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Changes in Free Amino Acid and Sugar Levels of Dried Figs during Aflatoxin B_1 Production by *Aspergillus flavus* and *Aspergillus parasiticus*

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An aqueous slurry of γ-irradiated sterilized dried figs was inoculated with toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. During incubation at 28 °C, pH, fructose, glucose, and free amino acids were determined by high-performance liquid chromatography and liquid chromatography (LC)/mass spectrometry, respectively, over 13 time points (1−20 days). At the same 13 time points using a LC/time-of-flight mass spectrometry screening method, aflatoxin B_1 and other secondary metabolites were simultaneously monitored. During the course of incubation, the pH significantly decreased and aflatoxin B_1 formation correlated with a reduction in proline content for both fungi. Of the 22 free amino acids that were monitored, only proline and cystine were found to be critical in supporting aflatoxin production. Levels of fructose and glucose steadily declined during incubation, until glucose was almost exhausted after 21 days. These time−course experiments confirmed the need for carbon and nitrogen sources for aflatoxin production in dried figs, and the favorable composition of figs as a fungal growth medium.

KEYWORDS: Dried figs; amino acids; sugars; aflatoxin B_1; secondary metabolites

INTRODUCTION

Since aflatoxins were first discovered, there have been many studies examining the conditions favoring fungal growth and stimulating mycotoxin production (*1, 2*), and the biosynthesis of aflatoxins has been reviewed (*3*). These studies have generally been carried out in well-defined liquid or solid culture media (*2*−*5*), partly being driven by the need to avoid the complexity of real biological samples, which historically have presented significant analytical challenges. Apart from understanding ecological and environmental factors such as fungal strain, water activity, pH, and temperature, these studies have provided insight into the critical composition of growth media (nutritional factors) and the events triggering secondary metabolism. Understanding the relationship between the composition of agricultural commodities and their ability to support fungal growth and toxin production is an important step in identifying susceptible commodities and in developing prevention and control strategies.

It is well-recognized that aflatoxin production is greatly affected by the nature and concentration of available carbon sources. Glucose, ribose, xylose, and glycerol have been shown to be good fungal substrates for *Aspergillus flavus* and *Aspergillus parasiticus* (*5*), confirmed by others who similarly found that glucose, ribose, fructose, sucrose, and maltose supported high levels of toxin production (*6*). From a carbon source perspective, as dried figs contain sucrose, fructose, and glucose at levels of around 0.2, 18.1, and 19.3 g/100 g fruit, respectively, they obviously present an excellent growth medium (*7*). As is the case with the carbon source, the types and concentrations of the nitrogen source in the growth medium are equally critical to supporting fungal growth and to subsequent aflatoxin production. It has been reported (*8*) that of the amino acids, proline (Pro) stimulates more aflatoxin production per gram of mycelium than asparagine (Asn), tryptophan (Trp), or methionine (Met) when *A. flavus* and *A. parasiticus* are grown in culture. Shake cultures have led to higher aflatoxin production than stationary cultures, indicating the importance of aeration for growth and toxin formation. Other important nutritional factors are, for example, the presence of critical levels of trace elements where it is known that potassium, iron, copper, magnesium, zinc, molybdenum, and calcium are essential for fungal growth (*9*), with zinc having an important role in secondary metabolism (*10*). Inorganic phosphate also plays a role in the enzymatic reactions in the fungal cell as well as in regulation of secondary metabolism, but both inhibitory and

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stimulatory activities have been reported, illustrating the complexity and influence of other apparently confounding factors (*11, 12*).

Recent developments in analytical techniques such as the introduction of combined liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) now enable analysis to be carried out on small samples from complex biological materials and for these to be rapidly screened for a wide range of secondary metabolites (*13*−*15*). Thus, instead of having to carry out biosynthetic studies restricted to simple but well-defined media, it is now possible to undertake similar experiments but simultaneously monitor levels of a number of parameters, which may be interacting and changing during the course of a time study. We have recently been studying the occurrence of mycotoxins in dried figs (*16, 17*), which are economically important in Turkey and have found not only the already well-established presence of aflatoxins and ochratoxin A but also previously unreported toxins (*17*). Dried figs thus appear to provide a particularly good medium to support the growth of a diversity of fungal species and contain a good balance of critical nutritional components to sustain secondary metabolism. To extend this work further, working with dried figs as a fungal growth medium, we have undertaken a time−course study using toxigenic strains of *A. flavus* and *A. parasiticus*, monitoring the relationship between the formation of aflatoxins and other secondary metabolites and the simultaneous depletion of sugars and amino acids.

