Kinetics of Growth and Accumulation of Aflatoxin B_1 by *Aspergillus parasiticus* in the Presence of Butylated Hydroxyanisole, Isoprothiolane, and Nystatin

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Growth of *Aspergillus parasiticus* and accumulation of aflatoxin B_1 in the medium that contained antifungal agents were monitored during the growth cycle of the mold. The antifungal agents tested are the food additive (antioxidant), butylated hydroxyanisole (BHA), the pesticide, isoprothiolane, and the antibiotic, nystatin. Growth of the mold was quantified using a newly developed criterion, named the growth coefficient (GC). The GC values were calculated from the growth parameters of the logistic function that fits the growth curve of the mold. At the range of concentrations of additives studied, maxima of growth inhibition were 31.3, 23.1, and 43.6% at 60 ppm of BHA, 70 ppm of isoprothiolane, and 45 units of nystatin/mL, respectively. The ability of the mold to accumulate aflatoxin B_1 in its medium [as measured by the accumulation rate constant (α)] in the presence of various levels of the antifungal agents was concentration-dependent. Sixty parts per million of BHA decreased the value of α by 71.7%, 30 units of nystatin/mL was the most effective concentration of that antibiotic and resulted in only 23.5% inhibition, and isoprothiolane at all its levels stimulated elaboration of aflatoxin B_1 by the mold.

INTRODUCTION

In previous studies,[1,2] we tested some antifungal agents and food additives for their effects on aflatoxin formation by *Aspergillus parasiticus*. An appreciable problem during those studies was to find appropriate methods for assessing inhibition or stimulation of mold growth and of the mold's ability to synthesize aflatoxin. These earlier studies emphasized the importance of monitoring both mold growth and production of aflatoxin throughout the growth cycle. After developing a suitable way to analyze those data, it was possible to compare the mold's ability to accumulate aflatoxin under different conditions with minimum interference from levels of mycelial growth.

A part of this study was devoted to assessing the growth behavior of the mold (stimulation or inhibition) and applying the developed criterion to cultures of mold that were grown in the presence of selected antifungal agents. The rate constant for accumulation of aflatoxin[2] was applied to measure the mold's ability to accumulate aflatoxin in the presence of butylated hydroxyanisole (BHA), isoprothiolane, and nystatin.

The BHA is an antioxidant that is added to some fat-containing processed foods. Beside being an antioxidant, BHA has antimicrobial activity against several bacteria and fungi.[3,4] Some studies were done to evaluate the inhibitory effect of BHA on growth of aflatoxigenic molds and their ability to produce aflatoxin.[3,5-7] Our review of these reports suggested that a more in-depth study was needed. Growth of mold and synthesis of aflatoxin in the presence of BHA can be monitored using a more quantitative approach for assay and data analysis than those used in the previous reports.

Isoprothiolane (diisopropyl 1,3 dithiolan-2-ylidene malonate) is a synthetic pesticide that is effective against rice blast (caused by the mold *Pyricularia oryzae*) and several species of plant hoppers.[8] Its minimum inhibitory concentration for *P. oryzae* was estimated to be 20-50 ppm. Isoprothiolane was reported to be one of the safest pesticides presently in use.[8] This prompted us to test this compound, as a potential food additive, for its effectiveness against growth and synthesis of aflatoxin by *A. parasiticus*. Some researchers[9] reported that isoprothiolane is an inhibitor of chitin synthetase. If its pesticidal action results from that property, use of isoprothiolane could be promising in controlling both molds and mites (both have chitin as a structural component) in cheese curing rooms.

Nystatin is an antifungal agent produced by *Streptomyces noursei*,[10] and is classified as a "polyene antibiotic."[11] It has the same mode of action on susceptible organisms as Amphotericin B, and acts against fungi by

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altering cell membrane function. Nystatin is currently used to treat serious fungal infections in man.[11] Recently, Gunasekaran[12] isolated an aflatoxigenic strain of *Aspergillus flavus* from a facial scar on the cheek of a patient with acute myelocytic leukemia. Accordingly, it may be worthwhile to test nystatin for its possible antifungal properties against aflatoxigenic molds and to determine if that is accompanied by any significant inhibitory effect on aflatoxin synthesis.

