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ORIGINAL ARTICLE

Development of a simple and high-throughput method for detecting aflatoxins production in culture media

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Abstract

Aims: To develop a simple, high-throughput and inexpensive procedure to detect and quantify aflatoxins into the culture media of growing mycelia.
Methods and Results: Fungal conidia (*Aspergillus flavus*) were inoculated into the wells of a microplate containing 200 μl of different formulations of coconut-derived liquid medium. Time-dependent production of aflatoxins in the culture media was evaluated by a procedure relying on the UV-induced fluorescence emission by the toxin, using a microplate reader. These data were validated by comparison with the outputs of a conventional HPLC-based procedure. Determinations of aflatoxin concentration, according to the fluorimetric procedure, were performed either by withdrawing samples from the plates or by direct 'in situ' readings, the latter method reinforcing the high-throughput feature of the procedure. Fluorescence enhancers (cyclodextrins) did not ameliorate the sensitivity of the procedure to low concentrations of the toxin into the medium. The efficacy of the procedure was also validated by testing the effect on toxin yield of adding an antioxidant agent (α-lipoic acid) to the medium.
Conclusions: We give evidence that our improved procedure is reliable and suitable to analyse aflatoxin accumulation time course in coconut-derived culture medium.
Significance and Impact of the Study: This study shows that our procedure may profitably be used to give insights into the mechanisms of regulation of mycotoxin production and, consequently, to implement different strategies for the containment of aflatoxin contamination of food and feed commodities.

Introduction

Aflatoxins (AF) as well as many other mycotoxins are representatives of a large group of substances originating from secondary metabolism of fungal cells. Aflatoxin B_1 (AFB_1), which is produced by several *Aspergillus* species, is considered one of the most potent hepatocarcinogenic agents in man and animals and its presence as food and feed contaminant gives cause for concern and is thus monitored by the health authorities in many countries (van Egmond and Jonker 2004). The cost of the procedures for preventing aflatoxin contamination, when possible, and of its detection are exorbitant. This situation has stimulated an extensive research activity addressed (i) to identify effective strategies with which to prevent aflatoxin contamination of crops in the preharvest and/or in the postharvest phases (storage and food processing) and (ii) to understand the genetic and physiological bases of AF production by the relevant fungal species that synthesize the mycotoxin. These two 'fields' are strictly connected as the insights that may obtained by unravelling the mechanisms that control toxin synthesis are obviously of fundamental importance in designing effective strategies with which to protect crops and food commodities from mycotoxin contamination. Intraspecific bio-competition is one of the prominent strategies that are taken in consideration to prevent AF contamination in the preharvest phase (for reviews see: Dorner 2004; Abbas *et al.* 2006; Yin *et al.* 2008). The implementation of this strategy relies (i) on the individuation of atoxigenic strains inside the

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populations colonizing the geographical area of interest and (ii) on assessing their efficacy as competitors *versus* the toxigenic strains. Isolation and validation of 'good' competitors is not an easy task and procedures may be required both in the field and in the laboratory. Development of simple, inexpensive and high-throughput methods for detecting AF production in culture media are thus highly desiderable. Several methods for detecting/quantifying AF in conventional media used in food mycology have been described (reviewed in Abbas *et al.* 2004). Many methods rely on UV-induced emission of blue-green light by AF. Moreover, several attempts were performed to increase fluorescence emission by the addition of different compounds (Fente *et al.* 2001; Ordaz *et al.* 2003; Rojas-Durán *et al.* 2007).

Among others, cultivation on coconut agar medium (de Vogel *et al.* 1965; Arseculeratne *et al.* 1969; Lin and Dianese 1976; Davis *et al.* 1987; Abbas *et al.* 2004) has been largely utilized as a preliminary test to detect AF production, by observing the formation of a fluorescent halo around the colony of growing mycelium. Recently, Degola *et al.* (2011) have shown that a liquid formulation of the above-mentioned medium may be used to detect AF by a microplate fluorescent reader-based assay. However, the procedure was devised to perform end-point detections of the final mycotoxin yields (but not the time course of mycotoxin production) in the culture medium.

