Interaction of temperature and microsomal peroxidase in aflatoxin degradation by *Aspergillus flavus* 102566

Ved Pal Singh

Applied Microbiology and Biotechnology Laboratory, Department of Botany, University of Delhi, Delhi 110 007, India

In *Aspergillus flavus*, mycelial yield (dry weight) and DNA content reach near maximum at day-2. The temperature-shift studies have shown that both growth as well as DNA profiles follow almost identical patterns. After the stationary phase of growth sets in, the decrease in mycelial dry weight and DNA levels is more prominent at 37°C than at 30°C (optimal growth temperature) and most at 45°C. With increase in temperature, there is a decrease in the levels of total aflatoxins, which in turn is correlated with the temperature-dependent increase in the activities of microsomal peroxidase. A possible role of microsomal peroxidase in aflatoxin degradation under the influence of temperature has been suggested.

THE aflatoxins constitute the most widely studied of all mycotoxins, and their toxic effect on humans and animals is well documented[1–3]. Almost all raw agricultural products and most foods and feeds are susceptible to invasion by the aflatoxigenic fungi, *Aspergillus flavus* and *A. parasiticus* with subsequent formation of aflatoxins at some stage of their production, processing and transportation[2,3]. Efficient degradation of aflatoxins in agricultural raw material used for foods and feeds could be of great significance to human and animal health. A number of physical and chemical methods of aflatoxin degradation have been developed but only a few have any practical application[2–5]. Aflatoxin degradation by other microorganisms has been extensively studied, which leads to realistic practical applications[5,6]. The ability of the toxin-producing fungi, *A. parasiticus*[7] and *A. flavus*[8,9] to degrade aflatoxins has been demonstrated. Intracellular mycelial substance(s)[10] and heat-labile ammonium sulphate precipitable factor(s)[11] participate in the degradation of aflatoxins.

All the aforementioned studies were, however, carried out for *in vitro* degradation of exogenous aflatoxins by the intact mycelia or cell-free extracts of the aflatoxin-producing fungi to show the role of enzymes, such as peroxidase[7] and cytochrome P-450 monooxygenase[8] in *A. parasiticus* and *A. flavus*, respectively. Studies have also shown that specific elevated temperatures can strongly influence the level of endogenous breakdown of aflatoxin by *A. flavus*[10]. The present investigation was undertaken to examine the relationship between *in vivo* degradation of aflatoxins and peroxidase activities in the microsomal as well as in the 35,000 *g* (3 h) supernatant fractions of *A. flavus* grown at different temperature regimes in shake culture.

Aspergillus flavus 102566, obtained from the CAB International Mycological Institute, Surrey, UK was used in the present study. The strain was grown at 30°C in slants of potato dextrose agar (PDA) medium. *A. flavus* cultures were prepared and harvested using the method of Hamid and Smith[8] by growing the fungus initially for 2 days at 30°C and subsequently trasferring the cultures to 37° and 45°C, keeping the control at optimal growth temperature (30°C). Mycelial dry weights were obtained by drying washed filtered mycelium at 80°C for 24 h. The procedures for the preparation of cell-free extracts and DNA estimation, described elsewhere[12,13], were used.

Cultures were extracted for aflatoxins by a modification of the procedures of Shih and Marth[14], where whole cultures were blended with 100 ml of chloroform in a Waring blender for 2 min. The same extraction procedures were used for both separated mycelia and culture filtrates. The quantitative estimation of aflatoxins B_1, B_2, G_1 and G_2 was made by the method of Nebney and Nesbitt[15].

For microsomal preparation, the mycelium (2 g wet weight) was homogenized by mortar and pestle in 5 ml ice-cold buffer (0.05 M tris-HCl, pH 7.0) using glass perlen (0.45–0.50 mm ϕ). The homogenate was centrifuged at 10,000 *g* for 30 min at 4°C and the precipitate discarded. The microsomes were sedimented by centrifugation at 35,000 *g* for 3 h at 4°C and the pellets containing microsomes resuspended in 3 ml ice-cold buffer (0.05 M tris-HCl, pH 7.0). The microsomal and 35,000 *g* supernatant fractions were stored under ice-cold condition and used for analysis of peroxidase enzyme activity and protein estimation.

Peroxidase activities in microsomal and supernatant fractions were assayed by the method of Polis and Shmukler[16], using dihydroxyphenylalanine (dopa) as hydrogen donor and H_2O_2 as an oxidant. Protein in the samples was estimated by Lowry's method[17], using bovine serum albumin (Sigma) as standard. The specific activity of peroxidase was expressed as the increase in the absorbancy unit (AU) at 475 nm due to the enzymatic oxidation of dopa per min per mg of protein (AU/min/mg protein).

