STUDIES ON THE PHYSIOLOGY OF CONIDIAL GERMINATION

BY

ASPERGILLUS FLAVUS

by

Ted Pass II

Thesis submitted to the Graduate Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Plant Pathology

APPROVED:

G. J. Griffin, Chairman

H. B. Couch

M. G. Hale

K. H. Garren

W. H. Wills

August, 1971

Blacksburg, Virginia

LD 5655 V856 1971 P38 c.2

STUDIES ON THE PHYSIOLOGY OF CONIDIAL GERMINATION BY ASPERGILLUS FLAVUS

by

Ted Pass II

Thesis submitted to the Graduate Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Plant Pathology

APPROVED:

G. J. Griffin, Chairman

H. B. Couch

M. G. Hale

K. H. Garren

W. H. Wills

August, 1971

Blacksburg, Virginia

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. G. J. Griffin, his major professor, under whose supervision and guidance this study was conducted. He also wishes to express his gratitude to Dr. H. B. Couch, Dr. M. G. Hale, Dr. K. H. Garren, and Dr. W. H. Wills for their helpful criticism and suggestions in the preparation of the manuscript.

Mrs. Joyce T. Sims has generously applied both her secretarial and editorial abilities to this manuscript.

The author is grateful to his wife, Ginny, for her patience, understanding, and encouragement during his graduate tenure.

The author extends his appreciation to the National Science Foundation, Plant Science Division, Agricultural Research Service, United States Department of Agriculture, and Virginia Polytechnic Institute and State University for supporting his study and research.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.	ii
LIST OF TABLES.	iv
LIST OF FIGURES	vi
INTRODUCTION.	1
LITERATURE REVIEW	3
Spore germination in axenic media.	3
Spore germination in soil.	18
MATERIALS AND METHODS	25
Conidial germination studies	25
Electron microscopy.	27
RESULTS	29
Effect of temperature and pH on germination. . . .	29
Exogenous carbon and nitrogen requirements for germination.	33
Electron Microscopy.	47
DISCUSSION.	50
SUMMARY	58
LITERATURE CITED.	60
VITA.	72

LIST OF TABLES

Table		Page
I.	Effect of temperature on percentage germination of conidia of *Aspergillus flavus* in B solution with glucose plus NH_4Cl or glucose plus peptone at 1 x 10^4 conidia/ml.	30
II.	Rate of germination of conidia of *Aspergillus flavus* in B solution with glucose plus NH_4Cl or glucose plus peptone at 1 x 10^4 conidia/ml	31
III.	Influence of pH on percentage germination of conidia of *Aspergillus flavus* in glucose plus NH_4Cl or glucose plus peptone and in two buffers at 1 x 10^4 conidia/ml.	32
IV.	Percentage germination of *Aspergillus flavus* in B solution with various carbon sources at two levels of equivalent carbon and at 1 x 10^4 conidia/ml	34
V.	Effect of growth and sporulation medium composition on the subsequent germination of conidia of *Aspergillus flavus* in doubly distilled water, B solution, or amino acid solution at 1 x 10^3 conidia/ml. . .	37
VI.	Percentage germination of conidia of *Aspergillus flavus* in B solution with glucose alone, glucose plus NH_4Cl, peptone, or an amino acid mixture supplied at constant amounts per conidium for three conidial densities	39
VII.	Percentage germination of conidia of *Aspergillus flavus* in B solution with three levels of glucose and glucose plus NH_4Cl at three conidial densities	41
VIII.	Percentage germination of conidia of *Aspergillus flavus* in B solution containing glucose with and without various nitrogen sources at equivalent nitrogen and 1 x 10^4 conidia/ml.	42

Table		Page
IX.	Percentage germination of conidia of *Aspergillus flavus* in B solution with glucose plus NH_4Cl or glucose plus an amino acid mixture in non-aerated media and media aerated with CO_2 containing air and CO_2-free air at 1×10^4 conidia/ml.	44
X.	Percentage germination of settling tower-deposited conidia of *Aspergillus flavus* in B solution and B solution with glucose plus NH_4Cl with and without surfactants at 1×10^3 conidia/ml	46

LIST OF FIGURES

Figure			Page
1.	A.	Typical germ tube of washed conidia of *Aspergillus flavus*.	49
	B.	Atypical thin germ tube of washed conidia of *Aspergillus flavus*.	49
	C.	Erlenmeyer flask apparatus used to determine influence of CO_2 on conidial germination of *Aspergillus flavus*. The inoculation tube (IT), vent tube (VT), and air supply tube (AST) are shown.	49
	D.	Electron micrograph of an ultrathin section through an ungerminated conidium of *Aspergillus flavus*. The outer hull (OH), spore wall (SW), intermediate space (IS), and spore membrane (SM) are shown.	49

INTRODUCTION

The soil-borne fungus *Aspergillus flavus* Link ex. Fries can colonize peanut (*Arachis hypogaea* L.) pods before and after harvesting (5, 7, 33, 34, 46, 89, 90, 91, 98, 105, 115, 120). The discovery in 1960 that *A. flavus* produces a class of toxins (aflatoxins) that are carcinogenic to animals (2, 6, 13, 47, 76, 96, 113, 126) has caused concern to peanut producers, processors, and health authorities in different parts of the world ever since.

Fungi generally survive in soil as dormant spores, and the initial event in the colonization of plant roots and other plant parts is the spore germination process (27, 56, 116, 117). Studies by Griffin (56, 59) have indicated that spores of *A. flavus* and *Fusarium* spp. may germinate under certain conditions within the layer (0.5 mm) of soil adjacent to peanut pegs or fruits (geocarposphere) (59) and roots (rhizosphere) (56). Germination of conidia of *A. flavus* was reported by Griffin (59) to be high in soil adjacent to fruits following mechanical injury of the shell. Knowledge of exogenous carbon and nitrogen requirements for conidial germination and the influence of pH and temperature on germination may be useful in understanding further the factors in the geocarposphere that restrict or stimulate conidial germination of *A. flavus*.

This investigation was undertaken to determine the exogenous carbon and nitrogen requirements for conidial germination and to determine the influence of pH and temperature on germination of conidia of *A. flavus*. Some aspects of the effect of surfactants and

incubation in water on conidial germination were also investigated.

REVIEW OF LITERATURE

Spore Germination in Axenic Media

There have been several studies investigating the effects of carbon and nitrogen compounds, aeration, relative humidity, temperature, and pH on growth and/or production of aflatoxins by *Aspergillus flavus* (1, 14, 29, 30, 31, 32, 40, 63, 64, 77, 93, 106, 114). The environmental factors influencing germination of *A. flavus* conidia have been neglected, however. An emphasis will be placed on these aspects as they affect fungal spore germination in the following discussion of the literature.

The physiology of spore germination has been reviewed by several workers (25, 48, 49, 122). Germination is affected by exogenous carbon and nitrogen compounds, spore density, carbon dioxide, temperature, pH, and several other factors. Investigations on carbon and nitrogen requirements for spore germination of soil fungi vary widely in their conclusions (18, 24, 54). Sisler and Cox (124) found medium-free conidia of *Fusarium roseum* grown on modified Tochinai's solution-agar required exogenous sources of carbon and nitrogen for germination. Marchant and White (85) have shown that both carbon and nitrogen sources were necessary for satisfactory germination of macroconidia of *F. culmorum* when grown on potato-sucrose agar. Griffin and Pass (60) recently demonstrated that washed macroconidia of *F. roseum* 'Sambucinum' grown on potato-dextrose agar (PDA) required an exogenous source of carbon for high germination, whereas only a partial dependence on

exogenous nitrogen was demonstrated. Similarly, high germination of washed macroconidia of F. oxysporum has been shown by Griffin (56) to be dependent on an exogenous source of carbon with only a partial dependence on nitrogen when grown on PDA-water agar. Conidia of Penicillium griseofulvum were found by Fletcher and Morton (43) to have a requirement for glucose to initiate germination. Maximum germination required the presence of nitrate and phosphate in addition to glucose. Gottlieb and Tripathi (51) have demonstrated that conidia of P. atrovenetum when grown on PDA required both exogenous carbon and nitrogen for germ tube formation. Recently, Martin and Nicolas (87) showed that exogenous carbon and nitrogen sources were necessary for the germination of washed conidia of P. notatum and Trichoderma lignorum when cultured on PDA. Cochrane et al. (24) demonstrated that in addition to an exogenous carbon and nitrogen source, macroconidia of F. solani f. sp. phaseoli required a factor in yeast extract which could be replaced by ethanol for germination when the fungus was grown on a yeast extract medium. Griffin (55), however, showed that medium-free conidia of Gliocladium fimbriatum and F. solani f. sp. phaseoli grown on PDA required only exogenous carbon and nitrogen sources for germination. Byther (18) has reported that washed macroconidia of F. solani f. sp. phaseoli when grown on PDA did not require exogenous nitrogen for complete germination when exogenous carbon was supplied as ethanol in a medium containing Mg and K salts.

The germination of conidia of A. niger (94, 149), and the sporangiospores of Rhizopus arrhizus (143, 145) and R. stolonifer (142)

have been shown to be strongly stimulated by L-proline. The conidia of *A. niger* were found to require L-proline or L-alanine, glucose and phosphate for complete germination by Yanagita (149). Miller (94) found L-proline and L-alanine could act at low temperature (21°C) as substitutes for high temperature (30°C) in stimulating germination of conidia of *A. niger*. At 30°C only glucose and citrate were required for good germination. For *R. arrhizus*, in the absence of phosphate, only L-proline proved effective in stimulating germination (145). However, in combination with phosphate, L-proline, L-ornithine, L-arginine, pipicolic acid, L-asparagine, as well as some hexose sugars were found to be stimulating. L-proline was the most stimulating of 60 compounds tested by Weber and Ogawa (145). Weber (142) earlier obtained similar results using sporangiospores of *R. stolonifer*. In addition, Weber and Ogawa (145) found L-glutamic acid to be ineffective. They felt this might be because of a block in its conversion to other amino acids. They went on to suggest that the need of proline as a sole nutritive source of *R. arrhizus* may be explained on the basis of a proline deficiency in the spore and the absence of enzymes for the conversion of glutamate to proline. Gottlieb (50) has similarly suggested that the explanation for the proline requirement in conidia of *A. niger* could be its deficiency in the spore or even its transformation to glutamate, since the formation of glutamate from proline is known (72).

The requirements for germination of spores are felt by some to be influenced by the cultural conditions present during spore formation (24, 58, 61). Grover (61) reported that the conidia of *A.*

flavus, harvested from Czapek's synthetic basal medium containing 10 amino acids, germinated in distilled water (78.7%). When one of the amino acids (i.e., β-alanine, glycine, DL-leucine, DL-methionine or L-tryptophane) was omitted from the Czapek's synthetic basal medium, the spores harvested from the media devoid of one of these five amino acids germinated poorly in distilled water (9.8 - 13.5%). Tryptophane seemed to be the most important amino acid among these, as its absence in the medium markedly affected spore germination. Also, when spores harvested from media devoid of one of these five amino acids were placed in a nutrient medium containing the deficient amino acid, normal germination was attained. Grover felt that this indicated that the presence of β-alanine, glycine, DL-leucine, DL-methionine and L-tryptophane in the Czapek synthetic basal medium used to culture the fungus seemed to be necessary for maximum spore germination in distilled water. Griffin (58) has recently shown that the carbon and nitrogen requirements for germination of chlamydospores of F. solani were dependent on nutritional conditions and the spore density at which chlamydospore formation occurred. Chlamydospores formed at high density (3.0×10^5 spores/ml) and in the presence of exogenous carbon and nitrogen required both exogenous carbon and nitrogen for high germination whereas chlamydospores at low density (3.0×10^3 spores/ml) and in the absence of exogenous carbon and nitrogen required only exogenous carbon for high germination.

Low spore densities of some fungi germinate more readily than at high spore densities (39, 43, 57, 80). The first evidence of self-

inhibition was observed by Edgerton (39) while working with conidia of Colletotrichum lindemuthianum. He noted that if there were 12 to 15 conidia or more per mm^3 of ordinary nutrient media, germination was drastically reduced as compared to the percent germination observed when there were six spores per mm^3 of medium. In 1911 Wallace et al. (141) observed that crowding had an inhibitory effect upon germination of conidia of Sclerotinia fructigena and that the inhibitory factor could be found in the water in which the spores had been suspended. Self-inhibitors have also been reported in other organisms, including conidia of Alternaria porri f. sp. solani (111), resting structures (microsclerotia) of Verticillium spp. (67) and uredospores of Puccinia graminis f. sp. tritici (3). Spores of Fusarium spp. have been reported to germinate to various degrees at low densities in the absence of exogenous carbon and nitrogen, but not at high densities (57, 58, 60, 102).

Allen (3) was the first to study the properties of self-inhibitory substances. He was able to demonstrate that the inhibitory substance produced by uredospores of P. graminis f. sp. tritici was water soluble and volatile. Forsyth (44) has indicated that trimethylethylene is the natural inhibitor produced by uredospores of P. graminis f. sp. tritici, since he found a parallel between the inhibitory activity of trimethylethylene and that of the natural inhibitor, and a similar absorption spectrum in acetone was also found. Van Sumere et al. (140) have isolated non-volatile inhibitors from uredospores of P. graminis f. sp. tritici. Of the compounds extracted with diethylether and identified by circular chromatography, the only one to cause inhibition

of germination of uredospores at concentrations ranging from 1 ppm to 400 ppm was ferulic acid. Other non-volatile compounds (aspartic and glutamic acids at 100 ppm), that inhibited the germination of uredospores of *Uromyces phaseoli*, have been described by Wilson (147). However, Bell (8) has since disputed this claim and reported that neutralized glutamic and aspartic acids did not inhibit uredospore germination, even at concentrations of 1000 ppm, whereas the neutralized inhibitor was active at 2 ppm. French (45) extracted an inhibitor from the uredospores of *P. graminis* f. sp. *tritici*. The inhibitor was not aromatic or carbohydrate in nature and was non-volatile. Woodbury and Stahmann (148) have reported that uredospores of both *P. graminis* f. sp. *tritici* and *U. phaseoli* carried film-forming materials which were released rapidly on contact with water surface. Oxidation of these materials, which was accelerated by light, resulted in the formation of products inhibitory to germination that were retained at the water surface.