In this study, the detailed analysis of an improved version of our previous procedure, suitable for assessing the AF production time course in the medium during fungal growth, and its possible application to the demands from different bio-research fields is described. Moreover, the possibility of extending the procedure to detect other toxins that are provided with natural fluorescence is suggested.

Materials and methods

Fungal strains, media and culture condition

Aspergillus flavus toxigenic (AT1, Fri2, Emi4) and atoxigenic (TOφ) strains used here were previously isolated from corn fields of the Po Valley, as described by Degola *et al.* (2011); the aflatoxigenic strain of *A. parasiticus* NRRL2999 was obtained from the Centraal Bureau voor Schimmelcultures (CBS, Utrecht, the Netherlands). Fungal cultures were maintained on YES-agar medium [2% (w/v) yeast extract (Difco, Detroit, MI), 5% (w/v) sucrose (Sigma, St Louis, MO), 2% (w/v) agar (Difco)] at 25°C in the dark, and long-term stored in YES-agar slants at −20°C. Spores suspensions were obtained from 7-day YES-agar cultures incubated at 28°C and conidia concentration (quantified by $OD_{600)}$) and viability (>90%) determined as described by Degola *et al.* (2011). Coconut milk-derived liquid medium (CLM) for microplate assays was obtained as described in Degola *et al.* (2011): briefly, coconut cream (a commercial brand purchased from a local store) was diluted to 30% (v/v) with bidistilled water, sterilized by autoclaving (10 min, 120°C) and used as it was. A modified version of CLM was also tested for its ability to detect aflatoxin. This new medium, here called Clarified Coconut Medium (CCM), was obtained as follows: the CLM-autoclaved medium was cooled at 4°C overnight. As a result of this cold incubation, the fatty phase solidifies and separated from the liquid phase, which was clarified by centrifugation for 15 min at 3200 ***g***. The residual floating material and the pellet were discarded, and the intermediate phase was then recovered and used as culture medium.

Microplates culture conditions and fluoresescence emission determination

Standard flat-bottom 96-well microplates (Sarstedt, Newton, NC, USA) were used. Suspensions of conidia were diluted to the appropriate concentrations in CLM or CCM and brought to the final concentration of 5×10^2 spores/well (final volume = 200 μl per well). (±)-α-Lipoic acid (1 mmol l^{-1} final concentration; Sigma), to be tested for its effect on AF biosynthesis was added to the medium, at 65, 72 or 89 h after conidia inoculation. The plates were incubated in the dark under stationary conditions for up to 10 days at 25°C. Visual inspection of mycelium development and sporification served as an indicator of the culture growth. Readings were performed following two procedures:

1. For CLM grown cultures, at the end of the desired incubation period, 100 μl of the culture broth were carefully removed from the wells of the plate (here defined culture plate) and transferred to a new plate (reading plate), avoiding as much as possible the floating mycelium being carried over
2. For CCM grown cultures, readings were performed either as described above or directly in the culture plate

The first procedure is henceforth referred to as 'sampled reading mode', whereas readings performed directly from the bottom of culture plate are referred to as 'direct reading mode'. In both cases, samples were analysed with a microplate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland) to quantify the UV-induced fluorescence (λ_{ex} = 360 nm and λ_{em} = 465 nm). Measurements parameters were set according to preliminary experiments (manual gain = 83; lag time = 0 μs; number of flashes = 3; integration time = 200 μs) and maintained constant throughout all the experiments described in this study. Readings were performed from the wells bottom.

