THE EFFECTS OF THREE SOIL-INHABITING FUNGI ON PEANUTS GROWN UNDER GERM-FREE CONDITIONS

by

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INTRODUCTION

Interactions among soil microorganisms and growing plants are varied. Certain microorganisms, such as nitrogen-fixing bacteria and mycorrhizal fungi, enter into a state of commensalism with roots and help supply nutrients to the plant. On the other hand, microorganisms may compete with each other and with roots of plants for essential substrates, or be actively parasitic on plant roots. It is, therefore, necessary to study the interactions among pathogens, other microorganisms, and plant roots to gain better understanding of disease development and plant growth. Also, it has been suggested that saprophytic microorganisms are capable of limiting the activity of plant pathogens (4, 15).

Reviews by Starkey (34), Katznelson, Lochhead, and Timonin (23), Timonin (35), and Clark (8) show that much is known about effects of plant roots on microorganisms. There are many publications concerning the effect of pathogens on roots, but little is known of effects of other rhizosphere organisms, the so-called saprophytes, on plant development or on disease development. Therefore, it is important to study the interactions of fungi in the rhizosphere of peanuts as a prelude to study of possible biological control of soil-borne pathogens of peanuts.

One area of research which has received an increasing

amount of attention is plant gnotobiology, the biology of plants grown under conditions in which unwanted forms of life are excluded from the environment of the plants under investigation. The purpose of this study was to determine the relationship of the 3 soil-inhabiting fungi, Aspergillus flavus Link ex Fries, Streptomyces griseus (Krainsky) Waksman and Henrici, and Trichoderma sp. to germ-free peanut seedlings, and to study effects of the 3 fungi introduced individually and in combinations to the root zone. Before studying the relationships of peanuts with soil-borne fungi, it was necessary to produce germ-free peanut seedlings. This required development of special techniques, which in turn involved the determination of the nutritional requirements of peanut embryos.

REVIEW OF LITERATURE

It was pointed out by Garrett (15) and Baker and Snyder (4) that the term rhizosphere was defined as the zone of soil influenced by the roots. A greater number of microorganisms were found in this region than in soil beyond the rhizosphere. Starkey (34) showed the rhizosphere of plants varied quantitatively and qualitatively from species to species. Numbers of microorganisms in the rhizosphere increased with the age of the plant and were affected by the temperature and rainfall.

Evans and Poole (11) were the first to report on fungi associated with peanut seeds and shells. Fusarium sp., Rhizoctonia sp., Botrytis sp., Pythium sp., Sclerotium bataticola Taub, and S. rolfsii Sacc. were the predominant fungi. They also mentioned that the peanut was a source of perpetuation and means of dissemination for fungi that were parasitic on many other crops.

Rankin (31) reported that fungi were parasitic inside peanut seeds. He also observed that several soil-inhabiting fungi, particularly S. bataticola and Rhizoctonia sp. penetrated the shells of the peanut during wet conditions. Higgins (18) stated that Rhizoctonia sp., Penicillium sp. and Rhizopus sp. were the common fungi isolated from the interior of peanut seeds. Crosier (9) reported on fungi in shipments of peanut seed. He noted

that only Fusarium sp. were highly pathogenic. Norton, Menon, and Flangas (30) reported that immature, unblemished Spanish peanuts were attacked by fungi. Diener (10) summarized the knowledge of peanuts in storage and used the term "seed-inhabiting fungi."

Ashworth, Langley, and Thames (3) showed that the use of seed infected by R. solani Kuhn led to serious stand depletions resulting from pre- and post-emergence death of plants. Jackson (20) stated, "Seed-borne species of A. niger van Tieghem and A. flavus were reported to cause field diseases. Planting seed infected with A. niger resulted in serious stand reduction due to pre-emergence rotting and Aspergillus crown rot." Norton, Menon, and Flangas (30) found that A. niger and A. flavus were abundant in peanut seed 6 weeks before maturity.

Other fungi frequently associated with peanut seed were reported to cause diseases, but the relationships between infected seed and development of the diseases in the field were not clear. None of the many species of Penicillium, Trichoderma, and Alternaria reported to be present in and on peanut seed have been shown to be the cause of a disease (20).

Garren and coworkers (12, 13, 14) stated that fungi were dominant in sound pods in early-season and invaded rotted pods in later-season. Trichoderma sp. was important

in both series from mid-season on. Fusarium sp. were possibly the only stable components of both series. In 1966, he reported that in Virginia, Trichoderma viride Pers. ex Fr. seemed a dominant and Penicillium sp. were sub-dominants of sound and rotting peanut pods. A. flavus and A. niger had a potential for causing damage, but they were minor and persistent. From his pathogenicity tests, he suggested the presence of a stage in which "some yet unidentified factors weaken the pods' natural barrier to invasion by rot-causing fungi so that pods are susceptible to several good competitive saprophytes from the geocarposposphere."

The presence of the specific fungi in peanut seed as related to the germination and growth of plants from infected seed has been studied in only a few cases. The fact that germinating seed and emerging seedlings were subject to attack by soil-borne fungi was established, but the question of the roles of seed-infecting fungi in peanut disease development in the field had not been completely solved (12).

Nilsson (29) stated, "In investigations concerning relations between plants and microflora in soil which are being carried on, it has on several occasions been proved necessary to apply techniques for cultivation of higher plants that permits the production of plant cultures entirely free from microbes. Such a technique is

indispensable, for example, in the study of the factors of plant origin which cause rhizosphere effects. Furthermore, the germ-free condition is desirable in studies of the influence of certain types of microbes on plants."

A number of investigations have shown that under axenic or gnotobiotic environment, microorganisms affect root respiration rate, morphology and water uptake. Khan (26) was the first to study the pathogenicity of R. solani on germ-free citrus seedlings. Sweet orange seedlings damped-off within 10 to 15 days after inoculation. Harley and Waid (17) indicated that microorganisms cause discoloration, loss of turgidity and reduced water uptake and induced changes in root morphology of intact roots growing in nutrient solution under germ-free environment. Lindsey (28) grew beans, corn, and tomatoes in a germ-free environment and compared them with those grown in non-germ-free conditions. Germ-free dwarf tomatoes grown in flexible film isolators and in an environment reinfested with Trichoderma sp. and Chaetomium sp. were significantly taller than those kept germ-free. The dry weights per plant were similar. Conversely, plants grown in an environment reinfested with F. roseum Link em. Snyder and Hansen and R. nigricans Ehr. had significantly greater dry weights per plant than the germ-free controls, but were similar in size.

Kerr (24) showed that Sclerotinia homeocarpa Bennett did not infect tomato, beet, radish, lettuce, subterranean clover, and wheat roots, but it produced substances which retarded germination and seedling growth of these plants.

Bowen and Rovira (5, 6) showed that the growth of roots and root hairs of subterranean clover grown in sand and agar was reduced by the presence of a general rhizosphere microflora. Tomato, Phalaris, and radiate pine were grown with a complete plant-nutrient solution in sterile treatments. There was a tendency for an increase in the concentrations of secondary roots with the non-sterile plants. Under the test conditions only radiate pine grown in sterile sand produced significantly greater top growth than those grown in the presence of microorganisms.

High populations of microorganisms and the increased microbial interactions--competitive, antagonistic and beneficial--could be especially important for soil-borne pathogens. Pathogens must penetrate the rhizosphere to initiate infection; however, the population of the rhizosphere is composed mainly of non-pathogenic microorganisms. These biological interactions might lead to the suppression of the pathogens, or they might even increase the effectiveness of the pathogen (1).