Criteria for Growth Behavior of Molds

Many researchers compare the growth behavior of microorganisms (stimulation or inhibition) under different environmental conditions by incubating cultures for an arbitrarily chosen period and then measuring growth attained at that point. Beside being a crude way to compare the effect of factors studied, this method can lead to conflicting results.[1] Monod[13] reported that growth of a bacterial culture can be characterized by three fundamental growth constants: total growth, exponential growth rate (or maximum specific growth rate), and growth lag. Researchers in different areas of biology have compared growth behavior of microorganisms using one of the growth constants, depending on the purpose of study. This report attempts to combine these growth constants in one expression to get a single criterion for general comparisons of growth behavior of molds under different environmental conditions.

In a previous study,[2] growth curves of molds were fitted using the logistic function:

$$X = \frac{K}{1 + \exp(A_1 - A_2 t)} \tag{1}$$

where X is the amount of growth, t is the incubation time, and K, A_1, and A_2 are constants.

In the following discussion, we will try to correlate the parameters of this function with the growth constants by Monod.

From eq. (1), it is obvious that K can be used as an estimate for maximum growth (X_{max}) attained by the mold's culture, i.e.

$$X_{max} \simeq K \tag{2}$$

The specific growth rate (μ), as expressed by the following equation,

$$\mu = \frac{dx}{dt}\left(\frac{1}{X}\right) \tag{3}$$

attains a maximum value (μ_{max}) at the logarithmic phase of growth.[14] Term μ_{max} is usually taken as a characteristic value of growth behavior. Since the logarithmic phase of the mold's growth in batch cultures is usually short,[15] it may be more convenient to take the value of μ at a reference point on growth curves (e.g. μ at the point of inflection or μ_i) as a characteristic value for growth. From eqs. (1) and (3),

$$\mu = A_2\left(1 - \frac{X}{K}\right) \tag{4}$$

and

$$\mu_i = 0.5A_2 \tag{5}$$

As can be noted in Figure 1, increasing the value of A_1 will increase the lag of growth curves. Since it is difficult to find an appropriate definition of lag period in the literature, it may be satisfactory at this point to consider the lag period (L) as a function of A_1, or

$$L = L(A_1) \tag{6}$$

Figure 1 shows how a hypothetical growth curve behaves when the magnitude of parameters of the logistic function vary.

An index to measure growth behavior will be termed the growth coefficient (GC). The GC value should be directly proportional to the specific growth rate and maximum growth of mold and inversely proportional to the length of lag period.

From eqs. (2), (5) and (6) it follows that:

$$GC = cK\frac{A_2}{2}\frac{1}{A_1} \tag{7}$$

where c is a constant. For a more meaningful GC, constant c may be assigned the value 1, accordingly

$$GC = \frac{K}{2}\frac{A_2}{A_1} \tag{8}$$

In this instance, GC equals the slope of the angle θ in Figure 2. In other words, at this special value of the constant C, the growth coefficient equals the average rate of growth during the incubation period that started with inoculation of mold spores and ended with the growth rate

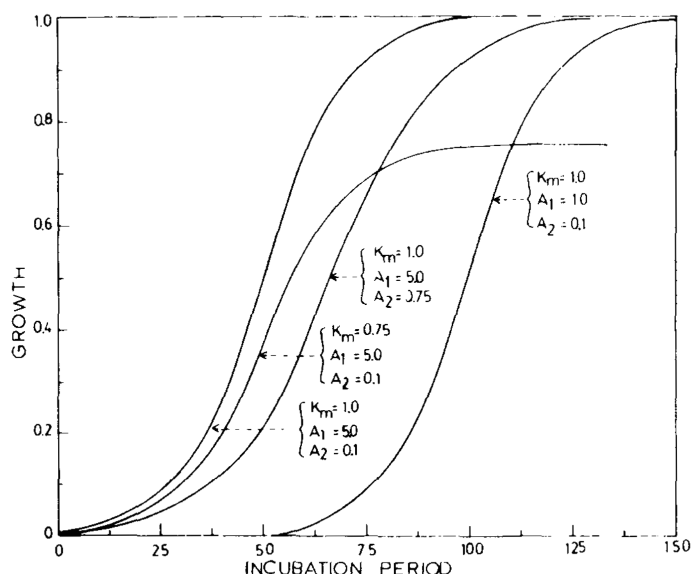

Figure 1. Hypothetical growth curves obtained at the indicated values of parameters of eq. (1).