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Fluorescence values reported in the figures are expressed as arbitrary units (a.u.). These were normalized either by subtracting the mean value of empty wells readings (see Fig. 1a) or by subtracting the values of a control atoxigenic culture (see Fig. 1b). Each inoculation experiment was performed at least in triplicate and repeated twice. Mean values ± SD were reported in the figures. Cyclodextrins to be assayed as fluorescence enhancers were added either to the samples resting in the reading plate (1·5% w/v final concentration) or directly to the culture medium (0·3% w/v final concentration) at the time of conidia inoculation. α-cyclodextrin and *β*-cyclodextrin were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas butyl substituted *β*-cyclodextrin was obtained from Fluka (Buchs, Switzerland).

For the validation of the microplate fluorescence-based procedure, the concentration of AF in a sample of culture medium (CLM inoculated with *A. flavus* Emi4 strain and incubated at 25°C for 5 days) was determined by the HPLC-based procedure described elsewhere. The AF containing medium was then serially diluted with fresh medium and the different diluted samples analysed for fluorescence emission by the microplate-based procedure.

Aflatoxin extraction, determination by HPLC-FLD and confirmation by UPLC-MS

Determination of AF concentration was conducted extracting the culture broth (300–500 *μ*l) with 2·5 volumes of 70% methanol (v/v), incubating the CLM-methanol mixture at room temperature under shaking conditions for 60 min and then centrifuging (10 min, 3000 ***g***). The supernatant was collected and aflatoxin concentration directly determined by HPLC coupled by a fluorescence detector, according to Chiavaro *et al.* (2001). The separation was achieved on a Waters XTerra C18 column (150 × 2·1 mm; 3 *μ*m) under isocratic conditions (CH_3OH: 6 mmol l^{-1} *β*-CD in water, 20 : 80 v/v). The Limit of detection (LOD) expressed as total injected amount was lower than 0·05 ng for AFs. Positive samples were further confirmed by UPLC-MS analysis, as reported by Degola *et al.* (2007). In this case, the chromatographic analyses were performed with an Alliance 2695 chromatographic system (Waters) equipped with ZMD single-quadrupole mass spectrometer (Waters). An XTerra™ C18 analytical column (250 × 2·1 mm, 3 *μ*m) was used. The chromatographic and ionization conditions applied were the same reported by Degola *et al.* (2007). The method was in-house validated as follows. Limit of detection was set at 0·1 *μ*g l^{-1} as signal to noise ratio 3 : 1. The linearity was found to be very good for all the main aflatoxins in the calibration range 0·5–50 *μ*g l^{-1} ($r^2 > 0·999$), and no matrix effect was observed. Accuracy and precision were evaluated by spiking a blank sample at 5 *μ*g l^{-1} of AFB_1. The analysis was performed in triplicates, finding a recovery of 95% and a mean CV% of 3·4%.

Statistical analysis

The data concerning Cyclodextrin experiment were analysed using the statistical and graphical function of PASW Statistics (SPSS Inc., Chicago, IL, USA). Differences were assessed using analysis of variance (ANOVA) followed by Dunnet-t *post hoc* test.

Results

Fluorimetric determination of aflatoxin

Data concerning the reliability of our fluorimetric procedure for AF determination in the culture medium have already been reported (Degola *et al.* 2011). However, in that case, validation was obtained by the use of a commercial Elisa kit. In Fig. 1c, a comparison of fluorescence measurements of AF concentrations (range, 100–2200 *μ*g l^{-1}) as obtained by our procedure and by a standard quantitative HPLC procedure is reported. A linear correlation was observed between the fluorimetric and the HPLC-based outputs. AF levels as low as 250 *μ*g l^{-1} were reliably detected by the fluorimetric procedure.

