotypes, if physical damage to the seed coat has not occurred.

Part II - Section II

Correlation of Peanut Seed Coat Surface Wax Accumulations with Tolerance to Colonization by *Aspergillus flavus*

INTRODUCTION

The use of peanut varieties resistant or tolerant to colonization by *Aspergillus flavus* Link ex Fries has been suggested as one method of reducing the incidence of aflatoxin in stored peanuts (6). A program to find peanut breeding lines with tolerance to colonization by *A. flavus* has been underway at the University of Florida for 3 years. Colonization ranged from less than 4 per cent up to 100 per cent for different peanut genotypes screened under similar environmental conditions. Three different statistical categories were determined by Duncan's new multiple-range test. Each category was significantly different at the 5 per cent level. Lines with less than 16 per cent colonization were referred to as tolerant. Those lines colonized from 16 per cent up to 60 per cent were considered moderately tolerant, while those having above 60 per cent colonization were considered highly susceptible. The mechanism of tolerance encountered among these breeding lines has been suggested to be purely mechanical (55).

MATERIALS AND METHODS

Four lines; UF71513, UF71104, UF71206, and UF711441, were used in this study. Based on the 1971-72 screening data, UF71104, UF71206, and UF71513 were tolerant while UF711441 was highly susceptible to colonization by A. flavus. All inoculations were performed as described previously (page 20).

Scanning electron micrographs were taken of hand shelled intact peanut seed from the above breeding lines. The seeds were observed in the mid-cotyledonary region, and were oriented perpendicular to the electron flow and parallel to the lens plane.

Portions of the peanut seed coat waxes were removed by extracting 15 g replicates of peanuts with 25 ml chloroform: methanol, 2:1 (v/v) at 45 C for 1/2, 1, or 5 minutes. The extracted and nonextracted (control) seeds were then washed for 1 minute in distilled water, air dried for 5 minutes, and inoculated. Three replicates of 10 noninoculated seed from each treatment were germinated as a test of viability. The amount of aflatoxin produced in each replicate of the nonextracted and the 5 minute extraction treatments, respectively, were assayed by a millicolumn technique. Scanning electron micrographs were taken of extracted peanuts as described previously.

One-tenth ml of an aqueous suspension of ca. 1 X 10^6 A. flavus conidia/ml was placed on the dried residue from a 5 minute chloroform extraction and from a 2 hour chloroform: methanol, 2:1 (v/v) extraction of intact tolerant peanut seed. The percentages of germinated

conidia were determined from 10 microscope fields. Samples were taken at random for each treatment and a distilled water control after 20 hours of incubation at 25 C.

All statistical analyses were performed on converted arcsin values from the percentage values using the Duncan's new multiple-range test at the 5.0 per cent level.

RESULTS

Scanning electron micrographs revealed that seed from UF71104, UF71206, and UF71513, which were highly tolerant to colonization by A. flavus, appeared to possess more wax-like accumulations than did UF711441, which was highly susceptible. Wax continuity appeared more uniform with fewer breaks in the cuticle of tolerant line UF71104 than in the cuticle of susceptible line UF711441 (Fig. 1).

A study of these surface waxes was conducted to determine their role in reducing colonization by A. flavus. After tolerant seeds had been soaked in a non-polar solvent for 5 minutes, the wax continuity appeared broken in scanning electron micrographs (Fig. 2). Tolerance was lost as a result of soaking seed of UF71206 in the non-polar solvent (Table 2). Mean colonization rates increased as the wax solvent extraction period was increased. A significant increase in average colonization percentage was observed between the control and the 1/2 minute extraction period, as well as the 1 and 5 minute extraction periods, respectively.

There was no statistical difference among any of the treatments for average per cent seed germination, indicating that viability was not affected by the solvent. A significant increase in mean aflatoxin levels occurred for the seeds in the 5 minute extraction treatment as compared to the control.