The observations on growth in terms of mycelial dry weight in mg per flask, containing 100 ml culture and DNA content in terms of µg per 100 ml culture of *A. flavus* are shown in Figure 1 *a*, *b*, respectively. The results indicate that both mycelial dry weights as well as DNA contents reached near the maximum level by day-2. However, the data further suggests that under the specific growth conditions such as media composition, op-

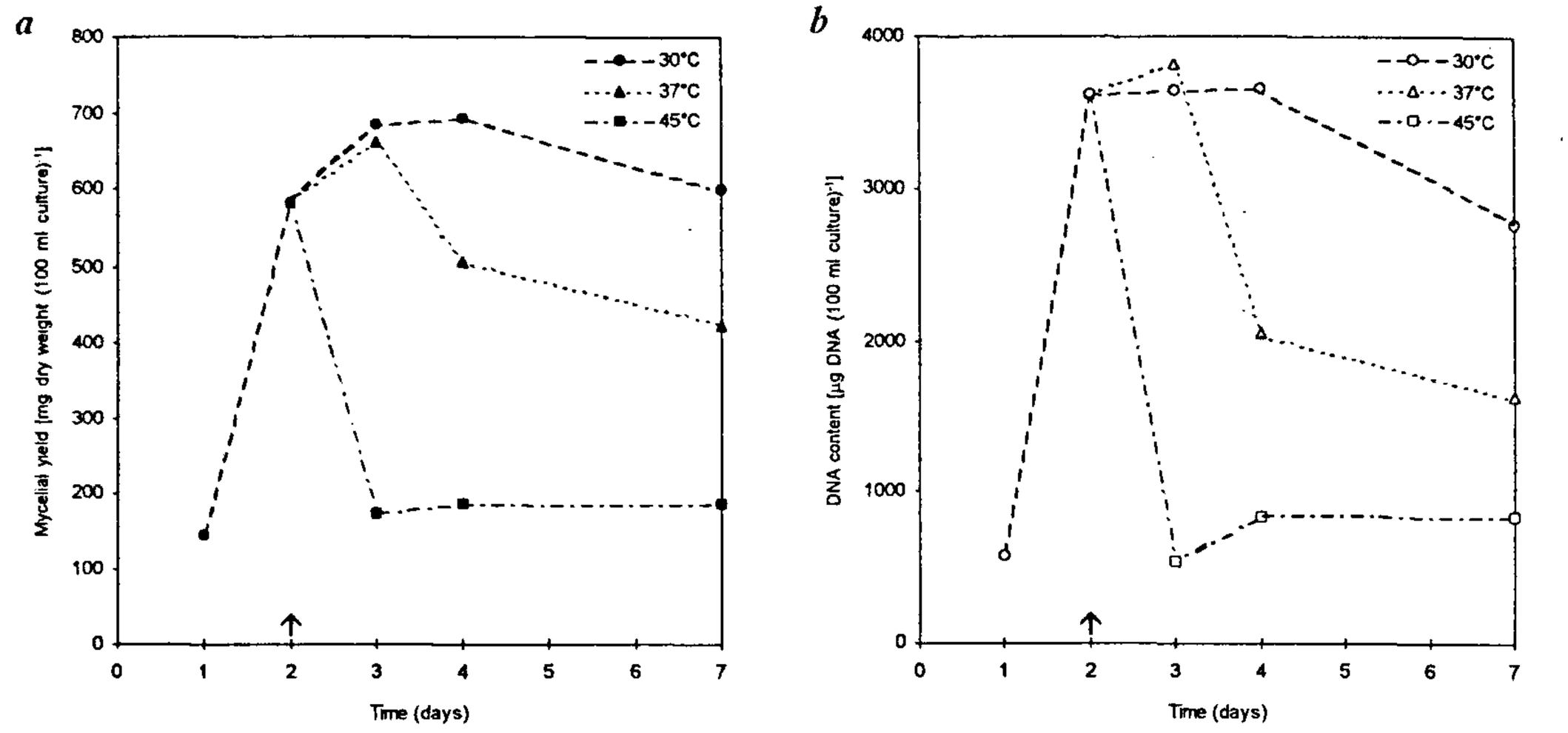

Figure 1. *a*, Mycelial yield of *Aspergillus flavus* at 30°, 37° and 45°. The time at which the cultures were transferred to different temperatures is indicated by an arrow. Each point in the figure represents mean value of triplicate determinations (reproducibility ±5%).

