MUCOR SP. ANTIBIOSIC INTERACTIONS TO *ASPERGILLUS PARASITICUS*, SPEARE

By

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MUCOR SP. ANTIBIOSIC

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CHAPTER 1

INTRODUCTION

A number of common, ubiquitous species of soil fungi produce metabolites which are highly toxic to animals, whereas other species produce metabolites without toxicity and sometimes with desirable antibiotic activity. Aspergillus parasiticus, Speare, produces a well known toxin, termed aflatoxin, which exerts an extreme toxicity to most animal species and is the most potent carcinogenic mycotoxin known. The growth of this fungi, resulting in synthesis of aflatoxin, is of considerable concern for improper handling and storage of farm commodities. It is likely that aflatoxin will continue to be found in food and feedstuffs wherever warm and moist weather conditions, faulty or inadequate storage facilities, or human error may produce circumstances favorable for fungal growth.

Oriental people have used selected fungi species in their food preparation for increasing the assortment and quality of their food. Among the common soil fungi employed extensively in these food fermentations are Mucor species. This fungi produces no known toxic substance, but apparently does synthesize antibiosic substances. The production of antibiosic substances by Mucor species may contribute to the increased nutritional value of the fermented foods.

Initial studies screening several fungi for their antagonistic effects on A. parasiticus indicated Mucor species inhibited aflatoxin synthesis.

The objective of this study was to develop techniques to evaluate Mucor induced antibiosic activity and to determine factors influencing biological control of this phenomena.

CHAPTER II

LITERATURE REVIEW

De Bary, in 1879, was among the first to emphasize the significance of the antagonistic relations among microorganisms (19). Various theories concerning the mechanism of antagonism were summarized by Waksman (19) as follows:

1. Exhaustion of nutrients in medium.
2. Physico-chemical changes produced by the growing organism in the medium, including changes in osmotic pressure, surface tension, oxidation-reduction potential and reaction.
3. Production of specific enzymes, either by the antagonist itself or as a result of autolysis of the antagonized cells.
4. Production and liberation of specific substances, which have a fungistatic and fungicidal action.
5. Certain types of reactions, which may be designated as, action at a distance.
6. Space competition antagonism.

Among these various types of antagonism, the most definite, and the one which is best understood, is the formation of antagonistic substances (19). The abundance of antagonistic substances produced by many fungi and bacteria is greatly influenced by the energy and nitrogen sources in the medium. Fungi may produce both growth-inhibiting and growth-promoting substances. With specific procedures, it is possible to separate one from the other (5,20).

Discovery of the aflatoxins and elucidation of the circumstances associated with their occurrence as food contaminates, as well as their potent biological effects, have collectively engendered many research investigations with diverse objectives and approaches (24). Legator and Withrow (14) reported aflatoxin suppresses mitosis and DNA synthesis in human lung cells. Gabliks, et al. (10) studied aflatoxin B_1 with embryonated eggs and found that duck embryos were four to five times more susceptible than chicken embryos. They also demonstrated that the cell number decreased and the protein, DNA, and RNA content per cell increased with increased aflatoxin concentration. In cell cultures, lethality has been reported at concentrations of 1-5 ug./ml. of medium. Inhibition of growth and mitotic rate has been reported at concentrations of 0.03 ug./ml. (15).

The production of aflatoxin is affected by environmental conditions (8). The optimum temperature for production of aflatoxin B_1 was $24^{\circ}C$, when incubated for a period of 5 to 10 days in nature. However, when the temperature is below $15^{\circ}C$ and the humidity is less than 75% the molds were found to no longer produce aflatoxin (2).

Chang and Lynd (6) illustrated that the peak of aflatoxin synthesis occurred with 72-96 hour incubations in saturated humidity at $30^{\circ}C$. Ciegler et al. (7) also suggested that yields were usually attained in 72 hours after which aflatoxin concentration declined rapidly. The aflatoxin concentration declined more rapidly than the rate of autolysis of mycelial material.

In general, it is considered that Phycomycetes build up relatively simple metabolic products and are not capable of synthesizing the complex molecules characteristic of many antibiotics (12,18). Within

this class of fungi, antibiotic materials have been isolated (16,18,22). One of the antimicrobial compounds is a polypeptide synthesized by Mucor pusillus NRRL 2543 (16). Wang et al. (23) suggest not only that Phycomycetes are capable of synthesizing antibiotics, but also that these biochemical functions may be a valuable criterion for supplementing morphological characters in taxonomy of fungi (11,17). The antibiotic producing ability of Phycomycetes, therefore, merits further study. Furthermore, it is well established that antibiotics, in addition to minimizing infections, also stimulate growth in animals, particularly those whose diets are deficient in any one of several vitamins, amino acids, or some growth factors still unknown. The finding of antibacterial compounds produced by molds commonly used in oriental food fermentations, therefore, offers a better understanding of the true value of fermented foods.