A comparison of A. flavus conidium germination in dried extracts of tolerant seeds and in sterile distilled water failed to reveal a germination inhibitor (Table 3). A stimulation in conidium germination occurred in the peanut extracts compared to distilled water. No significant difference in conidium germination occurred in the extracts from the 5 minute and the 2 hour extraction periods.

Table 1. Mean per cent colonization by A. flavus (N.R.R.L. 2999) of four lines with corresponding arcsin conversions

Breeding lines	Mean per cent colonization	Mean arcsin 1/
UF71513	3.7	11.1 a
UF71104	13.2	21.3 a
UF71206	15.6	23.3 a
UF711441	90.3	71.9 b

1/ All mean arcsin values followed by the same letter are not significantly different at the 5.0% level by Duncan's new multiple-range test.

Table 2. Effect of differential wax extraction of intact peanut seed of UF71206 on A. flavus colonization, aflatoxin production, and seed viability

Extraction period(min)	Mean % colonization 1/	Mean % seed germination	Mean toxin level 2/
Control	28.8 a	96.7 a	31.7 a
0.5	37.9 b	93.3 a	
1.0	42.4 b	100.0 a	
5.0	51.3 c	90.0 a	50.0 b

1/ All treatment means followed by the same letter are not significantly different at the 5.0% level by Duncan's new multiple-range test.

2/ Toxin values are ppm aflatoxin B_1.

Table 3. *A. flavus* conidium germination on non-polar extract residues from tolerant seed of UF71206 vs. distilled water

Treatment 1/	Mean % conidia germination 2/
5 minute extraction	66.7 a
2 hour extraction	78.0 a
sterile distilled water	52.7 b

1/ All treatment means followed by the same letter are not significantly different at the 5.0% level by Duncan's new multiple-range test.

2/ Conidia are considered germinated if the germ tubes extend approximately one spore diameter.

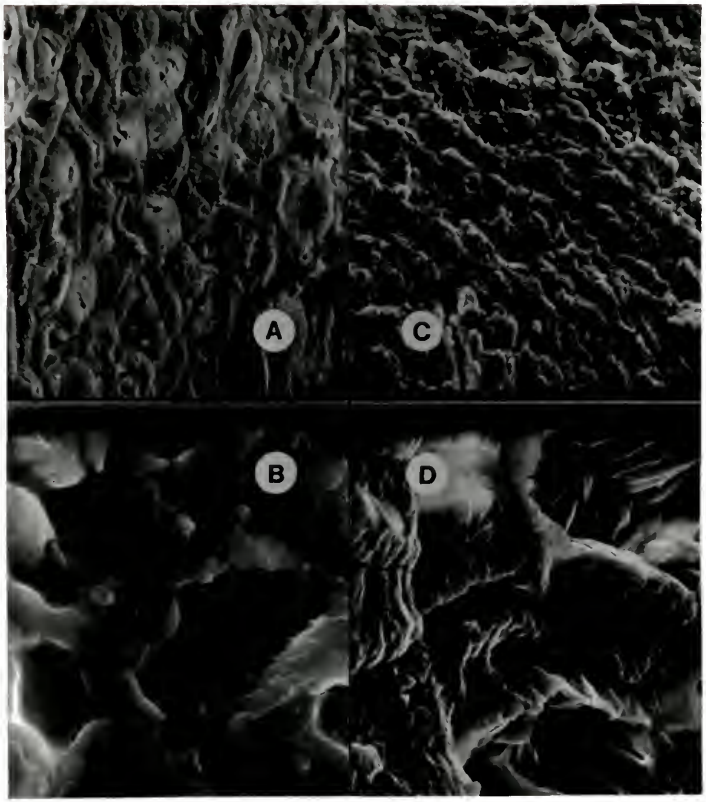

Figure 1. Scanning electron micrographs of the surface of intact peanut seeds. A and B are UF711441 (highly susceptible to A. flavus colonization) at 500 and 2150 X, respectively. C and D are UF71104 (highly tolerant to A. flavus colonization) at 500 and 2150 X, respectively.

