Comparative studies on growth patterns and metabolic status of aflatoxin-producing and non-producing strains of *Aspergillus flavus*

Four species of *Aspergillus* – *A. flavus*, *A. parasiticus*, *A. nomius* and *A. tamarii* produce aflatoxins[1,2]. The former two being more common, have been studied the most. Almost all isolates of *A. parasiticus* produce aflatoxins[3], which is not true for *A. flavus*, although the latter has been characterized with respect to its biological control property[4]. Repeated subculture of *A. flavus* strains on synthetic media leads to loss of toxigenicity[5], but whether or not growth and metabolic status play a role in aflatoxin production, is not known. The present investigation was, therefore, carried out to understand the nature of strain-specific aflatoxin production in *A. flavus* with respect to its growth and metabolism.

A. flavus CMI 102566 (toxigenic) and *A. flavus* CMI 93803 (non-toxigenic) strains used were maintained on slants of Czapek-Dox agar (pH 7.2) (Himedia, Mumbai) at 30°C. The cultures were grown on sucrose, low salt (SLS) medium (pH 4.5)[6] in 50 ml of liquid medium in 250 ml Erlenmeyer flasks. From 7-day-old cultures, 5×10^5 spores were inoculated in flasks and incubated at 30°C for 3, 5, 7, 9, and 11 days under static culture conditions.

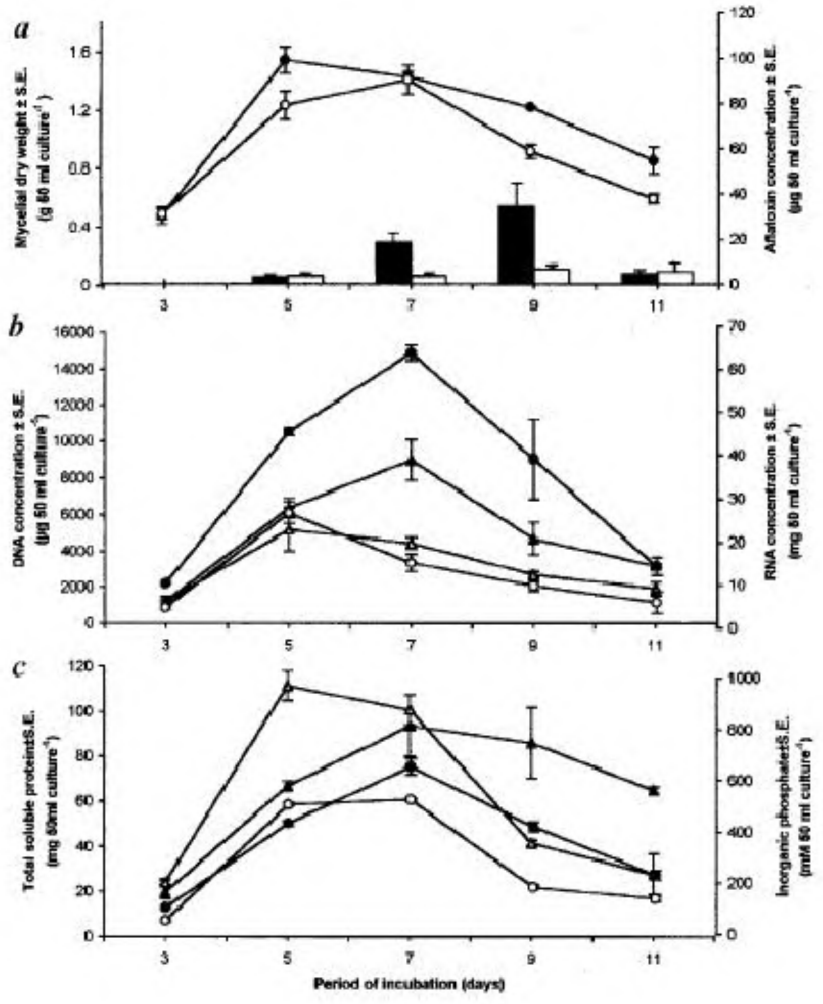

Figure 1. Effect of incubation period on growth and different metabolites in *Aspergillus flavus* at 30°C. ***a***, Mycelial dry weights of the toxigenic (●) and non-toxigenic (○) strains as well as concentrations of aflatoxin B_1 (closed bar) and aflatoxin B_2 (open bar) in the toxigenic strain; ***b***, Concentration of DNA (▲△) and RNA (●○) in the toxigenic (▲●) and non-toxigenic (△○) strains; ***c***, Content of total soluble protein (▲△) and inorganic phosphate (●○) in the toxigenic (▲●) and non-toxigenic (△○) strains.