Procedures are outlined (1,3,13) for quantitation of antagonistic effects between microorganisms. Johnson and Curl (13) suggest that probably the most important factor in the selection of a test method for a certain pathogen is the time lag between the application of the test organism and the antagonist. A pure culture of an organism was inoculated upon an artificial medium; after several days incubation, another organism was added and the specific effect was determined (20,21). These tests may indicate the selective nature of the antagonistic action and give some quantitative estimates about the intensity of this activity. Further studies might require growing the antagonist in liquid culture and test filtrates for antimicrobial activity (13). Procedures using filter papers (3,13,23) for the antimicrobial assay are practiced.

CHAPTER III

METHODS AND MATERIALS

Procedures will be presented in three parts to conform with the sequence of the progressive phases for this study.

1. Methods for the Mucor sp. x A. parasiticus culture inhibition and antibiosic interactions.
2. Separation and quantitation of Mucor antibiosic factor.
3. Statistical analysis for per cent inhibition and probit estimate.

1. Methods for the Mucor sp. x A. parasiticus culture inhibition and antibiosic interactions.

Initial studies involved numerous trial and error culture composition experiments that systematically established suitable broth media composition and culture conditions for near optimum growth of both organisms, yet suitable for high aflatoxin synthesis by A. parasiticus.

The standardized broth media contained per liter:

Steam (H_2O) soluble extract from 25.6 ground whole oats

Steam (H_2O) soluble extract from 12.8 ground bermuda grass hay

53 grams yeast extract

49 grams malt extract

The challenge cultures were prepared with 20 ml. broth media per 500 ml. wide mouth Erlenmeyer flask, in triplicate per treatment, with

sixteen treatments, and 48 cultures per experiment. Media was autoclaved for 15 minutes at 15 psi, cooled to room temperature and inoculated. One-half of the cultures were inoculated with Mucor mycelia suspension, approximately 10 mg/flask. The remaining half was inoculated with A. parasiticus spore suspension, approximately 5×10^6 spores/culture. The opposite organism as a challenge was inoculated into base cultures at successive 12 hour intervals.

Another study utilized both organisms inoculated at the same time resulting in an initial competition from time zero for these cultures. The mycelial growth was determined at 24 hour intervals or at the designated time intervals required in challenge culture studies. The mycelial pads were carefully removed from the culture flasks and the excess liquid media removed before drying at 70°C and weighing. Challenge culture effects were measured by the amount of mycelia produced as compared to the check cultures.

The quantities of aflatoxin produced were also used as another measure of the activity of A. parasiticus influences by Mucor. An aflatoxin analysis procedure was developed for the residual liquid broth from these challenge cultures as follows:

A. Add 15 ml. 20% lead acetate, heat to boiling temperature for 15 minutes and filter.

B. Filtrate extracted with $CHCl_3$.

C. Evaporate $CHCl_3$ fraction to dryness and then bring to quantitative volume with $CHCl_3$.

D. Separate $CHCl_3$ soluble fraction for aflatoxins with TLC, nonfluorescent silica gel, 25 micron thickness, developed with 12% acetone in $CHCl_3$.

E. Quantitate fluorescent aflatoxin spots with Turner Fluorometer TLC scanner attachment and determine concentration with prepared standard curves.

2. Separation and quantitation of Mucor antibiosic factor.

Extractions from Mucor mycelia were screened using both filter paper and TLC silica gel plates for chromatographic separations of antagonistic components. Mucor mycelium was produced in large quantities with bulk culture technique. After 72 hours of growth, Mucor mycelium was harvested from shallow (1mm) broth media grown in 2 liter flasks, 50 ml./flask. The fresh mycelia was then extracted with Bloor's lipid extraction procedure that included ethanol:diethyl ether (3:1), 100 ml./1.gram dry mycelia (first extraction); diethyl ether:ethanol (3:1), 100 ml./1.gram dry mycelia (second extraction). The total solvent of the extraction was removed using a rotary vacuum evaporator. The remaining extraction residue was first washed with non-polar lipid solvent (hexane:diethyl ether:acetic acid, 90:10:1) to remove the non-polar components. A polar lipid solvent (chloroform:methanol:water, 65:35:3) was then used to remove the polar lipids. These crude extracts were brought to a quantitative final volume equivalent to the weight of the dry mycelia extracted (1.gram = 1 ml.).

These lipid extracts were spotted in bands on fluorescent, 1.mm thick silica gel plates, and developed with the non-polar solvent system, figures 1 and 2. The plates were removed after separation, air dried, and saturated with A. parasiticus spore suspensions in Czapek-Dox broth. The inoculated, developed plates were placed in saturated humidity at 30°C for 24 hours. Visualization was accomplished by spraying the plate with a 2% iodine in ethanol solution. The areas that remained unstained were those areas of A. parasiticus inhibition that contrast with the dark iodine stained areas of mycelial growth, figures 3 and 4.

The silica gel was then removed from the areas of inhibition for extraction of antibiosic components. The silica gel antibiosic areas were extracted with polar solvent. The polar solvent extraction was evaporated under an air stream where a bilayer was formed. The two layers were separated and the volumes of the two layers were adjusted to be equal to a 10 fold dilution of the crude extract.