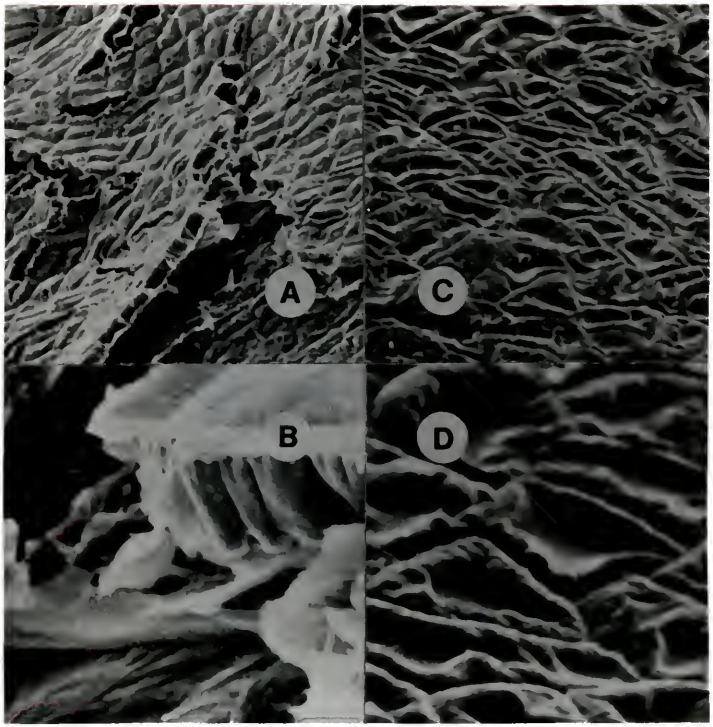

Figure 2. Scanning electron micrographs of intact and solvent soaked peanut seed. A and B are UF71513 seed soaked in chloroform: methanol 2:1 (v/v) at 45 C for five minutes. C and D are UF71513 (highly tolerant to A. flavus colonization) intact, not soaked in wax solvent, at 500 and 2150 magnifications respectively.

DISCUSSION

Heavier cuticular wax accumulations occurred on selected peanut cultivars highly tolerant to an isolate of A. flavus capable of producing high quantities of aflatoxin. These wax accumulations were partially removed by soaking the seed in chloroform at 45 C for up to 5 minutes. The differential removal of surface waxes increased susceptibility to colonization by A. flavus. Subsequent production of aflatoxin was also increased. Extraction of waxes did not significantly affect seed germination, although the 5 minute extraction period resulted in the lowest average seed germination.

The possibility of the presence of a conidium germination inhibitor in the cuticular wax extract of tolerant seed was tested. Residues from the evaporation of seed extracts were not fungistatic or fungitoxic, even when the extraction period was increased to 2 hours.

The mechanism of tolerance exhibited by these breeding lines has been shown to be expressed in their intact seed coats (55). The wax present on the surface of intact peanut seed coats prevented penetration and subsequent colonization by A. flavus. Peanuts from tolerant lines seemed to possess more wax with less breaks in the cuticle than peanuts from susceptible lines or solvent extracted tolerant lines.

Results from previous work have further suggested that the seed coats of tolerant peanut lines are less permeable to aqueous solutions of 2, 3, 5-triphenyl-2H-tetrazolium chloride (T.Z.C.) than seed coats

of susceptible peanut lines (55). The differences in wax accumulation as noted in this study could account for these permeability differences. If decreased seed coat permeability is indeed correlated with increased wax accumulations, then a determination of the permeability characteristics of peanut seed could be used to indirectly determine the tolerance characteristics of a given peanut breeding line. Permeability characteristics of peanut seed can be determined readily with a 1.0 per cent solution of T.Z.C. as described previously (page 21) or by determination of electrolyte leakage rates. These determinations can be performed more expediently than could colonization differences obtained from the inoculation technique previously described (page 20).

Part III - Appendix No. 1

The Influence of Peanut Moisture Content on the Reliability of the Screening Technique Used to Determine Tolerance of Breeding Lines.