Rishbeth (32) reported failure of Fomes annosus (Fr.) Cooke to grow in the root zone of pine where T. viride

was established. Gregory et al. (16) observed that damping-off caused by Pythium could be controlled with strains of Trichoderma and Streptomyces. According to Alexander (1), selected actinomycetes reduced the severity of root rot of wheat caused by Helminthosporium sativum Pamm., King and Bakke. Kerr (25) reported that the root rot-Fusarium wilt complex of peas was caused by Pythium sp. and Fusarium oxysporum Snyder and Hansen. Either fungus alone produced only slight symptoms, but together they produced typical symptoms of Fusarium wilt.

The use of inocula of antibiotic-producing strains has been shown to control seed and seedling infecting pathogens. Alexander (1) cited successful work in Russia in controlling the Fusarium damping-off of Scots-pine seedlings by treating the seeds with antibiotic-forming bacteria. Wright (37) reported that Pythium infection of white mustard seeds was controlled by dusting the seeds with spores of Trichoderma viride and other fungi.

MATERIALS AND METHODS

I. The Microorganisms Associated with Peanuts

In order to study the microorganisms associated with peanuts, it was necessary to make a preliminary study of the microorganism associated with pods. The following method was employed in this experiment.

One gram each of shells, seed coats, cotyledons, and embryos of fresh peanuts (Virginia Runner variety) were aseptically ground in 99 ml sterile water in a Waring blendor for 3 minutes. Ten ml of each suspension were aseptically pipetted into a 90 ml sterile water blank, and successive 10-fold dilutions were made to 10^{-7} g/ml. One ml aliquots of each dilution were transferred into 2 kinds of unsolidified agar plates.

(A) Synthetic acid agar (35)

Czapek-dox + 0.5% yeast extract acidified with phosphoric acid to pH 4.0.

(B) Jensen's agar (22)

2.0g dextrose, 0.2g casein (dissolved in 10 ml of 0.1 NaOH), 0.5g $MgSO_4$, trace $FeCl_3$, 15g agar, and 1 liter distilled water adjusted with NaOH or HCl to pH 6.5 to 6.6.

One ml samples of each dilution were applied to each of 3 plates. The plates were incubated at room temperature for 7 days. Fungi, actinomycetes, and bacteria were counted.

Each different fungus and actinomycete culture was isolated and preserved for identification.

II. The Production of Germ-free Peanut Seedlings

Early attempts to produce germ-free seedlings by single surface sterilization of seed were unsuccessful; therefore, an experiment on chemical treatment to eliminate internal bacterial contamination was instituted.

Dried, unstained peanut seeds were washed in 1% NaOCl for 5 minutes and rinsed 3 times with sterile, distilled water. Then they were treated with the following chemicals:

(a) 0.05% Hyamine

(b) 0.125% Roccal (Benzalkonium chloride)

(c) 10, 25, and 50% NaOCl

(d) 0.1% $HgCl_2$

(e) 0.01% streptomycin

(f) 1 and 2% dimethylsulfoxide (DMSO)

(g) Mixture I (0.025% Hyamine + 1% DMSO)

(h) Mixture II (0.06% Roccal + 1% DMSO)

(i) Mixture III (0.01% streptomycin + 1% DMSO)

(j) Mixture IV (0.025% Hyamine + 0.01% streptomycin + 1% DMSO)

(k) 2% peracetic acid

Peanut seeds were soaked in each of the above chemicals for 5, 10, 15, 25, and 30 minutes, then plated on

potato-dextrose-yeast agar (PDY) plates to check for sterility and germination. Because none of the above procedures were successful, it was decided to attempt to grow plants from excised embryos in a sterile environment.

Seed from 4 varieties--Florigiant, 61-R, N.C.-2, and N.C.-47--were cut across the cotyledons with a razor blade. The cotyledons were carefully separated by hand and the embryo removed with forceps. Embryos were soaked in 1% NaOCl for 5 minutes and then rinsed with sterile water and plated on PDY plates in order to check sterility and germination before transplanting on solid media.

III. Embryo Nutrition Studies

The dissected embryos were nutritionally deficient; therefore, a study of embryo nutrition was conducted.

Modified Hoagland and Arnon's solution (19) was combined with different carbon and nitrogen sources, growth factors and rooting media to find the proper combination of nutrients. These different materials are listed as follows:

(A) Basic mineral elements (Sol. A)

1. 435.0 ppm K_2SO_4
2. 12.6 ppm $Ca(H_2PO_4)_2$
3. 270.0 ppm $CaSO_4$
4. 484.0 ppm $MgSO_4$
5. trace FeEDTA plus minor elements (Zn, Mo, B, Mn, Cu, and Cl)

(B) Carbon sources

1. 0.5%, 1.0%, and 2.0% glucose
2. 0.5%, 1.0%, and 2.0% sucrose

(C) Nitrogen sources

1. 500 ppm, 1,000 ppm, and 4,000 ppm KNO_3
2. 570 ppm, 1,140 ppm, and 4,560 ppm $(NH_4)_2SO_4$
3. 320 ppm, 640 ppm, and 2,500 ppm $(NH_2)_2CO$

(D) Growth promoting chemicals

1. 0.003 ppm, 0.03 ppm, and 0.3 ppm gibberellic acid
2. 0.003 ppm, 0.03 ppm, and 0.3 ppm kinetin

(E) Growing media

1. Agar
2. Vermiculite
3. Weblite (expanded granular shale)

Excised peanut embryos were first transferred onto PDY plates. Then after 7 days they were again transferred to 9-inch sterile test tubes containing approximately 30 mm of one of the solid media (E) moistened with Sol. A and different combinations of (B), (C), and (D).

The tubes were placed under continuous illumination with fluorescent lights. The cotton plugs were covered with aluminum foil to reduce evaporation. Data on growth of the embryos were collected after 30 days.

IV. Effects of Introducing 3 Soil-inhabiting Fungi on Germ-free Peanut Seedlings

Excised peanut embryos were sterilized by treatment with 1% NaOCl for 5 minutes and placed in 250-ml flasks containing 110g Weblite moistened with 85 ml nutrient solution. Nutrients consisted of Sol. A, 500 ppm KNO_3, 1% sucrose, and 0.03 ppm gibberellic acid. Plants were grown 20 days in flasks at room temperature under continuous illumination. When the plants were 2 to 3 inches tall, a 0.5 ml suspension of a mixture of spores and mycelia was placed in each flask in the root zone. The suspension was obtained by rinsing the surface of a single 7-day-old slant with 20 ml distilled water. Inoculations were replicated 5 to 9 times. A. flavus, S. griseus, and Trichoderma sp. were used singly and in all possible combinations as inoculum.

Attempts to use the dry weight of plants as an index of pathogenesis caused by the introduction of fungi were not satisfactory because of callus tissue formation around the cotyledon scars and fungal mycelium that had invaded the plant organs. These factors contributed more to the plants' dry weight than did the roots and shoots of the plants.

This method of evaluation of pathogenesis was discarded and a scale was employed. On the scale, 1 represented the most severe pathogenesis--death within 15 days

after inoculation--and 5 represented the least pathogenesis--a healthy appearance and good growth 15 days after inoculation. See Fig. 1.

After inoculation, the peanut seedlings were observed daily. The grading system for pathogenesis was as follows:

1--killed 15 days after inoculation

2--killed 20 days after inoculation

3--leaves necrotic 20 days after inoculation

4--growth retarded 20 days after inoculation

5--the best growth 20 days after inoculation

V. Histological Studies

Histological studies were made to determine the relationship of each of the microorganisms with the peanut seedlings. Roots were fixed in Navaschin's solution, dehydrated in tertiary butyl alcohol series, sectioned 10 μ thick on a rotary microtome and stained with safranin and fast green (33).