MATERIALS AND METHODS

Chemicals. Alanine (Ala), arginine (Arg), Asn, aspartic acid (Asp), cysteine (Cys), cystine (Cys-Cys), glutamic acid (Glu), glutamine (Gln), glycine (Gly), histidine (His), hydroxyproline (Hyp), leucine (Leu), isoleucine (Ile), lysine (Lys), Met, phenylalanine (Phe), Pro, serine (Ser), threonine (Thr), tyrosine (Tyr), Trp, and valine (Val) standards (99%) were supplied by Aldrich (Milwaukee, WI). Mycotoxin standards aflatoxins B_1, B_2, G_1, and G_2 and kojic acid were obtained from Biopure (Tulln, Austria), and the internal standard (IS) (benzophenone) was from Merck (Germany). Glucose, fructose, and saccharose were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Formic acid (98%), acetic acid (glacial), and isopropanol were analytical grade and were obtained from Merck (Darmstadt, Germany). Ethyl acetate was supplied by J. T. Baker (Holland). Ultrapure water prepared with a Milli-Q system (Millipore, Bedford, MA) was used throughout the experiments. Malt extract agar (MEA), potato dextrose agar (PDA), and yeast extract sucrose (YES) agar from Merck KGaA (Darmstadt, Germany) were used as fungal growth media.

Fungal Strains. Toxigenic strains of *A. parasiticus* (NRRL-2999) and *A. flavus* (200-138) were provided from the culture collection of TUBITAK Marmara Research Center (Gebze, Turkey).

Sterilized Fig Sample Preparation. Dried fig samples of the Sarilop variety were collected from fig processing areas near to İzmir. Samples were maintained at refrigeration temperature (4 °C) prior to the study. Homogenized figs (2 kg) were sterilized by γ-irradiation for 8 h (9.36 kG) by the Turkish Atomic Energy Authority (Saraykoy Nuclear Research and Training Center, Turkey).

Fungal Growth Experiments. *A. flavus* and *A. parasiticus* cultures stored at 4 °C were activated twice by inoculation onto DRBC agar, and subsequently, plates were incubated at 28 °C for 1−20 days. For the purpose of fresh spore production, the environmental conditions were usually set at the optimum for growth, in terms of water activity [80% relative humidity (RH)] as well as temperature. Fresh spores of *A. flavus* and *A. parasiticus* were mixed with 20 mL of sterile distilled water, filtered, and poured into a 100 mL flask to provide spore numbers of around 10^6. Fungal colonies were identified by spread-plate technique on PDA and MEA. Fresh spores were obtained after the mycelium was grown on media, after 7 days of incubation, and were confirmed visually by microscopic examination. The number of spores was counted by thoma slide. Stock cultures had a cell density of 1.7 10^6 and 5.4 10^6 CFU/mL, respectively, for each strain.

Dried fig samples were mixed with sterile distilled water with a ratio of 1:1 and homogenized by Ultra-Turrax. The mixture (25 g) was poured into each of 84 Petri dishes (duplicate for each *Aspergillus* strain) and spread by a glass spreader. A total of 150 μL of activated *A. parasiticus* and *A. flavus* cultures were spread for incubation within an amount of 30 μL on five separate points, four in a circular range and one in the center by a glass spreader. Petri dishes were incubated at 28 °C, 80% RH, for 1−20 days.

Sampling. The pH, fungal metabolite, amino acids, and sugar levels were determined at 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 14, 17, and 20 days of incubation. Duplicate inoculations were carried out, and experiments were repeated in triplicate throughout the study. For sugar and fungal metabolite analyses, a total of 1 g of sample was taken from four different fungal hyphae growth points, together with the underlying culture media, by vertically cutting 6 mm diameter plugs using a cork borer, and 0.5 g of sample was taken for amino acid analyses by the same method from each half of the Petri dish.