Mycelial dry weights were obtained by harvesting the mycelium on pre-activated (at 80°C for 24 h), and pre-weighed Whatman no. 1 filter paper. The mycelium was dried at 80°C for 48 h in an oven and weighed again. The difference between initial and final weight was taken as dry weight. Cell-free extract was prepared by grinding the freshly harvested, washed (thrice in distilled water, for 5 min each time) and blot-dried mycelium in distilled water in the ratio, 1 : 10 (w/v), and centrifuged at 20,000 *g* (ref. 7). The supernatant was used as the cell-free extract.

The aflatoxins were extracted from the culture filtrate with chloroform and separated by thin layer chromatography using the solvent system: toluene–ethylacetate–chloroform–90% formic acid (70 : 50 : 50 : 20, v/v)[8,9]. Individual aflatoxin bands (B_1 and B_2) were eluted and estimated spectrophotometrically[10]. Quantification of DNA and RNA was done using the diphenylamine assay[11] and Orcinol test[12] respectively; total soluble proteins and inorganic phosphate (Pi) were estimated according to Lowry *et al.*[13] and Heinonen and Lahti[14] respectively.

Each experiment was repeated at least twice and performed in triplicate. The results are discussed on the basis of mean values of data obtained and analysed statistically for test of significance.

In toxigenic strain, growth was maximum at day-5, but the level of DNA, RNA, protein and inorganic phosphate increased until day-7; this suggests continued synthesis of these primary macromolecules compared to non-toxigenic strain. Such an increase on day-5 could be a result of cell division and growth (in terms of DNA level) as well as synthesis of new proteins for the induction of aflatoxin biosynthesis (Figure 1 *a*). Earlier workers[15,16] have reported synthesis of new proteins during the induction of

aflatoxin production. Since these processes are energy-intensive, increased concentration of inorganic phosphate and associated macromolecules is understandable. However, reports suggest decreased mitochondrial activity and respiration[17,18], which may lead to decline in ATP level and an increase in the concentration of AMP, ADP and inorganic phosphate[19,20]. This further supports the view that higher concentration of inorganic phosphate observed is a result of higher rate of ATP utilization. The enhanced rates of metabolism as well as aflatoxin production might lead to depletion of primary metabolites, which could have possibly caused a decrease in the biomass of the organism. The metabolic rate was maximum on day-7, at which time the aflatoxin concentration increased sharply and reached a maximum on day-9. After 7 days, however, the concentration of nucleic acid, protein and inorganic phosphate decreased as a result of induction of the autolysis phase, which otherwise becomes faster after 9 days of incubation. The aflatoxin levels decreased after 9 days on account of autolysis or due to induction of aflatoxin-degrading system[8,9,21].

In non-toxigenic strain, maximum growth was observed on day-7, but the levels of DNA, RNA, protein and inorganic phosphate were found to increase only until day-5. This suggested an increase in cellular growth, as shown by DNA level (Figure 1 *b*) till day-5, and a uniformly constant growth later due to synthesis and storage of primary metabolites. This is corroborated by occurrence of uniformly increased levels of protein and inorganic phosphate concentration till day-7. Studies carried out by Bu'Lock[22] and Borrow *et al.*[23] have shown increased carbohydrates, fats and amino acids in non-dividing cells, resulting in increased biomass.

The present study shows that toxigenic and non-toxigenic strains of *A. flavus* follow different growth and metabolic patterns. The growth rate of the toxigenic strain was higher than that of the non-toxigenic one. In toxigenic strain, aflatoxin biosynthesis starts before initiation of stationary phase, with longer trophophase in comparison to non-toxigenic strain, thus providing the former a greater opportunity to prepare for secondary metabolism.

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