INTRODUCTION

Moisture content of the peanut seed is the primary preconditioning factor to colonization of stored peanuts by A. flavus (43). The moisture content of the peanut required for colonization ranges between 12 and 35 per cent (15). The inoculation technique (described on page 20) was performed by adjusting each 15 g replication of seeds collectively at a 20 per cent moisture content prior to inoculum introduction. After inoculation and incubation, each seed of each replication was observed for colonization by A. flavus. If the moisture content of any seed within a replication is not within the range required for infection at the time of conidia germination and penetration (ca. 48 hours incubation) colonization will not take place although that seed may be susceptible. The objective of this study was to determine the variation in water percentage among individual seed within replications of tolerant and susceptible breeding lines.

MATERIALS AND METHODS

Thirty seeds each of a highly tolerant (UF70201) and a highly susceptible (UF70214) line that had been force air dried to ca. 6.5 per cent water content and stored for at least 60 days were carefully hand-shelled and inspected to insure against mechanical damage. These 30 seeds were divided into three replications of 10 seeds each. All the seeds of each replication were numbered with indelible ink and weighed to four decimal places. Water content of each replication was adjusted by soaking the seeds in sterile distilled water for 15 minutes prior to weighing and adding enough water to equal a 25 per cent increase in weight after shelling. After soaking, seed of each replication was transferred to a 20 X 200 mm petri plate where the additional water was added. The petri plates were swirled to equally distribute the additional water to all seeds in each replication. After incubation at 25 C for 48 hours, each seed in all replications was weighed, dried in an oven at 90 C for 72 hours and weighed again.

RESULTS

The moisture content of each seed after shelling and incubating was calculated from the difference between their weight after shelling and incubation and their weight after oven drying. Seed numbers shown for each replication in Table 1 correspond with seed numbers shown in Table 2.

The water content of the seeds after shelling ranged from 6.16 to 7.25 per cent for the tolerant line (UF70201) compared with 5.60 to 7.16 per cent for the susceptible line (UF70214). All seeds of both lines were well below the range for colonization by A. flavus before soaking in water.

The water content of the seeds after adjusting and incubating ranged from 20.08 to 51.52 per cent for seeds of the tolerant line compared with 15.54 to 61.96 per cent for seeds of the susceptible line. All 30 seeds of the tolerant and susceptible line were above the low critical value of 12 per cent for A. flavus colonization. Three seeds of both the tolerant and susceptible lines (shown with asterisks in Table 2) were above the high critical value of 35 per cent for A. flavus colonization.

Table 1. Moisture content immediately after shelling seeds of lines UF70201 and UF70214, tolerant and susceptible to A. flavus colonization, respectively

	UF70201 (per cent)			UF70214 (per cent)		
Seed Number	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3
1	6.43	6.89	7.25	5.99	6.14	5.67
2	6.31	6.86	6.68	5.82	5.78	6.08
3	6.50	7.23	7.43	6.11	5.88	5.68
4	7.07	7.18	6.16	6.20	5.87	6.05
5	6.29	6.49	7.06	5.76	5.84	6.12
6	6.71	6.55	6.97	5.99	5.77	5.60
7	6.98	7.07	6.87	5.81	6.32	5.77
8	6.63	6.40	6.34	6.17	6.07	5.97
9	6.57	6.25	6.96	6.27	6.05	5.68
10	6.87	6.92	6.40	6.13	7.16	6.32

Table 2. Moisture content after incubating seeds of line UF70201 tolerant, and UF70214 susceptible to A. flavus colonization

	UF70201 (per cent)			UF70214 (per cent)		
Seed Number	Rep.1	Rep.2	Rep.3	Rep.1	Rep.2	Rep.3
1	22.07	21.77	26.34	22.22	20.02	22.50
2	26.15	25.01	22.88	24.70	22.57	24.57
3	20.47	30.07	22.37	21.93	21.08	17.02
4	23.40	24.56	20.30	22.10	22.81	22.61
5	48.95*	23.26	24.96	28.13	15.54	40.69*
6	24.98	22.01	23.06	22.87	23.54	22.09
7	28.28	25.63	49.32*	38.75*	22.45	18.10
8	24.27	20.08	23.12	30.22	19.53	26.13
9	23.41	31.82	22.89	26.47	20.24	23.81
10	21.40	51.52*	20.29	23.64	61.96*	20.37

* Indicates that the seed is not in the susceptibility range (12-35 per cent water content) for A. flavus colonization.

