FUNGICIDAL INHIBITION OF *ASPERGILLUS FLAVUS* LINK EX FREIS, ON *ARACHIS HYPOGAEA* L. VARIETY, ARGENTINE

By

JIMMIE WARREN BLAYLOCK

Bachelor of Science

Oklahoma State University

1965

Submitted to the faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
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Thesis Approved:

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

The production of harmful mycotoxins has been recognized as a serious problem in the feed industry. These toxins result from fungus invasion and there is an increased interest in recent years for expanding knowledge of toxins produced by fungi on peanuts.

Peanuts provide a high gross income per acre in Oklahoma and cultural practices that will improve peanut quality are important to agriculture of the state.

Fungus invasion on this crop can be attributed to the fact that peanuts are formed in the soil and are in intimate contact with the soil microflora. The moisture content of peanuts is normally high following removal from the soil and may remain high during conditions unfavorable for rapid curing. Fungus invasion of the nuts may occur at that time.

Control of fungus proliferation in peanuts by fungicide application has been explored to a limited extent. The research included in this study was initiated to determine the feasibility of fungicide treatment of freshly dug peanuts (Arachis hypogaea) to inhibit mycotoxin formation.

LITERATURE REVIEW

Aflatoxin is the most publicized of the mycotoxins associated with moldy peanuts and consists of four identified components. These toxic compounds may be separated with thin layer chromatography and are designated as aflatoxin B_1, B_2, G_1, and G_2. These four fractions are found in various combinations with each having different toxicity levels (8). A recent bibliography of current literature on the general topic of aflatoxins has been published by Skau (11).

Phelps (6) reported occurrence of aflatoxin fractions in the following ratios: 15 B_1:1 B_2: 15 G_1:1 G_2 (5) on a weight basis, and, on a molar basis 118 B_1:6 B_2: 11 G_1:1 G_2 (1).

Approximately 132 different fungi have been found associated with peanut pods (6). Many fungi which occur on peanuts are capable of producing toxins. Twelve strains of Aspergilli and one Penicillium are known aflatoxin producers. Undoubtedly, the list will become larger as more species are examined.

Aspergillus flavus, Link ex Freis, is reported to be one of the most active producers of aflatoxin. However, Austwick (2) found that not all A. flavus isolates are capable of producing aflatoxin and the amount produced varied with the strain.

The asexual stage of Aspergilli, with which we are dealing in the production of aflatoxin by A. flavus, is characterized by a vegetative mycelium consisting of septate, branching hyphae on the growth

mycelium from which spore-bearing, conidial structures arise aerially. The spores or conidia are produced on specialized stalks (conidiophores) growing usually perpendicular to the surface of the substrate. Conidial varying greatly in color, size, shape, and markings, are born in chains on the tips of the sterigmata. The conidia of A. flavus vary from yellowish-green to dark green (9).

Invasion of peanuts by A. flavus apparently does not occur on healthy living tissue. Fungus proliferation on unblemished, intact and mature pods in the soil apparently is very limited. Diener (4) found that moribund pods and kernels were most susceptible to invasion by A. flavus.

Moisture is a critical factor determining mold invasion in peanuts and most fungus species remain dormant until high humidity or high moisture content prevails. Dickens and Pattee (3) found that moisture contents between 10 and 30 per cent were most conducive to fungal growth and aflatoxin production. At moisture contents greater than 30 per cent, fungal growth was inhibited and bacterial degradation occurred.

Development of chemical treatments in the field for control of pod invasion by fungus has been studied to a limited extent. Jackson (7) used a modified disk bioassay method to evaluate 18 fungicides for control of fungi commonly found on peanuts. This work was an indirect approach to the problem and no reports have been published on a direct control of aflatoxin in peanuts with fungicides.

METHODS AND MATERIALS

Three incubation procedures were used in these experiments with modifications incorporated as improvements in technique became apparent.

The purpose of the work in experiment one was to extensively screen materials that may have promise for A. flavus inhibition with an emphasis on prevention of fungal growth or aflatoxin production.

Field cured peanuts from the 1964 harvest were obtained and sound mature pods with 12 per cent moisture were hand selected for these studies. Approximately 25g of peanuts were placed in whirl-pack plastic pouches with one ml of 100 ppm a.i. inhibitor solution. A suspension of A. flavus spores in distilled water was added to adjust the moisture content of the peanuts to 25 per cent. Distilled water was added to the noninoculated untreated check which consisted of nonsterilized peanuts adjusted to 25 per cent H_2O with no spores or inhibitor added. The plastic pouches were closed and incubated at 32^{o} C for six days. After incubation the peanuts were assigned a visual rating proportional to the amount of mycelia and spores observed on the pod surface. The rating was numerical, (0-4), with no apparent fungal growth at 0 and the heaviest obvious mycelial development at 4.