Determination of pH of Growth Media. pH values were measured for the whole sample for each Petri dish using a spear tip solid probe with a calibrated pH meter. Measurements were carried out by dipping the probe into five individual points. Values at fixed points were recorded, and the average was calculated.

LC/MS Analysis of Free Amino Acids. The LC/atmospheric pressure chemical ionization (APCI)-MS analyses for the quantification of 22 free amino acids were as described elsewhere (*18*). A stock solution of 1000 μg/mL amino acids was prepared by dissolving 25 mg of each amino acid in 25 mL of distilled water. A 100 μg/mL amount of intermediate mixed standard solution was prepared by diluting 1 mL of stock solution to 10 mL. Working standards were prepared by diluting the 100 μg/mL mixed standard of amino acids to concentrations of 0.05−5.00 μg/mL with 0.2 mM acetic acid. Stock solutions were kept at 4 °C for a week for daily use and kept at −18 °C for longer term needs. Working standards were prepared daily before analysis. Homogenized sample (1 g) was weighed into a 10 mL glass centrifuge tube with a cap, and 0.2 mM acetic acid (10 mL) was added. After it was mixed in a vortex mixer for 2 min, the mixture was centrifuged at 5000 rpm for 10 min at −5 °C. The clear supernatant was quantitatively transferred into a vial, avoiding the top oil layer if present, and filtered through 0.45 μm nylon syringe filter. LC/MS analysis employed an Agilent 1100 high-performance liquid chromatography (HPLC) system (Waldbronn, Germany) consisting of a binary pump, an autosampler, and a temperature-controlled column oven, coupled to an Agilent 1100 MS detector equipped with an APCI interface used for analysis. The analytical separation was performed on a 100 mm × 2.1 mm i.d., 3.5 μm, Zorbax Bonus-RP, narrow-bore column (Agilent Technologies, Wilmington, DE) using an isocratic mixture of 0.01 mM acetic acid in 0.2% aqueous solution of formic acid at a flow rate of 0.2 μg/mL. Data acquisition was performed in selected ion monitoring (SIM) mode using the interface parameters: drying gas (N_2) flow of 4 L/min, nebulizer pressure of 55 psig, drying gas and vaporizer temperatures of 320 °C, a capillary voltage of 3 kV, a corona current of 8 μA, and a fragmenter voltage of 55 eV.

LC Analysis of Glucose, Fructose, and Saccharose. Stock solution of sugars (15 mg/mL) were prepared by dissolving 150 mg of each in 10 mL of water. Working standards were prepared by diluting the stock solution of sugars to concentrations of 500−5000 μg/mL with 50% acetonitrile. Stock solutions were kept at 4 °C for a week for daily uses and kept at −18 °C for longer term needs. Working standards were prepared daily before analysis. Finely homogenized sample (0.5 g) was weighed into a 10 mL glass centrifuge tube with a cap, and 50% acetonitrile (5 mL) was added. After it was mixed in a vortex mixer for 2 min, the mixture was centrifuged at 5000 rpm for 10 min at −5 °C. The clear supernatant was quantitatively transferred into a vial, avoiding the top oil layer if present. It was filtered through a 0.45 μm nylon syringe filter. LC analysis was carried out using an Agilent 1100 series liquid chromatograph equipped with a refractive index detector (RID), ALS autosampler, and thermostatted column module. The mobile phase was acetonitrile:water (75:25, v/v) with a 150 mm × 2 mm i.d. Shodex Asahipak NH2P-50 2D LC column operated at 35 °C at a flow rate of 0.2 mL/min. The injection volume was 20 μL.