In a detailed study of the self-inhibitor from uredospores of *U. phaseoli*, Bell and Daly (9) showed that the inhibitor was extractable with water and appeared in two zones on paper chromatograms. The partially purified inhibitor reduced germination 50 percent at a concentration of 2 μg/ml. Recently, Macko et al. (83) reported the isolation and identification of two germination inhibitors from water extracts of bean rust uredospores which had the properties of the self-inhibitors described earlier by Bell and Daly (9). The two germination inhibitors from uredospores of *U. phaseoli* were identified, by gas chromatography and mass spectral analysis, as the

cis and *trans* isomers of methyl 3, 4-dimethoxycinnamate. Each isomer was found to be equally toxic, and germination of 50 percent of the spores was inhibited by 5×10^{-3} µg/ml. Ettel and Halsquth (41) isolated trimethylamine from teliospores of *Tilletia caries* and showed that it was capable of inhibiting germination at low concentrations. They went on to suggest that trimethylamine was the endogenous inhibitor of smut spores. Later, Singh and Trione (123) working with bunt teliospores of *T. caries* and *T. controversa* found an inverse relation of the percentage germination to the density of teliospores per (a) unit area (b) unit volume of the medium, and the inhibition of the teliospore germination by aqueous extracts of the same teliospores. However, trimethylamine inhibited teliospore germination only at very high concentrations when the synthetic compound was tested, precluding its role as an inhibitor. Volatile compounds have also been isolated from liquid cultures and agar medium preparations of *F. oxysporum* and *R. stolonifer* by Robinson and Park (109) that are inhibitory to the germination of their own spores. The analysis of gas chromatographs and melting point determinations indicated that one of the main inhibitory factors was acetaldehyde. Robinson et al. (110) have since reported that volatile substances and a non-volatile vacuolation factor, which were produced in cultures of a wide range of fungi that inhibited spore germination. This inhibition of germination was generally lessened as the pH or the glucose content of the medium was increased.

Evidence has been presented that indicates that the nutritional requirements for spore germination may be dependent on spore density.

Farkas and Ledingham (42) showed that respiration as well as germination decreased with increased spore densities of P. *graminis* f. sp. *tritici*. They felt this was caused by the accumulation of inhibitory substances. Self-inhibition of uredospores was counteracted by both butyrate and propionate. Lingappa and Lingappa (80) reported that the percentage of germination of washed conidia of *Glomerella cingulata* decreased with increasing densities of the conidia in distilled water. At low density (30 conidia/mm^2) greater than 90% germination was obtained, whereas at high density (3000 conidia/mm^2) less than 3% germination was observed. Inhibition at high densities was nullified by the addition of 3% peptone. Glucose (7.5%), however, did not stimulate germination at the higher densities. Onurah (102) has shown that conidia of F. *culmorum* at high densities (1.0 x 10^5 conidia/ml) did not germinate in distilled water; low germination (9%) was observed by lowering the density of the conidia to 2.0 x 10^4 conidia/ml. High germination (100%) was obtained by suspending the conidia in a 1.3% sucrose solution. Griffin and Pass (60) have observed that washed macroconidia of F. *roseum* 'Sambucinum' germinated (10.0%) in the absence of exogenous carbon and nitrogen, whereas no germination was observed at higher densities. Griffin (56) has also shown that washed macroconidia of F. *oxysporum* did not germinate in the absence of exogenous carbon and nitrogen at a conidial density of 7.0 x 10^5 conidial/ml, but did germinate (3.5%) at a conidial density of 1.4 x 10^5 conidia/ml. Complete germination occurred at high conidial density for both F.

oxysporum and F. roseum 'Sambucinum' in the presence of exogenous carbon and nitrogen (56, 60). An extensive study by Griffin (57) has shown that the exogenous carbon and nitrogen requirements for the germination of washed macroconidia of F. solani were dependent on conidial density. When the density of the conidia was 3.1×10^3 conidia/ml or lower, complete germination of washed macroconidia of F. solani occurred in a phosphate buffered inorganic salts medium (pH 5.7) lacking exogenous carbon and nitrogen. As the density of the conidia was increased, percentage germination decreased. Complete dependence on exogenous carbon and partial dependence on exogenous nitrogen were first observed in the range of 1.6 to 3.2×10^5 conidia/ml. Complete dependence on both exogenous carbon and nitrogen was observed near a density of 1.0×10^6 conidia/ml. Griffin has also found a correlation between density of conidia and the amount of exogenous carbon and nitrogen required to overcome inhibition. Recently, Griffin (58) reported that chlamydospores of F. solani when formed in a high conidial density system (3.0×10^5 conidia/ml) required exogenous carbon and nitrogen for high germination but did not require exogenous nitrogen for high germination when the density of the chlamydospores was reduced from 3.0×10^5 to 3.0×10^4 spores/ml. High germination was observed at 3.0×10^3 spores/ml in the absence of both exogenous carbon and nitrogen. Chlamydospores formed in low conidial density system (3.0×10^3 conidia/ml) required only an exogenous source of carbon for high germination. Arthrospores of Geotrichum candidum have been reported by Park and Robinson (103) to germinate (86.0%) in the absence of exogenous nutrients at low

spore densities (5.7 x 10^2 spores/ml) but at high spore densities (3.5 x 10^6 spores/ml) showed self-inhibition. They did show that nutrients could overcome the self-inhibition, and also found a relationship between spore density and the amount of nutrients required to overcome the inhibition. Blakeman (11) has demonstrated that at high spore densities, self-inhibition of germination of pycnidiospores of *Mycosphaerella ligulicola* increased with rise in temperature (12°C to 27°C) at which the spores were formed. Spores that were formed at 15°C showed little self-inhibition at high spore densities, whereas spores formed at 26°C showed marked self-inhibition. Inhibition of 26°C spores was overcome by washing ten times with deionized water. Diffusates collected from dense suspensions of 26°C spores increased germination and growth of 26°C spores, but had no effect on germination of 15°C spores. However, diffusates from 15°C spores prevented germination of 26°C spores but not 15°C spores where growth of germ tubes was increased. The inhibitory substance from 15°C spores was not readily volatile and not affected by high temperature in solution. Volatile inhibitors were not detected from either 15°C or 26°C spores. He concluded the inhibitor causing self-inhibition of 26°C spores was different in nature from that produced by 15°C spores. These effects of spore density suggest that auto-inhibitors of spore germination may be produced by *Fusarium* spp. and other fungi which are overcome by exogenous carbon and nitrogen.

Carbon dioxide concentration is known to affect conidial germination of a number of fungi, but the optimum concentration for germination varies from species to species (15, 24, 43, 62, 136, 139,

146, 149).

Brown (15) found that the germination of conidia of Botrytis cinera was completely inhibited by CO_2 concentrations of 20% and above, whereas in turnip extract a similar inhibitory effect was noticed only at a CO_2 concentration of 30% or higher. Rippel and Bortels (108) reported in 1927 that the swelling and sprouting of the conidia of A. niger were completely inhibited by passing CO_2-free air into the culture flasks. Yanagita (149) confirmed Rippel and Bortels' work in 1957. He, too, was able to show that in the absence of CO_2, both swelling and germ tube formation of conidia of A. niger were completely inhibited while being cultured in L-proline medium for 9 hours. A similar response to the absence of CO_2 has been reported for basidiospores of Schizophyllum commune (62). Germination of conidia of A. nidulans (136) and P. griseofulvum (43) has been found to be significantly reduced when CO_2-free air was bubbled through static liquid cultures which contained glucose as the sole carbon source. The germination of macroconidia of F. solani f. sp. phaseoli was shown by Cochrane et al. (24) to be significantly accelerated by removal of metabolic CO_2 from the atmosphere after 5 and 6.5 hours of incubation, but the final percentage germination was not affected.

Fixation of CO_2 by germinating fungus spores has been demonstrated in Aspergillus spp. (12, 23, 66, 100, 137, 150), B. cinera (75), F. culmorum (86), Puccinia recondita (129), and U. phaseoli (127, 128, 129).

Yanagita (150) demonstrated that the incorporation of CO_2 in A. niger occurs in both germinating and dormant conidia. When conidia were placed in phosphate buffer, most of the incorporated ^{14}C was found in the trichloroacetic acid soluble fraction in which ATP was revealed to be the major substance labeled. Soluble protein and nucleic acid were also labeled within a short period of time (45 min). Studies carried out earlier by Hoshino et al. (66) on ^{14}C-alanine metabolism in the early phase of germination of conidia of A. niger showed that the alanine taken up was instantly deaminated to give ammonia and pyruvate, which in turn was decarboxylated gradually with the formation of CO_2. Their pattern of labelling was quite similar to the labelling observed in $^{14}CO_2$ fixation experiment reported later by Yanagita (150). Bloom and Johnson (12) have indicated that the CO_2 fixation product in conidia of A. niger was oxaloacetate. Tsay et al. (137) investigated the low molecular weight products derived from CO_2 fixation by conidia of A. oryzae. Their results indicated that CO_2 was fixed into dicarboxylic acids such as malate (via oxaloacetate) and that glutamic acid derived from such dicarboxylic acids may be one of the key substances synthesized at the initial phase of germination.

Carbon dioxide fixation by conidia of B. cinera was relatively slow during the initial stage of germination; fixation increased as time of incubation increased (75). Most of the labelling was detected in amino acids (68%) and organic acids (30%) at the end of germination. Staples and Weinstein (129) have reported on the dark fixation of carbon dioxide by uredospores P. recondita and U. phaseoli.

^{14}C in malate was only in the carboxyl carbons, the fixation was presumed to proceed by the reaction of carbon dioxide with phosphoenolpyruvate to form oxaloacetate, then to malate. However, Rick and Mirocha (107) found malic enzyme in uredospores of U. phaseoli. Malic enzyme catalyzed the formation of pyruvic acid from L-malic acid and synthesized malic acid from pyruvic acid and CO_2. The enzyme was found to be NADP specific and dependent on manganous ions for activity. Weber (144) reported that $^{14}CO_2$ was incorporated by spores of R. arrhizus into amino acids with the following distribution: unknown B (46%), arginine (31%), aspartic acid (20%), and glutamic acid (2%). Unknown B was found to react with ninhydrin and pisatin. Infra-red spectra and gas chromatographic-mass spectrometry analyses indicated unknown B was a modified hydroxyproline.

Temperature has been found to not only affect percentage germination of conidia (2, 19, 149), but the time required for germination (18, 60), and the rate at which germ tube elongation takes place. Chi and Hanson, (19), using two isolates of F. solani, found that both isolates germinated over the range of 12° to 36°C, reaching maximum germination (100%) at 28°C. Byther (18) found that the optimum temperature for conidial germination of both F. solani f. sp. phaseoli and F. roseum f. sp. cerealis was 25°C. He also showed that conidia of F. roseum were more tolerant than conidia of F. solani to lower temperatures, and that conidia of either species did not germinate at 35°C. Conidia of A. niger were shown by Yanagita (149) to germinate (90%) in about 9 hours at 30°C, and germination

was suppressed (less than 50%) at 33°C, but not inhibited.

Another limiting factor for conidial germination is the pH of the environment, though it is felt by Gottlieb (48) and Cochrane (25), that only under extremely acid or alkaline conditions, is pH a natural limiting factor for spore germination. The effects of pH on conidial germination has been studied by several workers. Chi and Hanson (19), working with F. solani found that macroconidia germinated well over the pH range investigated (2.4 to 10.0), with maximum germination (96 to 97%) occurring at pH 5.0 to 6.0. Macroconidia of F. roseum f. sp. cerealis and F. solani f. sp. phaseoli were both shown by Byther (18) to have maximum germination (100%) at pH 6.0 in a sodium phosphate buffer supplemented with Czapek's Dox broth. Yanagita (149) demonstrated that conidial germination of A. niger was optimal (83%) at pH 6.2 and that a pH of 7.5 markedly retarded germination (6.0%) in phosphate buffer. Gottlieb and Tripathi (51) found that conidia of P. atrovenetum germinated over a range of 2 to 12, with maximum germination (90%) occurring between pH of 4.0 to 7.5. Martin and Nicolas (87) reported that the optimal pH range for germination for conidia of both P. notatum and T. lignorum was between 5 and 6 in a citrate-phosphate buffer.

The conidial cell wall of A. niger has been examined by means of electron microscopy. Tsukahara et al. (138) found the conidia cell walls of A. niger to be composed of an outer hull, an inner spore wall, an intermediate space and spore membrane. They found in some ultrathin sections of A. niger conidia, separation of the

outer hull from the spore wall. Further, during the stage of swelling, the hull disappears and the spore wall becomes uncovered prior to spore germination. They feel that since it appears that the outer hull loosely covers the inner spore wall which lies directly beneath the hull, the hull may serve to protect conidia from the harmful effects of physical and chemical agents until spore swelling takes place. This is in agreement with the findings of McKeen et al. (92) who also showed that the conidia of A. niger has a dark outer wall, about 0.2 μ thick, that had an undulating contour and encompassed a large relatively empty space which was limited inwards by a slightly thinner opaque wall, about 0.1 μ thick, which had a more or less regular contour. Inside the inner opaque wall was a relatively thick electron-transparent wall about 0.2 μ thick, which surrounds the protoplast.

The chemical composition of the conidial cell wall of A. oryzae was shown by Horikoshi and Iida (65) to exhibit a complex chemical composition consisting of phosphate, ash, nucleic acid, protein and polysaccharides of glucose, galactose, mannose, and glucosamine (chitin), as well as a β-1, 3-linked laminarin-like material which existed as a polymer of glucose. However, the chemical properties of an insoluble residue in 6N HCl was not elucidated. No qualitative differences were found between the hyphal cell walls and the conidial cell walls of A. oryzae. The protein content was found to be higher in the conidial cell wall than in the hyphal cell wall.

Burges (16, 17), while studying movement of wet and dry spores in soil, pointed out that fungi which have dry spores (spores with

waxy, non-wetting coats), such as penicillia, remained at the surface of the soil even after prolonged washing with water. However, this was found true only for freshly formed, fully matured spores. Young spores, according to Burges (17), can be detached before they have developed their hydrophophic coat and these immature spores will wet more easily. He also stated that old spores appear to lose their waxy coat after being in the soil for some time.

Surfactants, e.g., Duponol, Monoxol, Nacconol, Nacconol-NR, Teepol, Triton X-100, and Tween 20 and 80 have been used to wet conidia in germination studies involving Aspergillus spp. (28, 56, 64, 73, 88, 130, 136, 149), Penicillium spp. (10, 51, 87) and the uredospores of Uromyces (84, 127, 130, 148). The effect of surfactants on germination have not been investigated, however.

Spore Germination in Soil

The term soil fungistasis was first introduced by Dobbs and Hinson (37) in 1953 to describe the inability of fungus spores to germinate in natural soil. Dobbs and Hinson (37) observed this inhibition of conidia of Penicillium frequentans in a variety of unsterilized English soils while using a buried cellophane method. Several other workers have observed that unamended natural soil provides an unfavorable environment for spore germination (20, 69, 82). The effects of soil on fungi include the inhibition of mycelial growth (38) as well as the inhibition of spore germination (20, 56, 69). These effects may be the result of a fungistatic factor(s) present in natural soil. There has been much done in an attempt to elucidate

this phenomenon of soil fungistasis. Fungistasis has become noted for its widespread occurrence in natural soils (21, 36, 37, 68, 69, 70, 81, 97, 99, 131, 134), association with microbial activity (37, 38, 52, 69, 79, 81, 118, 132), and its dissipation in the presence of nutrients (20, 22, 37, 38, 56, 95, 119).

