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A composite medium for the Standardised Measurement of Aflatoxin accumulating capacity of *in-vitro* culture systems

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Abstract : Conventional methods which have used single incubation periods for the study of aflatoxin production in solid substrates, have the disadvantages of not taking into account, significant variations of toxin content that occur with time. A single step method, using a double layered composite medium is described here for the determination of the maximum quantity of aflatoxin accumulated in experimental culture. This method is based upon the capacity of silica gel to adsorb aflatoxin and remove it from degradative mechanisms which operate in the substrate, and which are probably responsible for the phasic variations of toxin content which occur in the adsorbent free medium. This method permits of standardisation in terms of adsorbent, substrate, inoculum, incubation conditions and extraction procedure.

1. Introduction

The measurement of aflatoxin production has been the basis of several important aspects of research on these biologically and economically important mycotoxins. These aspects include (1) the capacity of substrates to support toxin production, upon which data, agronomically useful substrates which show resistance to aflatoxin accumulation can be selected for propagation ; (2) the capacity of fungal strains to produce aflatoxin. This data could be used to select high yielding strains for the laboratory production of aflatoxin for experimental purposes, for the differentiation of genera, species and strains of fungi and in field studies on the accumulation of aflatoxin under natural conditions ; (3) the effects of cultural conditions, inhibitors and promoters on toxin production ; (4) the biosynthesis and metabolism of aflatoxins.

Most studies done *in vitro* have hitherto used cultures of aspergilli, incubated for arbitrarily selected single incubation periods, which have generally been between the fifth and tenth day. Some studies have used determinations over a few days while only a few have been made serially over three to four weeks.

We have reported earlier[3] that cultures of *Aspergillus flavus* and *A. parasiticus* on grated coconut showed multiple peaks of aflatoxin content when replicate cultures were assayed serially for two to three weeks. The patterns shown by different culture systems may differ in respect of :—

- the time relations of the peaks of toxin content
- the occurrence of monophasic or multiphasic patterns
- the patterns shown by the individual aflatoxin components
- shifts produced by modifications of the substrate, the inoculum or the conditions of incubation.

The results of van Walbeek *et al*[8] also showed that a similar phenomenon was occurring in one of their experiments at the time of termination of their observations ; they however did not comment on this phenomenon. Lafont and Lafont[6] described similar phasic variations of aflatoxin content in a synthetic liquid medium and the phenomenon was also reported by Applegate and Chipley.[1]

It would therefore appear that an assay of a conventional culture at a given incubation period without data on the time course of the variation of toxin content, may provide erroneous conclusions regarding the identity or concentration of the components of aflatoxin which a culture system can produce. Examples of possible errors were pointed out earlier. The assay of several replicate cultures will therefore have to be made before the patterns characteristic of a given culture system are defined and for obtaining valid conclusions on the capacity of the system to accumulate toxin. This approach however entails the cumbersome use of replicate cultures over prolonged periods.

The technique of de Vogel *et al*[4], for the screening of fungi for aflatoxin production, seemed to provide a basis of an abbreviated method. Their technique used hyflo-supercel as an adsorbent with an overlying culture medium on which the strain under investigation was inoculated. These authors considered that the removal of the toxin into the underlying adsorbent would have differentiated the greenish or greyish fluorescence of the invading mycelium in the agar medium, from the fluorescence of the aflatoxin in the adsorbent, when the adsorbent side of the plate was viewed under ultraviolet light. Their cultures were examined on the third or fourth day after inoculation.

We used the techniques of de Vogel *et al*[4] to screen wild strains of aspergilli for aflatoxin production and to study the time course of aflatoxin accumulation which was determined semiquantitatively with reference to standard solutions of quinine sulphate, over prolonged periods. These plots showed that strains which produced phasic variations of toxin content with time on conventional culture, gave patterns which consisted of a rise of the toxin content to a maximum within a few days ; this maximum was maintained for two or three weeks as a plateau and then declined gradually. Apparently the toxin was removed from the culture by the adsorbent which thus prevented the degradation or inactivation of the toxin and hence the multiple peaks which would have occurred in the adsorbent free medium.