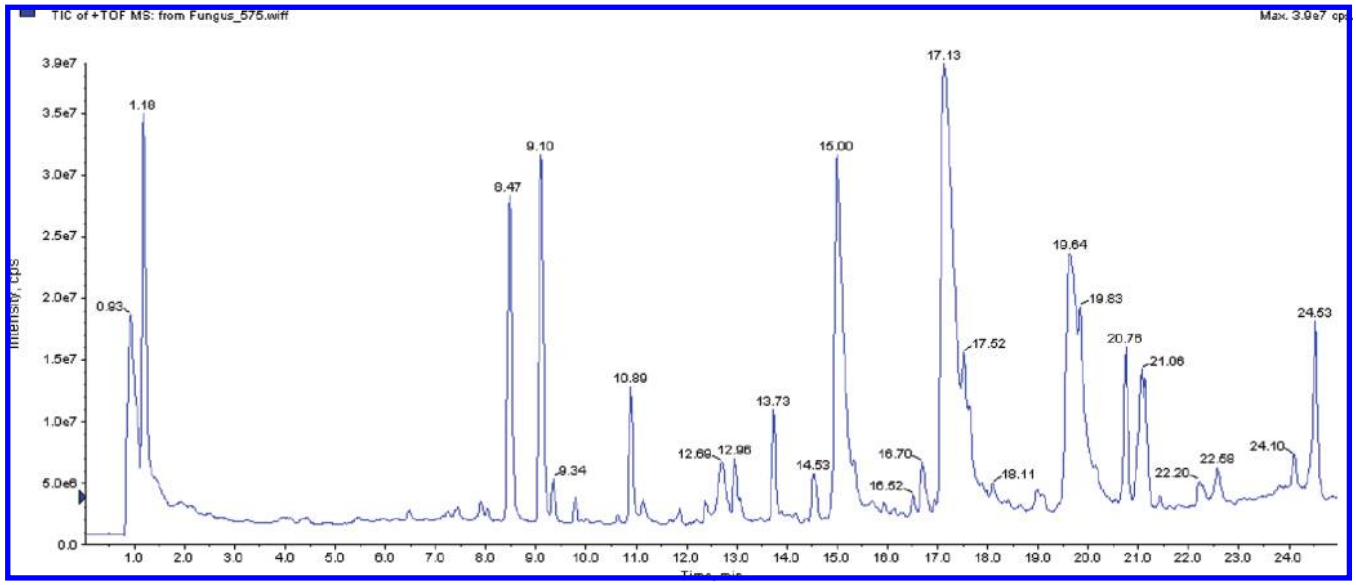

Figure 1. TIC for LC-TOF/MS analysis of a fungal extract from *A. parasiticus*. By searching accurate mass data, peaks were found corresponding to kojic acid (1.08 min), aflatoxin G1 (8.40 min), aflatoxin B_1 (9.04 min), and 5-methoxysterigmatocystin (10.89 min).

LC/TOF-MS Analysis of Fungal Metabolites. Two grams of sample taken as plugs was transferred to 5 mL disposable screw-cap bottles. Extraction conditions were modified from previous published methods (*19, 20*). The plugs were extracted twice with 3 mL of acetonitrile and 2 mL of 1% formic acid in ethyl acetate. Extracts were collected in a tube and evaporated gently to dryness under a nitrogen stream at 45 °C (*21*). The fungal extracts were spiked with 100 μL of benzophenone (10 μg/mL) as an IS. The residues in both cases were dissolved in 1 mL of methanol, ultrasonicated for 10 min, and passed through a 0.2 μL disposable filter prior to LC/TOF-MS analysis. The relative amounts of the fungal metabolites were monitored on the basis of changes in peak areas.

An Agilent 6210 TOF-MS coupled to an Agilent 1200 Series HPLC was employed for LC/TOF-MS analyses. The separation of mycotoxins and other fungal metabolites was carried out using an HPLC system [consisting of vacuum degasser, autosampler with thermostat, binary pump, and diode array detection (DAD) system] equipped with a 100 mm × 2.1 mm i.d., 1.8 μm, reversed-phase Zorbax Eclipse XDB C18 column (Agilent Technologies) and precolumn. The TOF-MS was equipped with a dual nebulizer electrospray source, allowing continuous introduction of reference mass compounds. All data recorded were processed with Analyst-QS software.