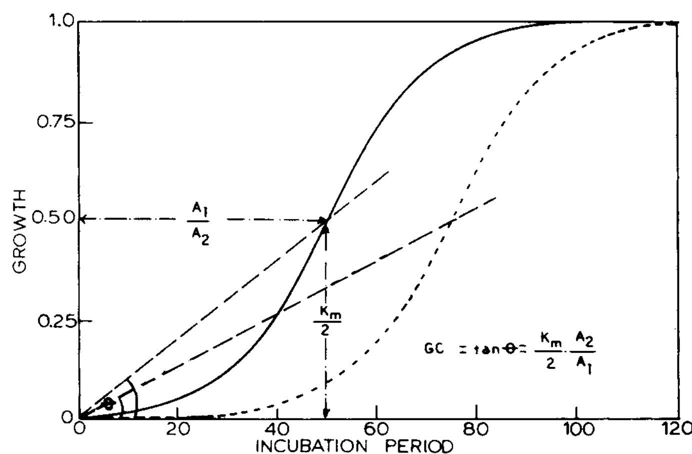

Figure 2. Growth coefficient (GC) as a function of the parameters of the logistic function [eq. (1)]. Values for growth and incubation periods are hypothetical.

being maximum. The form of GC depicted in eq. (8) will be used to evaluate the growth of *A. parasiticus* at different levels of the antifungal agents to be tested in this study.

MATERIALS AND METHODS

A glucose-salts-yeast extract medium (previously designated as M_2)[1] was adjusted to pH 5.5. One of the following antifungal agents was added to the medium:

1) BHA (Nutritional Biochemical Corp., Cleveland, OH); a series of concentrations of this material in dimethyl sulfoxide (DMSO) was prepared to give 15, 30, 40, or 60 ppm of BHA when 1 mL of solution was added to the medium;

2) isoprothiolane (Nihon Nohyaku Co., Ltd., Tokyo, Japan) was also dissolved in DMSO to give a concentration of 10, 30, 50, or 70 ppm when 1 mL of solution was added to the medium;

3) nystatin (Sigma, St. Louis, MO); aqueous suspensions of this material were prepared so that 1 mL of the suspensions, when added to the medium, gave 15, 30, 45, or 60 USP units of nystatin/mL of medium. A series of flasks of medium to which 1 mL of DMSO was added served as control for BHA and isoprothiolane, whereas the control for nystatin was prepared by adding 1 mL of water to another series of flasks of medium.

Decisions about the just mentioned range of concentrations of test chemicals were made after testing wider ranges of concentrations in a preliminary experiment. A reasonable length of incubation and solubility of test materials were considerations when those decisions were made.

A series of 125-mL Erlenmeyer flasks, each containing 28 mL of sterilized medium, was inoculated each with 1 mL of a spore suspension (10^4-10^6/mL) of *A. parasiticus* NRRL 2999 (spore suspension was prepared as indicated before[1] except that distilled water instead of the solution of Tween 80 was used to harvest and dilute the spores). Solutions of antifungal agents or carrier solvent (1 mL each) were then added, bringing the volume of cultures to 30 mL/flask. Cultures were incubated quiescently at 28°C ± 1. At intervals during incubation, mycelium and liquid medium were separated and mycelial dry weight was determined as reported earlier.[2] Liquid medium-chloroform mixtures were stored frozen for later analysis. The mixtures taken from cultures two days before to two days after the growth was maximum were thawed, and the remainder of the extraction and aflatoxin analysis procedures were done as described in our earlier report.[1] Aflatoxin B_1 in extracts was determined using HPLC.[2] The maximum amount of aflatoxin B_1 produced was estimated by selecting the highest value obtained over the four-day interval that brackets maximal growth of the mold. The accumulation rate constant (α) for aflatoxin B_1 was estimated as follows[2]:

$$\alpha = \frac{\text{maximum amount of aflatoxin } B_1 \text{ accumulated (mg/flask)}}{\text{maximum amount of dry mycelium observed (g/flask)}} \tag{9}$$

