EFFECTS OF FUNGAL INFECTION AND AGRO-CHEMICALS ON THE CHEMICAL COMPOSITION OF SOME SEEDS AND AFLATOXIN PRODUCTION

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An experiment was conducted to envisage the influence of some fungal metabolism and growth on the chemical composition of some seeds or grains. The data show that *Aspergillus flavus* behaved differently according to the principal chemical constituents of the growth media. The aflatoxin distribution in some cereal seeds which differ greatly in their composition was measured. Statistical analysis showed a significant correlation between aflatoxin production of *A parasiticus* and carbohydrate (C) + lipid (L)/protein (P) ratio. The seeds with high C + L/P ratio produced high amounts of aflatoxins and vice versa.

Model systems were prepared to study the effect of the widely used herbicides, insecticides, plant hormones and anti-oxidants on *A. parasiticus* growth and aflatoxin production. Generally, at the recommended application rate, with the exception of indol acetic acid and treflan, all the compounds used suppressed the mold growth and aflatoxin production. Butylated hydroxy toluene and dedecyl gallate produced a remarkable increase in the aflatoxin production.

The effect of some essential oils and their basic compounds on growth and aflatoxin production of *A. parasticus* was studied. Sage, rosemary, caraway and clove oils generally prevented mold growth and aflatoxin accumulation only at high levels. Cumin and thyme oils reduced mold growth at all levels. Very low concentrations (0,125 - 12,0 mg/ml) of the essential oils were quite sufficient to prevent the growth of various bacteria representing Gram-positive bacteria, Gram-negative bacteria, and acid fast bacteria and *S. cerevisiae*. In general, the oils of thyme and clove had strong potent antifungal and antibacterial effect. Hence, these oils can be applied practically as antimicrobial agents and as treatments which will prevent deterioration of stored foods by bacteria or fungi.

A danger exists with foodstuff caused by fungi which produce mycotoxins which, in turn, are known to be carcinogenic. We have been studying this subject since 1975 and the work can be divided into four main subjects :

1 - The influence of fungi on the chemical composition of seeds.
2 - Effects of varied substrates on aflatoxin production.
3 - Effects of agricultural practices on aflatoxin production.
4 - Prevention of aflatoxin production.

Table 1 shows the effect of *Aspergillus flavus* metabolism and growth on the very different substances, i.e., wheat, sesame and soybean seeds. One is carbohydrate rich, the other is lipid rich and the later is a source of lipids and proteins. The data show that fungus behaved differently according to the principal chemical constituents of the seeds or the growth media. For instance, *A. flavus* caused no change in lipid content and an increase and decrease in protein and carbohydrate contents, respectively of wheat kernels (the starchy crop). The fungus caused no change in the protein content of sesame seeds (high lipid content) but it increased and decreased the carbohydrate and lipid contents, respectively. These results demonstrate that the fungus utilized the basic compound of seeds for its growth. The differences in crop composition were mainly due to the influence of the pathway to use the major energy source of each seed, whether lipid (sesame) or carbohydrate (wheat).

Table 2 shows the amylase, lipase and protease activities of healthy and infected wheat, sesame and soybean seeds. The activities of these enzymes were much higher in infected seeds than the healthy ones (1, 4, 6, 7).

Table 3 shows some physical and chemical characteristics of oils extracted from various healthy and infected seeds. In general, the *A. flavus* infection caused:

1) Deeping in oil colour so that drastic bleaching is required before use,

2) A remarkable increase in the acid value so that neutralization of acidity is necessary before use,

3) An increase in the amounts of unsaponifiable matter due to the extraction of fungal pigments along with oil,

4) An increase in the peroxide value and consequently these oils can only be used for certain industrial purposes (3,8).