This paper reports a technique based upon the use of silica gel as an adsorbent, which eliminated the phasic variations of toxin content and which produced a plateau which represented the maximum amount of toxin which had accumulated over two or three weeks, during which period the plateau was maintained. During this period, a single assay provided an estimate of this maximum.

We prefer to use the term aflatoxin 'accumulation' rather than aflatoxin 'production' since the aflatoxin content of a culture at a given period of incubation, is the resultant of production and inactivation or loss and because the method which we have described here, measures the maximum content of toxin which was accumulated upto the time of assay.

2. Experimental

Test strains and inocula. *A. parasiticus* NRRL 2999 was used in all the experiments in establishing the method. *A flavus* F209 and F218 in our collection, isolated from mouldy copra, were used in addition, to test the system.

Spores from 3 week old cultures on potato dextrose agar ('Difco') slopes were suspended in sterile 0.1% Tween 80 in distilled water (autoclaved at 115° C/10 min.) and the spore count was made in a blood cell counting chamber. The inoculum used was 0.2 ml of suspensions containing 20,800 spores/c.mm. *Culture medium.* Agar media containing peanut or other substrates, homogenised as in the method of de Vogel *et al*[4] were replaced by the substrate alone in as finely divided a state as possible. Agar media had the disadvantage of producing relatively low levels of toxin with only 10% of the homogenised substrate as used in the original method. On the other hand, natural substrates alone had the advantage of yielding data which was more indicative of the levels of toxin contamination under natural conditions in the field.

Pulses and grains were pulverised in a mortar or mill. With these substrates it was possible to obtain particles with a sieve size of BS 22 or even 44. Oilseeds such as peanut and coconut were minced and used as particles of approximately BS 10 mesh size. Preliminary drying at 40°C to 50°C of the oilseeds made it easier to reduce their particle size; oil extrusion which occurred with more intensive grinding was avoided. Substrates in replicate tests were obtained from the same nut or batch of seeds.

Adsorbent.

Silica gel H ('Merck') was found to produce higher yields of toxin and more consistent results than hyflosupercel.

Preparation of the medium.

Weighing bottles of 3 cm diameter and 6 cm height provided a suitable container for the double layered composite medium. The composite medium was prepared as shown in Figure 1 by packing the silica gel powder into a uniform layer at the bottom of the bottle. The finely divided substrate was then lightly packed over the adsorbent. In early experiments 2 g of the adsorbent and 4 g of the substrate were used but in later experiments the amount of substrate was reduced to 1.5 g.

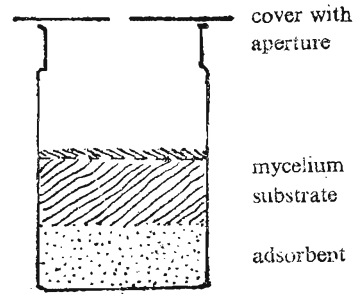

Figure 1. Diagramatic representation of the weighing bottle containing the double layered composite medium.

With both unautoclaved and autoclaved (115°C/10 min.) composite media, 2 ml of sterile distilled water was added after packing of the components. There was no increase in the moisture content (as determined by weighing) of the composite medium after autoclaving. There was no alteration of the pH of the substrate in the sterilised medium. The bottle was covered with a glass plate which had a central aperture (3 to 4 mm in diameter) which allowed of inoculation and access to air. Duplicates were used for each test. The preparation and inoculation of the composite medium was done as aseptically as possible with sterile solutions and glassware.

Incubation.

The bottles were incubated in a moist chamber at 24°C in a box which while excluding direct sunlight, permitted intermittent observation of the bottles.

Assay of toxin.