Six commercially available fungicides found to be effective for inhibition of A. flavus were used in a revised procedure for incubation.

Approximately 25g of non-cured peanuts, frozen immediately after digging to prevent deterioration, were washed and treated with 1 ml of 100 ppm, (a.i.), fungicide solution. The peanuts were placed in seed germination boxes, covered with plastic food wrap, and incubated at 30° C for six days.

A revised procedure for treatment of peanuts with fungicides was used to attain data shown in Tables III, IV, V, VI, and VII. The peanuts were soaked in the fungicides five minutes and the excess solution removed. New solutions were made for each series to prevent chemical hydrolysis of the unstable compounds. The peanuts were placed in plastic cartons, covered with plastic wrap and incubated 2 days at 30° C.

Mixtures of fungicides were used in the last experiment (Table IX) consisting of Captan, Phalatan, Difolatan, and Du-Ter in all possible combinations with an equal amount (v:v) of each being used. The five minute soaking procedure was used with 48-hour incubation at 30° C.

Thin layer chromatography analysis for aflatoxin B_1 were made by Dr. M. E. Mason, Biochemistry Department, and the results are shown in Tables III and IV.

A Friedman rank test analysis of variance was calculated and the results shown in Tables II, III, IV, and VI (10).

RESULTS AND DISCUSSION

Screening of thirty-four chemical materials was completed at 100 ppm level to determine fungicidal characteristics favorable for inhibiting A. flavus proliferation on peanuts. The materials included fungicides, experimental fungicides, herbicides, insecticides, and inorganic salts. No consistent inhibitory characteristics were found in materials not formulated as fungicides. Results are shown in Table I.

A. flavus inhibition appeared most promising with Captan, Phalatan, Difolatan, Du-Ter, TH093F, Orthocide, and Ortho 7680 in these initial studies. Other materials indicating some inhibiting properties were Zineb, Pipron, Sulquin, Tutane, TH174F, FL565, and Stopmold B.

The incubation procedure was altered to provide near-optimum conditions for fungus invasion and proliferation on the peanuts. Results shown in Table II indicate only some reduced development of A. flavus on peanut pods with the fungicides used. Of the six fungicides used, Difolatan and Ortho 7690 gave low average ratings of .25. Intermediate ratings were .83 for Orthocide, 1.0 for Du-ter and 1.2 for Phalatan; Captan average rating of 1.7 was high as compared to the inoculated check mean rating of 2.7. These results indicated that the procedure for treatment should be revised to obtain more uniform inhibitory effects with the fungicides.

The data shown in Tables III, IV, V, VI, and VII are results with a treatment procedure in which the peanuts were soaked for a

TABLE I

RELATIVE INHIBITOR RATINGS AT 100 PPM ON PEANUTS

Treatment	100 ppm Rating	Rank	Treatment	100 ppm Rating	Rank
Tordon	2.0	Poor	$CaSO_4$	3.7	Poor
Chlorodane	2.0	Poor	$CuSO_4$	4.0	Poor
$NaClO_3$	2.7	Poor	Elemental S	4.0	Poor
Prefar	2.0	Poor	Guanadine	3.0	Poor
TCA	2.0	Poor	Na Propionate	3.0	Poor
N-Serve	2.7	Poor	Na Benzoate	3.7	Poor
Paraquat	2.0	Poor	Pipron	1.0	Fair
PCNB	2.3	Poor	Sulquin	1.7	Fair
Amiben	3.0	Poor	Germix	3.3	Poor
Trifluralin	3.3	Poor	Ca Cyanmid	3.0	Poor
Amitrol	2.7	Poor	Ar Sulfa	3.3	Poor
Zytron	2.3	Poor	Tutane	1.0	Fair
Captan	0.0	Good	Du-Ter	0.0	Good
Phalatan	0.0	Good	TH174F	1.0	Fair
Zineb	1.3	Fair	TH204F	3.5	Poor
Biuret	2.3	Poor	TH265F	2.3	Poor
KCl	2.7	Poor	TH214F	3.7	Poor
$NaSO_3$	3.0	Poor	TH093F	0.0	Good
NaCl	3.3	Poor	TH184F	2.0	Poor
CaO	3.3	Poor	F1565	1.0	Fair
F1564	2.3	Poor	Na Acetate	4.0	Poor
F1562	2.7	Poor	Stop-Mold B	1.0	Fair
F11856	2.3	Poor	Difolatan	0.0	Good
GS-11783	3.0	Poor	Orthocide	0.0	Good
F1542	3.0	Poor	Ortho 7680	0.0	Good
Fl-3359	3.3	Poor	Sevin	2.0	Poor
Fl-3497	4.0	Poor	Acet-Dione	4.0	Poor

Rating figures are mean values from 3 replicate cultures.