DISCUSSION

Any seed that is not found to be within the critical moisture content range after incubation would be capable of giving erroneous results in the screening technique. Both breeding lines showed only 3 seeds out of 30 that could give erroneous results. Therefore, a 90 per cent efficiency level can be placed on results obtained from this technique, since only 10 per cent error can be attributed to variations in water uptake by the peanuts. A 10 per cent error in results should be negligible compared to the wide range of colonization noted between genotypes (page 25).

This screening technique may well have limitations in terms of making selections based on these results. As the rate of colonization increases on peanuts of a given breeding line, the chance of selecting a peanut that is not in the susceptibility range increases. Therefore, only those peanuts of breeding lines where very low colonization is observed should be selected.

Appendix No. 2

Preconditioning Factors Affecting Colonization by Aflatoxin Producing *Aspergillus* spp.

INTRODUCTION

Several peanut genotypes possess a tolerance to colonization under laboratory conditions by *A. flavus* isolates capable of producing aflatoxin (55,56,66). Certain variable preconditioning factors occur under ordinary growing and storage conditions that could influence expression of tolerance. Factors considered in this study were: (a) nematode damage and pod rot, (b) digging time or seed maturity, and (c) shelled versus in-shell storage. The first two factors are known to vary widely from field to field wherever peanuts are grown.

The effects of these variable factors on the tolerance of genotypes must be determined before the usefulness and limitations of the tolerance mechanism can be established. The effects of shelled versus in-shell storage of tolerant peanuts on colonization by *A. flavus* should be useful in the determination of storage requirements for these genotypes as well as providing a further insight into the tolerance mechanism.

MATERIALS AND METHODS

Nematode Damage and Pod Rot Study

A natural field infection of Florunner peanuts with varying levels of pod rot was used as a source of samples. The fungi and nematodes contributing to the pod rot symptoms were *Pythium* spp., *Rhizoctonia* spp., and *Hellicotylenchus sp.*, respectively. Random samples were obtained from different field locations at the time of optimum maturity. The samples were divided into five replications at each of three different levels of pod rot. Each replication consisted of 50 g of unshelled peanuts at a 30 per cent average moisture level. Each replication was placed in a 25 X 150 mm petri dish and inoculated with 2.0 ml of aqueous conidial suspension (*A. flavus* N.R.R.L. 2999). The conidial suspension consisted of 5 per cent Tween 20 in sterile distilled water (v/v) at a concentration of 8.0×10^6 conidia per ml. After inoculation, all samples were incubated at 85-90 per cent R.H. and 25 C for 14 days. Fungus colonization rates and aflatoxin accumulation were then determined for all three levels of pod rot. Toxin concentrations were determined by a millicolumn technique (47).

Digging Time or Maturity Effects

Breeding line UF71206 (tolerant to *A. flavus* colonization) was grown under field conditions. Samples were harvested at five different dates; three times before optimum maturity, at optimum maturity, and one time after optimum maturity. A total of 25 plants were harvested at

random for each digging date. Five groups of five plants each were used for replications. After each harvest the plants were allowed to air dry in a shed for 1 week prior to hand removal of peanuts from the vines. The peanuts were further dried in a forced-air oven for 24 hours at 45 C before hand shelling. The shelled kernels were divided into five replications; taking one replication of 15.0 g from each group of five plants as originally grouped at harvest. Only the most mature (largest and firmest) peanuts were used for samples from each harvest date. All 15.0 g replications were inoculated, incubated, and results recorded as previously described for the screening technique.