The upper layer was diluted as needed for analysis of its antagonistic effects on A. parasiticus. Small portions were placed in test tubes and dried under an air stream. Each component sample was brought to a final volume of 100 ul. This total sample was spotted on Whatman #4 filter paper disks (4.25 cm.) with the 100 ul. placed in the center of the paper as a single droplet. The filter disk was supported only at the outer edge by placing it on the top of a class cylinder for the spotting procedure. This technique allowed the solvent droplet to diffuse uniformly from the disk center. The dried filter disk was then saturated with an A. parasiticus spore Czapek Dox broth medium suspension using an apparatus consisting of circular cut indentations and channels in plexiglass. This apparatus allowed the suspension to uniformly diffuse toward the center of the filter disk from the edge when it was kept flat by another circular cut piece of plexiglass. This apparatus is shown in figure 5.

The inoculated disk was placed in a petri dish and these culture dishes randomly placed within a filter paper lined glass tank having a free water surface. The developing tank was incubated at 30°C for 72 hours.

After incubation, the filter disks were submerged in 2% iodine in ethanol solution, the iodine solution was then drained from the disks

and readings taken following 3 minutes and 30 minutes reaction time. The developed disks were placed in the color analyzer attachment of the Bausch & Lomb Spectronic 20 for absorbance readings. Absorbance readings were proportional to the amount of inhibition with numerical values correlated to the amount of inhibition. These values were compared to standard values obtained by this same quantitation procedure using concentrations of the fungicide N - (trichloromethylthio) - 4 - cyclohexene - 1,2 - dicarboximide (Captan).

The color analyzer attachment of the Bausch & Lomb Spectronic 20 used for the iodine stain quantitation is shown in figure 6.

Duplicate filter disks were determined for each treatment with three experiments for each extraction and three experiments for the standard fungicide, N - (trichloromethylthio) - 4 - cyclohexene - 1,2 - dicarboximide (Captan), with duplicates for each treatment level.

3. Statistical analysis for percent inhibition and probit estimate.

The raw data, recorded as absorbance (Abs), was punched into IBM computer cards on an IBM Keypunch 029. Each absorbance value was converted to % transmission by the formula: Trans. =2.30259(2.0-Abs.). Calculation of the probit was found by the formula:
PROBT = Probit(Trans. x 0.01).

The slope-ratio analysis (9) was assumed valid when the print outs indicated a significant F value for linear effects and non-significant F values for the quadratic, cubic, and quartic effects. When curvature occurred, the curves were plotted to find these trends.

Linear response trends were used to calculate the computed linear regression values to estimate the relative potency and confidence limits (9). The analyses were made using the probit and transmission values for comparing the relationships between these two responses.

CHAPTER IV

RESULTS AND DISCUSSION

Antagonistic interactions between Mucor sp. and A. parasiticus were demonstrated by antibiosic studies evaluating their metabolic inhibition apparent with several different variables. The antibiosic procedures, involving challenge cultures, gave important clues to the actions of each microorganism with cultural competition.

Challenges with Mucor sp. as the base culture, when inoculated with A. parasiticus, had no significant effect on the Mucor. The growth remained approximately the same for each set of challenge cultures with exception to the challenge at the initial inoculation time. This treatment resulted in both an increased mycelial weight and aflatoxin levels produced from the small amounts of A. parasiticus mycelia. Even though aflatoxin was produced in the zero time challenge, shown in Table I, there was an inhibitory effect on the A. parasiticus mycelial growth.

Table I also illustrates definite antagonistic action when Mucor sp. is inoculated into an A. parasiticus base culture. The inoculation of the challenge initially, and after 24 hours of base culture growth, produced aflatoxin in higher concentrations than the 0 time. The two treatments with antagonistic activity indicated that small quantities of Mucor are stimulatory to aflatoxin production with the 24 hour challenge; whereas, with the initial time, a larger quantity

TABLE I

EFFECTS OF INCUBATION TIME FOR BASE CULTURE, PRECEDING CHALLENGE CULTURE INOCULATION, ON GROWTH AND AFLATOXIN SYNTHESIS

Base Culture	*Mucor* Base *A. para.* Challenge		*A. para.* Base *Mucor* Challenge	
Time (Hr.)[1]	Mycelial[2] dry wt. g	Aflatoxin[3] ug/ml	Mycelial dry wt. g	Aflatoxin ug/ml
0	0.53	0.00	0.88	11.98
1	0.57	39.57	0.47	21.65
12	0.54	0.00	0.89	214.15
24	0.36	0.00	0.88	16.75
36	0.44	0.00	0.88	10.41
48	0.40	0.00	0.90	8.94
60	0.39	0.00	0.93	4.15
72	0.44	0.00	0.91	7.99