Parameters of the logistic function [eq. (1)] as fitted to growth curves were estimated using the nonlinear regression subroutine of the Madison Academic and Computing Center (University of Wisconsin-Madison) and the Univac-1100-82 Computer. Data for the growth curve up to maximum growth were used to fit the logistic function. Parameters of the logistic function were used to calculate the GC values [eq. (8)].

RESULTS

Growth Behavior

Growth, expressed as mycelial dry weight, was fitted to the logistic function [eq. (1)] as reported earlier. Parameters of the logistic function for growth curves, values of the growth coefficient (GC), and values of the aflatoxin accumulation rate constant (α) are given in Table I. To check the repeatability of the GC and α values, two other independent trials were prepared as in the control for the nystatin treatment. Growth parameters, growth indices, and aflatoxin accumulation indices for these two trials were estimated as before. A total of three trials were thus obtained, which were: 1) control for nystatin treatment (designated as C_1)—data for that control are in Table I; 2) the second trial (designated C_2)—K, A_1, and A_2 for this trial were 1.069, 4.086, and 0.0710, respectively, and values of GC and α were 9.288×10^{-3} and 0.803, respectively. 3) the third trial (designated C_3)—K, A_1, and A_2 for this trial were 1.073, 3.953, and 0.0795, respectively, and values of GC and α were 10.790×10^{-3} and 0.901, respectively. The average GC value for the three trials was 10.566×10^{-3} and the standard deviation (SD) was 1.182×10^{-3}. It is noteworthy that GC

Table I. Growth parameters [eq. (1)], growth and aflatoxin B_1 accumulation indices [eqs. (8) and (9), respectively] of *A. parasiticus* in the presence of different levels of BHA, isoprothiolane, and nystatin.

Concentration of additive[a]	BHA					Isoprothiolane					Nystatin				
	Growth parameters			Indices		Growth parameters			Indices		Growth parameters			Indices	
	K	A_1	A_2	GC ($\times 10^{-3}$)	α	K	A_1	A_2	GC ($\times 10^{-3}$)	α	K	A_1	A_2	GC ($\times 10^{-3}$)	α
Control[b]	0.897	4.475	0.0651	6.525	0.226	0.932	3.180	0.0445	6.521	0.652	1.061	4.848	0.1062	11.621	0.803
10						0.916	5.630	0.0771	6.272	0.690					
15	0.916	4.483	0.0668	6.825	0.186						1.075	4.873	0.0759	8.372	0.705
30	0.789	4.036	0.0587	5.738	0.162	0.877	5.487	0.0701	5.602	0.781	1.065	5.017	0.0697	7.398	0.614
45	0.776	6.502	0.0899	5.365	0.136						1.152	6.637	0.0755	6.552	0.725
50						0.887	5.007	0.0597	5.288	1.438					
60	0.725	5.286	0.0654	4.485	0.064						1.219	3.517	0.0396	6.863	0.771
70						0.885	4.262	0.0483	5.015	1.163					

[a] Concentrations were expressed as ppm for BHA and isoprothiolane and USP units/mL for nystatin.