Time course of aflatoxin production in CLM

When incubated in CLM (Fig. 1a), a time-dependent increase in medium fluorescence was observed for the toxigenic ($afla^+$) strains only. At any time considered, the fluorescence values for the atoxigenic ($afla^-$) strains were almost constant (<5000 arbitrary units; a.u.). Henceforth, the values for the $afla^-$ strains (used as controls in each experiment) were subtracted from those of the $afla^+$ strains and the resulting values reported on graphs (compare Fig. 1a,b). The kinetic of toxin accumulation could be readily determined, starting from 50 to 60 h, when cultures were incubated at 25°C. In these conditions, the toxin concentration progressively increased for up to 85 h and then reached a 'plateau' value. Aflatoxin accumulation in the medium was dependent on the inherent ability of the $afla^+$ strain to synthesize it. The slope of the curves was different for the two $afla^+$ strains, reported in Fig. 1b, indicating that the rate of toxin accumulation/release into the medium during the AF productive phase was, at least in part, the cause of the different final yields.

In Fig. S1, the time course of AFs production for an *Aspergillus parasiticus* and an *A. flavus* strain are compared. We were interested in determining when aflatoxin production really started during the first 50–60 h following

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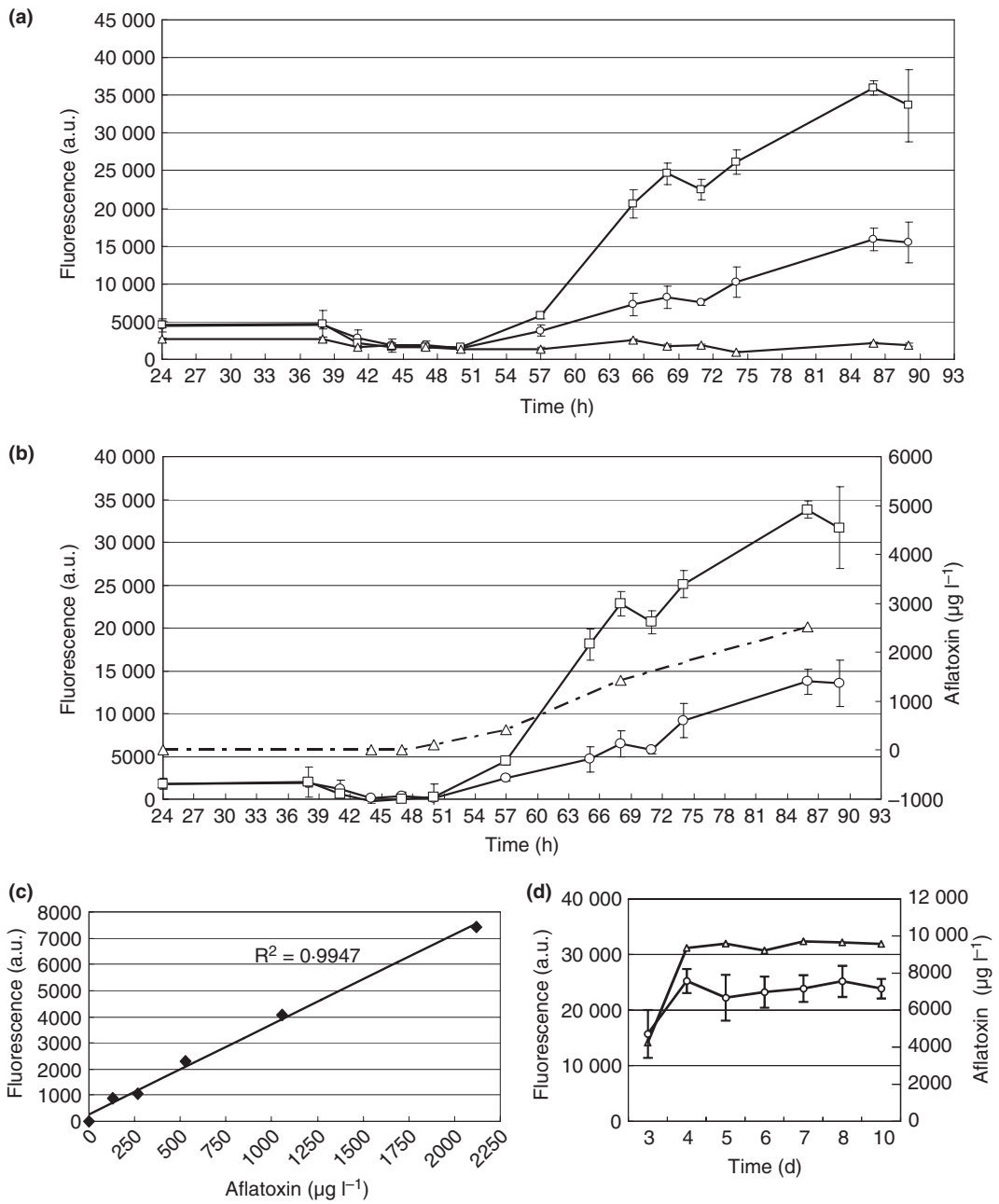