It has been observed that spores of many fungi fail to germinate in soil or soil extracts, but will germinate upon the addition of an available carbon source (37, 134, 135) or when the soil extracts are passed through a microbiological filter (38, 53, 134). Glucose, when added to soil in concentrations of 0.1%, has been found by Dobbs and Hinson (37) to allow germination of conidia of P. *frequentans*. Similarly, as little as 10 ppm glutamic acid, yeast extract and banana sap have been shown by Stover (134) to stimulate germination of macroconidia of F. *oxysporum* f. sp. *cubense* in soil extracts. Several sugars and amino acids were investigated by Jackson (71) to determine their affect on the germination of conidia of P. *citrinum* on agar discs over soil. Glucose was found most effective at low concentrations (0.01%) of those compounds examined. Dobbs et al. (38) found that in the two soils they studied, one supported germination of conidia of P. *frequentans* with only 0.1% glucose solution while the other required 4.0% glucose solution. They also noted that the addition of inorganic nutrients had no effect on the germination of conidia of P. *frequentans*.

Griffin (55) made the first attempt to relate the exogenous carbon and nitrogen requirements for spore germination in distilled water to the capacity of the soil solution to support germination.

Griffin reported that washed conidia of both F. solani f. sp. phaseoli and Gliocladium fimbriatum required exogenous carbon and nitrogen for germination in distilled water. Filtered aqueous soil extracts of non-amended soil supported the germination of conidia of both of these fungi. However, in non-filtered soil extracts washed conidia of G. fimbriatum failed to germinate and washed conidia of F. solani f. sp. phaseoli germinated. Macroconidia of F. solani f. sp. phaseoli and other fusaria germinate in artifically infested soil (55, 97) and are considered less sensitive to soil fungistasis. Griffin suggested that this indicated that some inhibitory factor(s) was present in the soil that prevented spore germination of G. fimbriatum when exogenous carbon and nitrogen nutrition was adequate.

Dix (35) showed that spores of P. nigricans, Doratomyces pupureofuscus, and Mucor plumbeus required exogenous carbon and nitrogen for high germination and that germination of spores of all three fungi occurred in filtered and sterilized aqueous soil extracts, but were considerably depressed in non-sterile aqueous soil extract. He also felt that this indicated that the germination of spores of P. nigricians, D. purpureofuscus, and M. plumbeus were highly sensitive to fungistatic factors in soil rather than any nutrient deficiency.

Dix (35) also determined the conditions of germination and sensitivity to soil fungistasis for spores of 12 species of fungi isolated from the rhizosphere and the surface and tissues of bean roots. He found that sensitivity to soil fungistasis, by indirect assay, varied from those that were unaffected to those that were

highly sensitive.

Ko and Lockwood (74) tested 18 fungi in order to relate the ability of spores to germinate in non-amended soil with their ability to germinate in the absence of exogenous nutrients. Generally, those fungi that germinated independently of exogenous nutrients (in distilled water) germinated when directly placed on non-amended soil. However, four failed to germinate on non-amended soil. They explained their sensitivity to soil fungistasis by suggesting that a steep concentration gradient of spore-exudate nutrients from the spore surface to the soil was a result of microbial assimilation at the spore surface. Evidence for this mechanism was the failure of these spores to germinate when leached with dripping water. Spores that did not germinate appreciably in distilled water did not germinate appreciably when placed on non-amended soil. However, they also showed that spores of _P_. _frequentans_, _A_. _fumigatus_, _F_. _solani_, and _M_. _ramannianus_ germinated in aqueous soil extracts, whereas in soil or distilled water, little or no germination was observed.

When macroconidia of _F_. _solani_ f. sp. _phaseoli_ were added to moist soil by Nash et al. (97) at high conidial density some germinated and formed chlamydospores. Griffin (55) also reported that washed macroconidia of _F_. _solani_ f. sp. _phaseoli_ germinated when added to non-amended soil. Macroconidia of _F_. _roseum_ 'Sambucinum' have been observed to germinate at high conidial density in artifically infested rewetted soil by Griffin and Pass (60). Macroconidia of _F_. _oxysporum_ (56) and _F_. _solani_ (57) have been shown by Griffin to

germinate in rewetted soil to form chlamydospores. However conidia of _A. flavus_ did not germinate in rewetted soil (56). Griffin and Pass (60) indicated that part of the germination stimulus in rewetted soil may be due to the increase in soluble carbon compounds that occurs when an air-dry soil is rewetted (104, 112, 125, 133).

Schroth and Snyder (117) has shown stimulation of germination (20-40%) of chlamydospores of _F. solani_ f. sp. _phaseoli_ by adding to the soil solutions of aspartic acid, asparagine, glucose, and sucrose and to some extent, fructose and maltose at a concentration of 1.0%. They found that by combining the amino acids and sugars, the concentration could be lowered to 0.2% without a noticeable decrease in chlamydospore germination. No germination was observed in controls.

Cook and Schroth (26) reported that chlamydospores of _F. solani_ f. sp. _phaseoli_ required exogenous carbon and nitrogen in soil for germination. When asparagine, glycine, glutamine, and phenylalanine were added to soil containing chlamydospores at high spore density, germination was three times greater than when simple sugars only were added. However, when sugars in soil were supplemented with inorganic nitrogen, spore germination was about the same as in soil amended with amino acids. Ammonium nitrogen was more stimulatory than the nitrate form.

When spores of _P. frequentans_ and _A. fumigatus_ were germinated on the surface of amended soils, conidia of _P. frequentans_ germinated in the presence of glucose plus ascorbic acid, but a concentration of 5% glucose alone failed to promote germination (74). Conidia of _A._

fumigatus required glucose, ascorbic acid and NH_4NO_3. Ko and Lockwood concluded that it appeared to them that nutrients were satisfying a requirement for germination rather than counteracting an inhibitor.

Griffin (56) has reported that upon the addition of nutrients to the soil, high germination (>50%) of chlamydospores of F. oxysporum was found to be supported by glucose (0.1%) plus NH_4Cl (0.01%), while conidia of A. flavus required peptone (0.5%) or glucose (0.25%) plus peptone (0.25%) for high germination (>50%). Neither chlamydospores of F. oxysporum nor conidia of A. flavus germinated in non-amended soil.

Jackson (71), using a buried glass slide technique, observed that macroconidia of Fusarium sp. germinated in soil to form chlamydospores. However, the chlamydospores did not germinate unless they were within 2.0 mm of the root surface of young pea plants. Conidia of Gliocladium roseum and Paecilomyces marquandii germinated only within 1.0 mm of the root surface.

Schroth and Snyder (117) were able to show that germination of chlamydospores occurred consistently in moist soil when in close proximity (1.0 mm) to germinating bean seeds and root tips of primary, lateral, and adventitious bean roots.

Cook and Snyder (27) observed that, at high spore density, chlamydospores of F. solani f. sp. phaseoli contiguous to bean seeds germinated (60%) within 16 hours after sewing the seeds. They also reported that the addition of nutrient compounds (glucose, sucrose, asparagine, glycine, glutamine) to soil, in the absence of the host, stimulated chlamydospore germination up to 40% in 16 hours.

Griffin (56), using a root-slide technique, observed germination of both chlamydospores and microconidia of *F. oxysporum* in the rhizosphere of primary roots of young peanut plants. High chlamydospore germination (>50%) was found to occur within 0.5 mm of the root surface. Germination of conidia of *A. flavus* was less than 0.2% in the rhizosphere of primary roots. He concluded that the requirement for high concentration of nutrient substrates for high germination in soil may explain in part the low germination of *A. flavus* conidia in the rhizosphere of peanuts.

Recently, Griffin (59) reported that conidia of *A. flavus* did not germinate in soil (0.5 mm) adhering to peanut pegs and only trace germination (< 0.1%) occurred in the geocarposphere (0.5 mm) of peanut pods. In contrast, chlamydospores of *F. oxysporum* germinated readily in soil adhering to the peanut pegs. However, when peanut pods were superficially injured and inoculated with infested soil, high germination (>50%) of conidia of *A. flavus* in the geocarposphere of the peanut pods was observed.

MATERIALS AND METHODS

Conidial Germination Studies

The strain of *Aspergillus flavus* used was isolated from peanut pods and was obtained from K. H. Garren (Tidewater Research Station, Holland, Virginia) and was identified as *A. flavus*. However, it was noted that biseriate heads were infrequent. Aflatoxin (B_1 & G_1) production was determined qualitatively by T. C. Campbell (Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia). The fungus was maintained on malt extract agar slants (1.5% maltose, 0.27% dextrin, 0.23% glycerol, 0.08% peptone, 1.5% agar, Baltimore Biological Laboratory, Baltimore, Maryland) at 27° to 29°C in room light. Conidia were harvested from 15- or 16-day-old cultures with a sterile inorganic salt solution (B solution) of the following composition: 0.01 M sodium phosphate buffer, 0.05% KCl, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 5.7). To free conidia of nutrients from the growth medium, the conidial suspension was washed by centrifugation four times with 45-ml portions of B solution plus 0.1 ml of 0.25% Tween 20 (polyoxyethylene (20) sorbitan monolaurate, Atlas Chemical Industries, Inc., Wilmington, Delaware) in sterile, capped centrifuge tubes for 20 minutes at 1300 r.p.m. (rotar radius-8cm.). This spore suspension was shaken on a Burrell wrist action shaker (Burrell Corp., Pittsburgh, Pa.) at position 10 for 10 minutes before and after centrifugation.

All glassware was cleaned in chromic acid solution for 12 hours before thorough washing and tap and distilled water rinsing. All

glassware was then given an additional rinse in doubly distilled water and placed in dust-free chambers to air dry before use. B solution was used also as a basal medium in all germination experiments. The solutions were prepared throughout with doubly glass-distilled water. Carbon and nitrogen sources were autoclaved at 121°C for 15 minutes separate from B solution and were combined after autoclaving to give the desired concentrations. Chemicals were reagent grade. Chemicals used in the study were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio except for D-glucose, D-arabinose, L-rhamnose, glycine, D-mannitol, peptone, KCl, $Na_2HPO_4 \cdot H_2O$ and NaH_2PO_4 which were obtained from Fisher Scientific Co., Fair Lawn, N. J. Glycerol, NH_4Cl, KNO_3 and $MgSO_4 \cdot 7H_2O$ were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Still culture experiments were conducted in 9-cm flat-bottom glass petri dishes containing 10 ml of medium. Conidial densities were standardized with a Spectronic "20" colorimeter (400 nm) (Bausch & Lomb Corporation, Rochester, N. Y.); determinations on the density of conidia were made by hemacytometer counts on a conidial suspension having a density near 1×10^6 conidia/ml (optical density = 0.13). The conidial densities used in each experiment were standardized with a spectronic "20" colorimeter (400 nm) and periodically confirmed by making hemacytometer counts; all other densities within an experiment were obtained by dilution or by pipetting the appropriate amounts of conidia and B solution or nutrient solutions. All experiments were conducted at 30°C and at pH 5.7 unless otherwise indicated.

For temperature studies petri plates, containing germination media, were pre-incubated at the appropriate temperatures for 30 min before being inoculated. Germination was stopped by autoclaving dishes or flasks after the specified periods of incubation. A conidium was considered germinated when a germ tube could be observed at a magnification of 250x. Conidia in clumps greater than three were not counted. All experiments were conducted three or more times in whole or in part.

Electron Microscopy

For preparation of ultrathin sections, conidia were harvested from 16-day-old malt extract agar slants and washed by centrifugation with B solution plus 0.1 ml of 0.25% Tween 20. The conidial suspension was centrifuged for 30 minutes at 10,000 r.p.m. in order to form a hard pellet. Portions of the pellet were then placed in liquid agar (45°C) which was allowed to solidify. Approximately 1 mm^3 sections of agar containing the conidia were fixed for one hour at 27° to 29°C in phosphate buffered glutaraldehyde and rinsed in a phosphate buffer to remove any excess glutaraldehyde and stored at 5°C for 72 hours. The conidia were post-fixed in a phosphate buffered osmium tetroxide (1% osmium buffered to pH 7.2 - 7.4). After fixing for 90 minutes at 27° to 29°C the conidia were dehydrated by passage through the usual graded series of ethanol. At this time the conidia were taken through 3 changes of propylene oxide, 3 minutes/change. The conidia were then placed into a 50:50 mixture of propylene oxide and Epon 812 and incubated for 30 minutes at 60°C

to insure infiltration. The mixture was introduced into a clean gelatin capsule (00) which was placed in a 60°C oven for approximately 15 to 20 hours in order for the conidia to become concentrated at the bottom of the capsule and to allow the resin in the capsule to polymerize.

Sections were prepared on a MT-1 type ultramicrotome ("Porter-Blum") using a glass knife. The sections were placed on a 200-mesh copper grid coated with formvar films stabilized with a carbon layer. The sections were counter stained for 5 minutes with uranyl acetate and for 15 seconds with lead citrate.

RESULTS

Effect of Temperature and pH on Germination

Germination of conidia of *A. flavus* was significantly influenced by temperature (Table I). In B solution containing glucose plus peptone maximum germination was observed at 30°C and 35°C after 12 h. In B solution containing glucose plus NH_4Cl maximum germination was observed at 30°C. Germination decreased sharply in both media between 25°C and 10°C. Neither germination nor swelling was observed at 5°C and 45°C in either medium. At 30°C rate of germination was higher in B solution containing glucose plus peptone than in glucose plus NH_4Cl (Table II). Complete germination was observed after 12 h of incubation in B solution containing glucose plus peptone, while in B solution containing glucose plus NH_4Cl complete germination was observed after 28 h of incubation.

The effect of pH on conidial germination was tested using citrate-phosphate buffer (0.005M citric acid, 0.01M sodium phosphate, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% KCl) and phosphate buffer (0.01M sodium phosphate, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% KCl). Exogenous carbon and nitrogen was supplied as either glucose plus NH_4Cl or glucose plus peptone. In the glucose plus peptone system, highest germination was observed from pH 3.0 to 7.5 (Table III). In the glucose plus NH_4Cl system, germination was best when initial pH was from pH 4.5 to 6.0. In the glucose plus NH_4Cl system the final pH was generally lower than the initial pH for values above pH 5.0. The drop in pH most likely accompanied the use of the ammonium ion. At all

Table I. Effect of temperature on percentage germination of conidia of *Aspergillus flavus* in B solution with glucose plus NH_4Cl or glucose plus peptone at 1 x 10^4 conidia/ml.