TABLE II

EFFECT OF PEANUT TREATMENT WITH 100 PPM (a.i.) FUNGICIDES TO INHIBIT A. FLAVUS

Treatment	Rating				Σ	$\bar{X}$
	I	II	III	IV		
Difolatan	0.0	0.0	0.0	1.0	1.0	0.25
Captan	1.7	2.0	1.0	1.0	6.7	1.7
Phalatan	0.7	2.0	1.0	0.0	3.7	1.2
Du-Ter	1.0	1.0	1.0	1.0	4.0	1.0
Ortho 7680	0.0	0.0	0.0	1.0	1.0	0.25
Orthocide	0.5	1.0	1.0	0.0	2.5	0.83
Inoc. Check	1.7	2.3	3.3	2.7	10.0	2.5

Calculated $X^2 = 46.92$*

five-minute period in fungicide solutions. Peanut absorption of the inhibitor solution was uniform with good duplication of fungus inhibition attained among treatments.

The concentration of the fungicides in each consecutive series III to VII was decreased by a factor of 1/2 and the fungicides were eliminated from the study when the minimum level of inhibition was indicated.

All fungicides effectively inhibited fungal growth at 100 ppm (Table III). Funigicide solution absorption was relatively uniform among all treatments averaging 10 per cent by weight.

At the 50 ppm level, Table IV, results indicated Difolatan, Du-Ter, and Ortho 7680 as effective inhibitors at that level although 0.2 ppm aflatoxin B_1 was detected with the Ortho 7680 treatment. Both Captan and Phalatan were not effective at this level. Orthocide exhibited some fungicidal activity but was not as effective at this level as at 100 ppm. Solution absorption was relatively uniform at about 10 per cent.

Fungicide effectiveness at 25 ppm is shown in Table V for Difolatan, Du-Ter, and Ortho 7680. All three were effective at this level with average solution absorption slightly less than 11 per cent.

Results with fungicide levels at 12.5 ppm as shown in Table VI indicated both Difolatan and Ortho 7680 effective at that level. Du-Ter was less effective with these cultures. Both Difolatan and Ortho 7680 were not effective inhibitors at the 6.25 ppm level shown in Table VII.

TABLE III

EFFECT OF PEANUT TREATMENT WITH 100 PPM (a.i.) FUNGICIDES TO INHIBIT A. FLAVUS

Treatment	Rating I	II	III	Σ	$\bar{X}$	A	B
Difolatan	0	0	0	0	0	.10	0.8
Captan	1	0	0	1	.3	.11	0
Phalatan	0	0	0	0	0	.10	0
Du-Ter	0	0	0	0	0	.10	.14
Ortho 7680	0	0	0	0	0	.09	.12
Orthocide	0	0	0	0	0	.10	.20
Inoc. Check	4	4	4	12	4	.10	.40

A Inhibitor solution absorbed g/g peanuts

B ppm Aflatoxin B_1

Calculated X^2 = 45.57*

TABLE IV

EFFECT OF PEANUT TREATMENT WITH 50 PPM (a.i.) FUNGICIDES TO INHIBIT A. FLAVUS

Treatment	Rating I	Rating II	Rating III	Σ	$\bar{X}$	A	B
Difolatan	0	0	0	0	0	.09	0
Captan	3	3	3	9	3	.10	-
Phalatan	3	3	3	9	3	.09	0
Du-Ter	0	0	0	0	0	.10	0
Ortho 7680	0	0	0	0	0	.11	.2
Orthocide	2	2	2	6	2	.09	-
Inoc. Check	4	4	4	12	4	.11	0

A Inhibitor solution absorbed g/g peanuts

B ppm Aflatoxin B_1

Calculated $X^2 = 61.19$*

TABLE V

EFFECT OF PEANUT TREATMENT WITH 25 PPM (a.i.) FUNGICIDES TO INHIBIT A. FLAVUS

Treatment	Rating I	II	III	Σ	$\bar{X}$	A
Difolatan	0	0	0	0	0	.11
Du-Ter	0	0	0	0	0	.11
Ortho 7680	0	0	0	0	0	.10
Check Inoc.	4	4	4	12	4	.10

A Inhibitor solution absorbed g/g/ peanuts

Aflatoxin determinations were not made on this series.