[b] Controls were prepared by adding 1 mL of additive's carrier (DMSO for controls of BHA and isoprothiolane and water for that of nystatin) to mold's medium.

values for the controls of BHA and isoprothiolane were almost equal in magnitude (Table I). These two controls were prepared by adding 1 mL of the solvent DMSO to the mold's medium. Solvent DMSO is frequently used in biological systems and some reports indicate that it does not inhibit growth of cultures.[16] In our experiment, the concentration of DMSO in media was ca 3.3%, which was higher than that reported in the previous study.[16] Average inhibition of mold growth because of the presence of DMSO in our experiment, as compared with DMSO-free controls (C_1, C_2, and C_3), was 38.3%. Since all GC values of each additive were compared with its own control, and assuming that DMSO did not exert any appreciable synergistic effect on growth, the inhibition caused by DMSO does not invalidate the results.

The relative GC values (percent of the control) rather than absolute values are depicted in Figure 3. Growth of *A. parasiticus* was inhibited by almost all levels of the agents tested, and inhibition was concentration-dependent. At the range of concentrations studied, the observed maxima for growth inhibition were 31.3, 23.1, and 43.6% at 60 ppm of BHA, 70 ppm of isoprothiolane, and 45 units of nystatin/mL, respectively. The response of growth inhibition to the changing level of various additives was different. Whereas inhibition was close to linear with the concentrations of BHA, it tended to level off as the concentration of isoprothiolane was increased and even diminished at the highest tested level of nystatin. The high concentrations of BHA were more effective in inhibiting the mold's growth than were those of isoprothiolane.

The GC values of growth curves obtained in earlier studies[1,2] are given in Table II. The results indicate that 200 ppm of sorbic acid inhibited growth of mold by 44.6%. Growth inhibition by PLL was concentration-dependent. With the exception of the highest level used, growth inhibition by dichlorvos was essentially concentration-independent.

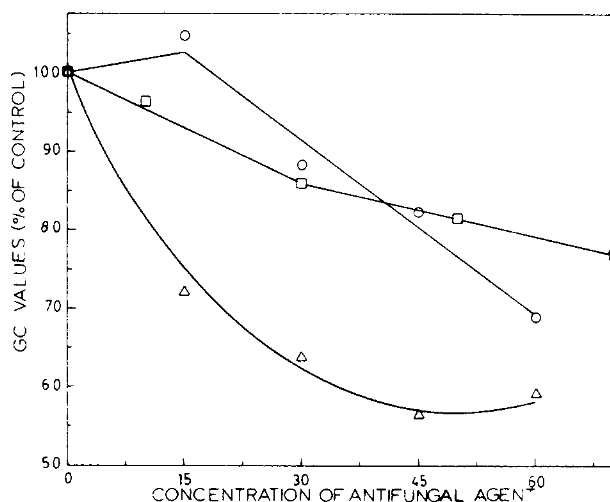

Figure 3. Growth coefficient, GC (percent of control) at different concentration of (○) BHA, (□) isoprothiolane, and (△) nystatin. Concentrations of BHA and isoprothiolane are expressed as ppm and that of nystatin in USP units/mL.

Table II. Values of GC [eq. (8)] for growth curves of *A. parasiticus* in the presence of sorbic acid, PLL, and dichlorvos.

Sorbic acid		PLL		Dichlorvos	
Concentration (ppm)	GC ($\times 10^{-3}$)	Concentration (ppm)	GC ($\times 10^{-3}$)	Concentration (ppm)	GC ($\times 10^{-3}$)
0	8.110	0	3.485	0	3.485
200	4.491	100	3.533	3	3.175
		200	3.244	6	3.174
		300	3.112	9	3.133
		400	2.587	12	3.134
		500	2.659	15	2.857

Accumulation of Aflatoxin B_1

The values for the aflatoxin B_1 accumulation rate constant, α, [eq. (9)] at different levels of additives are given in Table I. The variation in values of α obtained from triplicate trials was evaluated as discussed in the first part of this report. The average value of α for the three trials (C_1, C_2, and C_3) was 0.866 and the SD was 0.055. Despite this reasonably small deviation, variation between α values for the controls of BHA and isoprothiolane was large (Table I). This large variation may be attributed to changes in inoculum size and/or temperature of incubation within the range reported in the Materials and Methods section. Since the experiments with BHA and isoprothiolane were not done simultaneously, changes in the physiological state of mold cultures used in those experiments may have contributed to some of the variability. These environmental changes, however, did not affect the GC values, as discussed before, which suggests that production of aflatoxin is influenced more than growth by alterations in the mold's environment. Aflatoxin is produced by fewer metabolic steps than is growth. Thus, it is more likely that environmental factors will alter secondary metabolism of the mold to a greater extent than growth.