TOF/MS scanning was from *m/z* 100 to 1000 for all samples at a scan rate of 1 cycle/s in 9429 transients/scan. This mass range enabled the inclusion of two reference mass compounds, which produced ions at *m/z* 121.050873 and 922.009798. The injected sample volume was 5 μL. The HPLC analysis used a mobile phase of acetonitrile and 2 mM ammonium acetate in an aqueous solution of 1% formic acid at a flow rate of 0.3 mL/min. The gradient elution started with 15% acetonitrile and reached 100% acetonitrile in 20 min. The Zorbax column and precolumn were washed with 100% acetonitrile for 5 min and equilibrated for 5 min between chromatographic runs. UV spectra were obtained using DAD scanning every 0.4 s from 200 to 700 nm with a resolution of 4 nm. The optimum TOF-MS conditions were previously described (*15*).

RESULTS AND DISCUSSION

This study for the first time has simultaneously used a combination of novel methodology (LC-TOF/MS) for metabolite and amino acid (LC/MS) analysis, together with conventional sugars analysis, which has enabled time−course measurements to be made of changes in carbon and nitrogen sources with the formation of secondary metabolites. These techniques, which have both high specificity and high sensitivity, have further enabled a real matrix (dried figs) to be used as a growth medium and for the analysis to be carried out on very small sample sizes. For the secondary metabolite analysis (including the determination of aflatoxins), about 15 major components were detected in the chromatograms at retention times from 1 to 24 min. However, as the matrix had not received any cleanup whatsoever, it contained significant coextractives, and these 15 major compounds were among over 50 minor components present with signal-to-noise ratios of greater than 10:1. A typical total ion chromatogram (TIC) is shown in **Figure 1**, illustrating the complexity of these extracts. Apart from the aflatoxins, other key metabolites that could be seen included kojic acid and 5-methoxysterigmatocystin, which were found manually/automatically using the database search facilities, giving a good match with the standards or reference spectra. Both of these compounds are well-known fungal metabolites that might be expected to be found to be produced by strains of *A. flavus* and *A. parasiticus*.

Table 1. Changes in pH of the Culture Media During Fungal Growth

	pH of growth media (fig slurry)	
days	*A. parasiticus*	*A. flavus*
1	4.11	4.12
2	4.10	4.14
3	4.20	4.22
4	4.16	4.24
5	4.28	4.10
6	4.15	3.84
7	4.14	3.72
8	3.70	3.40
9	3.60	3.35
11	3.70	3.36
14	3.70	3.10
17	4.14	3.11
20	4.28	3.35

In **Table 1**, the results are shown for monitoring the pH of the fig slurry during the course of fungal growth and secondary metabolism. For both organisms, it can be seen that there was a decline in pH from an initial level of 4.1 to around 3.1−3.7, with the decrease starting around day 6 and corresponding to the onset of secondary metabolism. After day 14, the pH seemed to increase again, which might be associated with commencement of autolysis (*1*). In defined media, an initial pH of 4.5

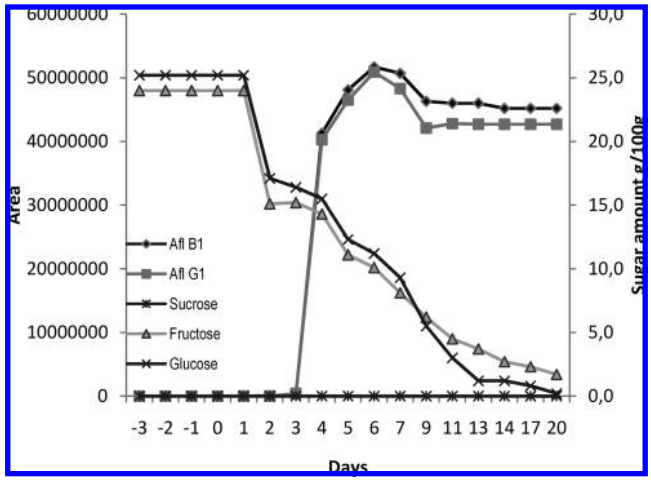

Figure 2. Relationship between levels of fructose, glucose, and sucrose and corresponding production of aflatoxin B_1 and G_1 in figs during the course of *A. parasiticus* secondary metabolism.

was found necessary to ensure good growth and high toxin production (*4*) for *A. parasiticus*, and thus, figs naturally have a pH that is close to the optimum for fungal growth and toxin production. There are conflicting reports in the literature concerning the change in pH, which occurs during secondary metabolism, with a fall from pH 4.00 to around pH 2.1−2.3 being reported in submerged culture (*22*) but a fall in pH followed by a rise after commencement of autolysis (*1*) being reported by others. However, it is difficult to directly compare the results reported here, as figs as a growth medium have both a physical and a chemical complexity not comparable to that of a defined and sometimes buffered liquid growth medium. There was, however, a clearly detectable decline in pH, which corresponded to the start of metabolite formation and continued presumably due to a progressive build-up acidic byproduct.