1. Number of incubation hours at 30°C preceding challenge culture inoculation to base culture.

2. Mycelial dry wt. as the mean of triplicate 20 ml. cultures.

3. Aflatoxin, pooled B_1 and G_1, synthesized per 20 mls. culture media.

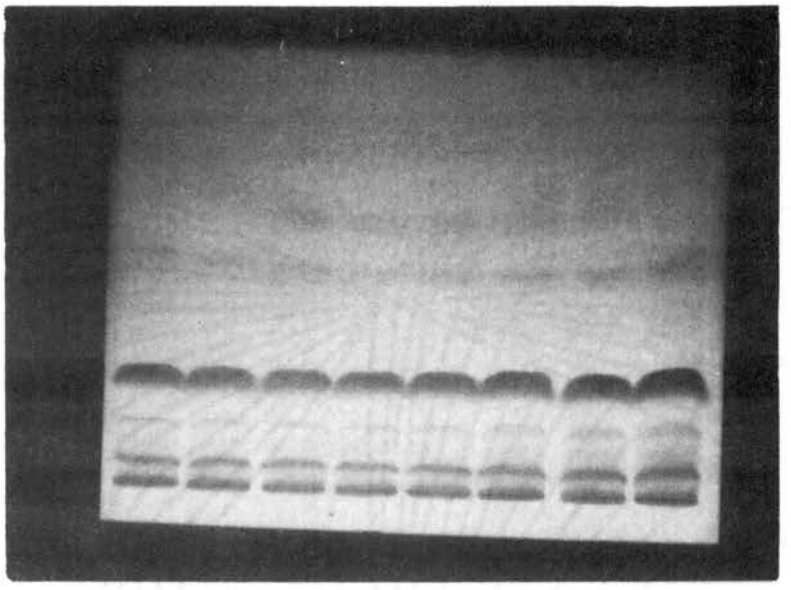

Figure 1. Nonpolar Solvent Separations of Nonpolar Mucor Mycelial Extraction with TLC Visualized in Long Wave Ultraviolet Light (360-366nm)

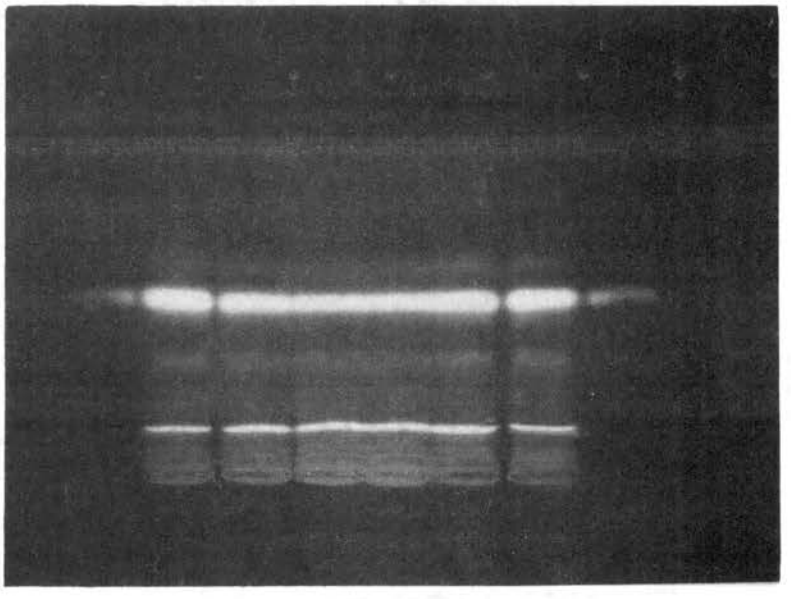

Figure 2. Nonpolar Solvent Separations of Nonpolar Mucor Mycelial Extraction with TLC Visualized by 2% Iodine-Ethanol Solution in Incandescent Light

TABLE II

EFFECTS OF INCUBATION TIME ON GROWTH AND SYNTHESIS OF AFLATOXIN FROM A. PARASITICUS CULTURES CHALLENGED WITH MUCOR

Incubation	Culture Yield	
Hours[1]	Mycelial[2] dry wt. g	Aflatoxin[3] ug/ml
36	0.13	0.00
48	0.22	0.00
60	0.26	0.00
72	0.29	0.00
84	0.28	0.00
96	0.34	9.65
108	0.44	11.85
120	0.54	33.61

1. Number of incubation hours for mixed base and challenge cultures before termination.

2. Mycelial dry wt. as the mean of triplicate 20 ml. cultures.

3. Pooled aflatoxin B_1 and G_1, synthesized per 20 mls. culture media.

of Mucor was produced resulting in an inhibition of A. parasiticus mycelial growth causing an inhibition of aflatoxin production.