After incubation, the bottles were steamed for 10 min. The entire composite medium was extracted by the aqueous acetone method of Pons *et al*[7], substituting three successive homogenisations in a 'MSE' homogenisor with an overhead drive, instead of shaking as in the original procedure. The extracts in chloroform were titrated on TLC plates (Silica Gel G 'Merck') run in two different solvent systems, methanol : chloroform, 3 : 97 v/v and in acetone : chloroform, 1 : 9 v/v respectively, by visual estimation against inocula of standard aflatoxin B1 and G1 solutions in chloroform, of known concentration. The results are expressed as micrograms of aflatoxin per gram of the wet weight of the original substrate.

3. Results

In the composite medium, fungal growth was visible on the surface of the substrate after three or four days of incubation. With autoclaved substrates, growth of contaminant fungi did not occur although with unautoclaved substrates, contamination occurred frequently.

Exposure of the bottles to uv light at 360 nm showed progressive diffusion of blue fluorescence downwards into the adsorbent. After 3 or 4 days of incubation and within a week, the fluorescence had permeated nearly the entire depth of the adsorbent and this suggested that with the degree of moisture in the substrate and the adsorbent there appeared to be a continuous aqueous phase between the substrate and the adsorbent. Separate analysis of the substrate and adsorbent after incubation, showed low amounts of aflatoxin in the substrate with large amounts in the adsorbent ; hence both substrate and adsorbent were extracted together for estimation of the total content of toxin in the composite medium.

There was no mycelial growth into the adsorbent, sufficient to bind the adsorbent particles into a matt as with the substrate. At the end of the incubation period, even with appreciable amounts of toxin, the particles of the adsorbent retained their discreteness as when originally packed.

Autoclaved or oven dried substrates showed both B1 and G1 components of aflatoxin with strain 2999 whereas with unsterilised substrates G1 was present in very low concentrations or was absent.

Strain NRRL 2999 was previously reported by us[3] to produce biphasic curves for both aflatoxin B1 and G1 in conventional culture on loose, freshly grated coconut. Figure 2 compares the patterns of the variations of aflatoxin content with time in cultures of this strain on adsorbent-free loose, grated coconut and in the substrate packed as a layer as in the composite medium, but without adsorbent. With both the loose and the packed substrates, biphasic curves with similar time relationships were obtained indicating that packing of the substrate had no effect on the phasic alterations of toxin content. The loose substrate yielded more aflatoxin ; this was probably due to greater accessibility of the particles in the loose medium, to mycelial spread.

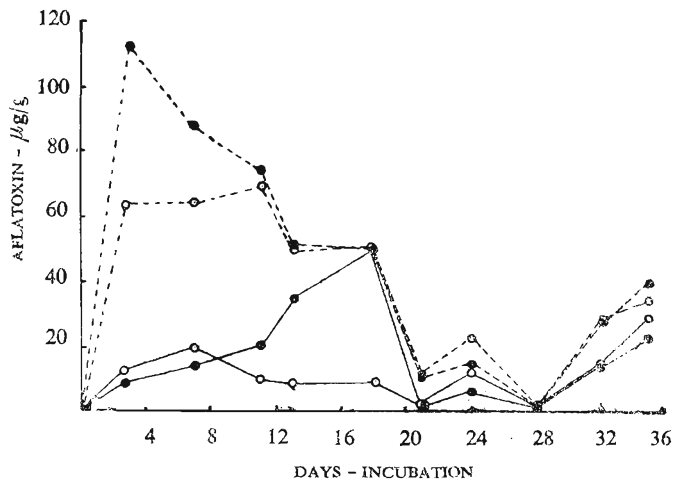

Figure 2. Pattern of variation of aflatoxin content of replicate cultures of *A. parasiticus* NRRL 2999 in grated coconut, in loose form (- - - - -), and as a packed layer (————), in the absence of adsorbent ; cultured at 24°C in semidarkness. Aflatoxin B1 –●–: Aflatoxin G1 –o–.