Figure 2 shows the results for the analysis of fructose, glucose, and sucrose plotted against the formation of aflatoxin B_1 in figs by *A. parasiticus*. In this figure, the time axis shows days −1 to −3 to indicate that the concentrations of sugars were constant prior to inoculation of the fig medium at day zero. Not unexpectedly, the sugars clearly provide the carbon source for both *A. flavus* and *A. parasiticus* with a steady decline in levels from the point of inoculation, through the onset of secondary metabolism to the point at which aflatoxin levels reached a steady state, about 5 days after the beginning of secondary metabolism. Interestingly, the consumption of fructose began to level out about 3−4 days before that of glucose, which appeared to continue to provide a carbon source beyond the end of aflatoxin B_1 production. Thus, clearly, there was other metabolite formation (e.g., kojic acid) continuing some 3−4 days beyond cessation of aflatoxin B_1 production. The level of sucrose was initially low and remained unchanged during the course of the experiment, indicating that no hydrolysis appeared to have occurred. These results in figs are completely consistent with studies in liquid culture, which clearly demonstrated that aflatoxin production is greatly affected by the nature and concentration of available carbon sources. Glucose, ribose, xylose, and glycerol were shown to be good fungal substrates for *A. flavus* and *A. parasiticus* (*5*), and others (*6*) have similarly reported that glucose, ribose, fructose, sucrose, and maltose supported high levels of toxin production.

Figure 3 shows the results for the analysis of the Pro, Arg, and Glu plotted against time for the formation of aflatoxin B_1 in figs for *A. parasiticus*. Pro levels were stable prior to inoculation of the fig medium (days −3 to zero), and then, a

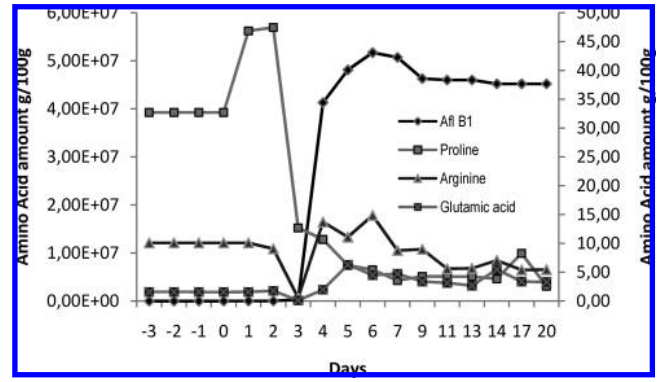

Figure 3. Changes in levels of Pro, Arg, and Glu and the corresponding production of aflatoxin B_1 in figs during the course of *A. parasiticus* secondary metabolism.

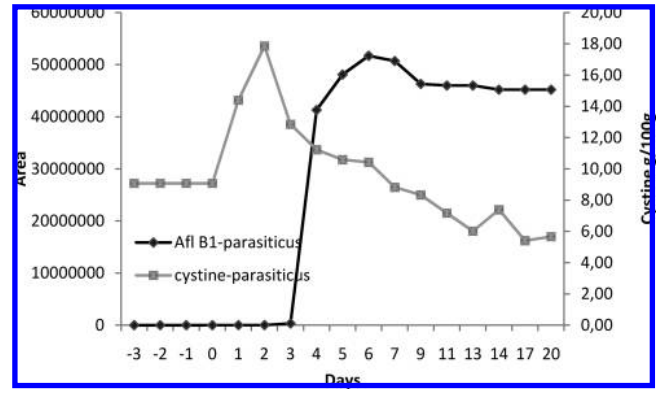

Figure 4. Changes in levels of Cys-Cys and the corresponding production of aflatoxin B_1 in figs during the course of *A. parasiticus* secondary metabolism.

