Phosphatases in aflatoxin-producing and non-producing strains of *Aspergillus flavus* Link.

Aspergillus flavus Link. is a common mould that is known for its strain-specific aflatoxigenicity[1–3]. Although ample work has been carried out on various aspects of aflatoxins such as their biosynthesis, metabolism and control, investigations with regard to strain-specific toxigenicity, which could otherwise help in understanding the regulation of aflatoxin production, are still few[4–11]. Inorganic phosphate has been reported to regulate the production of aflatoxins[12,13]. However, there have been no studies so far on the enzymes regulating the phosphorus concentration in the mycelia of the aflatoxin-producing and non-producing strains of *A. flavus*. It was against this backdrop that the present investigation was undertaken to study nonspecific acid and alkaline phosphatases and ATPase of toxigenic (CMI 102566) and non-toxigenic (CMI 93803) strains of this fungus, so as to establish a relationship, if any, between the activities of these enzymes and the concentrations of inorganic phosphate as well as aflatoxins produced by its toxigenic strain.

The stock cultures of *A. flavus* CMI 102566 (toxigenic) and *A. flavus* CMI 93803 (non-toxigenic) strains were maintained in slants on Czapek–Dox agar medium as described elsewhere[9–11]. Both the strains were grown separately for different time durations (i.e. for 3, 5, 7, 9 and 11 days) at 30°C under static culture conditions[10]. Mycelium from each flask was harvested from the medium by filtering through Whatman No. 1 filter paper.

Preparation of cell-free extract for enzymatic studies and extraction of aflatoxins from the culture filtrates and their analysis were done as described earlier[9,10,14,15]. Protein and inorganic phosphate estimations in the cell-free extracts were done by the methods of Lowry *et al.*[16], and Heinonen and Lahti[17] respectively.

Nonspecific acid and alkaline phosphatases were assayed according to the method described by Plummer[18], with certain modifications[15,19], using *p*-nitrophenyl phosphate as the substrate. The specific activity of these enzymes was expressed as mmol *p*-nitrophenol released/min/mg protein. The release of inorganic phosphate (Pi) from the substrate (ATP) was a measure of adenosine triphosphatase (ATPase) activity[15]. The specific activity of ATPase was expressed as μmol Pi released/min/mg protein[20].

The observations presented in Table 1 regarding the production of aflatoxins by *A. flavus* CMI 102566 indicate that the synthesis of aflatoxins B_1 and B_2 started at day 5 and their concentrations reached maximum level at day 9, followed by a decrease in their concentrations at day 11.

In toxigenic and non-toxigenic strains of *A. flavus*, there was no marked difference in inorganic phosphate levels at different days of incubation, except that in the former case the rate of inorganic phosphate concentration build-up was higher than that of the latter at day 3, and it decreased towards the start of aflatoxin biosynthesis (day 5; Table 1, Figure 1 *a*).

The activities of both acid and alkaline phosphatases in non-toxigenic strain (CMI 93803) of *A. flavus* exhibited an inverse relationship with inorganic phosphate profile up to day 9 (Figure 1). However, in the toxigenic strain (CMI 102566), increase or decrease in the activities of these enzymes was independent of inorganic phosphate concentrations. However, the activity of acid phosphatase decreased in toxigenic strain from day 5 to day 9 with increase in aflatoxin concentration (Table 1 and Figure 1 *b*), indicating its sensitivity to inhibition by aflatoxins produced by this strain itself. In contrast, the activity of alkaline phosphatase exhibited a sharp increase up to day 5 and remained relatively stable during the period (day 5 to day 9) of aflatoxin biosynthesis, followed by a slight decrease in its activity at day 11.

In non-toxigenic strain of *A. flavus*, a gradual increase in the specific activity of ATPase was observed up to day 11 (Figure 1 *d*), irrespective of the inorganic phosphate levels (Figure 1 *a*). In toxigenic strain, increase in ATPase activity was observed up to day 5 (when aflatoxin synthesis starts), followed by a gradual decrease in its activity up to day 9 (when aflatoxin concentration was found to be maximum). At day 11, there was again an increase in ATPase activity (Figure 1 *d*) with a sharp decrease in aflatoxin concentration (Table 1). These observations indicate that ATPase activity of the toxigenic strain was sensitive to inhibition by aflatoxins produced by *A. flavus* itself.