Figure 3 compares the patterns of variation of aflatoxin content, with the packed substrate but without the adsorbent, with that in the composite medium with the adsorbent. The elimination of the variations and their replacement by the plateau in the latter system indicate that it was the adsorbent which was responsible for the modification of the pattern. The packing of the substrate apparently merely served to provide a continuous phase for the rapid diffusion of toxin through the substrate into the adsorbent.

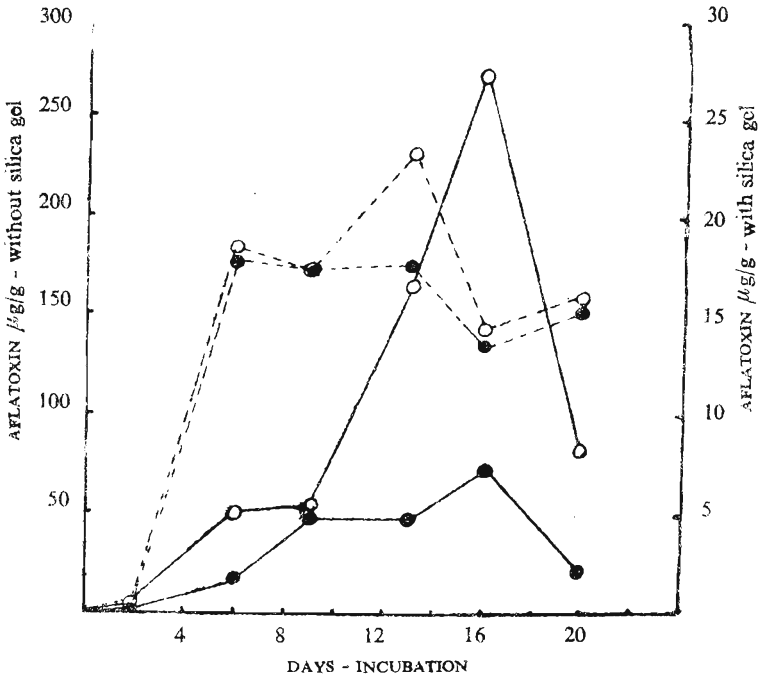

Figure 3. Pattern of variation of aflatoxin content of replicate cultures of *A. parasiticus* NRRL 2999 in packed, grated coconut with adsorbent (- - - - - -) and without adsorbent (————), cultured at 24°C in semidarkness. Aflatoxin B1 –●–; Aflatoxin G1 –o–.

This effect parallels the patterns observed on plates with hyflosupercel, used in the screening of strains for toxigenicity. The composite medium produced approximately ten-fold more toxin than the adsorbent free medium.

The Table records the aflatoxin levels obtained in replicate cultures on the composite medium containing 4 g of the substrate, indicating an acceptable degree of inter-replicate variation and reproducibility of the method.

TABLE 1. The aflatoxin content of replicate cultures of *A. parasiticus* NRRL 2999 on grated, pulverised coconut in the composite medium containing 4 g of substrate and 2 g of adsorbent, cultured at 24°C in semidarkness.

Aflatoxin content ug B1/g, in replicate cultures	mean	coefficient of variation
487.5 475 462.5 437.5 450 475	464.6 ± 18.4	3.9

The plateau pattern was also produced by four other substrates incorporated in the composite medium, cow-pea and Lanka dhal (Figure 4) and Green-gram and peanut (Figure 5), with strain NRRL 2999. Only aflatoxin B1 was detected in these cultures. These four substrates however produced much lower levels of aflatoxin in comparison with coconut which was earlier shown by us[2] to be an excellent medium for the production of very high levels of toxin. This strain NRRL 2999 was found to produce biphasic curves with crushed peanuts in conventional culture (Arseculeratne, unpublished data).

