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GROWTH AND AFLATOXIN PRODUCTION BY *ASPERGILLUS PARASITICUS* WITH CO-OCCURRING FUNGI AND BACTERIA

H.K. CHOURASIA* AND A.K. ROY
University Department of Botany, Bhagalpur University, Bhagalpur-812 007
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Aspergillus parasiticus was grown in the presence of *A. niger, Rhizopus nigricans, Saccharomyces cerevisiae, Corynebacterium rubrum* and *Flavobacterium aurantiacum* and aflatoxin concentration was determined after 3,5,7 and 10 days of incubation at 28°C. *A. niger* and *R. nigricans* exhibited complete inhibition of growth and aflatoxin production by *A. parasiticus*. *S. cerevisiae* and *F. aurantiacum* caused moderate inhibition, whereas *C. rubrum* stimulated growth and aflatoxin production by *A. parasiticus*.

Key Words : Aflatoxin production, *Aspergillus parasiticus*, fungi, bacteria.

Aflatoxin, a secondary metabolite produced by certain strains of *Aspergillus flavus* and *A. parasiticus* contaminates field crops (Anderson *et al.*, 1975; Sinha, 1990) and stored agricultural products (Ahmad & Singh, 1991; Mishra & Daradhiyar, 1991) destined for animal and human consumption. Production of aflatoxin can be influenced by several factors including temperature, water activity, pH, available nutrients and competing microflora (Jarvis, 1971).

Growth of other microorganisms may change available nutrients or produced volatile and/or non-volatile end products which may stimulate, inhibit, detoxify or have no influence on growth of fungi (Moore-Landecker & Stotzky, 1972; Fries, 1973) or on mycotoxin production (Barr, 1976; Roy and Chourasia, 1990). Only limited research has been done on how competitive growth influences aflatoxin production even though mold growth and aflatoxin production often would occur in a competitive environment. The objective of this study was to determine the effect of peanut geocarposphere fungi and bacteria on growth and aflatoxin production by *A. parasiticus*.

MATERIALS AND METHODS

Organisms and inocula : Six microflora comprising fungi *(Aspergillus parasiticus, A. niger, Rhizopus nigricans* and *Flavobacterium aurantiacum)* were obtained from peanut geocarposphere taken from peanut research plots at the wiregrass substation of the Alabama Agricultural Experiment Station, Alabama (USA). *A. parasiticus, A. niger* and *R. nigricans* were grown on slants of Potato Dextrose Agar (PDA) for 8 days at 28°C. Spores were harvested with sterile deionized water and spore concentration was determined by diluting 1 ml of spore suspension with 0.1% peptone water, plating with PDA and counting colonies after incubating plates for 2 days at 29°C.

S. cerevisiae, C. rubrum and *F. aurantiacum* were grown on slants of an appropriate medium (Mycological agar (Difco) for *S. cerevisiae*, nutrient agar (Difco) for *C. rubrum*, Tryptic Soy Agar (TSA) for *F. aurantiacum*) for 3 days at 29°C, harvested with sterile deionized water and diluted to a final concentration of approximately 10^7 cells/ml.

Culture conditions and quantitation of aflatoxin : Flasks containing 100 ml of yeast extract (2% wt/vol) sucrose (20% wt/vol) broth (YES) were inoculated with 1 ml of spore suspension of *A. parasiticus* (10^7 spores) and an equal number of spores of *A. niger, R. nigricans* or cells of *S. cerevisiae, C. rubrum* or *F. aurantiacum*. In trials with bacteria, broth containing yeast extract (2% wt/vol) and sucrose (10% wt/vol) was used so they could grow well. Each trial included duplicate flasks of mixed culture and control flasks of each culture. Inoculated flasks were incubated quiescently at 28°C for 10 days. After 3,5,7 and 10 days of incubation, aflatoxin concentration in the broth and mycelial mat, amount of growth and pH of the broth were determined.

Aflatoxin in 50 ml of filtrate from cultures and from mycelial mats was extracted using the method described by Shih & Marth (1971). Extracts containing aflatoxin were stored in glass-stoppered volumet-

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ric flasks at 4°C until the amount of aflatoxin was determined. Aflatoxin B_1 and G_1 were separated by spotting 5.0 μl of the chloroform extract along with standard solutions on thin layer chromatographic plates pre-coated with 250 μ thick Silica gel G (E. Merck). Plates were developed in an equilibrated tank containing chloroform : methanol : water (98:1:1, v/v/v). The concentration of Afl-B_1 and G_1 was measured by fluorometric procedures described by Shih & Marth (1969).

Cell concentration was determined by diluting 1 ml of broth with 0.1 % peptone water, plating with the appropriate medium and counting colonies after incubating plates at 28°C for 3 days. Mold growth was determined as dry weight of mycelium. Mold cultures were filtered under reduced pressure through Whatman No. 1 filter paper, washed twice with deionized water, dried at 50°C for 24 h and weighed.