Table 1 and Figure 1 *a* indicate that the lower levels of inorganic phosphate favour the synthesis of aflatoxins, showing that the synthesis of secondary metabolites needs a narrow tolerance for concentrations of inorganic phosphate[13], and its excess concentration during the production phase could lead to the inhibition of their synthesis[12]. In toxigenic *A. flavus* (CMI 102566), biosynthesis of aflatoxins starts before exhaustion of phosphorus, which is due to longer period of primary metabolism during the growth of this strain compared to non-toxigenic *A. flavus* (CMI 93803)[10]. The specific activities of acid and alkaline phosphatases appear to be inhibited by inorganic phosphate concentrations in non-toxigenic strain of *A. flavus*, which is due to competitive inhibition of phosphatase by inorganic phosphate, suggesting a possibility of the occurrence of a repressible system of phosphatase regulation in this strain. Similar phenomenon of phosphatase regulation has also been reported in yeast[21], *Neurospora*[22] and *Escherichia coli*[23]. However, such a relationship of phosphate concentration with phosphatase activities was not observed in the

Table 1. Aflatoxin concentration in cultures of toxigenic strain (CMI 102566) of *Aspergillus flavus* at different days of incubation

	Aflatoxins (μg/50 ml of the culture medium)*		
Incubation period (days)	B_1	B_2	Total (B_1 + B_2)
3	Non-detectable	Non-detectable	Non-detectable
5	3.194 ± 1.10	2.682 ± 1.41	5.876
7	18.459 ± 4.30	2.69 ± 1.40	21.149
9	34.468 ± 19.31	6.80 ± 1.61	41.268
11	4.531 ± 1.90	5.625 ± 3.82	10.156

*Mean ± standard error of means.

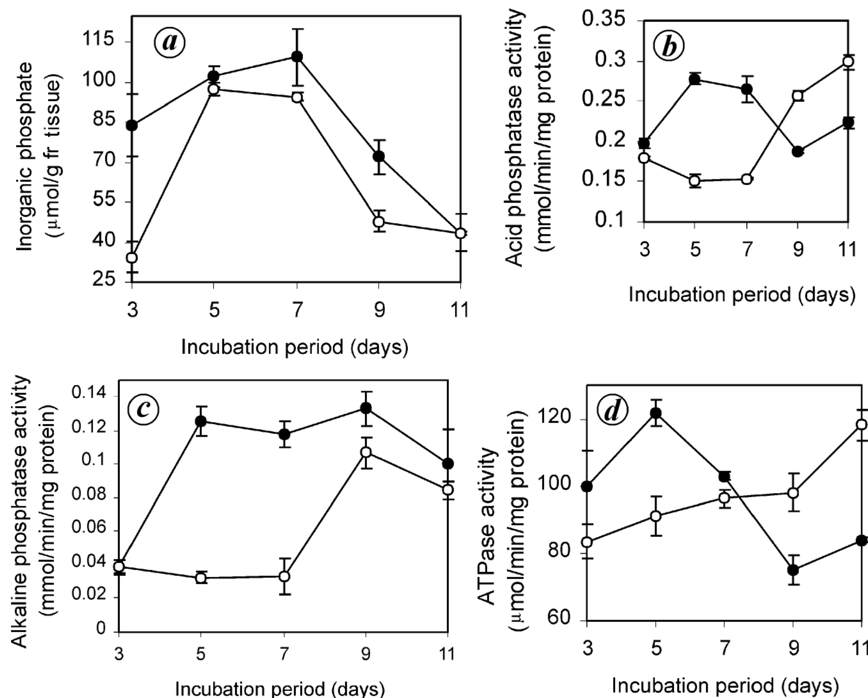

Figure 1. Profiles of inorganic phosphate concentrations (***a***), and activities of acid phosphatase (***b***), alkaline phosphatase (***c***) and ATPase (***d***) of toxigenic (●) and non-toxigenic (○) strains of *Aspergillus flavus* at deferent days of incubation. Each point is based on the mean of duplicate readings of three independent determinations. Vertical line at each point indicates standard error of mean (SE).

toxigenic strain (CMI 102566) of *A. flavus*, as the increase or decrease in its phosphatase activity was independent of phosphate concentration. A relatively stable alkaline phosphatase activity at the time of aflatoxin production in the toxigenic strain indicates a possible regulatory role of this enzyme on the biosynthesis of aflatoxins. In toxigenic strain, acid phosphatase and ATPase enzymes were sensitive to inhibition by increased aflatoxin concentrations, which is due to the binding of aflatoxins to these enzymes, thereby making them inactive. Findings of the present study with respect to ATPase activities are in agreement with those of Gupta *et al.*[12], who observed that in the toxigenic strain, the ATP level increased during the exponential phase of growth and decreased during the stationary phase, where aflatoxin biosynthesis occurs. Such a decrease in ATP levels was not observed during the same time period in the non-toxigenic strain[24].

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ACKNOWLEDGEMENTS. The research was supported by the Centre of Advanced Study in Botany, University of Delhi. I.S. thanks Council of Scientific and Industrial Research, New Delhi for financial assistance in the form of Senior Research Fellowship.

Received 14 February 2007; revised accepted 4 September 2007

ISHWAR SINGH
VED PAL SINGH*

Applied Microbiology and Biotechnology Laboratory,
Department of Botany,
University of Delhi,
Delhi 110 007, India
**For correspondence.*
e-mail: vpsingh_biology@rediffmail.com