In addition to these changes, the fatty acid and unsaponifiable matter patterns of infected seeds were significantly altered. One would expect that the previously mentioned changes in the corn oil were due to inherent lipids of various fungi and not to the fungal effects. This assumption is totally wrong since the fatty acid composition of various fungi is quite different from that of corn oil extracted from deliberately infected embryos. Thus, some short chain fatty acids (8:0 and 10:0) were found in infected corn oil but not in the fungi. Furthermore, the infected corn oil did not contain arachidic acid which was found as a major constituent in every fungus (2,5).

Aflatoxin contamination of several products is a major problem throughout the world. These mycotoxins are hepatocarcinotens, mutagens and toxins. Almost

all foods are susceptible to mould growth during some stages of production, processing, storage or transport. We measured the distribution of aflatoxins in some cereal seeds which differ greatly in their composition. Table 4 shows the aflatoxin distribution of sterilized and non-sterilized grains or seeds inoculated with *A. parasiticus*. Four types of aflatoxins were detected, i.e., B_1, B_2, G_1 and G_2 and G_1 were usually greater than the respective dihydro derivatives. Sterilized seeds infected with the fungus generally contained greater amounts of aflatoxins than those infected without previous sterilization. Sterilization process plays a dual fonction by causing the breakdown of the outer shell and destroying some of the competitive microorganisms associated with the seed crops (9, 10).

The chemical composition of the seeds under study was determined and the results are shown in table 5. The total carbohydrate (C) and lipid (L)/protein (P) ratio was calculated to deduce the relationship between aflatoxin production and the chemical composition of the seeds. The values of C + L : P ratios of the seeds under study were arranged in the decreasing order : wheat (starchy seeds) > sesame (oily seeds) > peanut (oily + proteiny seeds) > faba bean (proteiny seeds) > soybean (proteiny seeds). Statistical analysis showed a significant correlation between aflatoxin production and C + L : P ratio ($r = 0,82$ at 5 % level). The seeds with high C + L : P ratio produced high amounts of aflatoxins and vice versa. Consequently, C + L : P ratio appears to play an important role in aflatoxin production.

We would like to prevent fungal growth and the production of aflatoxins. Nowadays, some chemicals are applied in agriculture practices for pest control, weed control, and growth promotion. These chemicals appear in foods during production, processing, packaging or storage. Consequently, model systems were prepared to study the effect of the widely used herbicides, insecticides and plant hormones on a *A. parasiticus* growth and aflatoxin production. Table 6 shows the effect of these substances. Generally, the addition of indol acetic acid (IAA) to the fungal medium increased the aflatoxin production more than gibberellic acid (GA_3). One would expect to achieve this result since IAA and not GA_3 contains an acetate group in its moiety and acetate is the starting material for aflatoxin synthesis.

The results of herbicides applications demonstrate that the increase in fungal growth was concomitant with the increase of aflatoxin production. Aflatoxin G group content in all cases was greater than the B group. Also, every herbicide at various levels behaved differently towards fungal growth and aflatoxin production. For instance, Treflan produces a highly significant stimulatory effect on both fungal growth and the aflatoxin production. While Stomp at low concentration possessed the reverse effect. The use of insecticides exhibited an inhibitory effect on *A. parasiticus* growth and aflatoxin production and the inhibitory effect followed the sequence : Guthion > Actellic > Malathion. The formulas presented in figure 1 show that these insecticides have in common thiophosphate ester group and differ in the rest of the molecule. The extent of inhibitory effect of the tested insecticides could be attributed to the presence of the aromatic nucleus and the inhibitory effect was increased with increasing the aromaticity.

Generally speaking, the compounds used in the present study caused dual function, i.e., each compound can be used for pest control, weed control or increase the plant growth and yield depending on the nature of the compound. At the recommended application rate, with the exception of IAA and treflan, all compounds suppressed the mold growth and aflatoxin production.