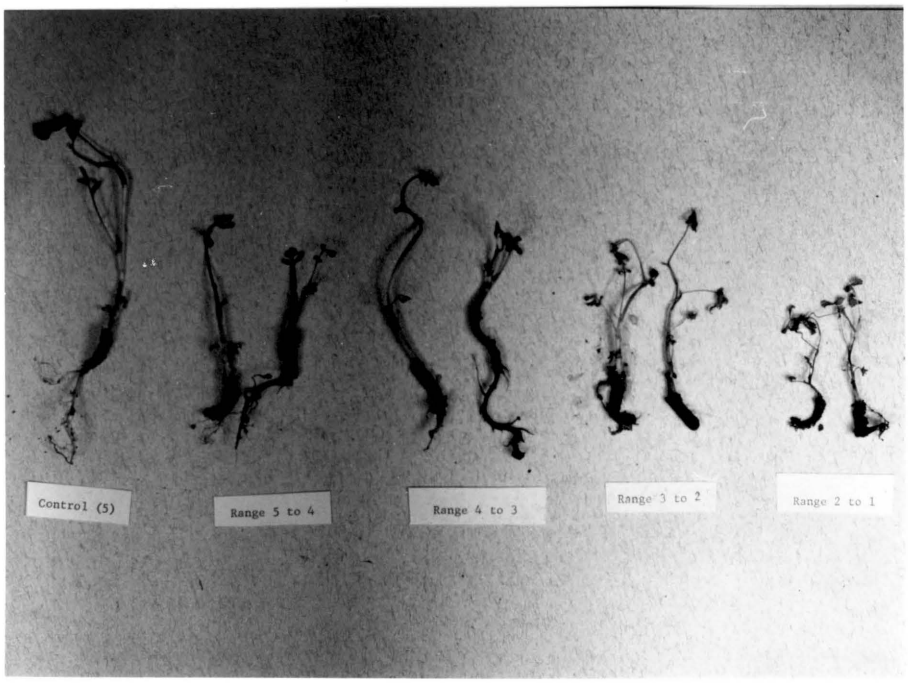


Fig. 1. Relative plant damage to peanuts inoculated with certain soil-inhabiting fungi. The degree of injury is used as a plant disease index scale of 1 to 5, based on injury 15 and 20 days after inoculation.

RESULTS

I. The Microorganisms Associated with Peanut Pods

The counts shown in Table 1 reveal that microorganisms were found in all parts of peanut pods.

Fifteen fungi were isolated and maintained in pure culture. They were identified to genus as follows: *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Mucor* sp., *Trichoderma* sp., *Alternaria* sp., and sterile mycelia. The cultures of actinomycetes were identified as 10 different strains of *S. griseus*.

A relatively high population of actinomycetes (Table 1) was observed on the shells, seed coats, and even on cotyledons; however, the observation was not previously reported in the literature.

II. The Production of Germ-free Peanut Seedlings

Despite the use of all reported methods of germ-free culture, 4 months of experimentation with seeds sterilized by chemical methods and with stem cuttings planted under conditions of proven sterility had failed to produce germ-free seedlings. This was due to bacterial contamination of the cotyledons.

Later, from 70 to 90% germ-free plants were obtained by using excised peanut embryos treated with 1% NaOCl and grown under controlled aseptic conditions. The percentages of germ-free peanut seedlings obtained from the different

Table 1. Frequency of microorganisms isolated from fresh peanuts

Part sampled	Media used	Microorganism frequency per one gram fresh tissue*		
		Bacteria	Fungi	Actinomycetes
Shells	SAA**	5.0×10^7	2.5×10^7	0.0
	JA***	1.0×10^7	0.0	1.0×10^7
Seed coats	SAA	2.0×10^3	1.0×10^3	0.0
	JA	6.5×10^3	0.0	1.0×10^3
Cotyledons	SAA	0.0	0.0	0.0
	JA	2.0×10^2	0.0	1.0×10^2

* The average number of microorganisms per sample calculated from the average of the number of plates.
** SAA = Synthetic acid agar.
*** JA = Jensen's agar.

treatments are listed in Table 2.

III. Embryo Nutrition Studies

In experiment I (Table 3) and experiment II (Table 4), the peanut roots were poorly developed; however, it was shown that 0.03 ppm gibberellic acid treatment promoted greater growth than that of the untreated control, and 1% sucrose resulted in further improvement of growth. In searching for a growth medium, experiment III, 0.8% agar was first tried as a medium to support the growth of peanut embryos. Since poor rooting resulted, vermiculite and Weblite were tried. Better results were obtained with Weblite (growth index was 4.1) than with vermiculite (growth index was 3.5). In experiment IV (Table 5), 1% sucrose and 0.03 ppm gibberellic acid and Weblite were used with 3 forms of nitrogen, KNO_3, $(NH_4)_2SO_4$, and $(NH_2)_2CO$, in different concentrations. The nitrogen concentration between 0.005N to 0.04N (KNO_3: 500 to 4,000 ppm, $(NH_4)_2SO_4$: 570 to 4,560 ppm, $(NH_2)_2CO$: 320 to 2,500 ppm) was found suitable to grow the peanut seedlings. When the concentration was above 0.01N, the peanut seedlings did not grow, except that the seedlings did grow in 0.04N KNO_3. The growth indices in all 3 experiments are shown in Tables 3 to 5.

Using the information from the preceding experiments, 1% sucrose, 0.03 ppm gibberellic acid and Weblite were used as the basic components in combination with 3 nitrogen

Table 2. Percentage of sterile peanut seedlings obtained by seed treatment and excised peanut embryos

Chemicals	Time of treatment	Percentages of sterile seedlings* Peanut varieties Florigiant	61-R	N.C.-2	N.C.-47
Seed treatment					
Hyamine	5-30 min	0	0	0	0
Roccal	5-30 min	0	0	0	0
$HgCl_2$	5-30 min	0	0	0	0
NaOCl	5-30 min	0	0	0	0
Streptomycin	5-30 min	0	0	0	0
DMSO	5-30 min	0	0	0	0
Mixture I	5-30 min	0	0	0	0
Mixture II	5-30 min	0	0	0	0
Mixture III	5-30 min	0	0	0	0
Mixture IV	5-30 min	0	0	0	0
Peracetic acid	5-30 min	0	0	0	0
Excised Embryos					
NaOCl	5 min	90	85	70	75

* Each treatment with 10 replications.

Table 3. Effect of gibberellic acid and kinetin at various concentrations on the elongation of peanut embryos

Basic components: Solution A, 500 ppm KNO_3, and 5% agar

	Chemicals		Growth index* (mean of 6 replicates)
	Gibberellic acid	Kinetin	
1)	0.03 ppm	0.03 ppm	1.36
2)	0.03 ppm	0.30 ppm	2.61
3)	0.03 ppm	3.00 ppm	1.83
4)	0.03 ppm		3.66
5)	0.30 ppm	0.03 ppm	1.36
6)	0.30 ppm	0.30 ppm	1.83
7)	0.30 ppm	3.00 ppm	2.50
8)	0.30 ppm		1.36
9)	3.00 ppm	0.03 ppm	2.00
10)	3.00 ppm	0.30 ppm	1.66
11)	3.00 ppm	3.00 ppm	1.83
12)	3.00 ppm		1.66
13)		0.03 ppm	2.36
14)		0.30 ppm	2.40
15)		3.00 ppm	1.80
16)	Check		1.36

* Growth Index:
1--no growth
2--0.1 mm to 0.5 cm above hypocotyl
3--0.5 cm to 1.0 cm above hypocotyl
4--1.0 cm to 2.0 cm above hypocotyl
5--above 2.0 cm of hypocotyl

Table 4. Effect of different combinations and concentrations of 2 carbon sources and 3 nitrogen sources on elongation of peanut embryos