Figure 1 Time course of aflatoxin production in Coconut milk-derived liquid medium (CLM). (a) Two toxigenic strains (□, Fri2, strong Aflatoxins (AF) producer; ○, Emi4, low AF producer) and one atoxigenic strain (Δ, TOφ) were incubated at 25°C. Fluorimetric readings (arbitrary units) were performed on 100 μl aliquots of medium sampled at the time indicated. (b) Values for the toxigenic strains were normalized by subtracting the corresponding values of the atoxigenic strain. AF concentrations (Δ, as determined by an HPLC-validated procedure; see Mat and Meth) in samples of medium inoculated with the low-producer strain. (c) Validation of the microplate reader-based procedure. A sample of CLM containing 2150 μg l^{-1} of AF (as determined by an HPLC-validated procedure; see Mat and Meth) was serially diluted and the diluted samples analysed by the microplate reader procedure. (d) Determination of AF production during the late phase of mycelium growth. Conidia of a toxigenic strain (Fri2) of *Aspergillus flavus* were inoculated in CLM and incubated at 25°C for up to 10 days. Fluorescence readings (○) and determinations of AF concentration (Δ) were performed in parallel. Fluorescence readings are reported as arbitrary units (a.u.) Vertical error bars indicate standard deviation of mean, based on three samples.

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conidia inoculation. As shown in Fig. 1b, the 'black window', that is, the time interval in which the toxin may be determined by HPLC but was not detectable by the fluorimetric procedure did not exceed 10 h. Analogously, as shown in Fig. 1d, the 'plateau' values of fluorescence emission displayed by samples of the late phase of fungal growth did correspond to constant concentrations of AF as determined by HPLC-based assay. Artefacts depending on the parallel accumulation and release into the medium of toxin and metabolites with fluorescence shielding capacity may thus be excluded.

Effect of cyclodextrins addition to the medium.

To increase the sensitivity of our procedure for the detection of AF in the culture medium the efficacy of fluorescence enhancers was tested. As shown in Fig. 2, all the cyclodextrins tested (α-, β- and butyl-β-cyclodextrin) were capable of increasing fluorescence emission when added to samples of medium containing different concentrations (200–2000 $\mu g\ l^{-1}$) of aflatoxin. α-cyclodextrin showed the best performance among all cyclodextrins tested in this survey (eightfold increase in fluorescence, as compared to the control). However, differences in efficacy were somehow obscured at low AF concentrations (<200 $\mu g\ l^{-1}$) in the medium). In the above-described experiments, cyclodextrins were added to samples just before they were transferred to the reading plate. The effect of adding β-cyclodextrin (0·3% w/v) to the culture medium at the time of conidia incubation was also tested. However, this modification of the procedure had no beneficial effect as compared with when cyclodextrin was added at the end of the incubation time (data not shown).