TABLE VI

EFFECT OF PEANUT TREATMENT WITH 12.5 PPM (a.i.) FUNGICIDES TO INHIBIT A. FLAVUS

Treatment	Rating I	II	III	Σ	$\bar{X}$
Difolatan	0	0	0	0	0
Du-Ter	2	2	2	6	2
Ortho 7680	0	0	0	0	0
Inoc. Check	4	4	4	12	4

Calculated $X^2 = 30.83$*

TABLE VII

EFFECT OF PEANUT TREATMENT WITH 6.25 PPM (a.i.) FUNGICIDES TO INHIBIT A. FLAVUS

Treatment	Rating I	II	III	Σ	$\bar{X}$
Difolatan	1	1	1	3	1
Ortho 7680	1	1	1	3	1
Inoc. Check	4	4	4	12	4

The minimum levels for effective inhibition with these procedures when conditions were favorable to the organism were: Difolatan 12.5 ppm, Captan 100 ppm, Phalatan 100 ppm, Orthocide 100 ppm, Du-Ter 25 ppm, and Ortho 7680 12.5 ppm (Table VIII).

Mixtures of the fungicides below their minimum level of inhibition consisting of an equal amount of each (v:v) is shown in Table IX. Combinations of the four fungicides did not indicate more effective inhibition than when the fungicides were used individually.

Thin layer chromatography determinations for aflatoxin B_1 were made on the treated peanuts shown in Tables III and IV. There was no consistent relationship between surface mycelial growth and presence of aflatoxin B_1. Internal invasion of the peanuts by A. flavus with no growth on the surface possibly explains the presence of aflatoxin.

Statistical analysis indicated a significant difference in treatment means for experiments II, III, IV, and VI.

TABLE VIII

MINIMUM FUNGICIDE LEVEL

FOR *A. FLAVUS* INHIBITION ON PEANUTS

Treatment	Min. Level ppm
Difolatan	12.5
Captan	100.0
Phalatan	100.0
Orthocide	100.0
Du-Ter	25.0
Ortho 7680	12.5

Levels determined from concentration studies.

TABLE IX

EFFECT OF FUNGICIDE MIXTURES WITH A. FLAVUS DEVELOPMENT ON PEANUTS

Treatment[1]	Rating $\bar{X}$
Difolatan, Ortho 7680	0
Phalatan, Ortho 7680	1.0
Captan, Phalatan	3.0
Du-Ter, Ortho 7680	1.0
Difolatan, Phalatan	0
Orthocide, Du-Ter	3.0
Phalatan, Du-Ter	2.7
Captan, Difolatan	2.7
Captan, Du-Ter	3.0
Captan, Ortho 7680	1.7
Phalatan, Difolatan, Du-Ter	1.6
Captan, Difolatan, Du-Ter	2.3
Captan, Phalatan, Du-Ter	3.0
Captan, Phalatan, Difolatan	3.3
Captan, Phalatan, Difolatan, Du-Ter	2.0

[1]Difolatan 6.25 ppm, Captan 50 ppm, Phalatan 50 ppm, Orthocide 50 ppm, Du-Ter 12.5 ppm, Ortho 7680 6.25 ppm.

Rating figures are mean values from 3 replications.

SUMMARY AND CONCLUSIONS

The objectives of this study were to develop chemical treatment procedures to inhibit A. flavus proliferation in Arachis hypogaea under post-harvest and storage conditions. The M-3 strain of the fungus and selected fungicidal materials were used with incubation procedures for these studies.

A. flavus is a common soil organism known to produce mycotoxin compounds when proliferating on grains and oil seed used in animal feeds.

Screening results with fungicide materials indicated seven promising inhibitors: Captan, Phalatan, Difolatan, Du-Ter, TH093F, Orthocide, and Ortho 7680.

The minimum fungicide levels required for inhibition determined by a peanut soaking procedure were: Difolatan 12.5 ppm, Captan 100 ppm, Phalatan 100 ppm, Orthocide 100 ppm, Du-Ter 25 ppm, and Ortho 7680 12.5 ppm.

Fungicide mixtures were found to be no more effective than the individual materials when used separately.

Thin layer chromatography determinations for aflatoxin B_1 were made on the treated peanuts and no consistent relationships between surface mycelial growth and presence of aflatoxin B_1 were found.

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