A Comparative Study of Toxigenic and Non-toxigenic Strains of *Aspergillus flavus*

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SUMMARY

The formation of kojic acid and aflatoxins by a toxigenic and a non-toxigenic strain of *Aspergillus flavus* was investigated using still and shake cultures on a glucose salts medium (AM medium) and a sucrose yeast extract medium (YES medium). YES medium supported better growth of both the strains and still cultures on this medium yielded the largest levels of kojic acid with both the strains. With the toxigenic strain, still cultures on this medium also produced the maximum amount of aflatoxins. The ratio of aflatoxins B to G and the ratio of aflatoxins in the mycelium to those in the medium were very low in YES medium still cultures. The significance of these data is discussed.

INTRODUCTION

Aspergillus flavus is known to invade agricultural products and produce a group of toxic metabolites known as aflatoxins (Wogan, 1966). Although several studies have been carried out on *A. flavus* with reference to aflatoxins (Hesseltine, Shotwell, Ellis & Stubblefield, 1966; Basappa, Sreenwasamurthy & Parpia, 1970; Hesseltine, Sorenson & Smith, 1970), a comparative study of toxigenic and non-toxigenic strains has not been reported. Such a study is here described.

METHODS

Aspergillus flacus strains ATCC15517 and ATCC9643, which are toxigenic and non-toxigenic respectively, were obtained from the Northern Regional Research Laboratory, Peoria, Illinois, U.S.A. Spores from 1 week cultures on a glucose+peptone agar medium were transferred to 100 ml. of liquid medium contained in 500 ml. Erlenmeyer flasks. The fungus was grown at 25° in stationary culture or on a rotary shaker. A 5.0% glucose+salts medium (AM medium) (Adye & Mateles, 1964) and a 20.0% sucrose+2.0% yeast extract medium (YES medium) (Davis, Diener & Eldridge, 1966) were used. All treatments were in triplicate; the average values are presented here.

Kojic acid was estimated by the method of Parrish, Wiley, Simmons & Long (1966). Mycelium and medium were separately extracted with chloroform, and the aflatoxins in the extract were separated by thin-layer chromatography on silica gel G with 2% methanol in chloroform. They were eluted with methanol and estimated by spectrophotometry as described by Nabney & Nesbitt (1965).

RESULTS AND DISCUSSION

Higher mycelial weights were obtained on YES medium, especially in shake cultures (Table 1). The increase in mycelial weight continued throughout the experimental period, whereas mycelial weight reached a maximum on 4th to 6th day on AM medium. This difference may have been at least in part due to the higher content of carbohydrate in YES medium.

Kojic acid yield ranged from 133 mg. to 3.4 g. depending upon the medium and the strain used. The amount of kojic acid in the medium generally continued to increase with age of the culture (Table 2), but whereas still cultures on YES medium gave very large yields of the order of 2.7 to 3.4 g./flask, shake cultures in the same medium produced negligible amounts of kojic acid. Still cultures on AM medium revealed a

Table 1. *Growth of toxigenic and non-toxigenic strains of Aspergillus flavus in still and shake culture in AM and YES media*

Days of growth	Wet weight of the mycelium (g./flask)			
	AM medium		YES Medium	
	Still culture	Shake culture	Still culture	Shake culture
Toxigenic strain ATCC 15517				
1	*	*	*	4.71
2	*	0.69	*	6.12
3	*	3.01	*	7.22
4	3.19	3.52	2.59	9.23
6	3.09	2.85	3.70	11.67
8	2.84	2.52	4.35	12.40
11	2.59	2.78	5.25	12.90
14	2.46	*	6.51	*
Non-toxigenic strain ATCC 9643				
1	*	*	*	4.15
2	3.14	0.56	*	4.92
3	*	2.91	*	8.05
4	3.83	2.59	2.74	8.60
6	3.71	2.04	4.59	10.39
8	3.49	2.33	5.15	10.80
11	2.96	2.55	7.05	11.83
14	2.76	*	8.29	*

* Denotes that estimations were not carried out.

remarkable difference between the two strains, the toxigenic strain yielding a maximum of 133 mg. of kojic acid and the non-toxigenic 1.05 g. Heathcote, Child & Dutton (1965) have suggested that kojic acid is a precursor of aflatoxins. Tulpule (1969) has put forward a similar view, based on incorporation studies using [^{14}C]kojic acid and on the finding that, on a glucose-salts medium, a toxigenic strain formed more kojic acid than a non-toxigenic strain. However, in the present study, the toxigenic strain formed less kojic acid than did the non-toxigenic strain in a glucose-salts medium. Kojic acid has been shown to be formed directly from [^{14}C]glucose (Arnstein & Bentley, 1953), whereas aflatoxins are formed from [^{14}C]acetate (Adye & Mateles, 1964). It therefore appears that kojic acid may not be a precursor of aflatoxins, although conditions of growth and media required for efficient production of aflatoxins and kojic acid are very similar.