Challenge by established flora : Flasks with 100 ml of YES broth were inoculated with *A. niger, R. nigricans, S. cerevisiae, C. rubrum* or *F. aurantiacum,* incubated for 3 days at 28°C, then inoculated with 1 ml of spore suspension (10^7 spores) of *A. parasiticus* and incubated for 7 days at 28°C. Aflatoxin concentration, cell numbers, mycelial dry weight and pH were determined by methods described previously.

RESULTS AND DISCUSSION

When *A. parasiticus* was grown in association with *A. niger* and R. nigricans, all molds grew but the amount of aflatoxin produced was much less in the competitive situation than when *A. parasiticus* grew alone (Table 1). The highest inhibition was noticed with *A. niger.* Growth of all molds was evident from the dry weight of mycelial mats in flasks as compared to those in control flasks. The reduction in the amount of aflatoxin may have been caused by *A. niger* or *R. nigricans* degrading or detoxifying aflatoxin as it was formed. Certain strains of *Rhizopus* have been found that metabolise performed aflatoxin B_1 and G_1(Cole &Kirksey, 1971; Cole *et al.,* 1972). Another possibility is that growth of *A. parasiticus* was not sufficient to result in an appreciable amount of aflatoxin being produced. The results with *A. niger* are in agreement with observations made by other workers (Horn & Wicknow, 1983; Wicklow *et al.,* 1980) with maize as a substrates.

Aflatoxin concentration in controls with *A. parasiticus* alone and in flasks with *S. cerevisiae* was essentially equal after 3 days, but after 5 days more aflatoxin (B_1 + G_1) was present in the control than in flasks with both cultures. This difference increased at 7 days and then decreased at 10 days even though the weight of the mycelium and pH were similar in both instances. More Afl-B_1 than G_1 was produced in the control and when the two organisms grew together. Production of G_1 was retarded by the presence of the yeast but production of B_1 appeared unaffected. Production of more B_1 than G_1 was expected since the pH of the medium would favour synthesis of this toxin (Davis *et al.,* 1966). The difference in aflatoxin production in the two environments appears to production of an end product by *S. cerevisiae* which might have served as a metabolic repressor for aflatoxin formation. This phenomenon was suggested by Fries (1973) and Barr (1976) in discussing the effect of volatile compounds produced by bacteria or fungi on fungal metabolism.

Dry weight of mycelial mats of *A. parasiticus* from the competitive and non-competitive environment were almost the same. *S. cerevisiae* in the competitive environment reached a population of approximately 10^5 cells/ml after 3 days and maintained this number for the 10 days incubation period. In the non-competitive environment a population of 10^7 cells/ml was reached and maintained by the yeast. The pH of both environments followed a similar pattern of decreasing first and then increasing.

When *C. rubrum* was the competitor, *A. parasiticus* grew and produced more aflatoxin than appeared in control (Table 1). *C. rubrum* could not be detected in flasks with both cultures at the 3 days plating, but the bacterium achieved a population of 10^7 cells/ml in the control. Patterns of fungal growth in the competitive and non-competitive environment were similar but growth was greater when both organisms were present. The pH of the medium in the control with *A. parasiticus* alone and in flasks with both cultures also followed a similar pattern, but pH of the medium decreased and then increased more rapidly when both organisms were present.

Total aflatoxin (B_1 + G_1) production was greater when *A. parasiticus* and *C. rubrum* grew together than when the mold grew alone. However, after 5 days of incubation more Afl-G_1 was produced by *A.*

Table 1: Grwth and aflatoxin production of *Aspergillus parasiticus* when grown with *A. niger*[1], *Rhizophus nigricans*[2], *Saccharomyces cerevisiae*[3], *Corynebacterium rubrum*[4], and *Falavobacterium aurantiacum*[5].