Basic components: Solution A and 5% agar

Carbon source		Nitrogen source			Growth index* (mean of 5 replicates)
Glucose	Sucrose	KNO_3	$(NH_4)_2SO_4$	$(NH_2)_2CO$	
1) 0.5%		1,000 ppm			2.2
2) 1.0%		1,000 ppm			2.2
3) 2.0%		1,000 ppm			1.2
4)	0.5%	1,000 ppm			2.2
5)	1.0%	1,000 ppm			4.0
6)	2.0%	1,000 ppm			2.6
7)	2.0%		570 ppm		1.2
8)	2.0%		1,140 ppm		2.0
9)	2.0%		4,560 ppm		1.0
10)	2.0%			320 ppm	2.6
11)	2.0%			640 ppm	1.0
12)	2.0%			2,500 ppm	1.0
13) Check					1.0

* Growth index:
1--no growth
2--0.1 mm to 0.5 cm above hypocotyl
3--0.5 cm to 1.0 cm above hypocotyl
4--1.0 cm to 2.0 cm above hypocotyl
5--above 2.0 cm of hypocotyl

Table 5. Effect of different concentrations of 3 nitrogen sources on elongation of peanut embryos

Basic components: Solution A, 1% sucrose, 0.03 ppm gibberellic acid and Weblite

	Nitrogen source			Growth index* (mean of 5 replicates)
	KNO_3	$(NH_4)_2SO_4$	$(NH_2)_2CO$	
1)	500 ppm			4.5
2)	1,000 ppm			3.5
3)	4,000 ppm			3.0
4)		570 ppm		4.0
5)		1,140 ppm		2.0
6)		4,560 ppm		1.0
7)			320 ppm	4.0
8)			640 ppm	1.0
9)			2,500 ppm	1.0
10)	Check			2.0

* Growth index:
1--no growth
2--0.1 mm to 0.5 cm above hypocotyl
3--0.5 cm to 1.0 cm above hypocotyl
4--1.0 cm to 2.0 cm above hypocotyl
5--above 2.0 cm of hypocotyl

sources to determine an optimum nutrient medium for the peanut embryos. The nitrogen sources were used at 0.005N concentration. This experiment included 16 treatments with 5 replications of each treatment. The results are found in Tables 6 and 7.

As shown by the data in Table 6, each of the 3 forms of nitrogen combined with sucrose and gibberellic acid significantly increased elongation, but potassium nitrate medium yielded the longest length of seedling. There were no differences among the other treatments. From the elongation measurement, the best medium was found to be 0.05N nitrogen source, 1% sucrose and 0.03 ppm gibberellic acid.

As seen from Table 7, there were no differences among the treatments which included nitrogen plus sucrose,or nitrogen plus sucrose plus gibberellic acid. The ammonium sulfate medium yielded the greatest dry weight of seedling especially in the presence of 1% sucrose. From the dry weight measurements, ammonium sulfate was the preferable nitrogen source for peanut embryos. Gibberellic acid did not increase the dry weight of seedlings when 1% sucrose was combined with any 3 forms of nitrogen in the medium.

From the results of embryo nutrition studies, 500 ppm KNO_3, 1% sucrose, and 0.03 ppm gibberellic acid were used as the standard components in the medium used in the studies on pathogenesis. Although $(NH_4)_2SO_4$ yielded the greatest

Table 6. Comparison of 3 forms of nitrogen combined with 1% sucrose and 0.03 ppm gibberellic acid to support growth measured as elongation of excised peanut embryos*

Basic components: Solution A and Weblite

Treatments**	Elongation (cm)					Average	MRT***
	1	2	3	4	5		
Check	8.5	6.3	3.9	3.6	3.2	5.10	b
G	12.0	9.0	5.5	1.7	1.0	6.48	b
S	12.0	7.5	6.5	4.6	2.3	6.58	b
G + S	10.2	8.5	4.0	3.3	3.2	5.84	b
K	8.1	5.5	4.0	3.3	3.1	4.80	b
K + G	8.5	6.5	4.8	6.0	4.8	6.12	b
K + S	8.0	7.5	5.0	4.0	1.0	5.10	b
K + G + S	11.5	10.0	11.5	11.0	6.5	10.10	a
U	8.0	7.5	7.2	7.4	1.8	6.38	b
U + G	7.3	6.9	6.4	6.2	2.5	5.86	b
U + S	10.5	5.7	4.5	2.3	2.5	5.10	b
U + G + S	13.0	10.0	9.6	9.5	1.2	8.66	a
A	5.8	5.7	2.4	2.3	2.2	3.68	b
A + G	8.9	5.3	4.5	4.0	1.2	4.78	b
A + S	9.0	7.0	6.0	3.0	3.0	5.60	b
A + G + S	14.8	12.5	10.0	10.5	2.2	10.00	a

* The analysis of variance is in Appendix Table I.

** G--0.03 ppm gibberellic acid, S--1.0% sucrose, K--500 ppm KNO_3, U--320 ppm $(NH_2)_2CO$, and A--570 ppm $(NH_4)_2SO_4$.

*** Duncan's multiple range test (Based on single nitrogen source) (27)
a--8.00 to 10.00
b--3.68 to 6.38

Table 7. Comparison of 3 forms of nitrogen combined with 1% sucrose and 0.03 ppm gibberellic acid to support growth measured as dry weight of excised peanut embryos*

Basic components: Solution A and Weblite

Treatments**	Dry weight (cm)					Average	MRT***
	1	2	3	4	5		
Check	18	17	16	14	14	15.8	c
G	24	23	21	19	14	20.2	b,c
S	32	34	24	22	21	26.6	b
G + S	38	34	28	25	24	29.8	a
K	22	21	19	15	15	18.4	c
K + G	22	18	16	22	15	18.6	c
K + S	25	24	22	21	16	21.6	b
K + G + S	30	27	25	22	20	24.8	b
U	23	17	17	16	14	17.4	c
U + G	28	23	18	17	17	20.6	b,c
U + S	30	29	22	18	15	22.8	b
U + G + S	32	23	22	21	10	21.6	b
A	24	20	19	18	18	19.0	b,c
A + G	26	25	20	18	16	21.0	b
A + S	34	32	29	24	20	27.8	a
A + G + S	43	34	34	26	18	31.0	a

* The analysis of variance is in Appendix Table II.

** G--0.03 ppm gibberellic acid, S--1.0% sucrose, K--500 ppm KNO_3, U--320 ppm $(NH_2)_2CO$, and A--570 ppm $(NH_4)_2SO_4$.

*** Duncan's multiple range test (Based on single nitrogen source) (27)
a--above 27.0
b--19.0 to 24.8
c--20.0 to 17.4

dry weight, it was not used as the nitrogen source in the growth medium for pathogenesis studies; KNO_3 was chosen as the sole nitrogen source because it supported the best appearance of growth of peanut seedlings.

IV. Effect of 3 Soil-inhabiting Fungi on Germ-free Peanut Seedlings.

The first indication of the effect of A. flavus or Trichoderma sp. on peanut seedlings was a discoloration of the lower leaflets. A. flavus affected the main branches which became chlorotic and wilted, and finally the plant was killed. Plants died within 10 to 17 days after inoculation with A. flavus. Chlorosis and wilting caused by Trichoderma sp. was slower, and plants were killed 20 days after inoculation. The roots of affected plants were decayed and fungi had colonized below the hypocotyl, especially around the cotyledon scar. Black or dark brown discoloration was seen below the hypocotyl. The plants inoculated with S. griseus alone had better root systems and larger leaves than the control plants.