Temperature °C	Supplement to B solution, % germination	
	Glucose[a] plus NH_4Cl[b]	Glucose[c] plus Peptone[d]
5	0.0[eg]	0.0[fg]
10	0.7	0.4
15	0.4	0.6
20	2.8	1.0
25	30.6	64.3
30	74.6	98.3
35	83.4	98.4
40	0.6	0.7
45	0.0	0.0

[a]Glucose supplied at 4.0 mg C/ml.

[b]NH_4Cl supplied at 0.26 mg N/ml.

[c]Glucose supplied at 2.0 mg C/ml.

[d]Peptone supplied at 5.0 mg/ml.

[e]After 16 h.

[f]After 12 h.

[g]Based on two counts of 100 conidia/count in each of two plates.

Table II. Rate of germination of conidia of *Aspergillus flavus* in B solution with glucose plus NH_4Cl or glucose plus peptone at 1×10^4 conidia/ml.

Incubation (Hrs)	Supplement to B solution, % germination	
	Glucose[a] plus NH_4Cl[b]	Glucose[c] plus Peptone[d]
4	1.0[e]	2.5
8	18.0	82.5
12	54.5	98.5
16	81.0	99.8
20	88.5	-
24	95.6	-
28	97.5	-

[a]Glucose supplied at 4.0 mg C/ml.

[b]NH_4Cl supplied at 0.26 mg N/ml.

[c]Glucose supplied at 2.0 mg C/ml.

[d]Peptone supplied at 5.0 mg/ml.

[e]Based on two counts of 100 conidia/count in each of two plates.

Table III. Influence of pH on percentage germination of conidia of *Aspergillus flavus* in glucose plus NH_4Cl or glucose plus peotone and in two buffers at 1×10^4 conidia/ml.

Initial pH	Citrate-phosphate buffer, % germination: Glucose[a] + NH_4Cl[b]	Final pH	Glucose[c] + Peptone[d]	Final pH	Phosphate buffer, % germination: Glucose[a] + NH_4Cl[b]	Final pH	Glucose[c] + Peptone[d]	Final pH
3.0	38.5[eg]	2.95	94.5[fg]	2.95	-	-	-	-
3.5	44.0	3.50	97.5	3.62	-	-	-	-
4.0	55.5	3.90	97.7	4.05	-	-	-	-
4.5	80.0	4.60	98.2	4.52	-	-	-	-
5.0	87.2	4.75	98.0	5.12	-	-	-	-
5.5	83.2	5.20	98.9	5.60	-	-	-	-
5.7	-	-	-	-	84.2[eg]	4.65	99.2[fg]	5.55
6.0	84.0	5.70	95.2	6.00	73.5	5.45	97.0	5.95
6.5	50.5	6.00	94.0	6.45	57.2	5.90	97.2	6.45
7.0	47.2	6.40	93.2	6.80	52.5	6.35	95.5	6.75
7.5	-	-	-	-	53.5	6.75	90.0	7.20
8.0	-	-	-	-	56.2	6.60	83.7	7.30

[a]Glucose supplied at 4.0 mg C/ml.
[b]NH_4Cl supplied at 0.26 mg N/ml.
[c]Glucose supplied at 2.0 mg C/ml.
[d]Peptone supplied at 5.0 mg/ml.
[e]After 16 h.
[f]After 12 h.
[g]Based on two counts of 100 conidia/count in each of two plates.

pH levels investigated germination was much greater in the glucose plus peptone system than in the glucose plus NH_4Cl system even though incubation time was shorter in the glucose plus peptone system.

Exogenous Carbon and Nitrogen Requirements for Germination

Various carbon sources were tested at two concentrations for their ability to support conidial germination (Table IV). At equivalent carbon, an amino acid mixture supported higher germination than a sugar-organic acid-alcohol mixture plus NH_4Cl. Of the carbon sources tested alone, proline and alanine supported higher germination than several other single amino acids or single sugars plus NH_4Cl tested. Glucose plus NH_4Cl was found to be the most stimulatory at both concentrations of the sugars tested. In order to allow for the difference in total nitrogen concentration obtained with amino acids used as carbon sources, B solution containing glucose plus NH_4Cl at nitrogen levels equivalent to the lowest and highest concentrations of amino nitrogen were tested. Increasing the concentration of NH_4Cl had little effect on percentage germination. Increasing the concentration of the carbon sources increased percentage germination. Typical germ tubes (Fig. 1A) were not observed in B solution alone or in B solution plus NH_4Cl. However, in B solution, in B solution plus NH_4Cl, in B solution containing glucose and in B solution containing glucose plus NH_4Cl generally less than 3% of the conidia possessed atypically thin, and usually short, germ tubes (Fig. 1B).

Table IV. Percentage germination of conidia of *Aspergillus flavus* in B solution with various carbon sources at two levels of equivalent carbon and at 1 x 10^4 conidia/ml.

	Concentration, % germination	
Supplement to B solution	0.4 mg C/ml	2.0 mg C/ml
No carbon or nitrogen source	0.0[hi]	0.0[hi]
NH_4Cl	0.0[a]	0.0[c]
Amino Acid mixture[f]	87.2	92.7
Sugar-organic acid-alcohol mixture[g] + NH_4Cl	46.2[a]	52.5[c]
L-Alanine	45.0	51.5
L-Proline	36.0	57.0
L-Aspartic acid	8.0	9.0
L-Glutamic acid	1.0	5.6
L-Leucine	0.0	0.0
D-Glucose + NH_4Cl	25.1[a]	50.0[c]
D-Glucose + NH_4Cl	27.0[b]	-
D-Glucose + NH_4Cl	29.7[d]	-
D-Glucose + NH_4Cl	-	54.0[e]
D-Xylose + NH_4Cl	10.7[a]	21.6[c]
D-Maltose + NH_4Cl	9.0[a]	14.7[c]
D-Fructose + NH_4Cl	6.0[a]	7.5[c]
Sucrose + NH_4Cl	5.7[a]	6.0[c]
Cellobiose + NH_4Cl	5.0[a]	7.9[c]
Malic acid + NH_4Cl	1.5[a]	3.5[c]
D-Mannitol + NH_4Cl	1.0[a]	1.0[c]
D-Galactose + NH_4Cl	0.0[a]	0.5[c]

[a] NH_4Cl supplied at 0.026 mg N/ml.
[b] NH_4Cl supplied at 0.09 mg N/ml.
[c] NH_4Cl supplied at 0.13 mg N/ml.
[d] NH_4Cl supplied at 0.23 mg N/ml.
[e] NH_4Cl supplied at 0.46 mg N/ml.
[f] L-proline, L-alanine, glycine, L-leucine, L-aspartic acid, L-glutamic acid HCl supplied at equivalent carbon.
[g] D-glucose, D-xylose, D-maltose, D-fructose, sucrose, cellobiose, malic acid, D-mannitol, D-galactose, D-arabinose, glycerol, ethanol, L-rhamnose, D-mannose, i-inositol, D-ribose supplied at equivalent carbon.
[h] After 16 h.
[i] Based on two counts of 100 conidia/count in each of two plates.

Typical germ tube formation was accompanied by swelling of the conidium, whereas swelling was not observed for atypical thin germ tube formation. Unless otherwise indicated conidial germination or germination refers hereafter to only typical germ tube formation.

It was also observed that the following amino acids were slightly stimulatory to germination when supplied alone at 0.4 mg C/ml: L-arginine HCl (9.0%), glycine (7.5%), L-ornithine HCl (3.5%), L-tryptophane (1.0%), and L-hydroxyproline (0.5%). Additional sugars and alcohols that were slightly stimulatory when supplied at a concentration of 0.4 mg C/ml with NH_4Cl (0.026 mg N/ml) were glycerol (7.0%), L-rhamnose (5.6%), i-inositol (4.0%), D-mannose (1.5%), ethanol (1.2%), and D-ribose (0.5%).

The requirements for germination of spores are felt by some to be influenced by the cultural conditions present during growth and sporulation. Grover (61) has reported that conidia of A. flavus, harvested from Czapek's synthetic basal medium containing 10 amino acids, germinated in distilled water (78.7%) in the absence of exogenous carbon and nitrogen sources. When one of these amino acids (ie. glycine or L-tryptophane) was omitted from the Czapek's synthetic basal medium, the spores harvested from the media devoid of only one of these amino acids would germinate poorly in distilled water (9.8 to 13.4%). In order to confirm the effect of amino acids on growth and sporulation of A. flavus and their carry-over for subsequent conidial germination, a critical portion of Grover's study was repeated.

Conidia of two isolates of *A. flavus* for this experiment were collected from 8-day-old cultures of Czapek's synthetic agar medium containing 100 µg N/ml of casein hydrolysate, washed two times with 45 ml portions of doubly distilled water. Isolate 1 is the isolate used throughout the present study. Isolate 3 differs from isolate 1 principally by the presence of more frequent biseriate sterigmata. One ml of 1 x 10^3 conidia/ml was placed in the following culture media: (a) complete medium (3.0% sucrose, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$, and 10 amino acids), (b) complete medium minus glycine, (c) complete medium minus tryptophane. The cultures were incubated for 8 days at 24° to 26°C in room light. After 8 days of incubation the conidia were carefully collected from the surface of cultures by means of a loop and washed as described above. A spore density of 1 x 10^3 conidia/ml was used for all germination tests. The conidia were incubated for 24 h at 24° to 26°C either in doubly distilled water, B solution, or amino acid solutions.

Little or no germination was observed when washed conidia were collected from the complete medium, or from the complete medium without glycine or tryptophane in doubly distilled water or B solution (Table V). Furthermore, conidia collected from media devoid of either glycine or tryptophane germinated poorly when glycine or tryptophane was added to doubly distilled water or B solution. When dry conidia were added by means of a loop to the germination media, instead of washed conidia, results were similar. Atypical thin germ

Table V. Effect of growth and sporulation medium composition on the subsequent germination of conidia of Aspergillus flavus in doubly distilled water, B solution, or an amino acid solution at 1×10^3 conidia/ml.

Growth and Sporulation medium	Germination medium, % germination			
	Doubly distilled water		B solution	
	Isolate 1	Isolate 3	Isolate 1	Isolate 3
Complete[a]	0.2[cde]	0.0	0.2	0.2
Glycine omitted	0.0	0.0	0.0	0.0
L-Tryptophane omitted	0.0	0.0	0.0	0.2
(L-Tryptophane omitted, but added to germination medium)[b]	2.8	4.8	4.1	5.5
(Glycine omitted, but added to germination medium)[b]	5.8	7.5	6.3	9.0

[a] B-alanine, L-arginine, DL-aspartic acid, L-glutamic acid, glycine, DL-histidine HCl, DL-leucine, L-lycine HCl, DL-methionine, and L-tryptophane supplied at equivalent nitrogen to give a total of 100 µg N/ml.

[b] L-tryptophane or glycine supplied at 100 µg N/ml.

[c] Incubated at 24° to 26°C.

[d] After 24 h.

[e] Based on three counts of 100 conidia/count in each of two plates.

tubes (<2.0%) were observed in B solution and doubly distilled water. Sporulation appeared to be best for both isolates in the culture medium minus tryptophane.

Conidial density greatly influenced the amount of conidial germination for A. flavus. When exogenous carbon and nitrogen sources were supplied for conidial densities at constant amounts/conidium, percentage germination of conidia was dependent on the density of the conidia (Table VI). The amounts of exogenous carbon and nitrogen/conidium were maintained the same for all conidial densities by dilution of the highest conidial density (1×10^5 conidia/ml) containing 20 mg C/ml and 1.3 mg N/ml with B solution. In B solution with glucose or glucose plus NH_4Cl percentage germination decreased as the conidial density decreased from 1×10^5 to 1×10^3 conidia/ml. In two of six experiments, decrease in germination was not observed in glucose alone. The difference in percentage germination between glucose and glucose plus nitrogen typically decreased as the conidial density decreased. Only a slight decrease was noted between 1×10^5 and 1×10^4 conidia/ml in glucose plus peptone, but between 1×10^4 and 1×10^3 conidia/ml percentage germination dropped sharply. However, only a slight decrease in percentage germination was observed in glucose plus an amino acid mixture between 1×10^5 and 1×10^3 conidia/ml. In other experiments where the density was increased to 1×10^6 conidia/ml percentage germination was nearly nil in 20 mg C/ml of glucose alone (0.5%) and low in 20 mg C/ml of glucose plus 1.3 mg N/ml of NH_4Cl

Table VI. Percentage germination of conidia of *Aspergillus flavus* in B solution and in B solution with glucose alone, glucose plus NH_4Cl, peptone, or an amino acid mixture supplied at constant amounts per conidium for three conidial densities.

Supplement to B solution	Conidia/ml, % germination		
	1×10^5	1×10^4	1×10^3
No glucose or NH_4Cl	0.0[ef]	0.0	0.0
Glucose[a]	27.0	22.0	4.2
Glucose + NH_4Cl[b]	52.1	36.0	6.5
Glucose + peptone[c]	98.7	94.2	49.0
Glucose + amino acids mixture[d]	95.5	94.0	85.5

[a]Glucose supplied at 20 mg C/ml supplied at 1×10^5 conidia/ml.

[b]NH_4Cl supplied at 1.3 mg N/ml supplied at 1×10^5 conidia/ml.

[c]Peptone supplied at 5.0 mg/ml supplied at 1×10^5 conidia/ml.

[d]L-alanine, L-aspartic acid, L-glutamic acid HCl, glycine, L-leucine and L-proline supplied at equivalent nitrogen.

[e]After 12 h.

[f]Based on two counts of 100 conidia/count in each of two plates.

(16.2%). Percentage germination was high in 20 mg C/ml of glucose plus 5 mg/ml of peptone (91.5%) and 20 mg C/ml of glucose plus 1.3 mg N/ml of an amino acid mixture (89.5%).

Percentage germination of conidia of _A. flavus_ was examined in B solution with three levels of glucose and glucose plus NH_4Cl, and at three conidial densities. At all levels of glucose plus NH_4Cl and glucose alone germination decreased as the density of the conidia increased from 1×10^3 to 1×10^5 conidia/ml (Table VII). However, at the higher levels germination generally decreased less rapidly in glucose plus NH_4Cl than in glucose alone. Although germination increased when the concentration of glucose alone was increased for all conidial densities, germination generally increased more rapidly when the concentrations of both glucose and NH_4Cl were increased. Highest germination was observed at 1×10^3 conidia/ml in glucose plus NH_4Cl supplied at the highest levels.