For better understanding the effects apparent with the initial challenge cultures, additional studies were undertaken to determine the transformations occurring at different stages of growth. The cultures were all challenged at zero time and harvested at 12 hour intervals for quantitation. Results of these studies are shown in Table II. No aflatoxin was produced at 72 hours and aflatoxin was produced in only small concentrations after 96 hours. Chang and Lynd (6) reported maximum aflatoxin production between 72 and 96 hours. In previous studies, mycelial weights were less than the unchallenged cultures of A. parasiticus and were comparable to the Mucor cultures. Data in Table II also shows that with increased time both mycelial weight and aflatoxin levels increased, indicating A. parasiticus developed some adaptation to inhibitory effects approximately 96 hours after the challenge occurred. This phenomena may result from the rapid growth of Mucor allowing early production of the inhibitory compound blocking A. parasiticus at early growth stages. Mucor decreased in growth vigor after 72 hours allowing the A. parasiticus to become more competitive. Apparently with the inhibitory compounds still present, the A. parasiticus apparently under a stress condition, resulted in more aflatoxin production per unit of mycelia. Thus at 96 hours the inhibitory component was not adequate to inhibit aflatoxin and mycelial production. At 120 hours, apparently the inhibitory component was less concentrated, but still inhibitory to mycelial growth although stimulatory to aflatoxin production.

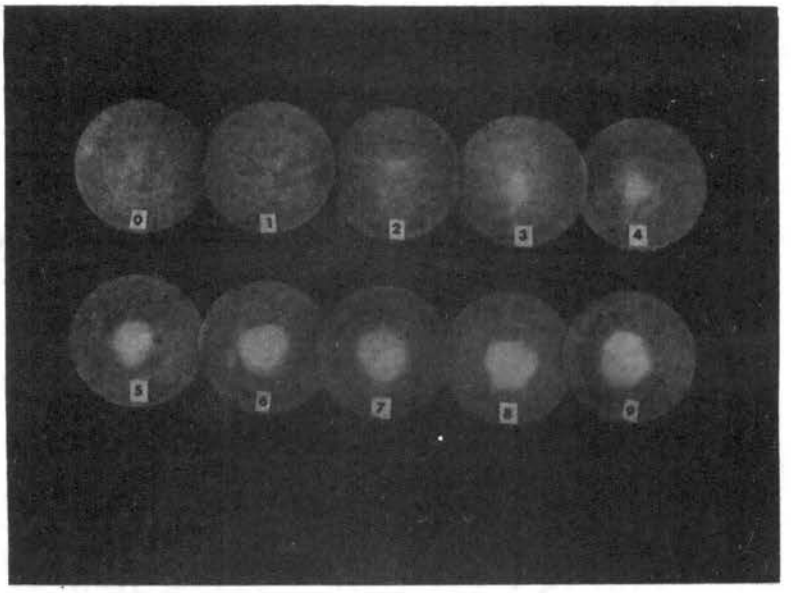

Figure 3. Inhibition of A. Parasiticus Growth on Filter Paper - Czapek Dox Media and Increased Captan Levels Visualized with 2% Iodine-Ethanol Solution

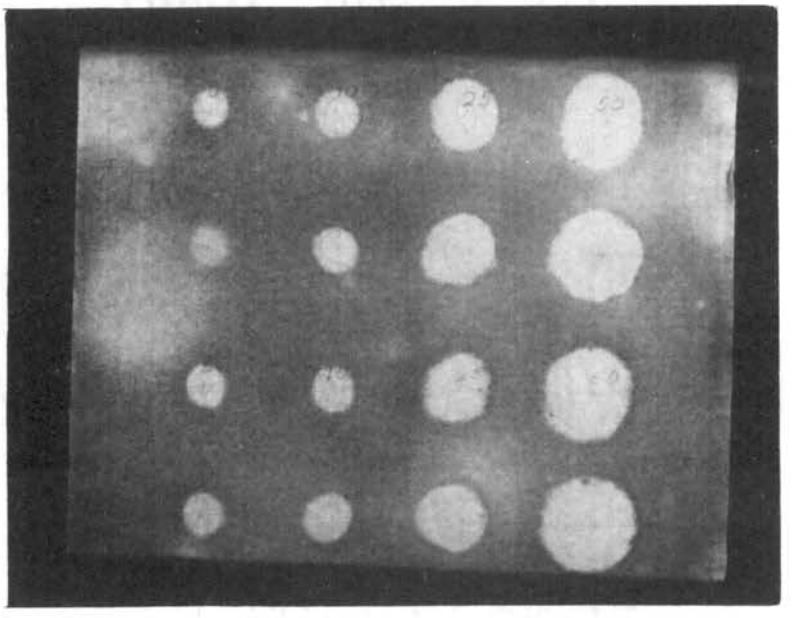

Figure 4. Inhibition of A. Parasiticus Growth on Filter Paper - Czapek Dox Media and Increased Levels of Antibiosic Mucor Component. Upper Paired Spots, First Incubation Series; Lower Paired Spots, Second Incubation Series

TABLE III

EFFECTS OF CAPTAN CONCENTRATION AND DETECTION TIME WITH IODINE QUANTITATION FOR INHIBITION OF A. PARASITICUS GROWTH

Captan Concentration [x]	% Transmission (560nm) [f(x)]	
ug/ml	3 min.	30 min.
Blank	25.1	43.7
25	24.5	39.8
50	36.3	58.8
100	58.9	87.1
200	58.9	93.4
400	58.9	89.1
800	67.6	95.5
Slope of the fitted straight line: $f(x) = a + b(x)$, $0 \leq x \leq 100$	.342	.381

F values: Effects due to treatment, Captan levels = 123.27
Effects due to reaction time = 304.01
F values were highly significant.

