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Influence of Menthol and Thymol on *Aspergillus parasiticus* Growth and Production of Aflatoxins in a Synthetic Medium

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Introduction

Aflatoxins are metabolites of the molds *Aspergillus flavus* and *A. parasiticus* which can grow on a wide variety of agricultural commodities and frequently contaminate foods and feeds inducing undesirable effects in both man and animals (1–5). There is no practical method to eliminate toxigenic molds from human and animal food resources, hence it is desirable to prevent aflatoxin formation or to inactivate aflatoxins in contaminated materials. Inhibition of the growth and the synthesis of aflatoxins by several means is the best approach to prevent contamination of foods with aflatoxins since their inactivation is very difficult (4, 6–10).

Recently, there has been considerable emphasis on the studies involving spices, spice oils and their constituents for inhibiting the growth and the synthesis of aflatoxins (11–22).

Menthol is the active principle of the essential oil of peppermint, and thymol is mainly present in the essential oils of thyme and origanum (17, 22–24).

This investigation was undertaken to determine growth and toxin production by *A. parasiticus* when menthol and thymol were introduced into a liquid medium.

Materials and Methods

Organisms

Strains of *Aspergillus parasiticus* NRRL 2999 and *Aspergillus parasiticus* CBS 26027 (ATCC 15517) were used. They were maintained on potato dextrose agar (PDA, Oxoid) slopes and stored in refrigerator.

Culture techniques

Yeast Extract Sucrose medium (YES) prepared according to *Davies* et al. (25) was used as basal medium throughout the study. The broth was dispensed in 10 ml quantities into test tubes and sterilized by autoclaving at 115 °C for 15 min. After sterilisation appropriate amounts of pure menthol, art. no 5995 and thymol, art. no. 8167 (Merck, Darmstadt, FRG) diluted in 1 ml of ethanol were added to separate tubes to give concentrations of 25, 50, 100, 150, 200, 300, 400 and 500 µg per ml. Ethanol containing no test material was added to culture media as a control. 0.1 ml portions of spore suspensions which were prepared according to the method described previously (21) were inoculated into 10 ml of culture medium. The tube cultures were slanted and incubated statically at 30 °C for 10 days. Experiments were carried out in duplicate.

Analyses

Mold growth was observed visually throughout the incubation period. After the incubation the cultures were autoclaved at 121 °C for 30 seconds to kill spores and mycelia. The mycelial weights were determined after filtering cultures through a sintered glass crucible No. 1 (Gallenkamp, Loughborough, U. K.) washing with distilled water and oven drying at 95 °C for 24 h.

Culture filtrates taken before washing were analysed for aflatoxin production according to the method of *Hitokoto* et al. (15), with use of Silica G-HR (Merck, Darmstadt, FGR) plates for thinlayer chromatography. The intensities of fluorescence of the separated aflatoxins were evaluated visually under UV at 366 nm.

Results and Discussion

Inhibition of growth by menthol and thymol was estimated qualitatively and quantitatively. Qualitative estimations were made visually using the scale explained at the bottom of table 1, whereas quantitative estimations were made on the basis of mycelial weight.

Although, as in the control tubes, growth of both strains initiated on the first day of incubation, growth rates were slowed down on media containing 25, 50, 100 µg/ml menthol (table 1). With level of menthol above 100 µg/ml, growth initiation retarded in both strains.

Growth inhibition increased with increasing concentrations of menthol and growth of both strains was completely inhibited on media containing 600 µg/ml. Similar results were observed when the dry weight of mycelial mats was used as a measure of growth (table 2).

At all concentrations, menthol caused slighter inhibition on the growth of *A. parasiticus* CBS 26027. Compared with the strain NRRL 2999 however, it will

also be seen from table 2 that up to the concentration of 300 µg/ml, menthol stimulated the aflatoxin production of *A. parasiticus* NRRL 2999, whereas its toxin-inhibiting activity against *A. parasiticus* CBS 26027 started at the level of 100 µg/ml (57% inhibition).

Table 1. Growth of *A. parasiticus* in the presence of menthol (visual estimation)

Level of menthol (µg/ml)	Incubation time (days) at 30 °C									
	1	2	3	4	5	6	7	8	9	10
A. parasiticus NRLL 2999										
0	∓	∓	++							
25	∓	∓	+	++						
50	∓	∓	+	++						
100	∓	∓	∓	+	++					
200	–	∓	∓	∓	+	+	++			
300	–	–	–	∓	∓	∓	∓	∓	+	++
400	–	–	–	–	–	–	–	∓	∓	∓
500	–	–	–	–	–	–	–	–	∓	∓
600	–	–	–	–	–	–	–	–	–	–
A. parasiticus CBS 26027										
0	∓	+	++							
25	∓	+	++							
50	∓	∓	+	++						
100	∓	∓	∓	+	++					
200	–	–	∓	∓	+	++				
300	–	–	–	–	∓	∓	∓	+	+	++
400	–	–	–	–	–	∓	∓	∓	∓	+
500	–	–	–	–	–	–	∓	∓	∓	∓
600	–	–	–	–	–	–	–	–	–	–