Table 8 shows the results of 4 experiments repeated on different dates. It shows plants inoculated with S. griseus alone were no different from the check plants. The plants inoculated with either A. flavus or Trichoderma sp. were variable. According to the multiple range test, they were divided into 3 groups. Only the control and

Table 8. Response of germ-free peanut seedlings within 15 to 20 days after inoculation by 3 soil-inhabiting fungi

Treatments	Exp. I In.*	MRT**	Exp. II In.	MRT	Exp. III In.	MRT	Exp. IV In.	MRT
Check	4.83	a	4.60	a	4.54	a	4.70	a
A. flavus	2.84	b	2.40	b,c	2.28	c	1.80	c
S. griseus	4.56	a	4.40	a	4.67	a	4.90	a
Trichoderma	3.00	b	3.00	b	1.83	c	2.80	b
A+S	2.73	b	1.80	c	2.11	c	1.80	c
A+T	2.78	b	2.40	b,c	2.00	c	2.00	b,c
S+T	2.51	b	1.80	c	2.22	c	2.40	b,c
A+S+T	3.22	b	2.20	b,c	3.00	b	2.80	b
F values	7.91***		9.81***		27.56***		20.18***	

* In. = Growth index
5--the best growth
4--leaves smaller than #5
3--plants chlorotic and necrotic
2--plants killed within 20 days after inoculation
1--plants killed within 15 days after inoculation.

** MRT = Duncan's multiple range test (27)
a--growth index between 5.00 to 4.00
b--growth index between 3.22 to 2.00
c--growth index between 2.40 to 1.80.

*** Significant of treatments at 1% level.

S. griseus-inoculated plants belonged to group a. The other combinations belonged to groups b and c; however, there was no clear-cut distinction between these 2 groups. On the average, inoculum containing 3 fungi caused less damage than any combination of 2.

All 3 soil-inhabiting fungi were reisolated from inoculated plants.

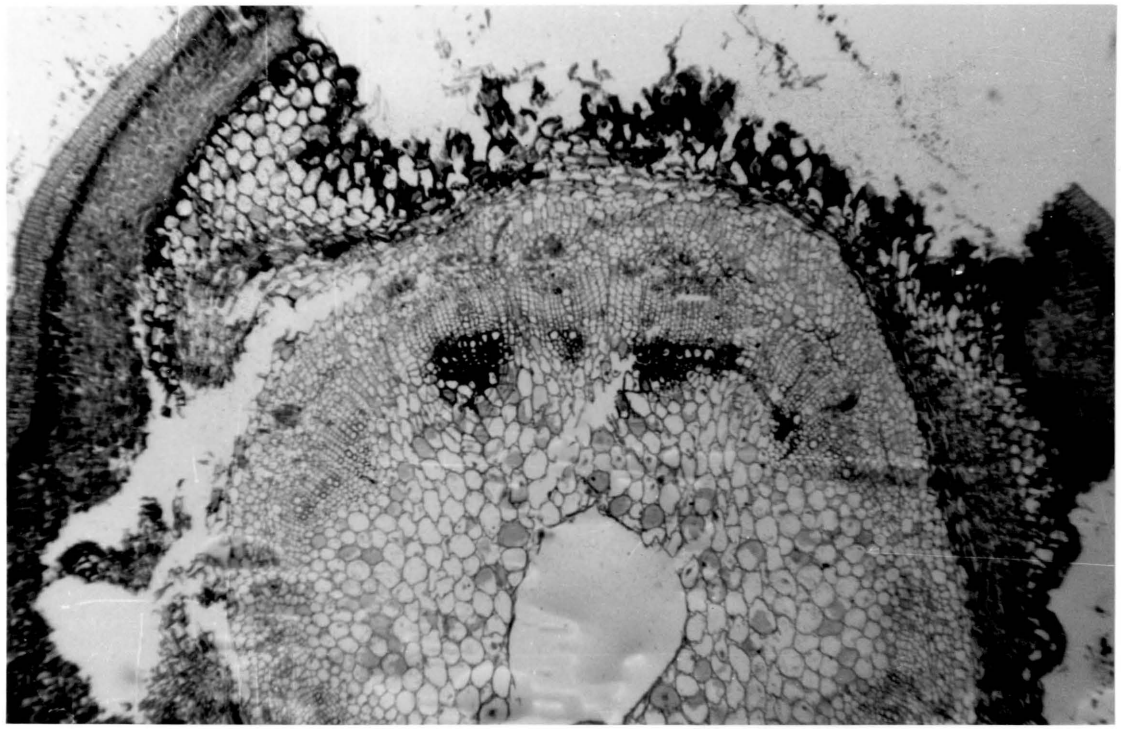
V. Histological Studies

Figure 2 shows the seedlings grown from the excised embryos. The wound and the cotyledon residues on the hypocotyl were vulnerable to penetration by the fungi. All 3 fungi attacked the root surface first, then penetrated into the cortex. The cotyledon residues had the highest density of fungal mycelia (Fig. 3).

Figures 2, 3, 4, 5, 6, 7, and 8 show that A. flavus or Trichoderma sp., either alone or in combination, penetrated the peanut roots and caused the brown discolorations. The fungi invaded the epidermis, attacked the cell walls, and eventually killed the plants. The hyphae of A. flavus were 5 to 8 μ in diameter outside of the plant tissue; the invading hyphae were 3 to 5 μ. The hyphae of Trichoderma sp. were 4 to 6 μ; the invading hyphae were 2 to 3 μ.

Microscopic examination of roots inoculated with S. griseus did not show this pattern of attack. The root surface remained white and smooth. However, S. griseus

(C)



(C)

Fig. 2. Cross section of cotyledon scars of seedlings grown from excised embryo showing Trichoderma mycelia colonizing through wounds. Note the food-rich cotyledon (C) residue which offers a very favorable food base for the fungus (100x).

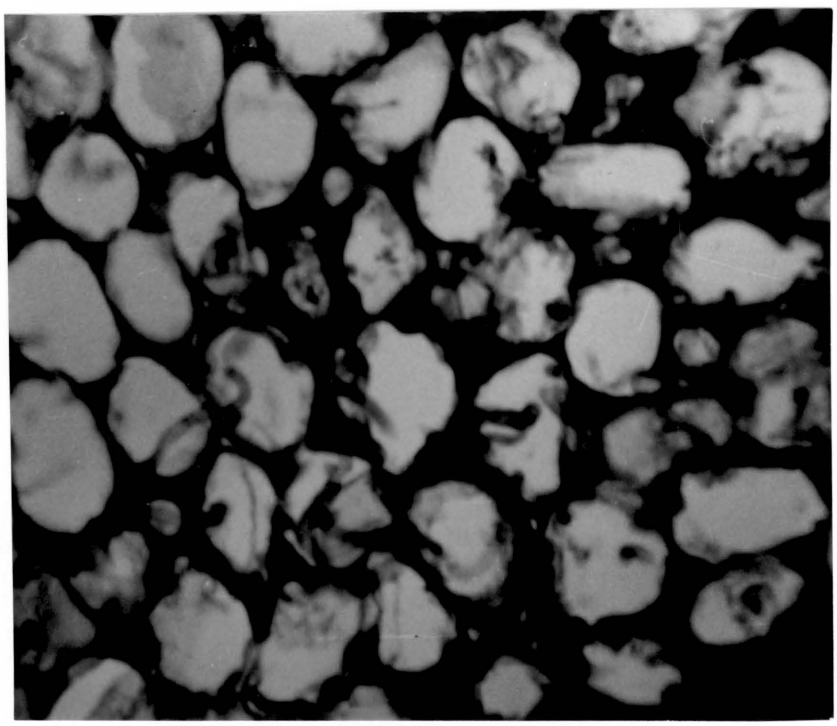


Fig. 3. A. flavus invading the cotyledon scars of peanut seedlings (450x).