Various nitrogen sources were tested for their effect on germination in the presence of glucose. All of the nitrogen sources tested, at equivalent nitrogen, except for KNO_3, supported higher germination than NH_4Cl in B solution (Table VIII). Two glucose levels were tested to allow for differences in total carbon obtained with amino acid sources of nitrogen. Proline plus glucose supported the highest germination of the individual nitrogen sources plus glucose tested. An amino acid mixture plus glucose supported the highest germination of all combinations and single nitrogen sources tested. Percentage germination was increased when aspartic

Table VII. Percentage germination of conidia of *Aspergillus flavus* in B solution with three levels of glucose and glucose plus NH_4Cl at three conidial densities.

Supplement to B solution	Conidia/ml, % germination		
	1×10^5	1×10^4	1×10^3
No glucose or NH_4Cl	0.0[ab]	0.0	0.5
Glucose (0.08 mg C/ml)	5.0	13.2	16.0
Glucose (0.08 mg C/ml) + NH_4Cl (0.0052 mg N/ml)	10.7	18.5	21.5
Glucose (0.8 mg C/ml)	7.2	20.2	39.5
Glucose (0.8 mg C/ml) + NH_4Cl (0.052 mg N/ml)	39.7	41.0	56.2
Glucose (8.0 mg C/ml)	13.2	44.2	69.5
Glucose (8.0 mg C/ml) + NH_4Cl (0.52 mg N/ml)	71.5	86.0	91.0

[a]After 18 h.

[b]Based on two counts of 100 conidia/count in each of two plates.

Table VIII. Percentage germination of conidia of *Aspergillus flavus* in B solution containing glucose with and without various nitrogen sources at equivalent nitrogen and 1 x 10^4 conidia/ml.

Supplement to B solution	% germination
No carbon or nitrogen source	0.0[f,g]
NH_4Cl[a]	0.0
Glucose[b]	40.1
Glucose[c]	41.9
Glucose[b] + KNO_3	46.6
Glucose[b] + NH_4Cl[a]	52.5
Glucose[c] + NH_4Cl[a]	53.1
Glucose[b] + L-hydroxyproline[d]	68.5
Glucose[b] + L-asparagine	71.5
Glucose[b] + L-arginine	74.5
Glucose[b] + L-ornithine	78.5
Glucose[b] + L-aspartic acid	86.0
Glucose[b] + L-glutamic acid	87.7
Glucose[b] + L-glutamic acid + L-aspartic acid	85.7
Glucose[b] + L-leucine	69.2
Glucose[b] + L-leucine + L-aspartic acid	83.2
Glucose[b] + L-leucine + L-glutamic acid	87.2
Glucose[b] + glycine	70.0
Glucose[b] + glycine + L-aspartic acid	86.2
Glucose[b] + glycine + L-glutamic acid	95.2
Glucose[b] + L-alanine	80.7
Glucose[b] + L-alanine + L-aspartic acid	87.5
Glucose[b] + L-alanine + L-glutamic acid	96.7
Glucose[b] + L-proline	94.5
Glucose[b] + L-proline + L-aspartic acid	96.0
Glucose[b] + L-proline + L-glutamic acid	96.0
Glucose[b] + amino acid mixture[e]	98.7

[a] NH_4Cl supplied at 0.26 mg N/ml.

[b] Glucose supplied at 4.0 mg C/ml.

[c] Glucose supplied at 8.0 mg C/ml.

[d] All amino acids supplied at 0.26 mg N/ml.

[e] L-alanine, L-aspartic acid, L-glutamic acid HCl, glycine, L-leucine, and L-proline supplied at equivalent nitrogen.

[f] After 12 h.

[g] Based on two counts of 100 conidia/count in each of two plates.

acid or glutamic acid was present in B solution containing glucose with leucine, glycine, or alanine; glutamic acid was generally more stimulatory than aspartic acid.

The influence of CO_2 on conidial germination was examined by passing air or CO_2-free air (CO_2 removed by bubbling air through 10% KOH) through still, liquid cultures (Fig. 1C). 1 ml of conidial suspension was added to 19 ml of medium after 30 min of equilibration. Removal of CO_2 inhibited germination and swelling of conidia in B solution containing glucose plus NH_4Cl and suppressed germination in B solution with glucose plus an amino acid mixture. Percentage germination was essentially the same in aerated and non-aerated treatments of both glucose plus NH_4Cl and glucose plus an amino acid mixture (Table IX). Thin germ tubes (<2.0%) were observed in all treatments except in B solution with glucose plus an amino acid mixture aerated with CO_2-containing air and non-aerated treatments.

Tween 20 was used to wet conidia of A. flavus during this study in order to harvest conidia from cultures, and to make possible the standardization of conidial density during the investigation. Low levels of Tween 20 may still be present after washing conidia even though the conidia were resuspended after the last centrifugation in Tween 20-free B solution. Since little is known about the effect of surfactants on germination, tests were conducted to determine whether or not surfactants affect percentage germination of conidia of A. flavus. To determine this a flat-bottom

Table IX. Percentage germination of conidia of *Aspergillus flavus* in B solution with glucose plus NH_4Cl or glucose plus an amino acid mixture in non-aerated media and media aerated with CO_2 containing air and CO_2-free air at 1×10^4 conidia/ml.

Supplement to B solution	% germination: Aerated (CO_2-free air)	Aerated (CO_2)	Non-aerated
Glucose[a] + NH_4Cl[b]	0.4[de]	51.0	54.0
Glucose + amino acid mixture[c]	34.0	97.2	98.2

[a]Glucose supplied at [illegible].0 mg C/ml.

[b]NH_4Cl supplied at 0.13 mg N/ml.

[c]L-alanine, L-aspartic acid, L-glutamic acid HCl, glycine, L-leucine, L-proline at equivalent nitrogen.

[d]After 12 h.

[e]Based on two counts of 100 conidia/count in each of two plates.

glass petri plate was divided in half with a glass partition. Divided plates containing B solution with and without glucose plus NH_4Cl and with and without two surfactants were used. Conidia were deposited on divided plates by gently tapping petri dish cultures above a settling tower (31.5 cm long and 9 cm in diameter). The densities of conidia obtained were estimated to be between 1 x 10^3 and 1 x 10^4 conidia/ml. Densities in individual plates were approximately the same in both halves of the plates.

In the absence of glucose and NH_4Cl and surfactants no germination was observed; however, in the presence of Tergitol NPX (Union Carbide Chemical Co., New York, N. Y.) alone or Tween 20 alone, low levels of typical germ tube formation were observed (Table X). Atypical thin germ tubes were not observed in B solution in the absence of surfactants but were observed in the presence of B solution plus surfactants (<2.0%). In B solution containing glucose, NH_4Cl and Tergitol NPX or Tween 20, germination was higher than in B solution containing glucose plus NH_4Cl without surfactants.

In order to determine if previous incubation of conidia of _A. flavus_ in aqueous media affects percentage germination, washed conidia were incubated in B solution at 1 x 10^6 conidia/ml (100 ml) at $30^{\circ}C$ for various time intervals (24 h to 3 wks). These treatments generally had little affect on percentage germination when conidia were placed in B solution containing glucose (4.0 mg C/ml) plus NH_4Cl (0.26 mg N/ml) at $30^{\circ}C$ and at 1 x 10^4 conidia/ml. However, in a few tests, percentage germination increased somewhat with time

Table X. Percentage germination of settling tower-deposited conidia of *Aspergillus flavus* in B solution and B solution with glucose plus NH_4Cl with and without surfactants at 1 x 10^3 conidia/ml.

Supplement to B solution	Surfactant[c], % germination			
	Tergitol NPX	No Tergitol NPX	Tween 20	No Tween 20
No glucose or NH_4Cl	3.0[de]	0.0	4.2	0.0
Glucose[a] + NH_4Cl[b]	55.9	37.4	58.6	45.5

[a]Glucose supplied at 4.0 mg C/ml.

[b]NH_4Cl supplied at 0.26 mg N/ml.

[c]Surfactants supplied at 0.01%.

[d]After 12 h.

[e]Based on three counts of 100 conidia/count in each of three plates.

of incubation. Conidial germination usually was not observed after 3 weeks of incubation in B solution alone at 1×10^4 to 1×10^6 conidia/ml. Trace germination (<3.0%) was occasionally observed at 1×10^3 and 1×10^2 conidia/ml in B solution alone after 3 weeks. Thin germ tubes (<3.0%) were observed at 1×10^2 to 1×10^4 conidia/ml.

Electron Microscopy

Light microscopic observations of sectioned conidia of *A. flavus* showed that the conidia are thick-walled, usually spherical to oval and generally 4.5µ in diameter. The conidia are characterized by a large number of conical warty projections over their entire surface.

By means of ultrathin sections and electron microscopy, it was found that the conidial wall was composed of three distinct components: an outer hull, a spore wall, and an intermediate space. The outer portion of the hull (OH) is composed of thin electron-dense material which surrounds an inner region of lesser electron density (Fig. 1D). In areas corresponding to the warty processes observed under the light microscope, the hull projects outward thickening the hull greatly. No boundry corresponding to a waxy coat could be observed. However, the waxy coat could have been dissolved by the solvents used during the preparation of conidia for electron microscopy. The spore wall (SW), an electron-dense structure, is visible just beneath the outer hull. The spore wall appears to be differentiated from both the outer hull and an intermediate

space of lower density. The intermediate space (IS), a uniformly electron transparent space, is constantly recognizable between the spore wall and an electron-dense line. This electron-dense line is believed by Tsukahara et al. (138) to be the spore membrane (SM) which directly surrounds the spore cytoplasm.

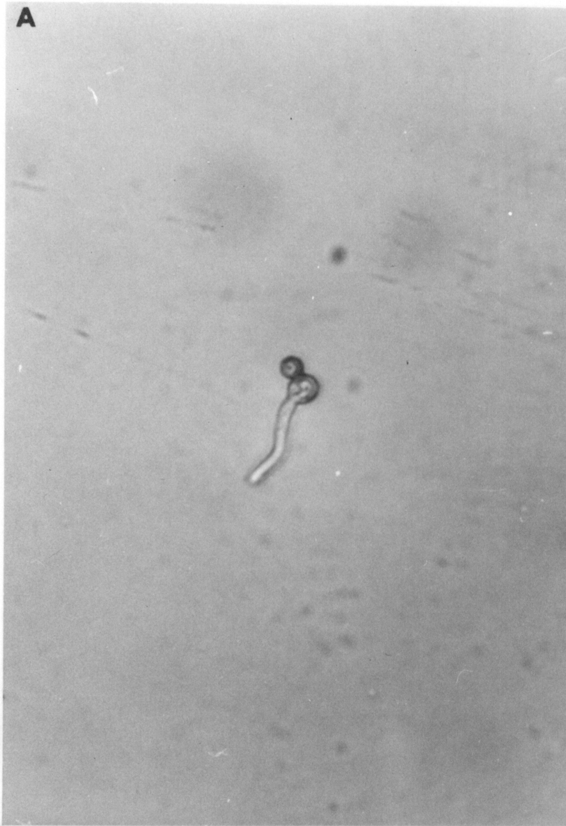
Figure 1

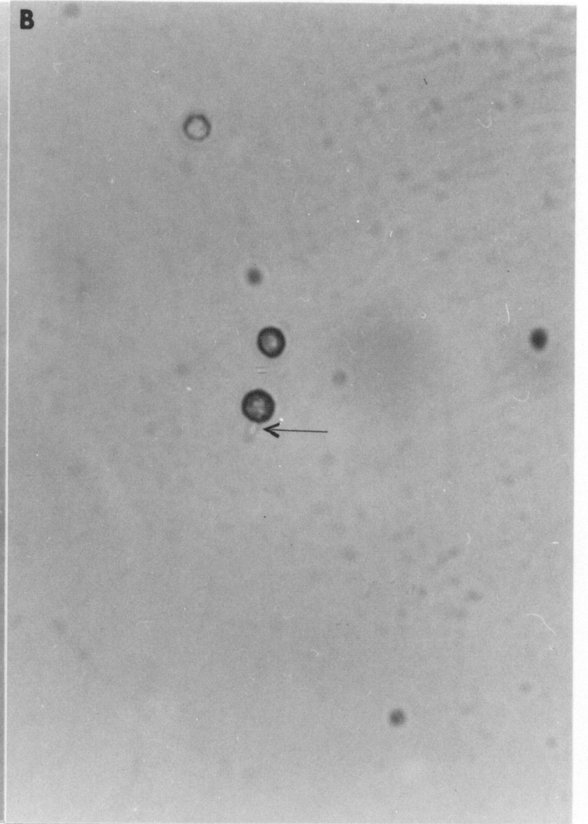
Figure 1. A. Typical germ tube of washed conidia of *Aspergillus flavus*. (Approximately 940x)

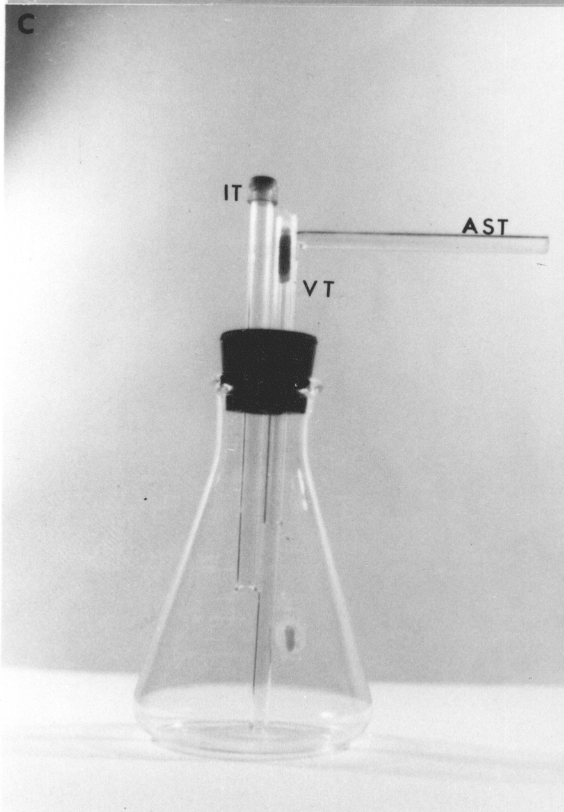
B. Atypical thin germ tube of washed conidia of *Aspergillus flavus*. (Approximately 1500x)

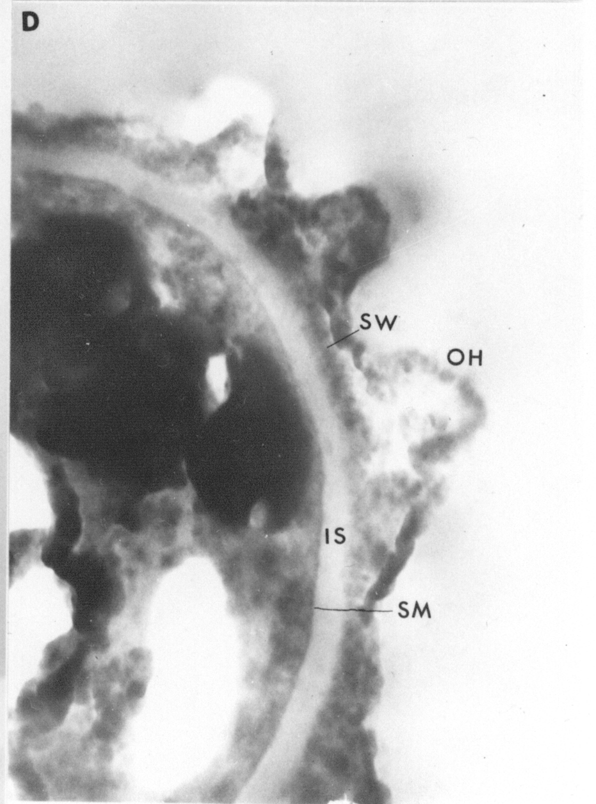
C. Erlenmeyer flask apparatus used to determine influence of CO_2 on conidial germination of *Aspergillus flavus*. The inoculation tube (IT), vent tube (VT), and air supply tube (AST) are shown.