– = no growth; ∓ = start of growth; + = scant growth; ++ = abundant growth

At concentrations of 200, 300, 400 and 500 µg/ml menthol respectively caused ca. 67, 98, 99, 100% inhibition of the aflatoxin production of *A. parasiticus* CBS 26027. The percentage inhibition of the aflatoxin formation by *A. parasiticus* NRRL 2999 was ca. 31, 40 and 98 for menthol levels of 300, 400 and 500 µg/ml, respectively. Menthol, at a concentration of 500 µg/ml, seems to be critical for both strains and it dramatically suppressed growth and toxin production.

Table 2. Inhibitory effects of menthol on growth and toxin production of *A. parasiticus* after 10 days at 30 °C

Menthol (μg/ml)	Mycelium (mg/ml)[a]		A. parasiticus NRRL 2999				
			Aflatoxins (μg/ml)				
			B_1	B_2	G_1	G_2	Total
0	20	(0)[b]	273	21	196	20	510 (0)
25	17	(15)	581	51	458	34	1124 (0)
50	17	(15)	555	52	357	34	998 (0)
100	19	(5)	515	44	250	41	850 (0)
200	14	(30)	365	30	142	24	561 (0)
300	13	(35)	301	30	11	10	351 (31)
400	Tr		210	24	57	13	304 (40)
500	Tr		8	1	2	0	11 (98)
600	0	(100)	0	0	0	0	0 (100)
	Mycelium (mg/ml)[a]		A. parasiticus CBS 26027				
			Aflatoxins (μg/ml)				
			B_1	B_2	G_1	G_2	Total
0	21	(0)[b]	89	9	53	14	165 (0)
25	20	(5)	92	4	72	3	171 (0)
50	19	(10)	132	6	49	4	191 (0)
100	20	(5)	55	3	13	0	71 (57)
200	15	(29)	48	1	6	0	55 (67)
300	12	(43)	6	0	1	0	7 (96)
400	2	(90)	1	0	0	0	1 (99)
500	Tr		0	0	0	0	0 (100)
600	0	(100)	0	0	0	0	0 (100)

a = Dry weight; b = Numbers in parentheses indicate percentage inhibition;
Tr = Trace growth

However, initiation of growth at the end of a 10 days incubation period was observed as seen from tables 1 and 2 which indicate that the organisms may overcome the inhibitory effects of menthol at this level. Peppermint contains 0.5–3.0% volatile oil with 50–78% menthol (16, 26). *Mabrouk* and *El-Shayeb* (16) tested the antimicrobial activity of several spices including mint against growth and toxin production of *Aspergillus flavus*. They reported that, although mint stimulated mycelial growth, it inhibited aflatoxin formation at the levels of 5.0 and 10.0%. Results in table 3 and 4 indicate that *A. parasiticus* CBS 26027 is more sensitive to thymol than *A. parasiticus* NRRL 2999. As it can be seen from table 4, antimicrobial activity of thymol against growth and toxin production of *A. parasiticus* CBS 26027 started at the lowest level and the amount of mycelia and to-

xins decreased by increased concentration of thymol whereas lower concentrations of thymol, up to 100 µg/ml, enhanced the aflatoxin production of *A. parasiticus* NRRL 2999.

Table 3. Growth of *A. parasiticus* in the presence of thymol (visual estimation)

Level of thymol (µg/ml)	Incubation time (days) at 30 °C									
	1	2	3	4	5	6	7	8	9	10
A. parasiticus NRRL 2999										
0	∓	∓	++							
25	∓	∓	+	++						
50	–	∓	∓	+	+	++				
100	–	–	–	∓	∓	∓	∓	+	+	++
150	–	–	–	–	–	–	–	–	–	–
A. parasiticus CBS 26027										
0	∓	+	++							
25	∓	∓	+	++						
50	–	∓	∓	∓	+	++				
100	–	–	∓	∓	∓	∓	∓	∓	+	+
150	–	–	–	–	–	–	–	–	–	–

However, thymol at a concentration of 150 µg/ml completely inhibited the growth of both strains. Thymol is a major constituent of the essential oil of thyme and origanum and the thyme contains 0.4—2.5% volatile oil with 20—60% thymol (15, 26).