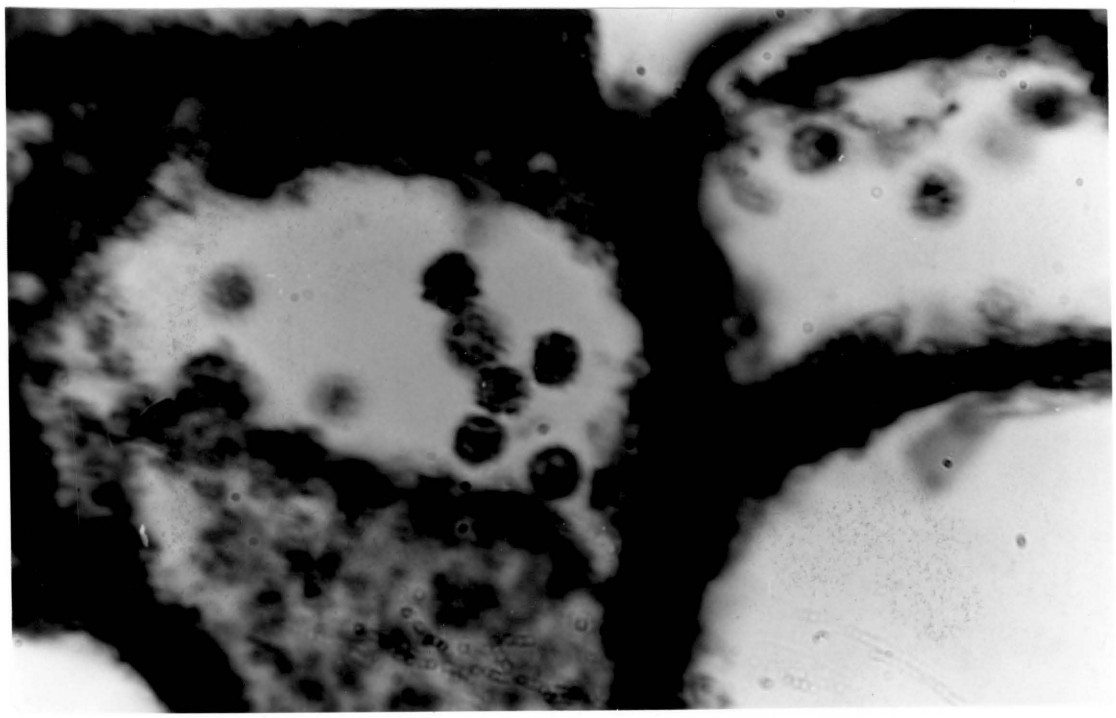
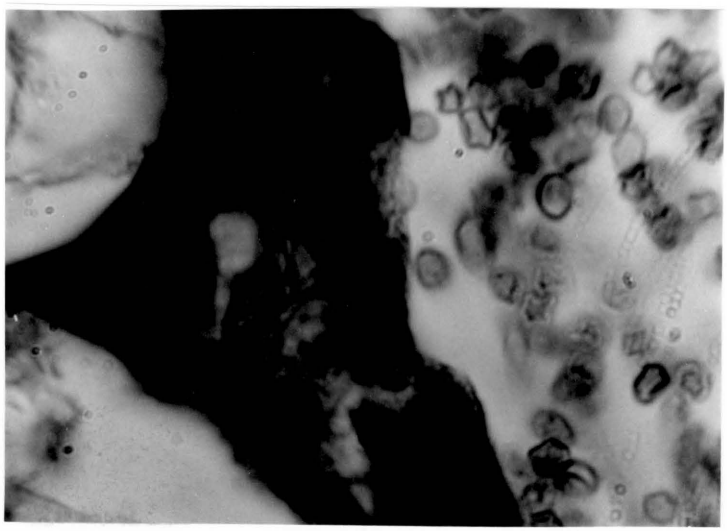
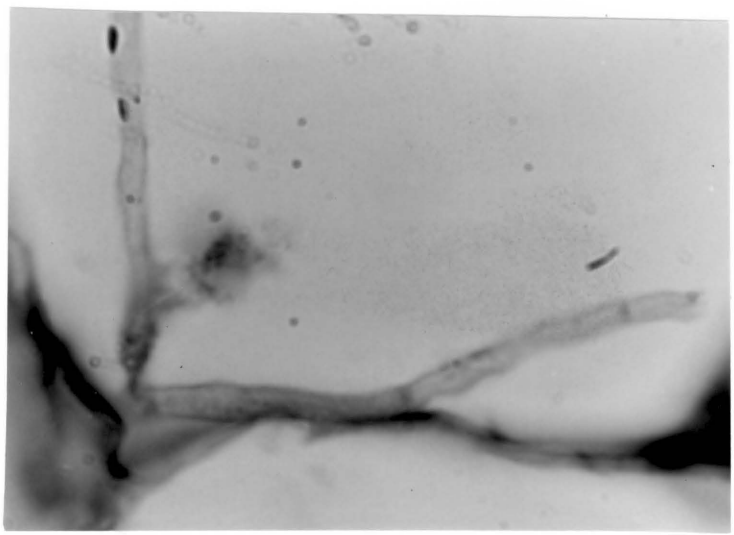


Fig. 4. A. flavus spores in the collenchyma cells of hypocotyl (900x).

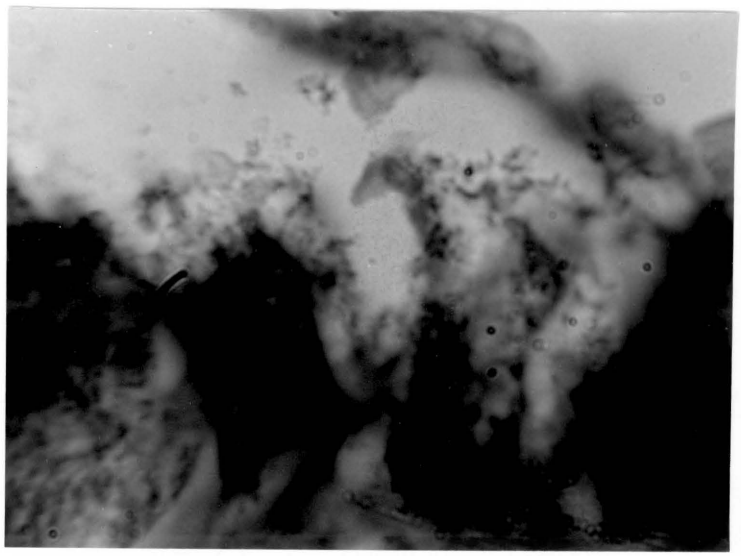


(a)

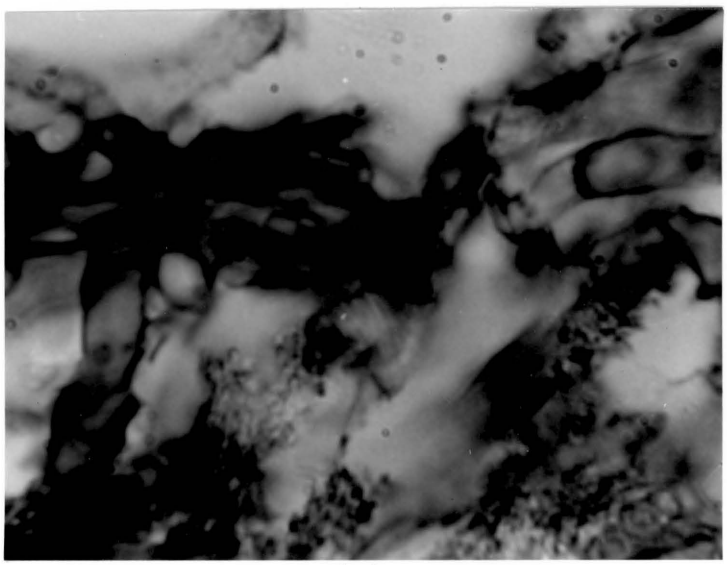


(b)

Fig. 5. Trichoderma sp. (a) sporulating at wound surface where the cotyledons were excised showing the penetration into the collenchyma cells, and (b) penetrating into the deep layer of collenchyma cells (900x).