D. Electron micrograph of an ultrathin section through an ungerminated conidium of *Aspergillus flavus*. The outer hull (OH), spore wall (SW), intermediate space (IS), and spore membrane (SM) are shown.


A


B


C
IT
AST
VT


D
SW
OH
IS
SM

DISCUSSION

Nearly full dependence on exogenous carbon was found for high germination of washed, Tween 20-treated conidia of *A. flavus* over a range of conidial densities. Low levels of exogenous carbon-independent germination of washed conidia were observed in B solution alone at 1×10^2 to 1×10^4 conidia/ml. Some typical and atypical thin germ tubes were observed at 1×10^2 and 1×10^3 conidia/ml. However, when dry conidia were deposited at approximately 1×10^3 to 1×10^4 conidia/ml in B solution alone, by means of a settling tower, neither typical germ tubes nor atypical thin germ tubes were observed, low germination was observed in B solution plus Tween 20. Possibly, the low frequency of typical and atypical thin germ tubes formed by washed conidia of *A. flavus* in B solution alone is due for the most part, to the presence of low amounts of Tween 20 used to prepare washed conidia of *A. flavus*. Effects on germination due to trace organic matter contamination do not appear to be important here. Thin germ tubes also have been observed by Griffin (unpublished data) when dry conidia of *A. flavus*, were applied to peanut pegs under greenhouse conditions. However, it is not known whether thin germ tubes are capable of continued elongation. Grover (61) has reported that conidia of *A. flavus* harvested from Czapek's synthetic basal medium containing 10 amino acids, germinated at a high level (76.7%) in distilled water alone. The results that were obtained here, using conidia of two isolates of *A. flavus*, did not confirm this finding.

At equivalent carbon, an amino acid mixture supported higher conidial germination of A. *flavus* than a sugar-organic acid-alcohol mixture plus NH_4Cl. Of the carbon sources tested alone, proline and alanine supported higher germination than several other single amino acids or single sugars plus NH_4Cl tested. Germination did not appear to be related to the differences in total nitrogen present in the medium. Proline alone has also been reported to support high germination of conidia of A. *niger* (94) and sporangiospores of R. *arrhizus* (143, 145) and R. *stolonifer* (142). "Amino acid carry-over" for conidial germination of A. *flavus*, as suggested by Grover (61), was not confirmed in these studies. Glucose plus NH_4Cl was found to be most stimulatory of sugars tested. Other sugars generally supported low germination. In contrast, several sugars supported complete chlamydospore and macroconidial germination by a peanut pod-colonizing clone of F. *solani* (57). Increasing the concentration of the various carbon sources at 1 x 10^4 conidia/ml generally increased percentage germination. Similarly, percentage germination increased when the concentration of glucose alone or glucose plus NH_4Cl was increased over a range of conidial densities. Percentage germination decreased as conidial density increased for each of the three levels of glucose alone or glucose plus NH_4Cl examined. Similar results have been observed for F. *solani* (57) and G. *candidum* (103). In soil or other natural environments where germination studies are conducted and where a given level of exogenous carbon and nitrogen compounds may be present, conidial density may

significantly influence the amount of germination observed.

When exogenous carbon was supplied as glucose, germination was more dependent on exogenous nitrogen, supplied as NH_4Cl as the conidial density increased from 1 x 10^3 to 1 x 10^5 conidial/ml. This was observed generally in both types of conidial density experiments (Tables VI and VII). Generally, exogenous nitrogen influenced percentage germination more, for each conidial density, as the concentration of exogenous carbon and nitrogen increased. Griffin (57, 58) has observed a more pronounced dependence on exogenous nitrogen with increasing spore density for both macroconidia and chlamydospores of F. solani. At equivalent nitrogen, proline or certain amino acid mixtures, such as glycine plus glutamic acid, supported higher germination than NH_4Cl, KNO_3, or several other single amino acids in the presence of glucose. Aspartic acid, glutamic acid, and leucine supported low or no germination as sole carbon sources when supplied at the high level, while high germination was observed when these compounds were used as nitrogen sources with glucose. The incubation period was longer for the carbon source experiment, but the total carbon in the medium was somewhat higher when amino acids were used in the nitrogen source experiment. Under these conditions, glucose plus NH_4Cl supported similar levels of germination in both experiments. Yanagita (149) has reported that in the presence of glucose, proline, and alanine were the most stimulatory of several compounds tested for germination of conidia

of A. niger. Glucose plus amino acid mixtures consistently supported higher germination of A. flavus than glucose plus NH_4Cl for all conidial densities examined. This greater effect of proline and other amino acids on germination, when supplied at equivalent nitrogen, appeared to be specific in nature and not due to a simple increase in total carbon supplied by the amino acids.

Removal of CO_2 from various germination media has been shown by several workers to inhibit or suppress spore germination of A. niger (139, 149), A. nidulans (136), P. griseofulvum (43), and S. commune (62). Germination of conidia of A. flavus was found to be almost completely inhibited in glucose plus NH_4Cl and suppressed in glucose plus an amino acid mixture upon removal of CO_2. As CO_2 levels in soils are higher than in the atmosphere inadequate CO_2 for germination in soil is unlikely.

Fixation of CO_2 by germinating fungus spores has been demonstrated in Aspergillus spp. (12, 23, 66, 100, 137, 150), B. cinera (75), F. culmorum (86), P. recondita (129), and U. phaseoli (127, 128, 129). Yanagita (150) demonstrated that the incorporation of CO_2 in A. niger occurs in both germinating and dormant conidia. When conidia were placed in phosphate buffer, most of the incorporated ^{14}C was found in the trichloroacetic acid soluble fraction in which ATP was revealed to be the major substance labelled. Soluble protein and nucleic acid were also labelled within a short period of time. Studies carried out earlier by Hoshino et al. (66) on ^{14}C-alanine metabolism in the early phase of germination of conidia of A. niger showed that the alanine taken up was instantly deaminated to give ammonia

and pyruvate, which in turn was decarboxylated gradually with the formation of CO_2. The pattern of labelling they observed was quite similar to the labelling observed in the $^{14}CO_2$-fixation study reported later by Yanagita (150). The presence of CO_2 in the early stages of conidial germination may be necessary for the synthesis of amino acids, purines, and pyrimidines which are felt by some to be key compounds responsible for the initiation of germination (66, 100, 137, 150). Incorporation of CO_2 appears to play a role in the initiation of conidial germination by A. flavus. Percentage germination decreased as the conidial density decreased from 1×10^5 to 1×10^3 conidia/ml when glucose plus NH_4Cl were supplied at each density at constant amounts/conidium. However, only a slight decrease in percentage germination was observed in glucose plus amino acid mixture, and a moderate decrease was observed in glucose plus peptone. Lower levels of metabolic CO_2 are probably produced as the conidial density decreases, and germination occurred in CO_2 free air-treated media in the presence of amino acids. Possibly, derivatives of CO_2 fixation products may be supplied to some degree by mixtures of amino acids.

Based on the data presented in this paper there is little evidence that conidia of A. flavus exhibit self-inhibition of germination. While complicated by the amount of metabolic CO_2 produced, the constant ratio experiment did not show evidence of self-inhibition of germination as the conidial density decreased from 1×10^5 to 1×10^3 conidia/ml. However, an increased dependency on exogenous nitrogen was observed as the conidial density increased

from 1 x 10^3 to 1 x 10^5 conidia/ml. As indicated, a similar effect was observed for macroconidial germination by _F. solani_, which also showed self-inhibition (57). Possibly, self-inhibition of conidial germination of _A. flavus_ occurs at higher conidial densities since low germination was observed at 1 x 10^6 conidia/ml when glucose plus NH_4Cl were supplied at a high level.

Surfactants have been used by several workers to wet conidia in germination studies involving _Aspergillus_ spp. (28, 56, 64, 73, 88, 130, 136, 149), _Penicillium_ spp. (10, 51, 87), and uredospores of _Uromyces_ spp. (84, 127, 130, 148). However, the effect of surfactants on germination were not investigated. The results of the present investigation indicate that surfactants used to harvest spores may increase percentage germination in the presence of glucose plus NH_4Cl or, as discussed above, may even support germination in the absence of exogenous carbon and nitrogen sources.

No germination of conidia of _A. flavus_ was observed in rewetted air-dry soil at high conidial density (56). Results of the present study suggest that at low or high conidial densities a capability for exogenous carbon-independent germination by conidia of _A. flavus_ in unamended soil is unlikely. Possibly, low levels of exogenous carbon-dependent germination may occur in rewetted air-dry soil at low conidial densities due to the increase of soluble carbon compounds that occurs when an air-dry soil is rewetted (104, 112, 125, 133).

The importance of amino acids for rapid conidial germination of

A. flavus in soil at high conidial density is suggested by the data of Griffin (56). In the present study, amino acids were shown to favor rapid and high conidial germination from 1 x 10^3 to 1 x 10^6 conidia/ml. Injury of the peanut pod surface has been shown to favor conidial germination of *A. flavus* in soil adjacent to the peanut fruit (59). Knowledge of the specific nature and amounts of carbon and nitrogen compounds, especially amino acid constituents, released from developing pegs and fruits in injured and non-injured conditions would help clarify the role of exudate components in conidial germination and colonization of peanut fruits by *A. flavus* in soil.

The results of this study indicate that the maximum germination of conidia of *A. flavus* occurred at 35°C in the glucose plus NH_4Cl medium. In the glucose plus peptone medium maximum germination occurred at 30°C and 35°C. At 30°C the rate of germination was higher in B solution containing glucose plus peptone than in glucose plus NH_4Cl. Conidia of *A. niger* were shown by Yanagita (149) to germinate best at 30°C in glucose plus proline medium. Brancata and Golding (14) found that a temperature of 30° to 35°C was best for growth of *A. flavus* when grown on malt agar, while 33° to 35°C was found to be the optimum for growth of *A. niger*. Schindler et al. (114), using two aflatoxin-producing isolates of *A. flavus*, observed that maximal growth of the *A. flavus* isolates occurred at 29°C and 35°C on wort medium. Maximal production of aflatoxin occurred at 24°C. Griffin (unpublished data) observed that a soil temperature of 35°C

supported the highest conidial germination by _A. flavus_ in soil adjacent to injured peanut fruits. It appears that conidial germination as well as growth of _A. flavus_ would be favored by high soil temperature.

A broad pH optimum range (pH 3.0 to 7.5) was found using both citrate-phosphate and phosphate buffers containing glucose plus peptone. In glucose plus NH_4Cl, however, a somewhat narrower initial pH optimum range (pH 4.5 to 6.0) was observed. Germination in the latter occurred over the entire pH range examined (pH 3.0 to 8.0). Yanagita (149) demonstrated that conidial germination of _A. niger_ was optimal at pH 6.2 and that a pH of 7.5 markedly retarded germination in phosphate buffer with glucose plus alanine. Gottlieb and Tripathi (51) found that conidia of _P. atrovenetum_ germinated over a pH range of 2.0 to 12.0, with maximum germination occurring between pH of 4.0 to 7.5. Brancato and Golding (14) found that the optimum pH for colony growth was between 3.4 to 5.5 for _A. flavus_, and between pH 4.4 to 7.5 for _A. niger_ when grown on malt agar. Davis et al. (30) reported that pH levels between 3.0 to 6.4 had little effect on aflatoxin production by _A. flavus_ when grown in yeast extract medium. Gottlieb (48) and Cochrane (25) both indicated that only under extremely acid or alkaline conditions is pH a natural limiting factor for spore germination. The pH of the acid soils planted to peanuts in Virginia would not appear to directly restrict conidial germination of _A. flavus_.

SUMMARY

The present investigation was undertaken to determine the exogenous carbon and nitrogen requirements for conidial germination and to determine the influence of pH and temperature on germination of conidia by _A. flavus_.

In a phosphate-buffered (pH 5.7) inorganic salts solution (B), nearly full dependence on exogenous carbon was found for high (90 to 100%) germination of washed, Tween 20-treated conidia of _A. flavus_ over a range of conidial densities. No exogenous carbon-independent germination was observed for settling tower-deposited dry conidia. At equivalent carbon, an amino acid mixture supported higher germination of washed conidia than a sugar-organic acid-alcohol mixture plus NH_4Cl; proline or alanine alone supported higher germinatio n than several other single amino acids or single sugars plus NH_4Cl tested. Glucose plus NH_4Cl was the most stimulatory of the latter. "Amino acid carry-over" for conidial germination of _A. flavus_ was not confirmed in these studies. When exogenous carbon was supplied as glucose, germination of washed conidia was more dependent, generally, on exogenous nitrogen, supplied as NH_4Cl, as the conidial density increased from 10^3 to 10^5 conidia/ml. At equivalent nitrogen, proline alone, or certain amino acid mixtures supported higher germination than NH_4Cl, KNO_3, or several other single amino acids tested in B solution plus glucose. Percentage germination decreased as the conidial density decreased from 10^5 to 10^3 conidia/ml when glucose plus NH_4Cl were supplied at

each density at constant amounts/conidium. A slight decrease in percentage germination was observed in glucose plus an amino acid mixture, and a moderate decrease was observed in glucose plus peptone. Percentage germination decreased as conidial density increased from 1×10^3 to 1×10^5 conidia/ml for each of three concentrations of glucose alone or glucose plus NH_4Cl examined. Removal of CO_2 from the germination medium almost completely inhibited germination and swelling in glucose plus NH_4Cl and suppressed germination in glucose plus an amino acid mixture. Two surfactants increased percentage germination of settling tower-deposited conidia in the presence of glucose plus NH_4Cl and supported low germination in B solution alone. Maximum germination occurred at $35^\circ C$ in glucose plus NH_4Cl medium, while in glucose plus peptone medium maximum germination occurred at $30^\circ C$ and $35^\circ C$. At $30^\circ C$ the rate of germination was higher in glucose plus peptone medium than in glucose plus NH_4Cl medium. A broad pH optimum range (pH 3.0 to 7.5) was found for conidial germination using both citrate-phosphate and phosphate buffers containing glucose plus peptone. In glucose plus NH_4Cl a somewhat narrower pH optimum range was observed. The possible relation of these findings to conidial germination in soil and in the geocarposphere of peanut fruit is discussed.