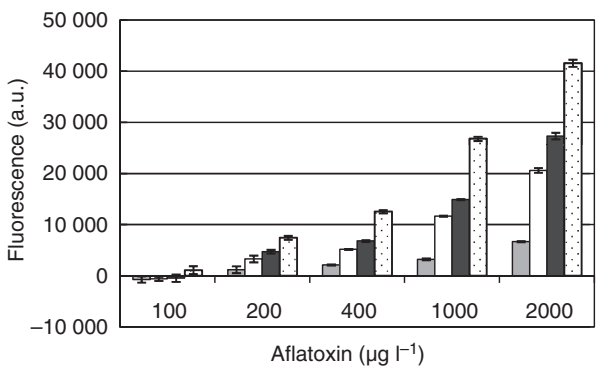

Figure 2 Effect of cyclodextrins on UV-induced aflatoxin fluorescence. α- (spotted), β- (black) and butyl β-(white) cyclodextrins were added (1·5% w/v final concentration) to sample of medium containing different aflatoxin B concentrations (100–2000 $\mu g\ l^{-1}$). Control (no cyclodextrin added, grey). Vertical error bars indicate standard deviation of mean, based on three samples. Differences between means (control *vs* cyclodextrins) were significant ($P < 0{\cdot}001$) at any Aflatoxins concentration but for 100 $\mu g\ l^{-1}$ ($P < 0{\cdot}05$ for α-cyclodextrin only).

Time course of aflatoxin production in CCM

Clarified Coconut Medium formulation was devised to obtain a less flocculating/turbid substrate than CLM. The rationale was to give the possibility to perform microplate readings directly from the bottom of the wells, thus avoiding having to withdraw, at each time, samples of medium from the culture plate and to transfer them onto the reading plate.

In Fig. 3, the time courses of aflatoxin accumulation into the medium by an $afla^+$ strain (Fri2) inoculated either in CLM or in CCM and incubated at 25°C is reported. In the latter case, two different readings were performed: (i) directly from the bottom of wells of the plate where mycelia were growing ('direct reading' mode) or (ii) sampling and transferring 100 μl of medium to a new plate before reading ('sampled reading' mode). The main observation is that growth in CCM elicits a delay of AF release into the medium as compared to that observed in CLM growing cultures. In fact, at the time of maximum AF production on CLM, AF production on CCM was about a half. However, similar toxin yields were reached in both media during the late phase of growth (114 h). Readings in CCM, conducted in the two conditions reported earlier, were substantially similar.

Effect of α-lipoic acid on aflatoxin production

α-Lipoic acid (LA) was tested for its effect on aflatoxin accumulation by two $afla^+$ strains of *A. flavus* (AT1 and Fri2) growing in CLM at 25°C. In a first set of experiments, different concentrations (0·2–2·0 $mmol\ l^{-1}$) of LA were added at the time conidia were inoculated into the

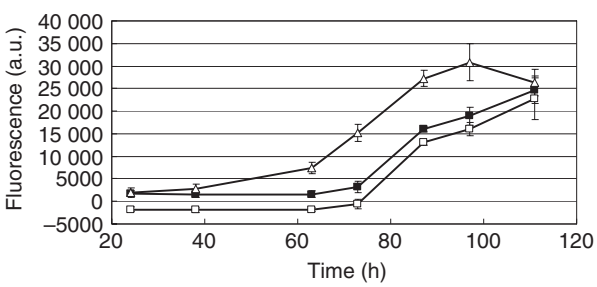

Figure 3 Time course of aflatoxin production in Clarified Coconut Medium (CCM). Conidia of a toxigenic strain (Fri2) of *Aspergillus flavus* were inoculated in Coconut milk-derived liquid medium (Δ) or CCM (□,■). Readings of CCM samples were performed either by the 'direct reading' mode (□) or by the 'sampled reading' mode (■).Vertical error bars indicate standard deviation of mean, based on three samples.