(a)



(b)

Fig. 6. Fungus mycelia (a) penetrating the cotyledon residues, and (b) growing on the surface of peanuts inoculated with a mixed inoculum of A. flavus, S. griseus, and Trichoderma (900x).

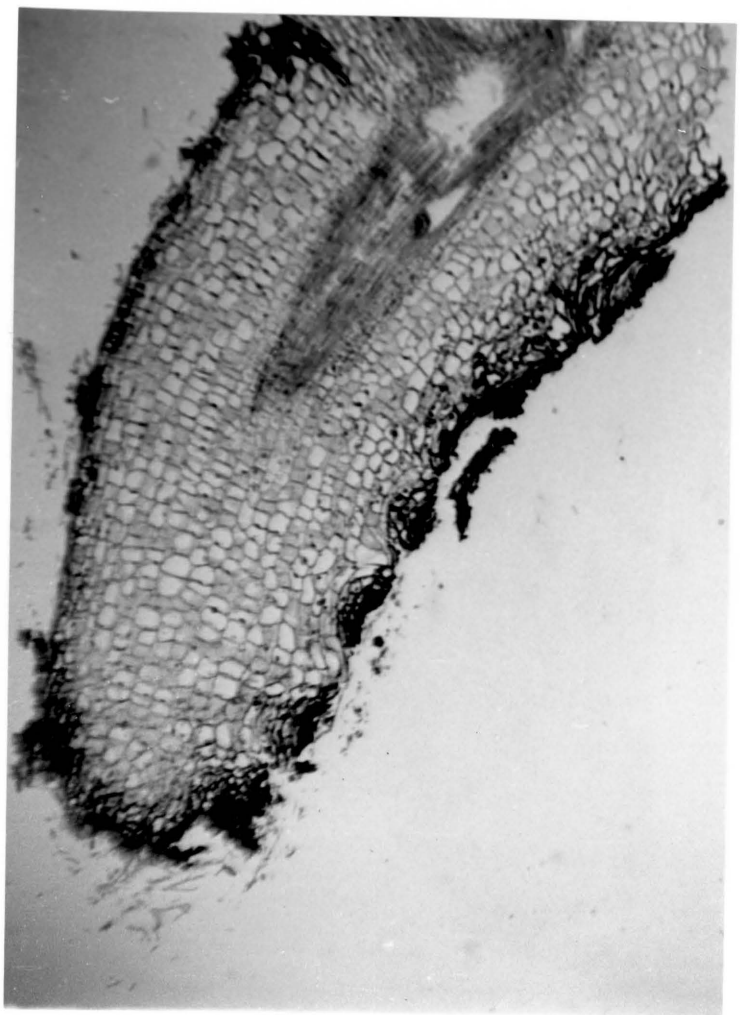


Fig. 7. Trichoderma sp. invading the peanut seedling root tip. Note the pattern of colonization in discrete colonies along the root surface (100x).

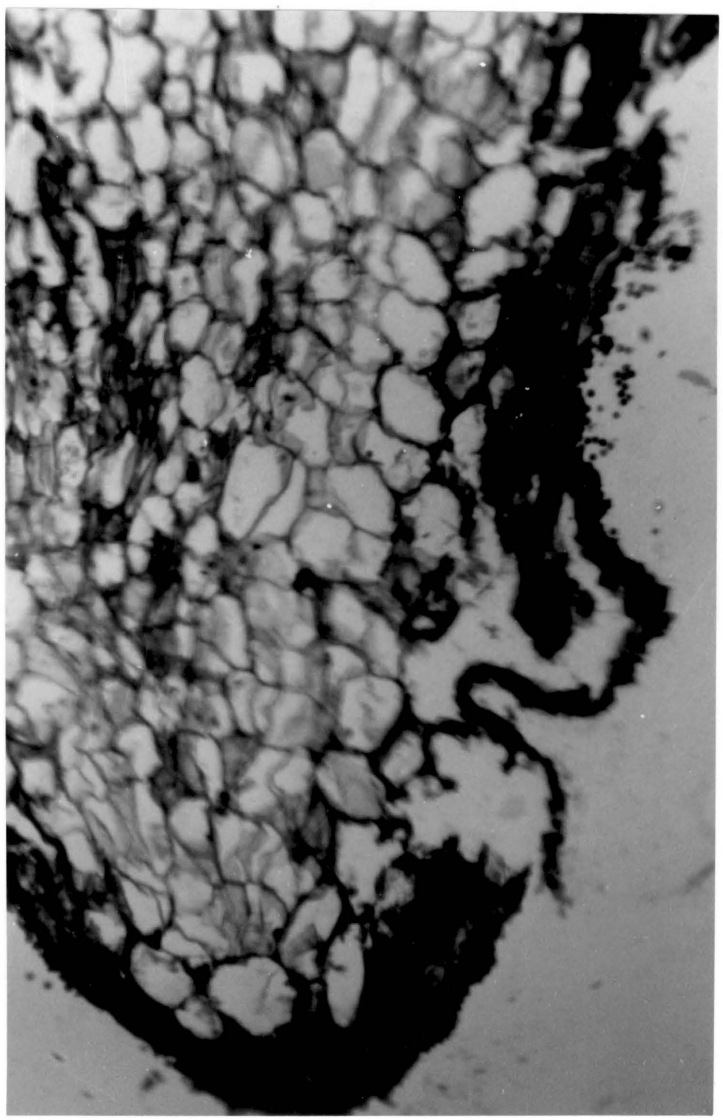


Fig. 8. A. flavus invading the root tip of peanut seedlings (450x).

attacked the surface of the plant and then penetrated into the cortex of the hypocotyl and roots as shown by microscopic observation. This is shown in Fig. 6. Nevertheless, only a few colonizations by Streptomyces on both the root and hypocotyl surfaces were observed.

DISCUSSION

In 1961, aflatoxin produced by A. flavus was observed as a contaminant of peanut meal (2). Since then many studies have been made of the effects of associated microflora on the production of peanuts. However, knowledge relating to the effects of microorganisms on peanut growth and pod formation is limited.

Soil-inhabiting fungi and seed-borne bacteria invading peanut seeds have been isolated and observed by many researchers, but no report has been found on the effect of actinomycetes on peanuts. In October of 1966, a relatively high frequency of actinomycetes on peanut shells, seed coats, and even on kernels was observed. All 10 identified actinomycetes were 10 strains of S. griseus. According to Bergey's Manual of Determinative Bacteriology (7), "Different strains of this organism produce different antibiotics. One of these, streptomycin, is active against a large number of bacteria and actinomycetes but not against fungi and virus." The finding of high populations of S. griseus associated with peanut pods influenced the remaining research.

Germ-free biological research has recently received great attention beyond medical applications; however, no

means are presently available for obtaining plant material free of seed-borne bacteria. Excising embryos, as was done in this study, seems the only presently available way to obtain peanuts free of seed-borne bacteria.

In the study of embryo nutrition, a medium composed of 1% sucrose, 0.005N nitrogen source and 0.03 ppm gibberellic acid could be substituted for the carbon, nitrogen, and growth-promoting chemicals of cotyledons in order to quickly grow seedlings to normal size. As seen from Appendix Table I, sucrose and gibberellic acid were 2 main factors influencing the elongation of peanut seedlings; however, there was no interaction between the 2 factors in elongation of the plants. Nitrogen alone did not significantly increase peanut seedling elongation, but did so when in any combination, either with sucrose or with gibberellic acid. The same problem also showed on the dry weight measurement (Appendix Table II). Statistical analysis showed all 3 factors increased dry weight of peanut seedlings, but no interaction was found between sucrose and gibberellic acid or sucrose and nitrogen although interaction between nitrogen and gibberellic acid was significant at 5% level. The interaction among nutritional factors needs further study. The relatively high F values of 2 replications from these 2 measurements might be caused by some uncontrolled and unrecognized factors from the environment or by lack of

growth uniformity of this peanut variety, Florigiant. This problem needs further consideration.