LITERATURE CITED

1. Agnihotri, V. P. 1964. Studies on Aspergilli XVI. Effect of pH, temperature, and carbon and nitrogen interaction. Mycopathol. Mycol. Appl. 24:305-314.

2. Allcroft, R. and R. B. A. Carnaghan. 1963. Toxic products in groundnuts--Biological effects. Chem. Ind. 53:50-53.

3. Allen, P. J. 1955. The role of self-inhibitors in the germination of rust uredospores. Phytopathology, 215:259-266.

4. Armolik, N. and J. G. Dickson. 1956. Minimum humidity requirement for germination of conidia associated with storage of grain. Phytopathology, 46:462-465.

5. Ashworth, Jr. L. J. and B. C. Langley. 1964. The relationship of pod damage to kernel damage by molds in spanish peanut. Plant Disease Reptr. 48:875-878.

6. Asplin, F. D. and R. B. A. Carnaghan. 1961. The toxicity of certain groundneat meals for poultry with special reference to their effect on ducklings and chickens. Vet. Rec. 73: 1215-1219.

7. Austwick, P. K. C. and G. Ayerst. 1963. Groundnut microflora and toxicity. Chem. Ind. (London) 41:55-61.

8. Bell, A. A. 1960. Partial purification of self-inhibitors of germination from uredospores of Uromyces phaseoli var. typica. Phytopathology, 50:629. (Abstr.)

9. Bell, A. A. and J. M. Daly, 1962. Assay and partial purification of self-inhibitors of germination from uredospores of bean rust fungus. Phytopathology, 52:261-268.

10. Bent, K. J. and A. G. Morton. 1964. Amino acid composition of fungi during development in submerged culture. Biochem. J. 92:260-269.

11. Blakeman, J. P. 1969. Self-inhibition of germination of pycnidiospores of Mycosphaerella liqulicola in relation to the temperature of their formation. J. Gen. Microbiol. 57:159-167.

12. Bloom, Sandra J. and M. J. Johnson. 1962. The Pyruvate carboxylase of Aspergillus niger. J. Biol. Chem. 237:2718-2720.

13. Blount, W. P. 1961. Turkey "x" disease. Jour. Brit. Turkey Fed. 9:52-58.

14. Brancato, F. P. and N. S. Golding. 1953. The diameter of mold colony as a reliable measure of growth. Mycologia, 45:848-864.

15. Brown, W. 1922. On the germination and growth of fungi at various temperatures and in various concentrations of oxygen and of carbon dioxide. Ann. Bot. 36:257-283.

16. Burges, A. 1950. The downward movement of fungal spores in sandy soil. Trans. Brit. Mycol. Soc. 33:142-153.

17. Burges, A. 1958. Micro-Organism in the Soil. Hutchinson & Co., Johannesburg, New York. 188p.

18. Byther, R. 1965. Ecology of plant pathogens in soil. V. Inorganic nitrogen utilization as a factor of competitive saprophytic ability of Fusarium roseum and F. solani. Phytopathology, 55:852-857.

19. Chi, C. C. and E. W. Hanson. 1964. Relation of temperature, pH, and nutrition to growth and sporulation of Fusarium spp. from red clover. Phytopathology, 54:1053-1058.

20. Chinn, S. H. F. 1953. A slide technique for the study of fungi and actinomycetes in soil, with special reference to Helminthosporium sativum. Can. J. Bot. 31:718-724.

21. Chinn, S. H. F. 1967. Differences in fungistasis in some Saskatchewan soils with special reference to Cochliobolus satius. Phytopathology, 57:224-226.

22. Chinn, S. H. F. and R. J. Ledingham. 1961. Mechanisms contributing to the eradication of spores of Helminthosporium sativum from amended soil. Can. J. Bot. 39:739-748.

23. Cleland, W. W. and M. J. Johnson. 1954. Tracer experiment on the mechanism of citric acid formation by Aspergillus niger. J. Biol. Chem. 208:679-689.

24. Cochrane, Joyce C., V. W. Cochrane, F. G. Simon, and J. Spaeth. 1963. Spore germination and carbon metabolism in Fusarium solani. I. Requirements for spore germination. Phytopathology, 53:1155-1160.

25. Cochrane, V. W. 1958. Physiology of fungi. John Wiley & Sons, Inc. New York. 524p.

26. Cook, R. J. and M. N. Schroth. 1965. Carbon and nitrogen compounds and germination of chlamydospores of Fusarium solani f. phaseoli. Phytopathology, 55:254-256.

27. Cook, R. J. and W. C. Snyder. 1965. Influence of host exudates on growth and survival of germlings of Fusarium solani f. phaseoli in soil. Phytopathology, 55:1021-1025.

28. Darby, R. T. and G. R. Mandels. 1955. Effects of sporulation medium and age on fungus spore physiology. Plant Physiol. 30:360-366.

29. Davis, N. D. and U. L. Diener. 1968. Growth and aflatoxin production by Aspergillus parasitious from various carbon sources. Appl. Microbiol. 16:158-159.

30. Davis, N. D., U. L. Diener, and D. W. Eldridge. 1966. Production of aflatoxins B_1 and G_1 by Aspergillus flavus in a semisynthetic medium. Appl. Microbiol. 14:378-380.

31. Davis, N. D., U. L. Diener, and K. E. Landers. 1964. Factors influencing the production of aflatoxin by Aspergillus flavus growing on laboratory media. Natl. Peanut Res. Conf. Proc. 3:114.

32. Davis, N. D., U. L. Diener, and V. P. Agnihortri. 1967. Production of aflatoxins B_1 and G_1 in chemically defined medium. Mycopathol. Mycol. Appl. 31:251-256.

33. Diener, U. L. 1965. Relation of Aspergillus flavus invasion to maturity of peanut at harvest. J. Alabama Sci. 36:21.

34. Diener, U. L., C. R. Jackson, W. E. Cooper, R. J. Stipes, and N. D. Davis. 1965. Invasion of peanut pods in the soil by Aspergillus flavus. Plant Disease Reptr. 49:931-935.

35. Dix, N. J. 1967. Mycostasis and root exudation: factors influencing the colonization of bean roots by fungi. Trans. Brit. Mycol. Soc. 50:23-31.

36. Dobbs, C. G. and M. J. Hash. 1965. Microbial and residual mycostasis in soils. Nature, 207:1354-1356.

37. Dobbs, C. G. and W. H. Hinson. 1953. A widespread fungistasis in soils. Nature. 172:197-199.

38. Dobbs, C. G., W. H. Hinson, and Joan Bywater. 1960. Inhibition of fungal growth in soils. In the Ecology of Soil Fungi. Edited by D. Parkinson and J. S. Waid. Liverpool University Press, Liverpool. pp. 130-147.

39. Edgerton, C. W. 1910. The bean anthracnose. La. Agr. Expt. Sta. Bull. 119:1-55.

40. Eldridge, D. W., N. D. Davis, U. L. Diener, and V. P. Agnihotri. 1965. Aflatoxin production by Aspergillus flavus in a chemically defined medium. Phytopathology. 55:498. (Abstr.)

41. Ettel, G. E. and W. Halbsguth. 1963. Beitr. Biol. pflanz. 39: 451-488. In The Fungi. Edited by G. C. Ainsworth and A. S. Sussman. 1966. Academic Press, New York, Vol. II, 805p.

42. Farkas, G. L. and G. A. Ledingham. 1959. The relation of self-inhibition of germination to the oxidative metabolism of stem rust uredospores. Can. J. Microbiol. 5:141-151.

43. Fletcher, J. and A. G. Morton. 1970. Physiology of germination of Penicillium griseofulvum conidia. Trans. Br. Mycol. Soc. 54:65-81.

44. Forsyth, F. R. 1955. The nature of the inhibiting substance emitted by germinating uredospores of Puccinia graminis var. tritici. Can. J. Bot. 33:363-373.

45. French, R. C. 1968. Some physical and chemical properties of an endogenous germination inhibitor from uredospores of wheat stem rust. Phytopathology, 58:884. (Abstr.)

46. Garren, K. H. and D. M. Porter. 1970. Quiescent endocarpic floral communities in cured mature peanuts from Virginia and Puerto Rico. Phytopathology, 60:1635-1638.

47. Garret, W. A., H. Heitman, Jr. and A. N. Booth. 1968. Aflatoxin toxicity in beef cattle. Proc. Soc. Expt. Biol. Med. 127: 188-190.

48. Gottlieb, D. 1950. The physiology of spore germination in fungi. Botan. Rev. 16:229-257.

49. Gottlieb, D. 1964. Germination of fungus spores. Endeavour, 23: 85-89.

50. Gottlieb, D. 1966. Biosynthetic processes in germinating spores. In The Fungus Spore Ed. M. F. Madelin. Butterworths Inc. Washington D. C. pp. 217-233.

51. Gottlieb, D. and R. K. Tripathi. 1968. The physiology of swelling shase of spore germination in Penicillium atrovenetum. Mycologia, 60:571-590.

52. Griffin, G. J. 1962. Physiological studies of conidial germination in soil. Ph.D. Dissertation. Colorado State University. 75p.

53. Griffin, G. J. 1962. Production of a fungistatic effect by soil microflora in autoclaved soil. Phytopathology, 52: 90-91.

54. Griffin, G. J. 1964. Influence of carbon and nitrogen nutrition on chlamydospore formation by Fusarium solani f. radicicola. Phytopathology, 54:886-913. (Abstr.)

55. Griffin, G. J. 1964. Long-term influence of soil amendments on germination of conidia. Can. J. Microbiol. 10:605-612.

56. Griffin, G. J. 1969. Fusarium oxysporum and Aspergillus flavus spore germination in the rhizosphere of peanut. Phytopathology, 59:1214-1218.

57. Griffin, G. J. 1970. Carbon and nitrogen requirements for macroconidial germination by Fusarium solani: dependence on spore density. Can. J. Microbiol. 16:733-740.

58. Griffin, G. J. 1970. Exogenous carbon and nitrogen requirements for chlamydospore germination by Fusarium solani: dependence on spore density. Can. J. Microbiol. 16:1366-1368.

59. Griffin, G. J. 1970. Conidial germination and population of Aspergillus flavus in the geocarposphere of peanut. Phytopathology, 60:1293. (Abstr.)

60. Griffin, G. J. and T. Pass. 1969. Behavior of Fusarium roseum 'Sambucinum' under carbon starvation conditions in relation to survival in soil. Can. J. Microbiol. 15:117-126.

61. Grover, R. K. 1964. The effect of amino acids on growth and sporulation of Aspergillus flavus and their carry-over for subsequent spore germination. New Phytologist. 63:12-20.

62. Hafiz, A. and D. J. Niederpruem. 1963. Studies on basidiospore germination in Schizophyllum commune. Am. J. Botany, 50: 614-615.

63. Hayes, A. W., N. D. Davis, and U. L. Diener. 1966. Effect of aeration on growth and aflatoxin production by Aspergillus flavus in submerged culture. Appl. Microbiol. 14:1019-1021.

64. Hesseltine, C. W., W. G. Sorenson, and Mabel Smith. 1970. Taxonomic studies of the aflatoxin-producing strains in the Aspergillus flavus group. Mycologia, 62:123-132.

65. Horikoshi, K. and S. Iida. 1964. Studies of the spore coats of fungi. I. Isolation and composition of the spore coats of Aspergillus oryzae. Biochim. Biophys. Acta. 83:197-203.

66. Hoshino, J., A. Nishi, and T. Yanagita. 1962. Alanine metabolism in conidiospores of Aspergillus niger in the early phase of germination. J. Gen. Appl. Microbiol. 8: 233-245.

67. Issac, I. and Q. MacGarvie. 1966. Dormancy and germination of resting structures of Verticillium spp. Trans. Brit. Mycol. Soc. 49:669-678.

68. Jackson, R. M. 1957. Fungistasis as a factor in the rhizosphere phenomenon. Nature, 180:96-97.

69. Jackson, R. M. 1958. An investigation of fungistasis in Nigerian soils. J. Gen. Microbiol. 18:248-258.

70. Jackson, R. M. 1958. Some aspects of soil fungistasis. J. Gen. Miciobiol. 19:390-401.

71. Jackson, R. M. 1960. Soil fungistasis and rhizosphere. In The ecology of soil fungi. Edited by D. Parkinsin and J. S. Waid. Liverpool University Press, Liverpool. pp. 168-181.

72. Johnson, Anne B. and H. J. Strecker. 1962. The interconversion of glutamic acid and proline. J. Biol. Chem. 237:1876-1881.

73. Johnson, D. E., G. E. N. Nelson, and A. Ciegler. 1968. Starch hydrolysis by conidia of Aspergillus wentii. Appl. Microbiol. 16:1678-1683.

74. Ko, W. and J. L. Lockwood. 1967. Soil fungistasis: relation to fungal spore nutrition. Phytopathology, 57:894-901.

75. Kosuge, T. and J. C. Dutra. 1963. Fixation of $C^{14}O_2$ by germinating conidia of Botrytis cinerea. Phytopathology, 53:880. (Abstr.)

76. Lancaster, M. C., F. P. Jenkins, and J. Mcl. Phillip. 1961. Toxicity associated with certain samples of groundnuts. Nature, 192:1095-1096.

77. Landers, K. E., N. D. Davis, and U. L. Diener. 1967. Influence of atmospheric gases on aflatoxin production by Aspergillus flavus in peanuts. Phytopathology, 57:1086-1090.

78. Lingappa, B. T. and J. L. Lockwood. 1961. The nature of the widespread soil fungistasis. J. Gen. Microbiol. 26:473-485.

79. Lingappa, B. T. and J. L. Lockwood. 1963. Direct assay of soils for fungistasis. Phytopathology, 53:529-531.

80. Lingappa, B. T. and Y. Lingappa. 1965. Effects of nutrients on self-inhibition of germination of conidia of Glomerella cingulata. J. Gen. Microbiol. 41:67-75.