Shelled Versus In-Shell Storage

Two treatments were employed with three 15 g replications each of the line UF70101, highly tolerant to A. flavus colonization. Peanuts of both treatments were dried to below 10 per cent moisture content and stored for 1 year at room temperature. Treatment one peanuts had been hand shelled prior to storage while treatment two peanuts were stored un-shelled until the time of inoculation. The kernels of both treatments were inspected carefully for obvious seed coat damage prior to inoculation and were discarded if visable damage was found. All replications were inoculated, incubated, and results recorded as previously described for the screening technique.

RESULTS

Nematode Damage and Pod Rot Study

The most prevalent fungi isolated from the pod rot peanuts were Pythium spp., and Rhizoctonia spp., while the most prevalent nematode was Hellicotylenchus sp. Nematodes were not recovered from all of the peanuts with pod rot symptoms, but a high population of the spiral nematode, Hellicotylenchus sp., was evident in the field. The most severely pod rotted peanuts appeared to be infected with Pythium spp. and the spiral nematode.

The three levels of pod rot; non-damaged, moderately damaged, and severely damaged were based on visual symptoms of shell necrosis (Fig. 1). These categories were established immediately before inoculation. Pod rot became much more severe at each pod rot level during the incubation period following inoculation with A. flavus conidia (Fig. 2). A minimum of 10 days was required for colonization by A. flavus to become apparent.

Mean colonization of A. flavus increased as pod rot severity increased under conditions that favor colonization and growth of A. flavus (Table 1). Mean toxin levels in the peanuts that were relatively non-damaged or moderately damaged by pod rot fungi were not significantly different. Peanuts severely damaged by pod rot before inoculation showed significantly more toxin accumulation than did those peanuts that were either non-damaged or moderately damaged with pod rot.

Digging Time or Maturity Effects

Optimum maturity occurred on 10/24 according to the number of days

after planting, pod color, size, and firmness. Digging when more pods were immature and late digging when pods were overmature resulted in higher percentages of colonized peanuts compared to digging at optimum maturity of the tolerant line UF71206 (Table 1). Heavy rainfall occurred 2 and 3 days prior to digging on 9/26. These peanut pods appeared to contain more water although moisture content of the seeds was not monitored during this experiment.

Shelled Versus In-Shell Storage

Peanuts of the tolerant line UF70101 stored in-shell were still tolerant to colonization after 1 year (Table 3). Those peanuts which were shelled before storage apparently lost their tolerance after 1 year.

Figure 1. One replication each of the non-damaged, moderately damaged, and severely damaged levels of pod rot as shown from left to right, before inoculation with A. flavus.

Figure 2. One replication each of the non-damaged, moderately damaged, and severely damaged levels of pod rot as shown from left to right seven days after inoculation with A. flavus.

Table 1. Mean colonization percentages by A. flavus (N.R.R.L. 2999) on the five replications of each level of pod rot

Level of pod rot	Mean % colonization 1/	Mean toxin level 1/
Non-damaged	19 a	22.0 ppm a
Moderately damaged	28 b	19.0 ppm a
Severely damaged	53 c	55.0 ppm b

1/ All mean per cent colonization values and mean toxin level values followed by the same letter are not significantly different at the 5.0% level by Duncan's new multiple-range test.

Table 2. Colonization by A. flavus (N.R.R.L. 2999) of the highly tolerant line UF71206 harvested on five different maturity dates.

Harvest dates	Mean colonization	Mean arcsin 1/
9/6	45.1	42.2 b
9/26	85.5	67.6 c
10/17	32.4	34.7 a
10/24	29.3	32.8 a
10/31	39.2	38.8 b

1/ All mean arcsin values followed by the same letter are not significantly different at the 5.0% level by Duncan's new multiple-range test.

Table 3. Colonization by A. flavus (N.R.R.L. 2999) of the tolerant line UF70101 stored for one year shelled versus being stored for one year in-shell

Stored shelled		Stored in-shell	
Mean % colonization	Arcsin mean 1/	Mean % colonization	Arcsin mean
84.0	66.4 a	18.1	25.2 b

1/ Arcsin means followed by the same letter are not significantly different at the 5.0% level by the lsd test.