Table 1: Chemical composition (%) of healthy and infected wheat kernels, sesame seeds and soybeans

Property	Wheat kernels			Sesame seeds			Soybeans		
	H	I	change	H	I	change	H	I	change
Proteins	14,47	23,82	+	22,11	23,63	±	35,17	36,63	±
Lipids	2,27	2,22	±	58,30	48,97	-	23,66	24,30	±
Total Carbohydrates	75,50	66,65	-	14,20	21,66	+	29,90	26,90	+
Crude fiber	3,79	3,54	±	2,13	2,61	±	5,13	5,90	±

H refers to healthy seeds
I refers to infected seeds

Table 2 : Amylase, lipase and protease activities of healthy and infected wheat, sesame and soybeans

Enzyme	Wheat kernels		Sesame seeds		Soybeans	
	H	I	H	I	H	I
Amylase	3,62	21,40	4,12	8,16	4,83	10,32
Lipase	0,83	20,83	0,70	18,17	0,89	15,00
Protéase	0,30	51,00	1,10	29,92	0,80	25,42

H refers to healthy seeds
I refers to infected seeds

Table 5 : The chemical composition (%) of peanut, fababean, soybean, wheat and sesame

Commodity	Proteins (Nx6,25) (P)	Lipids (L)	Total Carbohydrate (C)	C+L:P ratio	Toxicity index[a]
Peanut	26,16	44,61	20,81	2,5:1	633
Fababean	30,61	1,10	51,04	1,7:1	527
Soybean	35,17	23,66	29,90	1,5:1	477
Wheat	14,47	2,27	75,50	5,4:1	1318
Sesame	22,10	58,30	14,20	3,3:1	686

[a] Toxicity index refers to the sum of the actual toxic amounts of the 4 aflatoxins when calculated as B_1

Table 6 : Aflatoxin pattern of some nonsterilized and sterilized grains or seeds infected by *A. parasiticus*

Commodity	Aflatoxin concentration (ppm) B_1	B_2	G_1	G_2	Toxicity index [a]
Nonsterilized substrates					
Peanut	175	12	121	13	235
Fababean	35	4	32	4	51
Soybean	117	8	323	25	270
Wheat	117	2	81	2	155
Sesame	47	1	16	1	54
Sterilized substrates					
Peanut	463	40	404	8	663
Fababean	351	60	323	105	527
Soybean	292	40	364	63	477
Wheat	877	80	882	126	1318
Sesame	526	50	323	8	686

[a] Toxicity index refers to the sum of the actual toxic amounts of the 4 aflatoxins when calculated as B_1

Several synthetic compounds are added to our food products in order to extend their shelf-life such as antioxidants. Consequently, another set of experiments was conducted to envisage the effect of the most common antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BAT), butylated hydroxy quinone (TBHQ) and dodecyl gallate (DDG) against the growth of some species of *Aspergillus*, *Penicillium* and *Trichoderma*. Also, the effect of these antioxidants on aflatoxin production by *A. parasiticus* in a synthetic medium was studied. The results in table 7 indicate :

1- BHA caused, in general, significant decrease in the mycelial dry weight and at the higher levels (200 - 400 ppm) completely prevented the fungi from growth.

2- Other antioxidants displayed anti-fungal and stimulatory activity depending upon the antioxidant concentration and the fungal type (13).

Tables 8 shows the influence of the antioxidants on aflatoxin production by *A. parasiticus* in yeast extract sucrose (YES) medium. The increase of BHA concentration in the medium caused a decrease in aflatoxin production. The reverse effect was noticed with BHT. DDG was more stimulatory compared with BHT. TBHQ at the high range of 50 - 400 ppm caused no significant effect on aflatoxin B content. Also, this antioxidant had no effect on aflatoxin G at concentrations of 50 - 100 ppm. At 200 and 400 ppm, TBHQ exhibited significant and highly significant decrease on aflatoxin content, respectively (13).

Recently, several publications demonstrate that BHT and DDG cause changs in rat thyroid, stimulation of DNA synthesis, that are toxic and carcinogenic. In addition, the compounds are already used as antioxidants in food products, caused a remarkable increase in the aflatoxin production.