small initial rise in concentration was seen presumably due to degradation of peptides by fungal action prior to the onset of secondary metabolism 3 days after inoculation. At the point at which aflatoxin B_1 formation was detected, a sharp decline in Pro concentration was evident, following the time course of toxin production leveling off after 3−4 days. Other amino acids (except Cys-Cys) showed no obvious changes in levels correlating with metabolite production. It is not possible to show the results for all 22 amino acids, but by way of illustration in **Figure 3**, the results for Arg and Glu are plotted showing some variability but no obvious pattern. However, the results for Cys-Cys shown in **Figures 4** and **5** for *A. parasiticus* and *A. flavus*, respectively, indicate after an increase in the level of this amino acid during initial fungal growth, there was then a rapid decline starting about a day before the onset of secondary metabolism.

This pattern was not evident for any of the other amino acids, indicating a clear preference by both *A. flavus* and *A. parasiticus* to use Pro and Cys-Cys as nitrogen sources for secondary metabolism. Arg concentrations were fairly constant throughout the time−course experiments from inoculation through secondary metabolism until the end of the experiment (**Figure 3**). Other amino acids such as Gln and Asn showed some changes in levels between time points with fluctuations in concentrations but overall an upward trend, whereas other such as Cys, Leu, Ile, and Tyr showed some decline in levels during secondary metabolism, albeit with not such an obvious direct correlation as that for Pro and Cys-Cys. The results that we have found with figs are fully consistent with previous observations (*8*) that of the amino acids, Pro stimulates more aflatoxin production per gram of mycelium than Asn, Trp, or Met when *A. flavus* and *A. parasiticus* are grown in culture. Pro and Asn are two

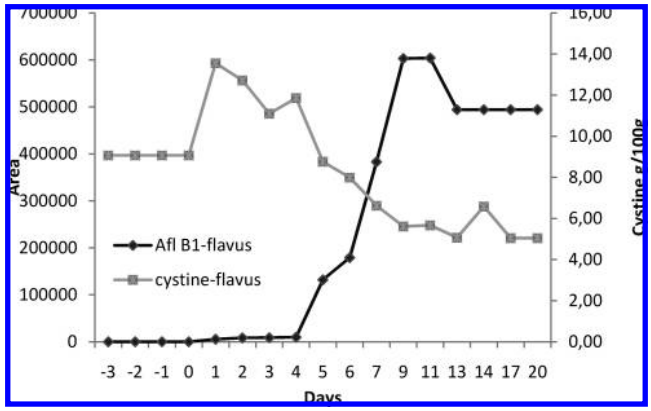

Figure 5. Changes in levels of Cys-Cys and the corresponding production of aflatoxin B_1 in figs during the course of *A. flavus* secondary metabolism.

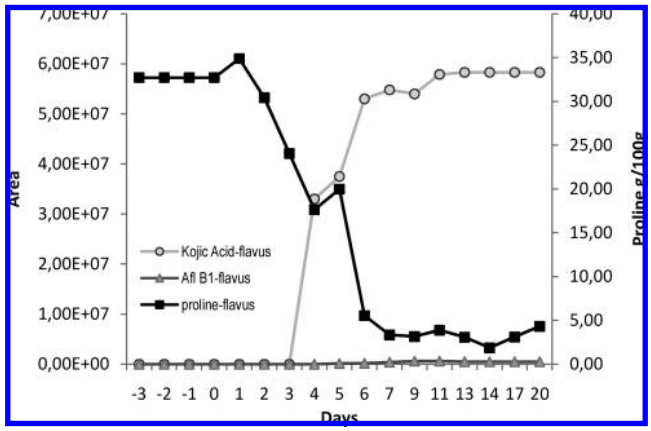

Figure 6. Changes in levels of Pro and kojic acid and the corresponding production of aflatoxin B_1 in figs during the course of *A. flavus* secondary metabolism.

of the principal free amino acids in figs at levels of 32.7 and 26.6 mg/100 g (*7*) being present at between two and 10 times higher concentrations than other free amino acids. For *A. flavus*, the production of aflatoxins B_1 was about 85 times lower than that of *A. parasiticus*, making the correlations more difficult to observe. However, for completeness in **Figure 6**, we have plotted for *A. flavus* the levels of kojic acid against those of Pro, showing again declining Pro levels correlating with increasing levels of kojic acid.