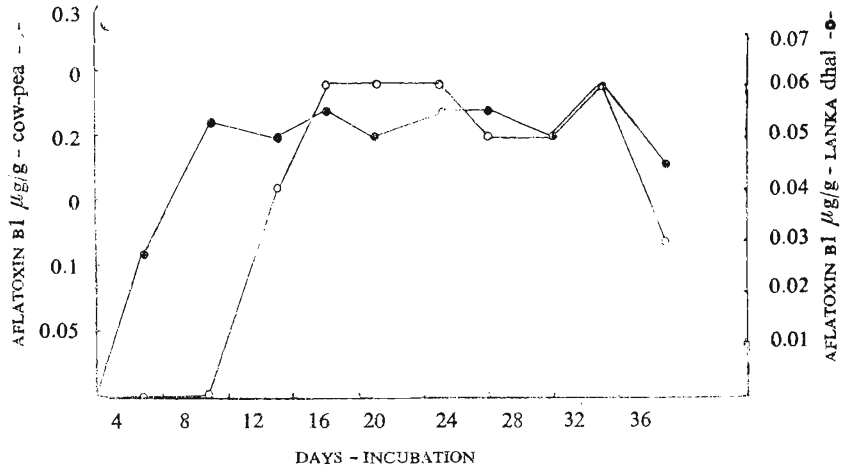

Figure 4. Pattern of variation of Aflatoxin B1 content of replicate cultures of *A. parasiticus* NRRL 2999 on the composite medium containing Lanka dhal -o- ; cow pea-●-. Cultured at 24°C in semidarkness.

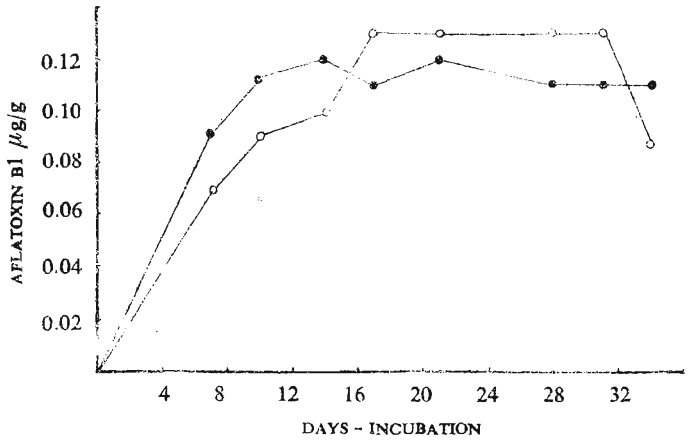

Figure 5. Pattern of variation of Aflatoxin B1 content of replicate cultures of *A. parasiticus* NRRL 2999 on pulverised green gram –●–; peanut –○–; in the composite medium at 24°C in semidarkness.

Figure 6 shows the patterns obtained with the strain F208 and F218 on the composite medium with grated coconut. The plateau of the curve with aflatoxin B1 was maintained for approximately ten days in both instances.

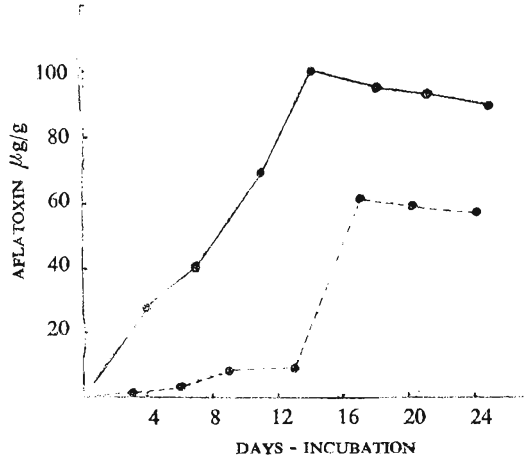

Figure 6. Pattern of variation of Aflatoxin B1 content of replicate cultures of *A. flavus* F208 (——) and F218 (- - - - - -) on grated coconut in the composite medium at 24°C in semi-darkness.