TABLE IV

CAPTAN CONCENTRATION AND IODINE DETECTION RESPONSE FOR STANDARD INHIBITION OF A. PARASITICUS

Captan Concentration ug/ml	% Transmission (560nm) Experimental Series			
	I	II	III	MEAN
Blank	30.9	42.2	39.8	37.6
0	30.2	43.2	41.2	38.2
10	42.6	41.6	40.3	41.5
20	71.6	46.2	50.7	56.2
30	88.1	62.4	63.1	71.2
40	88.1	67.6	69.2	75.0
50	89.1	76.7	87.1	84.3
60	92.3	89.1	83.2	88.2
70	90.2	88.1	93.3	90.5
80	89.1	94.4	93.3	92.3
90	86.1	93.3	93.3	90.9
100	89.1	93.3	93.3	91.9

% Transmission value for each experiment is the mean of duplicate cultures after 30 minutes reaction time with iodine solution.

The procedure for determining inhibition was evaluated by using a standard fungicide, N - (trichloromethylthio) - 4 - cyclohexene - 1,2 - dicarboximide (Captan) and these results obtained were compared for response trend distortions and the concentrations required for maximum inhibition. The filter disks, allowed to react only 3 minutes with iodine, were discolored by the residual iodine giving distorted readings, Table III. Following 30 minutes reaction time, the residual iodine had diffused from the filter disks, except for the I_2 - unsaturated lipid stained areas, allowing a direct proportional relationship between the amount of inhibition and % transmission. Captan concentrations above 100 ug/g active ingredient equivalent, gave complete inhibition with constant minimum readings approaching 100% transmission (0 absorbance); therefore, the standard curve for fungicide inhibition was developed with concentrations below 100ug/g.

Table IV presents values for the standard inhibition response determined with Captan levels. The higher concentrations have approximately equal values because at these higher inhibition levels the color analyzer reflectance attachment of the Spectronic 20 is most sensitive to a relatively small central portion of the filter disk, Figure 3 and 6. The concentration F values were highly significant with C. V. ranging from 2 to 10%. The experimental series I of Table IV had overall high absorbance levels which were less desirable for statistical evaluations. Analysis of variance indicated a small quadratic effect on the 5 level analysis, although the linear effect was highly significant. In the 3 level analysis the quadratic effect was removed.

The Mucor components were extracted and purified for determination of the antagonistic inhibition of A. parasiticus, Fugures 1 and 2.

TABLE V

RELATIVE INHIBITION OF MUCOR EXTRACT TO A. PARASITICUS WITH IODINE SOLUTION QUANTITATION

Mucor Component ug/ml	Incubation Series % Transmission (560 nm)		
	I	II	MEAN
Blank	46.5	40.9	43.7
0	47.5	40.7	44.1
1	43.2	45.0	44.1
2	67.9	66.3	67.1
3	82.9	82.5	82.7
4	90.9	90.2	90.6
5	93.0	93.0	93.0

% transmittance values are means of duplicate cultures from three repeated experiments for each Mucor incubation series.

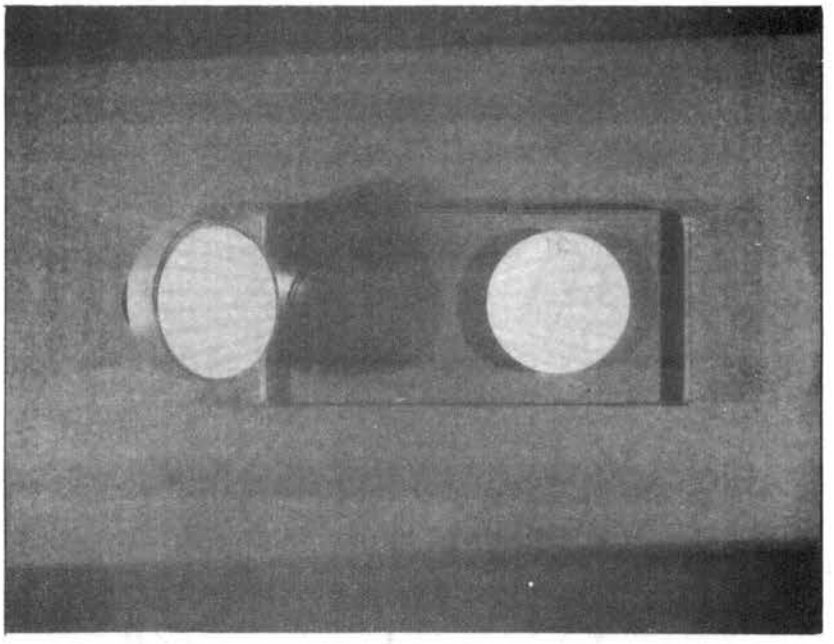

Figure 5. Transparent Plastic Base with Depressed Circular Groove Patterns for Uniform Impregnation of Filter Paper Disks with A. Parasiticus Spore Suspensions