In this study, we have confirmed previous findings in liquid culture that indicate the importance of fructose and glucose as carbon sources for secondary metabolism by *A. flavus* and *A. parasiticus*. Furthermore, we have clearly shown the importance of free Pro as a nitrogen source for secondary metabolism, with a strong correlation between aflatoxin B_1 formation and declining concentrations of Pro in figs. These results were confirmed when concentrations of other aflatoxins such as aflatoxin G_1 were similarly plotted against amino acid levels as shown in **Figure 2**. The results for the production of all four aflatoxins (B_1, B_2, G_1, and G_2) by *A. parasiticus* are shown in **Figure 7** where comparable levels of aflatoxins B_1 and G_1 were found with relatively minor amounts of aflatoxins B_2 and G_2. These results show that in terms of composition, figs provide a very good nutritional source to support *Aspergillus* fungal growth and subsequent aflatoxin production. To date, we have only studied the relationship between aflatoxin formation and concentrations of sugars and amino acids in figs during time−course experiments. However, the LC-TOF/MS methodology employed

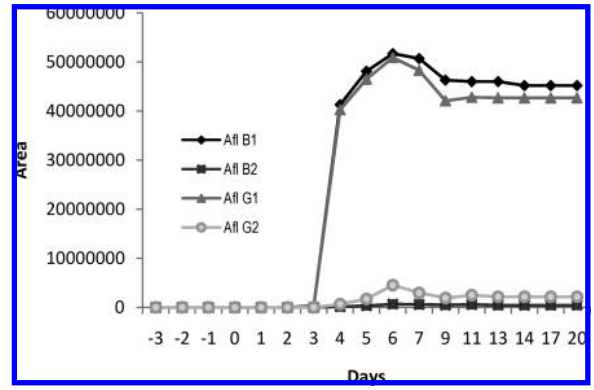

Figure 7. Production of aflatoxins B_1, B_2, G_1, and G_2 in figs inoculated with a toxigenic strain of *A. parasiticus*.

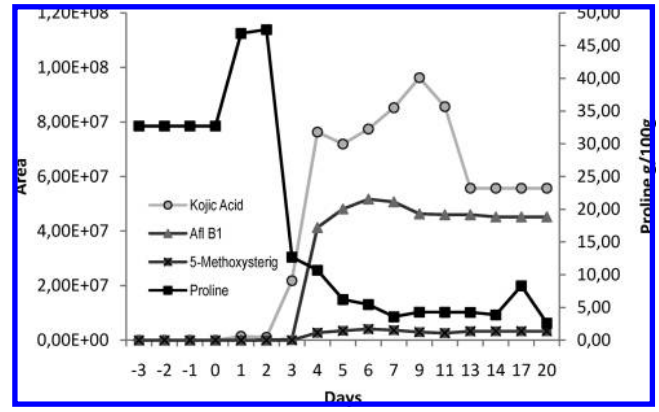

Figure 8. Changes in levels of Pro with the corresponding production of aflatoxin B_1, kojic acid, and 5-methoxysterigmatocystin in figs and during the course of *A. parasiticus* secondary metabolism.

for metabolite analysis is capable of providing for the first time considerable further insight into the metabolic pathway by allowing the complex rise and fall in levels of different metabolites to be followed. At a single time interval, the concentrations of presumptive aflatoxin precursors such as sterigmatocystin can be monitored, providing a time course of the metabolic pathway. This is illustrated in **Figure 8**, where the time course for the formation of aflatoxin B_1, kojic acid, and 5-methoxysterigmatocystin is shown plotted against the decline in Pro levels with time. Additionally, although to date we have only so far studied the behavior of *A. flavus* and *A. parasiticus*, it would also be useful to apply this new methodology to ochratoxigenic strains of other fungi, to see whether in figs the influence of similar nitrogen and carbon sources can be confirmed as has recently been reported for studies in liquid cultures with fungal strains isolated from grapes (*23*).

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