Table 1. The levels of aflatoxins (B_1, B_2, G_1 and G_2) in the mycelia and the culture filtrates of *Aspergillus flavus* under different temperature regimes. Each result is based on mean value of triplicate determinations (reproducibility ± 5%)

Growth temperature (°C)	Days of harvest	Mycelial aflatoxins (ppm/100 ml culture)					Culture filtrate aflatoxins (ppm/100 ml culture)					Total aflatox concentratic (I + II)
		B_1	B_2	G_1	G_2	Subtotal (I)	B_1	B_2	G_1	G_2	Subtotal (II)	
	1	–	–	–	–	–	–	–	–	–	–	–
	→ 2	0.343	0.189	0.176	–	0.708	0.093	0.026	0.324	–	0.443	1.151
30	3	0.422	0.385	0.472	0.428	1.707	0.551	0.104	0.574	0.366	1.595	3.302
	4	0.128	0.261	0.463	0.214	1.066	0.415	0.287	0.287	0.202	1.201	2.267
	7	0.336	0.058	0.370	–	0.764	0.336	0.320	0.166	–	0.822	1.586
37 (after the	3	0.214	–	0.222	–	0.436	0.243	0.183	0.101	–	0.527	0.963
transfer of cultures	4	0.107	–	0.092	–	0.199	0.135	–	0.120	–	0.255	0.454
at day-2)	7	0.021	–	–	–	0.021	0.042	–	–	–	0.042	0.063
45 (after the	3	0.021	0.058	0.185	–	0.264	0.085	0.045	0.083	0.028	0.261	0.525
transfer of culture	4	0.135	0.091	0.074	–	0.300	0.415	0.215	0.231	0.135	0.996	1.296
at day-2)	7	0.021	0.006	0.018	–	0.045	0.186	0.032	0.064	0.091	0.301	0.346

→ Time of temperature shift of cultures.

timal temperature (30°C), and shaking speed (200 rpm), the stationary phase of growth sets in after a very short period of time (i.e. day-2 onwards), which is conducive to aflatoxin biosynthesis by *A. flavus*. The data also indicates that in control cultures of *A. flavus* (i.e. the cultures raised at optimal growth temperature, 30°C), mycelial yield continued to increase but DNA content remained unchanged up to day-4, followed by gradual decrease of both the components up to day-7. At 37°C, on the other hand, there was a continuous increase in both mycelial yield and DNA content only up to day-3 whi gradually decreased through day-4 up to day-7 stage growth of *A. flavus*. However, after the transfer of c tures to 45°C at day-2, both mycelial yield and DI content decreased drastically up to day-3. But at the sa temperature (45°C), at a more advanced stage of grov (i.e. at day-4 stage), there was a slight increase in t mycelial yield as well as DNA content; the magnitude such increase remained almost same up to day-7. Su periodic increase or decrease in fungal biomass a

Table 2. Peroxidase activities in the microsomal as well as supernatant (35,000 *g*, 3 h) fractions of mycelia of *A. flavus* grown at different temperatures. Values are based on mean of triplicate determinations (reproducibility ± 5%)

Growth temperature (°C)	Days of harvest	Specific activity of peroxidase (AU/min/mg protein)	
		Microsomal fraction	Supernatant fraction
30	1	0.75	0.01
	→ 2	1.26	0.02
	3	1.26	0.02
	4	1.72	0.03
	7	2.57	0.06
37 (after the transfer of cultures at day-2)	3	2.83	0.02
	4	2.85	0.02
	7	3.05	0.02
45 (after the transfer of cultures at day-2)	3	3.93	0.05
	4	3.45	0.00
	7	4.26	0.00

→ Time of temperature shift of cultures.

DNA content, therefore, appeared to be growth-phase dependent, similar to the observations made by Smith and Moss[2] and Arseculeratne and Bandunatha[18] for various toxigenic aspergilli, in which the decrease in mycelial yield and the contents of various biomolecules has been attributed to their autolytic degradation and their increase at more advanced phase of growth has been attributed to resynthesis of the cellular as well as biomolecular materials[2,18].