From the pathogenicity studies, it was found that S. griseus alone had a saprophytic relationship with peanut roots and even stimulated plant growth; however, when S. griseus was combined with Trichoderma sp. and introduced on peanuts, it acted synergistically to cause damage on plants. On the other hand, it did not increase the pathogenesis when it was introduced with A. flavus. Because of the multiple interactions of S. griseus with other organisms, further studies on the interrelationship between the soil-inhabiting microorganisms are urgently needed. S. griseus seems a possible microorganism to control the soil pathogens of peanut plants, if further information about the interactions of S. griseus and other organisms is obtained.

When 1% sucrose was included in the medium, A. flavus and Trichoderma sp. were individually pathogenic to the seedlings grown from excised embryos. They invaded the roots and finally killed the plant. However, the lack of increased pathogenesis when combinations of these fungi were introduced, suggested the possibility of competition between the 2 fungi. The antagonism of these 2 fungi might be caused by Trichoderma sp. alone. It had been reported many times that T. viride could control pathogenic fungi (1, 11, 35). It was found that the pathogenesis caused by

introducing A. flavus and S. griseus was due to A. flavus alone. Therefore, there was no synergism or antagonism between them.

Inoculations with A. flavus, S. griseus, and Trichoderma sp. caused less damage than inoculations in paired combinations, but the difference was not significant at the 5% level. There was little doubt that the reduced pathogenesis was due to the antagonistic behavior of either S. griseus or Trichoderma sp. in the inoculum containing these 3 fungi.

It seems probable that pathogenesis caused by inoculations of A. flavus, S. griseus, and Trichoderma sp. (either singly or in combination) would vary in accordance with the stage of growth of the plant at the time of inoculation, probably due to the degree of healing of the abscission zone of the cotyledon scars. In addition, sucrose, which was introduced in this study as a nutrient substitute for the carbon source of cotyledons, is not normally found in the habitat of peanut plants. This was another factor beneficial to fungi in this study. It accelerated the growth of fungi in the vicinity of peanut seedlings and also the pathogenesis to the plant.

It is important to have more information on the soil-inhabiting microorganisms in their relationship to plant growth. With this information, the soil-borne plant

pathogens may be controlled by means of altering the rhizosphere microorganism population, quantitatively and qualitatively, in order to render the host roots inimical to pathogens.

SUMMARY

The investigations were conducted on the frequency of microorganisms associated with fresh peanut pods, the production of germ-free peanut seedlings, growth of the plants under germ-free conditions, and the effects of 3 soil-inhabiting fungi on the germ-free seedlings under gnotobiotic conditions.

The frequency of microorganisms in 1g fresh peanut tissues was between 10^2 and 10^7. Bacteria occurred in the highest density, and actinomycetes followed next. There were no fungi associated with fresh, clean peanut cotyledons. Ten actinomycetes were identified as strains of S. griseus. The fungi were identified as to genus. They were Penicillium, Aspergillus, Fusarium, Alternaria, Trichoderma, Mucor, and miscellaneous fungi.

Several attempts to produce germ-free plants by chemical treatment of the peanut seeds failed. In an effort to produce the plants free from microorganisms, seedlings were grown from excised embryos treated with 1% NaOCl for 5 minutes. Then the treated embryos were removed and planted on potato-dextrose-yeast agar plates for checking the sterility and germination of the embryos. Seven days later, the germ-free plants were placed in 250-ml flasks containing nutrients and solid medium. Agar and vermiculite failed to permit proper root development of seedlings.

Only expanded granular shale (Weblite) permitted the growth of healthy plants. Hoagland's solution amended with 0.005N KNO_3, 1% sucrose and 0.03 ppm gibberellic acid provided the best nourishment.

When the plants were 2 to 3 inches tall, the flasks were inoculated in the root zone singly and in all combinations with A. flavus, S. griseus, and Trichoderma sp. All 3 organisms invaded the root tissue. When compared with uninoculated check plants, S. griseus individually caused no measurable damage, but in combination with either of the other 2 fungi it caused stunting and chlorosis. A. flavus alone killed plants within 15 days after inoculation. Trichoderma sp. singly killed plants within 20 days; however, in combinations it killed plants within 15 days. Inoculations of plants with all 3 fungi in combination caused less damage than any combination of 2 organisms.

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Recognition is given to of the University of North Carolina for the identification of the actinomycetes and to the author's committee members, , , and , for their service.

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APPENDIX

Appendix Table I. Analysis of variance for treatments of comparison of 3 forms of nitrogen combined with 1% sucrose and 0.03 ppm gibberellic acid to support growth measured as elongation of excised peanut embryos†

Analysis of variance for treatments

Source of variation	Degree of freedom	Sum of squares	Mean squares	F
Replications	4	457.49	114.37	45.93**
Treatments	15	252.84	16.85	6.77**
Sucrose	1	65.98	64.98	26.10**
Gibberellin	1	69.00	69.00	27.71**
Nitrogen	3	7.21	2.40	--
S x G	1	28.68	28.68	11.52**
G x N	3	30.30	10.10	4.06*
S x N	3	27.27	9.09	3.65*
S x G x N	3	25.40	8.47	3.40*
Error	60	149.56	2.49	
Total	79	859.89		

† The details of experimental results are in the text, Table 7.
* Significant at 5% level.
** Significant at 1% level.

Appendix Table II. Analysis of variance for treatments of comparison of 3 forms of nitrogen combined with 1% sucrose and 0.03 ppm gibberellic acid to support growth measured as dry weight of excised peanut embryos†

Analysis of variance for treatments

Source of variation	Degree of freedom	Sum of squares	Mean of squares	F
Replications	4	1308.88	327.22	46.74**
Treatments	15	1515.99	101.07	14.44**
Sucrose	1	945.31	945.31	135.04**
Gibberellin	1	63.01	63.01	9.00**
Nitrogen	3	227.84	75.95	10.85**
S x G	1	2.12	2.12	--
G x N	3	178.34	59.45	8.49**
S x N	3	30.44	10.15	1.45
S x N x G	3	68.93	22.98	3.28*
Error	60	420.32	7.00	
Total	79	3245.19		

† The details of experimental results are in the text, Table 8.
* Significant at 5% level.
** Significant at 1% level.

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THE EFFECTS OF THREE SOIL-INHABITING FUNGI ON PEANUTS GROWN UNDER GERM-FREE CONDITIONS

by

Chen-hong Ting Chang

ABSTRACT

Excised peanut embryos were sterilized by treatment with 1% NaOCl for 5 minutes, and placed in 250-ml flasks containing nutrients and expanded granular shale. Nutrients consisted of Hoagland's solution supplemented with 1% sucrose and 0.03 ppm gibberellic acid. Plants were grown for 4 weeks in flasks placed between tubes of fluorescent lights at room temperature.

When the plants were 2 to 3 inches tall the flasks were inoculated in the root zone singly and in all combinations with Aspergillus flavus, Streptomyces griseus, and Trichoderma sp. All 3 organisms invaded the root tissue. When compared with uninoculated check plants, S. griseus individually caused no measurable damage, but in combinations caused stunting and chlorosis. A. flavus and Trichoderma sp. alone killed plants within 15 and 20 days, respectively, after inoculation; while in combination they killed plants within 15 days. Inoculations of plants with all 3 fungi in combination caused less damage.