Total aflatoxins increased throughout the experimental period in still cultures on YES medium (Table 3). The maximum yields were 6.4 mg./flask compared to only 258 µg. in AM medium. This is in agreement with the findings of Davis *et al.* (1966). In both media, still cultures gave higher yields than shake cultures, though the differences in AM medium were not so marked as in YES medium. Aflatoxin levels reached a maximum after 4 to 6 days depending upon the medium, and thereafter decreased, except in YES medium still culture. This is in agreement with the findings of other workers (Ciegler, Peterson, Logoda & Hall, 1966; Schroeder, 1966).

Table 2. *Production of kojic acid by toxigenic and non-toxigenic strains of Aspergillus flavus in still and shake culture in AM and YES media*

Days of growth	Kojic acid (mg./flask)			
	AM medium		YES medium	
	Still culture	Shake culture	Still culture	Shake culture
Toxigenic strain ATCC 15517				
1	*	*	*	Nil
2	*	Nil	*	Nil
3	*	Nil	*	32.0
4	Nil	Nil	155.0	43.7
6	13.4	137.5	504.0	91.9
8	28.5	628.8	1396.8	117.7
11	107.5	780.0	2560.0	224.2
14	133.1	*	3376.0	*
Non-toxigenic strain ATCC 9643				
1	*	*	*	Nil
2	307.0	Nil	*	Nil
3	*	Nil	*	15.7
4	440.4	Nil	620.0	36.4
6	435.3	130.6	1550.0	108.1
8	1050.0	320.6	2522.0	131.7
11	921.7	507.9	2677.0	185.0
14	691.3	*	2677.0	*

* Denotes that estimations were not carried out.

Ciegler *et al.* (1966) studied the degradation of aflatoxins in submerged cultures and showed that increased aeration increased their breakdown. This would explain the low levels obtained in YES medium in shake cultures as compared to still cultures. Aflatoxin G was present in only traces in mycelia grown in shake cultures apparently because it was degraded more.

The ratio of aflatoxins B to G was dependent on the medium. It was low in still cultures in YES medium. In still cultures in AM medium the ratio was much greater in the mycelium than in the medium. The reverse was true in still cultures in YES medium. It appears that the proportion of aflatoxin G was higher when large amounts of aflatoxin were produced. This view is supported by the data of Schroeder (1966) and Schindler, Palmer & Eisenberg (1967).

In still cultures in YES medium, the ratio of aflatoxins in the mycelium to those in the medium was very low and of the order of 0.07 to 0.1. Under all the other conditions employed, the ratio was comparatively high and ranged from 0.35 to 1.84. The relative amounts of aflatoxins present in the medium and mycelium have been reported to

vary with the media and growth conditions (Schroeder & Ashworth, 1966; Frank, 1968). These workers' data also showed a decrease in the ratio of aflatoxins in the mycelium to those in the medium when aflatoxin production was high. Since aflatoxin

Table 3. *Production of aflatoxins B and G by a toxigenic strain of Aspergillus flavus in still and shake culture in AM and YES media*

	Aflatoxin ($\mu\text{g./flask}$)									
	AM medium					YES medium				
	Mycelium		Medium		Total	Mycelium		Medium		Total
Days of growth	B	G	B	G		B	G	B	G	
	Still culture									
4	34.6	8.5	42.7	65.9	151.7	15.1	130.6	462.0	1225.0	1832.7
6	64.2	19.2	102.0	72.4	257.8	28.3	235.8	826.0	1837.0	2927.1
8	37.1	7.3	52.0	41.8	138.2	43.8	234.7	1386.0	1960.0	3624.5
11	22.4	4.7	40.3	33.8	101.2	65.5	376.9	2971.0	3307.0	6420.4
14	16.0	20.0	29.3	16.9	82.2	51.1	45.5	2846.0	3430.0	6372.6
	Shake culture									
1	*	*	*	*	*	Trace	Trace	16.4	16.4	32.8
2	Trace	Trace	14.8	16.0	30.8	Trace	Trace	28.6	20.3	49.2
3	24.9	Trace	46.5	22.8	94.2	4.0	Trace	26.5	21.2	51.7
4	52.3	Trace	30.7	20.5	103.5	39.9	Trace	45.2	28.9	114.0
6	60.8	22.31	23.6	21.9	128.6	84.2	Trace	37.3	22.7	144.2
8	20.1	Trace	19.2	17.4	56.7	34.6	Trace	24.9	11.6	71.1
11	28.1	Trace	20.3	Trace	48.4	153.7	Trace	81.1	81.1	315.9

* Denotes that estimations were not carried out.

appears to be excreted with ease into the medium, large amounts of toxins can be expected to accumulate in the medium over a period of time. Apparently there is a limit to the amount of toxins that can be held in the mycelium and hence mycelium: medium ratio is very low when toxin formation is high.

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