The observations that both mycelia as well as culture filtrate showed similar patterns of total aflatoxin levels consistently under different temperature regimes (Table 1) suggest that there is no reabsorption of aflatoxins by mycelia at any of the stages of growth and temperatures tested. The decrease in total aflatoxin levels in both mycelia as well as culture filtrate after day-3 was, therefore, due to degradation of aflatoxins. A slight increase in aflatoxin concentration in 4-day-old cultures grown at 45°C appeared to be growth-phase mediated phenomenon in *A. flavus*, as is evident from the experimental observations presented in Figure 1 *a*, *b* and Table 1. Similar growth-phase mediated phenomenon has also been observed for aflatoxin synthesis and degradation by Huynh *et al.*[1] and Huynh and Lloyd[11] in another species of toxigenic *Aspergillus – A. parasiticus*. Aflatoxin B_2 in the mycelium was found to be completely degraded at 37°C, but in the culture filtrate it was sensitive to degradation after day-3 at this temperature. On the other hand, aflatoxin G_2 in the mycelium was found to be completely degraded both at 37° and 45°C, but in the culture filtrate it was most sensitive to degradation at 37°C; even at 30°C (normal growth temperature of *A. flavus*), this aflatoxin appeared later and degraded earlier than all other aflatoxins in both mycelium as well as culture filtrate. However, aflatoxin G_1 was found to be completely degraded at 45°C in both mycelium as well as culture filtrate at day-7. The decrease in total aflatoxin level at day-7 and at 45°C might be associated with the redegradation of the newly-synthesized aflatoxins.

In aflatoxigenic aspergilli, more than one phase of rise and decline of aflatoxin levels, in association with growth stages have been reported[2,18]. This is also in agreement with the fact that there was an increase in total aflatoxin concentration from day-3 to day-4 (at 45°C), where both mycelial yield (Figure 1 *a*) and DNA content (Figure 1 *b*) increased. However, Table 2 indicated that most of the peroxidase activity is associated with microsomal fraction of *A. flavus*, and only a negligible amount of the enzyme activity was observed in the supernatant fraction of this toxigenic fungus. The data further indicated that, at initial stages of growth of *A. flavus* at 30°C, the aflatoxin levels continued to increase up to day-3 (Table 1) and there was no change in specific activity of microsomal peroxidase from day-2 to day-3, thereby enabling the biosynthesis of aflatoxins to continue during this period of growth. At day-4 and day-7, however, the increase in microsomal peroxidase activity (Table 2) could possibly be responsible for the increased rates of aflatoxin degradation. But after the shifting of cultures from 30°C to 37°C at day-2, the higher rates of aflatoxin degradation on subsequent days might be due to a substantial amount of increases in microsomal peroxidase activities. However, the transfer of *A. flavus* cultures to 45°C at day-2 resulted in marked (about 54%) decrease in aflatoxin concentration at day-3, which appears to be due to degradation of this toxin by the microsomal peroxidase enzyme. It is quite apparent that even a slight decrease in the specific activity

of microsomal peroxidase (i.e. from 3.93 to 3.45 AU/min/mg protein) could possibly lead to the production of high amounts of total aflatoxins at day-4; and again an increase in the specific activity of this enzyme at day-7 (i.e. from 3.45 to 4.26) resulted in enhanced degradation of newly-synthesized aflatoxins, thus lowering their concentrations in *A. flavus* cultures. However, the relationship of aflatoxin degradation with increased microsomal peroxidase activities was stage-specific, with respect to both growth phase as well as temperature regimes (Figure 1 *a*, *b* and Tables 1 and 2). It is evident that the decrease or increase in the total aflatoxin level, under the influence of temperature, was consistently in accordance with the respective increase or decrease in the specific activity of microsomal peroxidase. The present findings suggested the participation of microsomal peroxidase of *A. flavus* in the *in vivo* degradation of endogenous aflatoxins under the influence of temperature. The potential role of temperature[4–6,8,9] in toxigenic aspergilli as well as of peroxidase of cell-free extracts of *A. parasiticus*[7] in aflatoxin degradation has been well documented. Moreover, the enzymatic preparations of liver microsomes have been found to degrade aflatoxins and detoxify them in various eukaryotic systems[19,20]. The role of microsomal enzymes, including peroxidase-related activity of cytochrome P-450 in biotransformation/degradation of various heterocyclic hazardous compounds in animal systems is well understood[21,22].

This paper highlights the effect of physiological growth conditions on aflatoxin production and degradation by *A. flavus*, with special reference to involvement of microsomal peroxidase in aflatoxin degradation under the influence of growth temperature from 30 to 45°C. The study also presents a possible mechanism for biological control of aflatoxins by implicating microsomal peroxidase of microbial origin.