4. Discussion

Although the original expectation of de Vogel *et al*[4] in the use of an adsorbent was that it would have prevented interference from the nor-aflatoxin greenish fluorescence of the mycelium in the agar medium with the fluorescence of the aflatoxins, the use of the adsorbent now appears to have a second advantage which is the removal of the aflatoxin from degrading or inactivating mechanisms which probably operate within the substrate. The evidence for this view is as follows : (1) the higher yields of toxin in the composite medium than in the adsorbent-free medium. An alternative explanation could be that substances which were inhibitory to aflatoxin production were removed by the adsorbent ; (2) the occurrence of phasic variations of aflatoxin content in the adsorbent-free medium whereas in the composite medium, these variations were eliminated and a plateau replaced the multiple peaks.

The reasons for our view that the phasic variations were not artefactual due to technical errors but were real variations are :—

(1) the variations were reproducible in degree and time relations.

(2) the variations were much larger (50 — 90%) than the general range of variation of the TLC values due to technical error (± 20%)

(3) the same assay procedure was applied to both adsorbent-free and the composite media while the levelling of the curves was seen consistently with the latter medium.

The use of substrates in a particulate form has several advantages. The use in conventional culture systems of seeds or kernels with seed coats which are impermeable to fungal invasion may produce false, low values of toxin content and may therefore not reflect the contamination as it would occur under field conditions or in storage. Under the latter conditions, fungal invasion and aflatoxin production occur mainly on seeds and kernels that have been damaged with the resultant exposure of the cotyledons or endosperm to fungal colonisation. A further advantage is that the uniformity of the plateau increased with the fineness of the particles of the substrate. This effect was probably due to the ease of mycelial penetration and aflatoxin production and the rapid transfer of the toxin into the adsorbent. Thus the composite medium would appear to measure the total aflatoxin accumulating capacity of the system under optimal conditions of accessibility of the substrate.

Conventional methods for the determination of the aflatoxin producing capacity of culture systems which use solid substrates incubated for arbitrarily selected, single incubation periods, have the disadvantage of not taking into account the significant variations of toxin content that occur with time. On the other hand the serial assay of replicate samples is a time consuming and cumbersome procedure. A further

disadvantage of conventional methods is the difficulty of standardisation to permit of valid comparisons between the results obtained in different laboratories or in the same laboratory on different occasions, in respect of both the toxigenic capacity of aspergilli on a standard substrate or of the capacity of a given substrate to support toxin production using a standard strain of *Aspergillus*.

The method described in this paper appears to provide a simple and convenient alternative procedure which would not only eliminate the need for performing serial assays, but would also allow of the standardisation of the determination of aflatoxin production, in respect of the following factors :—

the container with cover, of specified dimensions

the substrate, of defined particle size, quantity and thickness of layer

the adsorbent, obtained from a single manufacturer and of defined quality, quantity and thickness of layer

the inoculum, a standard toxigenic strain is grown on a specified medium, cultured under defined conditions, and its spores are suspended in 0.1 % Tween 80 to a standard spore count. A specified inoculum is used. To prevent loss of toxigenicity on repeated subculture on laboratory media, the standard strain may be periodically passaged through moist crushed peanuts.[4]

Incubation. The composite medium could be incubated under defined conditions of temperature, light, humidity and atmosphere. From our observations on the 5 different substrates tested, it would appear that an interval of three weeks after inoculation would coincide with approximately the midpoint of the plateau. Hence the single assay could be performed after an incubation period of three weeks, after inoculation of the composite medium.

Extraction and assay procedure, for aflatoxins could be prescribed in terms of solvents, methods of extraction, purification and titration of extracts.

The use of such standard conditions, and the measurement of the cumulative maximum amount of aflatoxin which the culture system has produced, may provide greater uniformity in the results obtained from studies on aflatoxin production. Hitherto, divergent results which are reported in the literature have been obtained by the use of conventional methods of culture. Examples of such controversial results include the claim that certain varieties of peanut are resistant to aflatoxin accumulation. It is possible that the occurrence of phasic variations of toxin content in conventional culture systems could have contributed to these discrepancies.

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