81. Lockwood, J. L. 1959. Streptomyces spp. as a cause of natural fungitoxicity in soils. Phytopathology, 49:327-331.

82. Lockwood, J. L. 1960. Lysis of mycelium of plant-pathogenic fungi by natural soil. Phytopathology, 50:787-789.

83. Macko, V., R. C. Staples, H. Gershon, and J. A. A. Renwick. 1970. Self-inhibitor of bean rust uredospores: methyl 3, 4-Dimethoxycinnamate. Science, 170:539-540.

84. Maheshwari, R. and A. S. Sussman. 1970. Respiratory changes during germination of uredospores of Puccinia graminis f. tritici. Phytopathology, 60:1357-1363.

85. Marchant, R. and M. F. White. 1966. Spore swelling and germination in Fusarium culmorum. J. Gen. Microbiol. 42: 237-244.

86. Marchant, R. and M. F. White. 1967. The carbon metabolism and swelling of Fusarium culmorum conidia. J. Gen. Microbiol. 48:65-77.

87. Martin, J. F. and G. Nicolas. 1970. Spore germination in Penicillium notatum and Trichoderma lignorum. Trans. Br. Mycol. Soc. 55:141-148.

88. Mazur, P. 1956. Studies on the effects of subzero temperatures on the viability of spores of Aspergillus flavus. I. The effect rate of warming. J. Gen. Physiol. 39:869-888.

89. MacDonald, D. and C. Harkness. 1963. Growth of Aspergillus flavus and production of aflatoxin in groundnuts. Part II. Trop. Sci. 5:143-154.

90. McDonald, D. and C. Harkness. 1964. Growth of Aspergillus flavus and production of aflatoxin in groundnuts. Part IV. Trop. Sci. 6:12-17.

91. McDonald, D. and J. A. Brook. 1963. Growth of Aspergillus flavus and production of aflatoxin in groundnuts. Part III. Trop. Sci. 4:208-214.

92. McKeen, W. E., N. Mitchell, Wm. Jarvie, and R. Smith. 1966. Electromicroscopy studies of conidial walls of Sphaerothecia macularis, Penicillium levitum, and Aspergillus niger. Can. J. Microbiol. 12:427-428.

93. Miller, D. D. and N. D. Golding. 1949. The gas requirements of molds. V. The minimum oxygen requirements for normal growth and for germination of six mold cultures. J. Dairy Sci. 32:101-109.

94. Miller, L. P. 1962. L-proline and L-alanine as substitutes for higher temperatures in the germination of fungus spores. Phytopathology, 52:743. (Abstr.)

95. Mircetich, S. M. and G. A. Zentymyer. 1969. Effect of carbon and nitrogen compounds on germination of chlamydospores of Phytophthora cinnamomi in soil. Phytopathology, 59:1732-1735.

96. Muller, R. D., C. W. Carlson, G. Semeniuk, and G. S. Harshfield. 1970. Response of chicks, ducklings, goslings, pheasants, and poults to graded levels of aflatoxin. Poultry Sci. 49(5):134-149.

97. Nash, S. M., T. Christou, and W. C. Snyder. 1961. Existence of Fusarium solani f. phaseoli as chlamydospores in soil. Phytopathology, 51:308-312.

98. Norton, D. C., S. K. Menton, and A. L. Flangas. 1956. Fungi associated with unblemished spanish peanuts in Texas. Plant Disease Reptr. 40:374-376.

99. Old, K. M. 1965. Fungistasis effects of soil bacteria on root-rotting fungi with particular reference to Helminthosporium sativum. Phytopathology, 55:901-905.

100. Ono, T., K. Kimura and T. Yanagita. 1966. Sequential synthesis of various molecular species of ribonucleic acid in the early phase of conidia germination in Aspergillus oryzae. J. Gen. Microbiol. 12:13-26.

101. Onuorah, P. E. 1963. The influence of sucrose and wheat root exudate on the germination and growth of conidia of Fusarium culmorum (W.S.G.M.) Sacc. Plant Soil, 25:27-32.

102. Park, D. 1963. Evidence for a common fungal growth regulator. Trans. Brit. Mycol. Soc. 46:541-548.

103. Park, D. and P. M. Robinson. 1970. Germination studies with Geotrichum candidum. Trans. Br. Mycol. Soc. 54:83-92.

104. Paul, E. A. and C. M. Tu. 1965. Alternation of microbial activities, mineral nitrogen and free amino acid constituents of soils by physical treatment. Plant Soil, 22:207-219.

105. Porter, D. M. and K. H. Garren. 1970. Endocarpic microorganisms of two types of windrow-dried peanut fruit (Arachis hypogaea L.) Appl. Microbiol. 20:133-138.

106. Rabie, C. J. 1965. Influence of temperature on the production of aflatoxin by Aspergillus flavus. Symp. Mycotoxins in food stuffs, Sess. 2: Agr. Aspects, Dept. Agr. Tech. Serv., Pretoria, p. 18-29.

107. Rick, P. D. and C. J. Mirocha. 1968. Fixation of carbon dioxide in the dark by the malic enzyme of bean and oat stem rust uredospores. Plant Physiol. 43:201-207.

108. Rippel, A. and H. Bortels. 1927. Vorlaufige versuche uber die allgermeine bedeutung der kohensaure fur die pfanzenzelle (Versuche an Aspergillus niger) Biochem. 2. 184:237-243.

109. Robinson, P. M. and D. Park. 1966. Volatile inhibitors of spore germination produced by fungi. Trans. Brit. Mycol. Soc. 49:639-649.

110. Robinson, P. M., D. Park, and M. K. Garrett. 1968. Sporostatic products of fungi. Trans. Brit. Mycol. Soc. 51:113-124.

111. Rotem, J. 1963. Inhibition of germination of spores of Alternaria porri f. sp. solani. Phytopathology, 53:1241.

112. Salonius, P. O., J. B. Robinson, and F. E. Chase. 1967. A comparison of autoclaved and gamma-irradiated soils as media for microbial colonization experiments. Plant Soil, 27:239-248.

113. Sargent, K., A. Sheridan, J. O'Kelly, and R. B. A. Carnaghan. 1961. Toxicity associated with certain samples of groundnuts. Nature, 192:1096-1097.

114. Schindler, A. F., J. G. Palmer, and W. V. Eisenberg. 1967. Aflatoxin production by Aspergillus flavus as related to various temperatures. Appl. Microbiol. 15:1006-1009.

115. Schroeder, H. W. and L. J. Ashworth Jr. 1965. Aflatoxins in spanish peanuts in relation to pod and kernel conditions. Phytopathology, 55:464-465.

116. Schroth, M. N. and D. C. Hildebrand. 1964. Influence of plant exudates on root infecting fungi. Anu. Rev. Phytopathology, 2:10-132.

117. Schroth, M. N. and W. C. Snyder. 1961. Effect of host exudates on chlamydospore germination of the bean root rot fungus, Fusarium solani f. phaseoli. Phytopathology, 51:389-393.

118. Schuepp, H. and E. Frei. 1969. Soil fungistasis with respect to pH and profile. Can. J. Microbiol. 15:1273-1279.

119. Schuepp, H. and R. J. Green. 1968. Indirect assay methods to investigate soil fungistasis with special consideration of soil pH. Phytopathol. Z. 61:1-28.

120. Sellschop, J. F. P. 1965. Field observations on conditions conductive to the contamination of groundnuts with the mold Aspergillus flavus Link ex. Fries. S. African Med. Journ. 39:774-776.

121. Semeniuk, G., C. M. Nagel, and J. C. Gilman. 1947. Observations on mold development and deterioration in stored yellow dent shelled corn. Iowa Exp. Sta. Res. Bull. 349:225-284.

122. Shaw, M. 1964. The physiology of rust uredospores. Phytopathol. Z. 50:159-180.

123. Singh, J. and E. J. Trione. 1969. Self-inhibition of the germination of bunt telliospores of Telletia carries and T. controversa. Phytopathology 59:15. (Abstr.)

124. Sisler, H. D. and C. E. Cox. 1954. Effects of tetramethyl-thiuram disulfide on metabolism of Fusarium roseum. Am. J. Botany, 41:377-390.

125. Soulides, D. A. and F. E. Allison. 1961. Effect of drying and freezing soils on carbon dioxide production, available mineral nutrients, aggregation, and bacterial population. Soil Sci. 91:291-298.

126. Spensley, P. C. 1963. Aflatoxin, the active principle in turkey "X" disease. Endeavour, 22:75-79.

127. Stallknecht, G. F. and C. J. Mirocha. 1970. Carbon dioxide fixation by urediospores of Uromyces phaseoli and its incorporation into cellular metabolites. Phytopathology, 60:1338-1342.

128. Stallknecht, G. F. and C. J. Mirocha. 1971. Fixation and incorporation of CO_2 into ribonucleic acid by germinating uredospores of Uromyces phaseoli. Phytopathology, 61: 400-405.

129. Staples, R. C. and L. H. Weinstein. 1959. Dark carbon dioxide fixation by uredospores of rust fungi. Contribs. Boyce Thompson Inst. 20:71-81.

130. Staples, R. C., R. Syamananda, V. Kao, and R. J. Block. 1962. Comparative biochemistry of obligately parasitic and saprophytic fungi. II. Assimilation of C^{14} labeled substrates by germinating spores. Contribs. Boyce Thompson Inst. 21:345-361.

131. Steiner, G. W. and J. L. Lockwood. 1969. Soil fungistasis: Sensitivity of spores in relation to germination time and size. Phytopathology, 59:1084-1092.

132. Steiner, G. W. and J. L. Lockwood. 1970. Soil fungistasis: Mechanism in sterilized, reinoculated soil. Phytopathology, 60:89-91.

133. Stevenson, I. L. 1956. Some observations on microbial activity in remoistened air-dried soils. Plant soil, 8:170-182.

134. Stover, R. H. 1958. Studies on Fusarium wilt of bananas. III. Influence of soil fungi-toxins on behavior of F. oxysporum f. cubense in soil extracts and diffusates. Can. J. Botany, 36:439-453.

135. Toussoun, T. A. and W. C. Snyder. 1961. Germination of chlamydospores of Fusarium solani f. phaseoli in unsterilized soils. Phytopathology, 51:620-623.

136. Trinci, A. P. J. and Christine Whittaker. 1968. Self-inhibition of spore germination in Aspergillus nidulans. Trans. Br. Mycol. Soc. 51:594-596.

137. Tsay, Y., A. Nishi and T. Yanagita. 1965. Carbon dioxide fixation in Aspergillus oryzae conidia at the initial phase of germination. J. Biochem. 58:487-493.

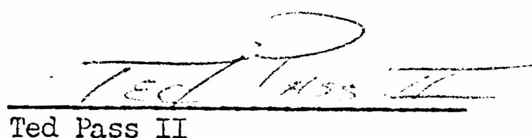
138. Tsukahara, T., M. Yamada, and T. Itagaki. 1966. Micromorphology of conidiospores of Aspergillus niger by electron microscopy. Jap. J. Microbiol. 10:93-107.

VITA

Ted Pass II was born on July 24, 1944 in Fleming, Kentucky. He is the son of Mr. and Mrs. Ted Pass of Jenkins, Kentucky. He is married to the former Ginny Kay Stucky.

The author graduated from Jenkins High School, Jenkins, Kentucky in May of 1962. In June of 1962 he entered Morehead State University, Morehead, Kentucky and received a Bachelor of Science degree in biology and chemistry in January of 1966. Immediately thereafter he was employed by Olive Hill High School, Olive Hill, Kentucky, as an instructor of biology. In June of 1966 he entered the Graduate School of Morehead State University, and was awarded the Master of Science degree in Biology in March of 1968. The author entered the Graduate School of Virginia Polytechnic Institute and State University, Blacksburg, Virginia in March of 1968 and is currently a candidate for the degree of Doctor of Philosophy in the Department of Plant Pathology and Physiology.

The author is a member of The American Phytopathological Society, The American Society for Microbiology, The American Institute of Biological Sciences, The Society of Sigma Xi, Phi Sigma, Gamma Sigma Delta, Blue Key, and Gamma Beta Phi.



Ted Pass II

STUDIES ON THE PHYSIOLOGY OF CONIDIAL GERMINATION

BY

ASPERGILLUS FLAVUS

by

Ted Pass II

(ABSTRACT)

In a phosphate-buffered (pH 5.7) inorganic salts solution (B), nearly full dependence on exogenous carbon was found for high (90 to 100%) germination of washed, Tween 20-treated conidia of *Aspergillus flavus* over a range of conidial densities. No exogenous carbon-independent germination was observed for settling tower-deposited dry conidia. At equivalent carbon, an amino acid mixture supported higher germination of washed conidia than a sugar-organic acid-alcohol mixture plus NH_4Cl; proline or alanine alone supported higher germination than several other single amino acids or single sugars plus NH_4Cl tested. Glucose plus NH_4Cl was the most stimulatory of the latter. "Amino acid carry-over" for conidial germination of *A. flavus* was not confirmed in these studies. When exogenous carbon was supplied as glucose, germination of washed conidia was more dependent, generally, on exogenous nitrogen, supplied as NH_4Cl, as the conidial density increased from 10^3 to 10^5 conidia/ml. At equivalent nitrogen, proline alone, or certain amino acid mixtures supported higher germination than NH_4Cl, KNO_3, or several other single

amino acids tested in B solution plus glucose. Percentage germination decreased as the conidial density decreased from 10^5 to 10^3 conidia/ml when glucose plus NH_4Cl were supplied at each density at constant amounts/conidium. A slight decrease in percentage germination was observed in glucose plus an amino acid mixture, and a moderate decrease was observed in glucose plus peptone. Percentage germination decreased as conidial density increased from 1×10^3 to 1×10^5 conidia/ml for each of three concentrations of glucose alone or glucose plus NH_4Cl examined. Removal of CO_2 from the germination medium almost completely inhibited germination and swelling in glucose plus NH_4Cl and suppressed germination in glucose plus an amino acid mixture. Two surfactants increased percentage germination of settling tower-deposited conidia in the presence of glucose plus NH_4Cl and supported low germination in B solution alone. Maximum germination occurred at $35^{\circ}C$ in glucose plus NH_4Cl medium, while in glucose plus peptone medium maximum germination occurred at $30^{\circ}C$ and $35^{\circ}C$. At $30^{\circ}C$ the rate of germination was higher in glucose plus peptone medium than in glucose plus NH_4Cl medium. A broad pH optimum range (pH 3.0 to 7.5) was found for conidial germination using both citrate-phosphate and phosphate buffers containing glucose plus peptone. In glucose plus NH_4Cl a somewhat narrower pH optimum range, was observed. The possible relation of these findings to conidial germination in soil and in the geocarposphere of peanut fruit is discussed.