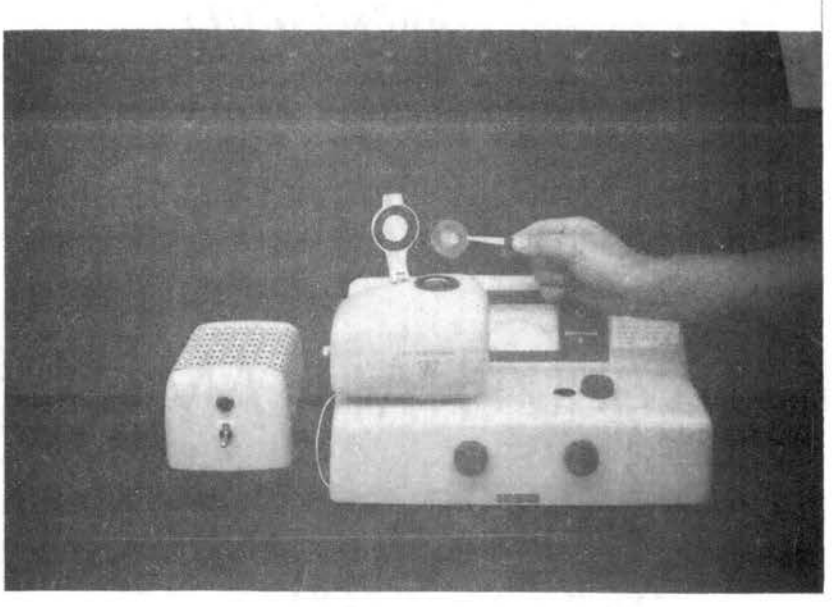

Figure 6. Adaptation of the Bausch and Lomb Spectronic 20 Color Analyzer Reflectance Attachment for Quantitation of A. Parasiticus Growth and Inhibition

TABLE VI

ESTIMATES OF MUCOR COMPONENT INHIBITION, AS PERCENT EQUIVALENT TO CAPTAN LEVELS ANTIBIOSIC TO A. PARASITICUS

Mucor Component	% Inhibition Incubation Series			Captan Level	% Inhibition
ug/ml	I	II	MEAN	ug/ml	
1	9.9	12.7	11.3	20	35.3
2	49.1	46.6	47.7	30	52.9
3	73.0	72.4	72.7	40	59.5
4	85.7	84.6	85.2	50	74.9
5	89.0	89.0	89.0	60	81.7

Mucor component figures shown are means of 6 cultures in each series. % Inhibition was estimated from % trans. readings calculated as: (%T-37) 1.59 = % Inhibition. The value 37 was % trans. with zero inhibition, 1.59 factor is assumed with 100% trans. resulting in complete inhibition.

TABLE VII

COMPARISONS OF EQUIVALENT INHIBITION BY GRAPHED ESTIMATIONS FOR PERCENT TRANSMISSION AND PROBIT ANALYSIS OF CAPTAN AND MUCOR COMPONENT ANTIBIOSIC TO A. PARASITICUS[1]

Five Level Slope-Ratio Analysis

CONC.	MUCOR SERIES #1				MUCOR SERIES #2			
			Estimated ug/g Captan Eqv.				Estimated ug/g Captan Eqv.	
ug/g	%Trans.	Probit	%Trans. Equation	Probit Equation	%Trans.	Probit	%Trans. Equation	Probit Equation
1	51.6	-0.12	10.0	5.6	51.5	0.00	10.0	10.0
2	63.5	0.41	25.5	26.2	63.8	0.41	25.9	26.3
3	75.7	0.96	41.7	46.8	76.0	0.82	42.0	42.1
4	87.8	1.49	57.6	68.0	88.0	1.23	57.8	58.4
5	100.0	2.01	73.4	88.0	100.0	1.63	73.5	73.5

Three Level Slope-Ratio Analysis

CONC.	MUCOR SERIES #1				MUCOR SERIES #2			
			Estimated ug/g Captan Eqv.				Estimated ug/g Captan Eqv.	
ug/g	%Trans.	Probit	%Trans. Equation	Probit Equation	%Trans.	Probit	%Trans. Equation	Probit Equation
2	68.9	0.68	35.0	40.5	67.0	0.52	33.2	38.5
3	79.3	1.11	45.4	57.3	78.0	0.95	44.1	52.0
4	89.6	1.53	59.5	71.3	89.0	1.38	58.8	66.3

1. Values shown are means of duplicate cultures in all experiments repeated three times. All concentration F values are highly significant.