The aforementioned compounds which are used commercially to control weeds and pests or for regulation of plant growth. However, these chemicals cannot be added to food products to prevent aflatoxin formation because of their hazardous effect on human health. The phenolic compounds BHT and DDG, already used as antioxidants in food products, present an increase in the health hazard to human beings and their use should be discontinued.

One of our recent investigations has been concerned with the use of natural essential oils to control fungal growth and aflatoxin production. In this respect, the effect of thyme, cumin, clove, caraway, rosemary and sage essential oils and their basic compounds on growth and aflatoxin production of *A. parasiticus* in a YES medium was studied. Table 9 shows the influence of these essential oils at 200, 400, 600, 800, 1000 and 2000 ppm on *A. parasiticus* growth and aflatoxin production. Sage, rosemary, caraway and clove oils generally prevented mold growth and aflatoxin accumulation at high levels and small stimulatory effect was observed at the lowest levels. Cumin and thyme oils reduced mold growth at all levels. In general, the inhibition effect of oils on *A. parasiticus* growth and aflatoxin production followed the sequence : thyme > cumin > clove > caraway > rosemary > sage. The inhibitory potency of the aforementioned essential oils on various bacteria representing Gram-positive bacteria, Gram-negative bacteria and acid fast bacteria and one yeast was evaluated. Table 10 shows the minimum inhibitory concentration (mg oil/ml medium) of the essential oils against various microorganisms. The data show Gram-negative bacteria were more resistant to various essential oils than Gram-positives. Very low concentrations (0,125 - 12,0 mg/ml) of the essential oils were quite sufficient to prevent the growth of all

Table 8 : Influence of some antioxidants on aflatoxin production by *Aspergillus parasiticus* in a synthetic medium

Antioxidant concentration (ppm)	Aflatoxin concentration			B : G ratio
	B	G	Total	
BHA				
0	89,52(a)	138,10(a)	227,62(a)	0,65 : 1
50	31,52(a)	39,98(c)	70,50(c)	0,79 : 1
100	20,68(c)	22,10(c)	42,78(c)	0,94 : 1
200	0,00	0,00	0,00	
400	0,00	0,00	0,00	
TBHQ				
0	89,52(a)	138,10(a)	227,62(a)	0,65 : 1
50	92,67(a)	125,10(a)	218,09(a)	0,74 : 1
100	88,76(a)	122,14(a)	210,90(a)	0,73 : 1
200	84,22(a)	105,57(b)	189,79(a)	0,80 : 1
400	79,23(a)	89,64(c)	168,87(b)	0,88 : 1
BHT				
0	89,52(a)	138,10(a)	227,62(a)	0,65 : 1
50	109,94(a)	179,22(c)	289,16(b)	0,61 : 1
100	122,05(b)	208,06(c)	330,11(c)	0,59 : 1
200	127,59(c)	222,18(c)	349,77(c)	0,57 : 1
400	119,52(b)	210,30(c)	329,82(c)	0,57 : 1
DDG				
0	89,52(a)	138,10(a)	227,62(a)	0,65 : 1
50	131,12(c)	147,30(a)	278,42(b)	0,89 : 1
100	161,89(c)	170,01(b)	331,90(c)	0,95 : 1
200	163,90(c)	168,78(b)	332,68(c)	0,97 : 1
400	157,45(c)	162,43(b)	319,88(b)	0,97 : 1

The numbers in the column followed by the same letter are not significantly different at p = 0,01 for each antioxidant

Table 9 : Influence of some spice essential oils on *A. parasiticus* growth and aflatoxin production in yeast extract sucrose medium