Figure 4 depicts the relative α values (percent of control) at various levels of each additive. All levels of BHA and isoprothiolane inhibited aflatoxin accumulation, whereas isoprothiolane stimulated it. The BHA almost linearly inhibited accumulation of aflatoxin B_1 in the mold's medium. A decrease of up to 71.7% in the value of α was achieved by 60 ppm of BHA. Nystatin also linearly inhibited accumulation of aflatoxin B_1 up to concentrations of 30 units/mL (23.5% inhibition); inhibition was reversed at the higher concentrations.

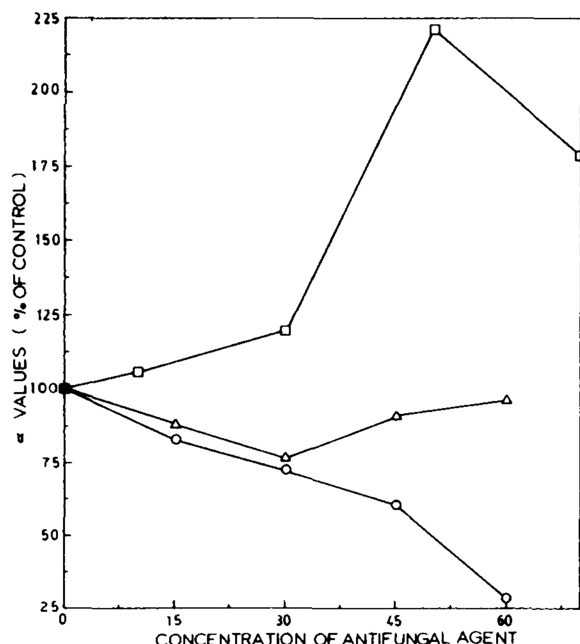

Figure 4. Aflatoxin B_1 accumulation rate constant, α (percent of control), at various levels of (○) BHA, (□) isoprothiolane, and (△) nystatin. Concentration of BHA and isoprothiolane expressed as ppm and that of nystatin as USP units/mL.

In contrast, isoprothiolane, up to 30 ppm, slowly increased accumulation of aflatoxin; this was accelerated at 50 ppm and diminished at 70 ppm. The greatest increase in α was 120.6% at 50 ppm of isoprothiolane. A previous study[2] on a different additive showed smoother curves, that were easier to model, than those depicted in Figure 4. Manual rather than computer estimation of growth and aflatoxin accumulation maxima may be responsible for the somewhat more erratic values of α in this study than those of the previous one.

DISCUSSION

Several investigators studied the effect of BHA on aflatoxigenic molds.[3,5-7] Their reports indicate that BHA is fungistatic toward aflatoxigenic molds and the amount of inhibition depends on the concentration of the additive. These investigators did not agree, however, about the extent of growth inhibition or how aflatoxin synthesis is affected by BHA in the mold's medium. Authors of the earlier reports used dissimilar methods to evaluate the efficacy of BHA on the aflatoxigenic molds. Some of the previous studies evaluated growth and aflatoxin synthesis in petri plates, using a technique similar to antibiotic susceptibility testing,[5,7] while others used different liquid media.[3,6] Temperature of incubation in those studies ranged between room temperature and 28°C. In all instances, growth and aflatoxin formation were determined after a fixed and arbitrarily chosen incubation period (single time points) that ranged between four and ten days. In most of those studies, both growth and aflatoxin detected after the incubation period were altered by BHA in the mold's medium. It was difficult to tell if the changes in the amounts of aflatoxin resulted from a change in the biomass or from alteration in the capacity of mold to form aflatoxin. In a previous study,[1] we demonstrated the possible contradiction of results obtained by measuring growth and aflatoxin accumulation after an arbitrarily chosen incubation period rather than monitoring them throughout the growth cycle. This discussion illustrates the difficulty of comparing our results with those reported earlier.