Qualitatatively the results are similar to those reported by *Hitokoto* et al. (15) *Buchanan* and *Shepherd* (17) and *Farag* et al. (22) who tested the antimicrobial activity of thymol against *A. flavus* and *A. parasiticus*. However, the *A. parasiticus* strains used in this study were substantially more sensitive to thymol than the *A. flavus* and *A. parasiticus* strains employed in earlier studies by other workers (15, 22). *Hitokoto* et al. (15) reported that a thymol concentration above 200 µg/ml was necessary to inhibit the growth and toxin production of *A. flavus*. Similarly *Farag* et al. (22) who tested the inhibitory effects of thyme oil and thymol on the growth and toxin production of *A. parasiticus* ATCC 120920 have reported that a medium containing either 400 µg/ml thyme oil or thymol completely inhibited the growth. It has been reported that a compound is considered a positive inhibitor if it reduces aflatoxin formation to 50% of that of control (16). Hence our results as well as other workers show that menthol and thymol if used in suf-

ficient quantities may be considered as effective inhibitors of aflatoxigenic fungi and consequently may offer some assistance in preventing of aflatoxin production.

Table 4. Inhibitory effects of thymol on the amount of growth and toxin production of *A. parasiticus* after 10 days at 30 °C

Thymol (μg/ml)	Mycelium (mg/ml)[a]	A. parasiticus NRRL 2999				
		Aflatoxins (μg/ml)				
		B_1	B_2	G_1	G_2	Total
0	20 (0)[b]	273	21	196	20	510 (0)
25	35 (0)	1124	53	253	30	1460 (0)
50	37 (0)	995	84	194	36	1309 (0)
100	14 (30)	124	9	14	19	166 (67)
150	0 (100)	0	0	0	0	0
	Mycelium (mg/ml)[a]	A. parasiticus CBS 26027				
		Aflatoxins (μg/ml)				
		B_1	B_2	G_1	G_2	Total
0	21 (0)[b]	89	9	53	14	165 (0)
25	20 (5)	88	2	11	1	102 (38)
50	18.7 (10)	28	2	5	1	36 (78)
100	3 (86)	2	0	0	0	2 (99)
150	0 (100)	0	0	0	0	0 (100)

a = Dry weight; b = Numbers in parentheses indicate percentage inhibition

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Summary

The antimicrobial and antitoxigenic activity of menthol and thymol which are active components of commonly used spices was investigated against two aflatoxigenic strains of *Aspergillus parasiticus*. Of the active components tested, thymol was the most effective against both strains leading to definite growth inhibition at the level of 150 μg/ml.

However, *A. parasiticus* CBS 26027 was more sensitive to thymol than the other strain. The growth and aflatoxin inhibition in this strain started at the lowest level of thymol (25 μg/ml); whereas low levels of thymol caused stimulation in growth and toxin accumulation by *A. parasiticus* NRRL 2999.

Both strains showed a similar inhibition pattern against menthol at the level of 600 µg/ml.

Zusammenfassung

In dieser Arbeit wurden die antimikrobiellen und antitoxigenen Einflüsse von Menthol und Thymol, die aktive Bestandteile von häufig verwendeten Gewürzen sind, bei zwei aflatoxigenen Stämmen von *Aspergillus parasiticus* untersucht. Thymol erwies sich bei beiden Stämmen als besonders wirksam.

Das Wachstum beider Stämme wurde bei einer Dosis von 150 µg/ml vollständig gehemmt, wobei *A. parasiticus* CBS 26027 empfindlicher reagierte. Bei *A. parasiticus* CBS 26027 begann das Schimmelpilzwachstum bei gleichzeitiger Hemmung der Toxinproduktion auf dem niedrigsten Niveau von Thymol (25 µg/ml). Paradoxerweise stimulierte dieselbe Thymolkonzentration das Wachstum und die Toxinbildung von *A. parasiticus* NRRL 2999.

Beide Stämme zeigten eine ähnliche Empfindlichkeit gegenüber Menthol, wobei ihr Wachstum bei 600 µg/ml vollständig gehemmt wurde.

Résumé

Cette étude a pour but d'examiner l'effet antimicrobien et antitoxigénique des deux principes actifs que sont le menthol et le thymol, que l'on trouve dans certaines épices, à l'encontre de deux isolats aflatoxigènes de *Aspergillus parasiticus.*

Le thymol s'est révélé particulièrement efficace: une solution de 150 µg/ml inhibe complètement la croissance de ces deux souches; cependant c'est *A. parasiticus* CBS 26027 qui a été le plus sensible au thymol.

La croissance de la souche et l'inhibition de la production d'aflatoxines ont commencé vers une concentration minimale de 25 µg/ml de thymol. Tandis que, paradoxalement, cette même concentration a provoqué chez *A. parasiticus* NRRL 2999 une stimulation de sa croissance et de sa production de toxines. Les deux champignons ont réagi de manière similaire à l'encontre du menthol, une concentration de 600 µg/ml de menthol a inhibé leur croissance.

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