DISCUSSION

Severe pod rot, caused by Pythium spp. and the nematode Hellicotylenchus sp. predisposed Florunner peanuts to greater susceptibility to colonization by A. flavus. The aflatoxin concentration in those peanuts with initially greater amounts of pod rot was also greater. Less aflatoxin was found in the moderately damaged peanuts compared to the non-damaged peanuts. These results could have been due to experimental error or because of a competitive interaction between A. flavus and other fungi present on those pods (11). Severely damaged peanuts were infected by greater numbers of nematodes than were moderately damaged peanuts. The greater number of nematodes might have affected the interaction mentioned above. In the latter case, heavy nematode damage may have provided the necessary wound allowing A. flavus to readily penetrate the peanut shell and testa before a competitive interaction could be established by other fungi. If these suggestions are correct, pod rots resulting primarily from severe nematode infections would be expected to lead to higher levels of aflatoxin contamination under natural situations than those caused primarily by fungi.

Data for the maturity test indicated that early as well as late digging lead to increased levels of A. flavus colonization as compared to digging at or close to optimum maturity. Environmental conditions can lead to the breakdown of a tolerant breeding line into a susceptible breeding line as shown by the average colonization noted for the 9/26 digging date. Heavy rainfall occurred 2 and 3 days prior to

digging at this date. Water content of the peanuts at digging time appears to greatly influence the expression of tolerance for this and possibly the other tolerant breeding lines.

The results of the storage experiment indicate that tolerance is lost after 1 year of storage of shelled peanuts compared with non-shelled peanuts of the same tolerant genotype. Storage environment is apparently capable of affecting the tolerance level exhibited by these tolerant genotypes.

Although the ability of certain peanut genotypes to minimize A. flavus colonization may be genetically determined, the environment can greatly influence the final outcome of the tolerance expression.

Part III - Appendix No. 3

Electrolyte Leakage Studies of Peanuts from Tolerant and Susceptible Lines.

INTRODUCTION

It has been shown that certain peanut genotypes express a tolerance to colonization by isolates of A. flavus capable of producing aflatoxin (55,56,66). The mechanism of tolerance has been suggested as being purely mechanical (55). A number of physical factors have been suggested as influencing tolerance. Peanut seed coats of tolerant cultivars have been suggested to be less permeable than those of susceptible cultivars. The thickness of the palisade-like layers, the size and arrangement of cells within these layers and the size and shape of the hila has been shown to affect the expression of tolerance among peanut cultivars (86). From a study of the structure of peanut seed coats, Deickert et al. (23) demonstrated the unique position of strands found within seed coats of tolerant cultivars. These strands were reported to block fungus hyphae from complete penetration through the seed coat. If the seed coat of peanuts of tolerant cultivars is indeed less permeable and serves as a physical barrier to fungal germ tube penetration, then electrolyte leakage should be less for peanuts of these cultivars compared with peanuts of susceptible cultivars.

MATERIALS AND METHODS

Electrolyte leakage from peanuts of line UF71513 (tolerant) was compared with peanuts of line UF711441 (susceptible). Colonization levels were determined by a screening study using the inoculation technique described previously (page 20). The average colonization levels for peanuts of these lines was 3.7 and 90.3 per cent, respectively.

All peanuts used in this study were hand shelled and carefully examined for physical seed coat damage prior to the test. Three replications weighing 4.3 g each of ten shelled seed were adjusted ca. 9 per cent water content. All replications were placed separately in clean, sterile, 50 ml beakers and 30 ml of sterile distilled water was added to each beaker. Conductivity of the water was measured periodically on a model Y.S.I. conductivity meter. Zero time interval readings were taken immediately after the water was added to each replication. All samples were placed on a shaker at 1500 R.P.M. and held at 10 C. Recording time was less than 5 minutes for each time interval.