Some researchers[17] suggested that the antimicrobial properties of the phenolic antioxidants (including BHA) may be related to activity on the cytoplasmic membrane. Because of the partial structural similarity between a phenolic ring in the aflatoxin molecules and that of the BHA molecule,[5] it could be that BHA competes with some intermediate compound in the aflatoxin synthetic pathway for a site on a key enzyme involved in synthesis of the toxin.

There is no agreement about the mechanism by which isoprothiolane inhibits growth of mold. While Leighton and co-workers[9] found that isoprothiolane acts as an inhibitor of chitin synthesis, work of other investigators[18,19]

suggests that it has no primary effect on chitin synthesis. It was reported that 5 ppm of isoprothiolane disturbed lipid metabolism and inhibited by more than 30% incorporation of [^{14}C]acetate into the total lipids of the fungus.[8] Furthermore, isoprothiolane inhibited uptake of sugars from a nutrient medium by fungal cells.[19] These findings may explain the inhibition of growth of *A. parasiticus* exerted by isoprothiolane in our study, but it cannot explain the significant increase in accumulation of aflatoxin B_1 in the mold's medium.

Nystatin alters cell membrane function by creating an osmotic disequilibrium between the cell and its environment. It binds principally to ergosterol, the main sterol in fungal membranes, causing leakage of intracellular contents of sensitive organisms and ultimately cell death.[11] This may explain the relatively strong inhibition of mold growth that was observed in this study. Furthermore, nystatin is unstable in solutions. Lack of stability can be further aggravated by storing solutions for a few days unrefrigerated or adjusting the pH to a value greater or lower than 7.0.[20] It can be concluded that cultural conditions, as those used to grow *A. parasiticus* in this study, are far from ideal for stability of nystatin. Beggs and coworkers[16] found that antioxidants synergistically increase the fungicidal action of amphotericin B. Since this compound is of the same class as nystatin, it is probable that the fungicidal action of nystatin can be improved by adding BHA to the mold's medium. Addition of BHA may also result in improving the ability of nystatin to inhibit aflatoxin accumulation, which was limited in this study. Speculations about the mechanisms by means of which BHA, isoprothiolane and nystatin alter aflatoxin synthetic activity are presented in another report.[21]

In conclusion, results of this study proved that BHA is effective against the aflatoxigenic mold *A. parasiticus.* Inhibition of growth and toxin accumulation were almost linear with the concentration of the additive. Nystatin was much more effective in inhibiting growth than accumulation of aflatoxin. Its effectiveness would probably be potentiated by the concomitant addition of BHA to media.

Isoprothiolane was not as effective in inhibiting mold growth as the other additives; furthermore it greatly enhanced toxin accumulation by the mold. The disappointing results obtained with this fairly safe pesticide suggests that it should not be thought of as a potential additive for foods on which growth of aflatoxigenic mold is possible. A limitation of using the proposed index of growth (GC) is the impracticality of using larger amounts of additives to show more dramatic inhibition than that obtained in this study. As the recent trend in the food industry is to keep use of additives to the minimum, it may be more reasonable to check the small amounts of additives, as was done in this study.

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NOMENCLATURE

α	aflatoxin B_1 accumulation rate constant [eq. (9)]
A_1, A_2	constants of eq. (1)
BHA	butylated hydroxyanisole
c	constant of eq. (7)
C_1, C_2, C_3	three independent trials, prepared as the control of nystatin
DMSO	dimethyl sufoxide
GC	growth coefficient of eqs. (7) and (8)
K	constant for eq. (1)
L	lag period of growth curve
PLL	N^{α}-palmitoyl-L-lysyl-L-lysine ethyl ester dihydrochloride
t	period of incubation (h)
μ	specific growth rate [eq. (3)] (h^{-1})
μ_i	specific growth rate at the point of curve inflection [eq. (5)] (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
X	amount of growth (g mycelial dry weight/30 mL medium)

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