Oil conc mg/ml)	Mycelial wt (g/50 ml)	Aflatoxin conc (µg/ml) B	G	Total
		Sage Oil		
Control	1,61(a)	80,4(a)	115,1(a)	195,5(a)
0,2	1,41(c)	97,6(c)	141,6(c)	239,5(c)
0,4	1,25(c)	104,3(c)	123,0(a)	227,3(b)
0,6	1,15(c)	95,5(a)	81,7(c)	175,2(a)
0,8	0,80(c)	61,5(b)	30,7(c)	92,2(c)
1,0	0,71(c)	41,6(c)	29,7(c)	71,3(c)
2,0[b]	0,20(c)	2,2(c)	1,3(c)	3,5(c)
		Rosemary oil		
0,2	1,67(a)	129,2(c)	153,9(c)	283,1(c)
0,4	1,54(a)	107,9(c)	104,9(a)	212,8(a)
0,6	1,33(c)	113,3(c)	84,6(c)	197,9(a)
0,8	1,28(c)	97,6(b)	79,2(c)	176,8(a)
1,0	1,21(c)	72,9(a)	44,9(c)	117,8(c)
2,0[b]	0,0	0,0	0,0	0,0
		Caraway oil		
0,2	1,24(c)	78,0(a)	119,5(a)	197,5(a)
0,4	0,35(c)	28,4(c)	16,3(c)	44,7(c)
0,6	0,11(c)	1,1(c)	0,5(c)	1,6(c)
0,8[b]	0,0	0	0	0
1,0	0,0	0,0	0,0	0,0
2,0	0,0	0,0	0,0	0,0
		Clove oil		
0,2	1,20(c)	87,6(a)	110,0(a)	197,6(a)
0,4	0,12(c)	0,4(c)	0,3(c)	0,7(c)
0,6[b]	0,0	0,0	0,0	0,0
0,8	0,0	0,0	0,0	0,0
1,0	0,0	0,0	0,0	0,0
2,0	0,0	0,0	0,0	0,0
		Cumin oil		
0,2	0,91(c)	50,0(c)	66,9(c)	116,9(c)
0,4[b]	0,09 (c)	1,2(c)	0,9(c)	2,1(c)
0,6	0,0	0,0	0,0	0,0
0,8	0,0	0,0	0,0	0,0
1,0	0,0	0,0	0,0	0,0
2,0	0,0	0,0	0,0	0,0
		Thyme oil		
0,2	0,25(c)	2,4(c)	3,6(c)	6,0(c)
0,4[b]	0,0	0,0	0,0	0,0
0,6	0,0	0,0	0,0	0,0
0,8	0,0	0,0	0,0	0,0
1,0	0,0	0,0	0,0	0,0
2,0	0,0	0,0	0,0	0,0

[a] Numbers in the column followed by the same letter are not significantly different at p=0,01

[b] Indicates the minimum inhibitory concentration (MIC) for both the neat oil and a maior component

tested bacteria and *S. cerevisiae*. In general, the oils of thyme and clove had strong potent antibacterial and antifungal effect (11, 12).

Each essential oil is characterized by having a different major compound, i.e., thymol, cumin aldehyde, carvone, eugenol, borneol and thujone for thyme, cumin, caraway, clove, rosemary and sage oils, respectively. The major compounds were added individually to the mold culture medium and microbes culture media at concentration similar to those in the neat oils and possessed an inhibitory effect and the minimum inhibitory concentrations of these compounds were equal to these obtained by the oils.

It appears that there is a relationship between the chemical structures of the most abundant compounds in the tested essential oils and the antimicrobial activities. Generally, the extent of the inhibitory effect of the oils could be attributed to the presence of aromatic nucleus containing a polar functional group. The wide spread use of phenol and chlorophenol and related compounds as disinfectants is well established. Borneol and thujone had little inhibitory effect compared to thymol due to the absence of an aromatic moiety. The higher inhibitory action of thymol might be due to the presence of phenolic OH groups. It is well known that the OH group is quite reactive and easily forms hydrogen bonds with active sites of target enzymes. The inductive effect of the isopropyl group must also be taken into consideration besides the aromaticity of the molecule (figure 2).

Our data show that the essential oils of thyme and clove can be applied partically as anti-microbial agents and 'as food treatments which will prevent deterioration of stored foods by bacteria or fungi. The spices used are feasible material because they are naturally occuring compounds, widely cultivated, cheap and safe, and used as flavouring agents.

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