TABLE VIII

TABLES OF ANALYSES OF VARIANCE

AOV's for Slope Ratio Analysis Using Five Levels

SOURCE	d.f.	MEAN SQUARES					
		Mucor I		Mucor II		Standard	
		%Trans.	Probit	%Trans.	Probit	%Trans.	Probit
Runs	2	108	1.19	14	0.01	882	1.03
Conc.	4	2520	4.25	2352	2.59	959	1.04
Runs x Conc.	8	55	0.48	20	0.03	71	0.07
Dup.	15	77	0.46	39	0.05	28	0.04
C. V.*		9.8	22.8	6.0	20.0	11.0	27.0

AOV's for Slope Ratio Analysis Using Three Levels

SOURCE	d.f.	MEAN SQUARES					
		Mucor I		Mucor II		Standard	
		%Trans.	Probit	%Trans.	Probit	%Trans.	Probit
Runs	1	68	0.23	23	0.02	56	0.08
Conc.	2	715	0.92	846	1.12	378	0.39
Runs x Conc.	2	66	0.07	30	0.03	28	0.05
Dup.	6	124	0.15	58	0.07	32	0.03
C. V.*		10.0	27.2	6.9	20.2	7.4	36.3

$$*\text{C. V.} = \frac{\sqrt{\text{Error Mean Square}}}{\text{Overall Mean}} \times 100$$

TABLE IX

POTENCY ESTIMATION OF MUCOR COMPONENTS WITH CAPTAN STANDARDS FOR A. PARASITICUS INHIBITION

Response Surface with Five Levels

Response Values as	Source	Regression	Rel. Potency	95% Limits Lower	Upper
%Transmittance	Captan	Y=44.010+0.778x			
	Mucor I	Y=39.591+1.2125x	1.559	1.159	2.189
	Mucor II	Y=39.935+1.189x	1.528	1.136	2.145
Probit	Captan	Y=-0.276+0.026x			
	Mucor I	Y=-0.617+0.053x	2.038	1.230	4.138
	Mucor II	Y=-0.403+0.041x	1.577	0.900	3.257

Response Surface with Three Levels

Response Values as	Source	Regression	Rel. Potency	95% Limits Lower	Upper
%Transmittance	Captan	Y=33.369+0.944x			
	Mucor I	Y=48.808+1.077x	1.141	0.058	2.746
	Mucor II	Y=45.080+1.163x	1.232	0.065	2.952
Probit	Captan	Y=-0.608+0.030x			
	Mucor I	Y=-0.198+0.039x	1.300	0.666	3.390
	Mucor II	Y=-0.377+0.043x	1.433	0.749	3.720

Calculated with slope ratio assay method proposed by Finney (9).

The values of Table V illustrate the inhibition of A. parasiticus by the components extracted from different increase cultures of Mucor. These values were first converted empirically to % inhibition with a direct interpolation that estimated fungicidal activity of the Mucor component as compared to the standard Captan response. Table VI summarizes the results of this method indicating the component as 12.5 times the Captan per equivalent weight in fungicidic effects to A. parasiticus growth.

Level of inhibition data may be considered as dose response and statistically analyzed by several methods. Table VI exhibits the observations that show the fungicical activity for the Mucor component to be 12.5 times as active as the Captan. A standard Captan level-response was used to estimate the fungicidal equivalents of Captan in Table VII and VIII. Probit values were determined for relative inhibition levels apparent with the mid-near linear portion of a sigmoidal plot. A comparison of % transmission and probits indicated little difference in relative potencies. It should be noted that the slope and intercept found by the % transmission lines were respectively different from slope and intercept found by using probits. When three levels of the concentration were used the quadratic trends were less than when five levels were used.

Relative potency was calculated as described by Finney (9). Limits of confidence determined for relative potencies showed the five level analyses gave smaller ranges than the three level analyses. The slope-ratio analysis described by Finney (9) allows calculations of limits for relative potency values, Table IX.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objectives of this study were: (1) determine antibiosic interactions between a Mucor sp. association and A. parasiticus, (2) develop procedures for determining inhibition of A. parasiticus growth and aflatoxin synthesis by Mucor components, and (3) statistically determine the relative potency using a standard fungicide, N - (trichloromethylthio) - 4 - cyclohexene - 1,2 - dicarboximide (Captan). Challenge cultures were used for determining antibiosis apparent between Mucor sp. and A. parasiticus. Mycelial weight and aflatoxin synthesis parameters were evaluated with the challenge cultures. A laboratory procedure was devised to determine relative inhibition of fungal growth, with I_2 - ethanol staining, using the color analyzer reflectance attachment of the Bausch & Lomb Spectronic 20. A slope-ratio analysis procedure described by Finney (9) was used to obtain the relative potency of the Mucor component.

Challenge cultures indicated that Mucor sp. do metabolize antibiosic components inhibiting A. parasiticus growth and aflatoxin synthesis. These materials were extracted and the antibiosic inhibition was determined by iodine visualization. A slope-ratio analysis showed that the Mucor component was established to be approximately 1.5 times the fungicidal activity to A. parasiticus than equivalent

weights of the active ingredient within a standard fungicide, N - (trichloromethylthio) - 4 - cyclohexene - 1,2 - dicarboximide (Captan).

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