RESULTS

The low temperature incubation was employed to retard possible microbial growth, while the shaker was used to evenly distribute the electrolytes being released. There was no difference in results when the shaker was not used.

Average conductivity readings were calculated for each time interval (Fig. 1).

Electrolytes were released much more rapidly by peanuts of the susceptible line than by peanuts of the tolerant line. The lowest rate of electrolyte leakage took place after 11 hours for both breeding lines, while the maximum rate took place between the 0 and 1 hour interval. The second highest rate of electrolyte leakage occurred between the 10 and 11 hour intervals, just before the lowest rate of release occurred.

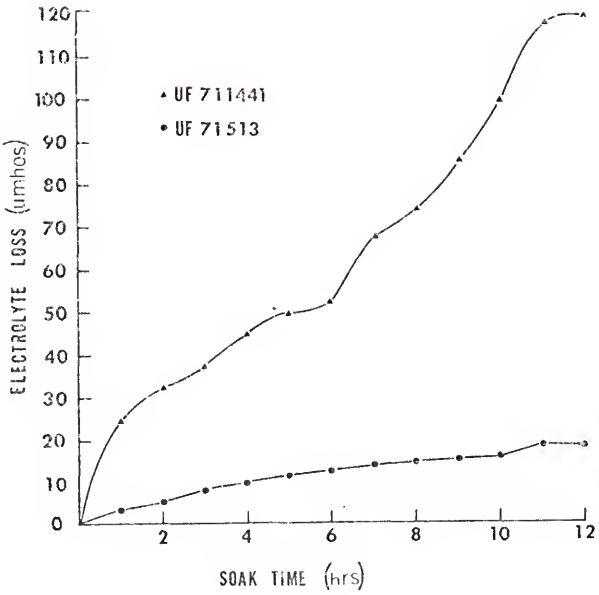

Figure 1. Mean electrolyte leakage (micro-mhos) for three replications of 10 seed each of the tolerant line UF71513 versus the susceptible line UF711441 monitored at hourly intervals of water soak time

DISCUSSION

Peanuts of the susceptible line UF711441 released electrolytes much more readily than the tolerant line UF71513. The difference in electrolyte leakage between the two breeding lines was considered to be due to the permeability characteristics of their seed coats. These permeability differences could be caused by differences in seed coat structure previously noted by other investigators (23,55, 56,86).

This technique might be used to screen peanut genotypes for tolerance to A. flavus colonization. One of the advantages of this technique is its rapidity, since no more than 48 hours would be required to complete the test. No inoculation is necessary, since the fungus is not used in determining electrolyte leakage characteristics. One disadvantage in using this technique for screening breeding lines is that all tolerant lines would be expected to have a similar mechanical tolerance mechanism. The limitations in their usefulness would remain as previously pointed out (page 35-36).

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BIOGRAPHICAL SKETCH

Jesse Cobb LaPrade was born in Bainbridge, Georgia, on April 1, 1941. Before school age, his family moved to Chatham, Virginia. He completed twelve years of school at Chatham High and was graduated in 1960. He attended Virginia Polytechnic Institute and received the degree of Bachelor of Science in ornamental horticulture in 1966. In 1968, he received the Master of Science degree at North Carolina State University with a major in plant pathology and a minor in botany. From 1968 to 1970, he was employed by the Extension Service and was Assistant County Agent in Collier County, Florida. In 1970, he began studies toward the degree of Doctor of Philosophy at the University of Florida. He is a member of the American Phytopathological Society, Alpha Zeta, Phi Sigma, and Phi Kappa Phi. He has accepted the position of Assistant Professor with Clemson University at the Pee Dee Experiment Station in Florence, South Carolina.

Jesse C. LaPrade is married to the former June Starling Davis of Fort Myers, Florida, currently employed by the Production Credit Association of Gainesville, Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jerry A. Bartz
Chairman
Assistant Professor of
Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Herbert H. Luke
Professor of Plant
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas A. Kucharek
Assistant Professor of
Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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