1. Huynh, V. L., Gerdes, R. G. and Lloyd, A. B., *Aust. J. Biol. Sci.*, 1984, **37**, 123–129.
2. Smith, J. E. and Moss, M. O., *Mycotoxins: Formation, Analysis and Significance*, John Wiley, Chichester, 1985, p. 148.
3. CAST (Counsil for Agricultural Science and Technology), *Task Force Report on Mycotoxins: Economic and Health Risks* (No. 116), 1989, CAST, Iowa.
4. Singh, V. P., Smith, J. E., Harran, G., Saxena, R. K. and Mukerji, K. G., *Indian J. Microbiol.*, 1992, **32**, 357–369.
5. Bol, J. and Smith, J. E., *Food Biotechnol.*, 1989, **3**, 127–144.
6. Faraj, M. K., Smith, J. E. and Harren, G., *Mycol. Res.*, 1993, **97**, 1388–1392.
7. Doyle, M. P. and Marth, E. H., *Eur. J. Appl. Microbiol. Biotechnol.*, 1979, **7**, 211–217.
8. Hamid, A. B. and Smith, J. E., *J. Gen. Microbiol.*, 1987, **133**, 2023–2029.
9. Faraj, M. K., Ph D thesis, University of Strathclyde, Glasgow, 1990.
10. Doyle, M. P. and Marth, E. H., *Eur. J. Appl. Microbiol. Biotechnol.*, 1978, **6**, 95–100.
11. Huynh, V. L. and Lloyd, A. B., *Aust. J. Biol. Sci.*, 1984, **37**, 37–43.
12. Singh, V. P. and Sinha, U., *Experientia*, 1982, **38**, 670–671.
13. Plummer, D. T., *Introduction to Practical Biochemistry*, Tata McGraw Hill, New Delhi, 1988, 3rd edition, p. 221.
14. Shih, C. N. and Marth, E. H., *Biochim. Biophys. Acta*, 1974, **338**, 286–296.
15. Nebney, J. and Nesbitt, B. F., *Analyst*, 1965, **90**, 155–160.
16. Polis, B. D. and Shmukler, H. W., *J. Biol. Chem.*, 1953, **201**, 475–500.
17. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1951, **193**, 265–275.
18. Arseculeratne, S. N. and Bandunatha, C. H. S. R., *J. Appl. Bacteriol.*, 1972, **35**, 43–52.
19. Patterson, D. S. P., *Food Cosmet. Toxicol.*, 1973, **11**, 287–294.
20. Patterson, D. S. P., *Pure Appl. Chem.*, 1977, **49**, 1723–1731.
21. Ruckpaul, K., Rein, H. and Blanck, J., in *Frontiers in Biotransformations: Basic Mechanisms of Regulation of Cytochrome P-450* (eds Ruckpaul, K. and Rein, H.), Taylor & Francis, London, 1989, vol. 1, pp. 1–65.
22. Singh, V. P. (ed.), *Biotransformations: Microbial Degradation of Health-Risk Compounds*, Elsevier, Amsterdam, 1995, p. 285.

ACKNOWLEDGEMENTS. I thank my post-doctoral supervisor, Professor J.E. Smith, of the University of Strathclyde, Royal College, Glasgow for inducting me into the field of mycotoxicology. I also thank the Head of the Department for providing me necessary facilities. The technical assistance provided by Mr. Krishan Lal and Mr. S. K. Dass is gratefully acknowledged.

Received 3 June 1997; revised accepted 4 August 1997

Uranium uptake by plants

Kedar Prasad Singh

Chemical Engineering Division, Regional Research Laboratory, Jorhat 785 006, India

This paper highlights the transport of uranium present in the soil to plants. An increase in the uranium content in soil enhances its transport in various parts of plants. The transport of uranium from the soil to the grain follows the order: black gram > maize > lentil > chick-pea > rice > wheat. In certain vegetables and fruits, this order is: spinach > carrot > radish > brinjal > banana > tomato > beet. In vegetables and fruits, the stem reflects minimum percentage of uranium present in the soil. The uranium transport is appreciably high in arecanut plant. The chances of uranium transport to the human organs, are expected to be more through consumption of crops grown in uranium-rich soil.

URANIUM is regarded as the heaviest[1] trace element found in nature, and probably is a normal constituent of all organisms. All the eleven known isotopes of uranium are unstable and, as they decay, emit alpha or beta particles. The alpha particles are regarded as hazardous to mankind. Prolonged exposure to alpha emitter may ac-