Culture	Days of incubation	Aflatoxin (mg/100 ml) B_1	G_1	Total ± S.D.	Mycelial dry wt. (g/100 ml)	Total aflatoxin (mg/g dry wt)	Cell concen. (log 10/ml)	pH
A. parasiticus	3	13.45	12.05	25.50 ± 5.72	2.60	6.10	-	4.22
	5	16.75	14.14	30.89 ± 5.92	3.75	7.25	-	4.30
	7	25.70	21.55	47.25 ±10.30	3.92	8.30	-	4.41
	10	30.15	20.05	50.20 ± 6.95	4.10	8.95	-	5.62
A. parasiticus	3	0.002	0.001	0.003 ± 0.001	2.41	5.85	-	4.11
+	5	0.006	0.002	0.008 ± 0.002	3.35	5.62	-	3.45
A. niger	7	0.011	0.008	0.019 ± 0.005	3.37	5.00	-	3.31
	10	0.019	0.009	0.028 ± 0.007	3.62	4.20	-	3.07
A. parasiticus	3	0.005	0.002	0.007 ± 0.001	2.45	6.00	-	6.41
+	5	0.012	0.009	0.021 ± 0.007	3.50	5.50	-	5.10
R. nigricans	7	0.028	0.015	0.043 ± 0.012	3.62	5.15	-	3.35
	10	0.035	0.012	0.047 ± 0.13	3.88	4.80	-	3.21
A. parasiticus	3	15.91	9.45	25.36 ± 3.06	2.50	6.12	5.30	4.25
+	5	16.37	9.46	25.83 ± 3.21	3.55	6.57	5.06	4.18
S. cerevisiae	7	18.21	11.21	29.42 ± 6.20	3.82	6.62	4.74	4.24
	10	32.75	12.92	45.67 ± 7.84	3.91	6.70	4.73	5.32
A. parasiticus	3	13.40	12.45	25.85 ± 3.07	2.73	6.12	2.40	3.88
+	5	23.83	12.15	35.98 ± 4.05	43.85	7.31	3.95	4.19
C. rubrum	7	33.24	20.55	53.79 ± 7.84	4.12	8.88	4.61	4.22
	10	39.61	19.32	58.93 ± 8.43	4.33	9.25	4.92	5.32
A. parasiticus	3	9.16	8.35	17.51 ± 1.72	2.22	5.07	2.12	4.35
+	5	12.67	8.88	21.55 ± 2.43	3.21	6.44	2.98	4.40
F. aurantiacum	7	15.25	10.40	25.65 ± 3.07	3.77	6.50	3.85	5.10
	10	290.42	11.55	31.97 ± 4.05	3.89	6.66	4.15	5.91

1. When *A. niger* grew alone, mycelial dry weight (g/100 ml) and pH of the medium were 1.17, 4.30; 1.92, 4.47; 2.15, 4.30 and 2.55, 4.10 after 3,5,7 and 10 days, respectively.
2. When *R. nigricans* grew alone, mycelial dry weight (g/100 ml) and pH of the medium were 1.05; 5.80; 2.15, 6.32; 1.85; 6.45 and 1.90, 4.95;after 3,5,7 and 10 days, respectively.
3. When *S.derevisiae* grew alone, the population (log_{10}/ml) and pH of the medium were 7.25; 4.70; 7.31; 4.55; 7.25; 4.70 and 6.80; 4.82 after 3,5,7, and 10 days, respectively.
4. When *C. rubrum* grew alone, the population (log_{10}/ml) and pH of the medium were 7.30; 3.40; 6.65; 3.20; 7.10; 3.12 and 6.50; 3.16 after 3,5,7, and 10 days, respectively.
5. When *F. aurantiacum* grew alone, the population (log_{10}/ml) and pH of the medium were 7.88; 5.90; 8.15; 6.33; 8.50; 6.72 and 8.65; 7.15 after 3,5,7, and 10 days, respectively.

parasiticus alone than in the mixed culture. In contrast, more Afl-B_1 was produced when both cultures grew together than when *A. parasiticus* grew itself. Data on amount of aflatoxin produced per gram of mycelium (dry weight) indicate that the increase in total aflatoxin level resulted from enhanced growth by *A. parasiticus* in the presence of *C. rubrum* rather than stimulation of aflatoxin production. This increase in growth by *A. parasiticus* could have been caused by the initial growth of *C. rubrum* creating conditions which favoured subsequent growth by the mold.

Culturing *A. parasiticus* in the presence of *F. aurantiacum* resulted in less growth and aflatoxin production than when the mold grew alone (Table 1). The pH of broth was always higher from flasks with growth of both organisms than in control with *A. parasiticus* alone. Data on total amount of aflatoxin produced per gram of mycelium (dry weight) indicate that this difference was a function of growth rather than degradation of aflatoxin by *F. aurantiacum* as had been reported by other investigators (Ciegler *et al.*, 1966).

These results indicate the great variability in aflatoxin production by *A. parasiticus* in a competitive environment even when the substrate is favourable for mold growth and aflatoxin production. According to

the principles of biological control stated by Cook & Baker (1983), for reducing aflatoxin contamination of peanuts, the antagonists should come from the geocarposphere, since this is the site of fungal invasion of the host. Since all microflora of the present investigation were isolated from the peanut geocarposphere and of these *A. niger, R. nigricans, S. cerevisiae,* and *F. aurantiacum* showed inhibition of growth and aflatoxin production by *A. parasiticus,* therefore, such microflora could be used as potential bilogical control agents against aflatoxigenic fungi on peanuts. However, detailed investigations are desirable to bring it to a field scale application.

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