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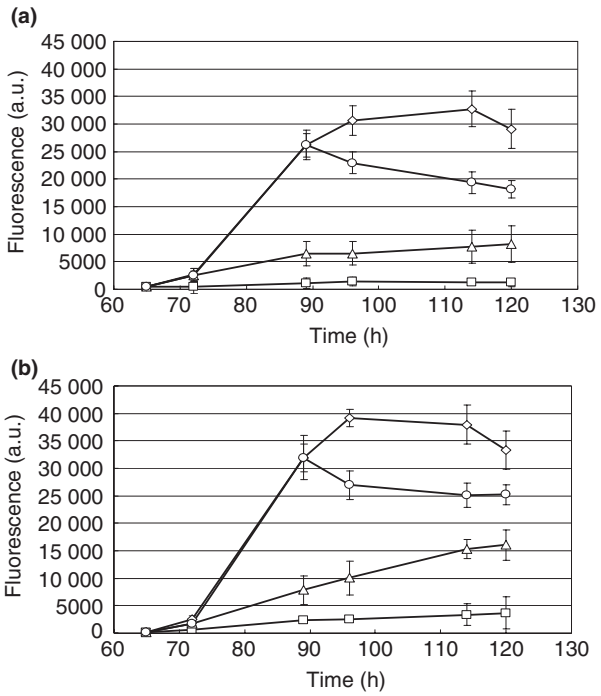

Figure 4 Effect of α-lipoic acid addition time on aflatoxin production. Conidia of Fri2 (a) and AT1 (b) toxigenic strains of *Aspergillus flavus* were inoculated in Clarified Coconut Medium (◇). α-lipoic acid was added to the medium at 65 h (□), 72 h (Δ) and 89 h (○) postinoculation. Vertical error bars indicate standard deviation of mean, based on three samples.

medium (data not shown). Visual observation of mycelium growth into the wells of the plate indicated that LA may have a negative effect on germination and/or on the initial phase of development if delivered during the first 2 days of cultivation (data not shown). The effect of delaying (65, 72, and 89 h postconidia inoculation) the addition of LA (1 mmol l^{-1} final concentration) to the medium was therefore analysed (Fig. 4). In control conditions, fluorescence started to increase in the 65- to 72-h time interval. Addition of LA at 65 h arrested AF production for at least the following 35 h of incubation. LA addition at 72 h was unable to arrest AF biosynthesis even if different levels of retardation of mycotoxin accumulation were observed for the two strains, in comparison with the control one. When delivered at 89 h, LA completely arrested AF release into the medium.

Discussion

Recently, Degola *et al.* (2011) described a microplate fluorescence reader-based procedure and the use of a coconut-derived liquid medium, to assess the bio-competition efficacy of different atoxigenic *A. flavus* strains In our experience, coconut-derived media have an advantage over the most commonly used synthetic media such as Yeast Extract Sucrose (YES), Potato Dextrose Agar, Czapek etc. because, while efficiently promoting AF production by moulds, they (CLM and CCM) are apparently deprived of those compounds that either fluoresce naturally or shield UV-induced fluorescence, thus preventing AF determination by fluorescence-based procedures (for a discussion on the topic see Rojas *et al.* 2005).

Cultural methods are generally regarded as being less sensitive than analytical methods (Abbas *et al.* 2004); however, even if this is true, evidence are given here that the time course of fluorescence emission was quantitatively correlated with that obtained by HPLC determination. In fact, AF released into the medium could be detected by the fluorimetric procedure at the very beginning of its accumulation: a time lapse of <10 h was observed between the effective start of toxin release into the medium (as determined by the HPLC-based procedure) and the first reliable values of fluorescence increase (as determined by the microplate procedure). Moreover, both CLM and CCM may be used to assess the time-dependent accumulation of AF by *Aspergillus* species. We would like to stress this point: the fluorimetric procedure is not intended to substitute one of the conventional HPLC- or ELISA-based assays for aflatoxin determination. Rather, it is particularly indicated for those experimentations requiring a multi-factorial analysis of the toxin production time course. The different phases of mycotoxin accumulation may be detected in 'real time'. In fact, AF concentration in the medium may be assessed rapidly (<1 min is required to read a microplate) and readings may be performed at short time intervals (<30 min) during the incubation. Moreover, if the 'direct' reading mode is used, the time course of AF biosynthesis may be determined in each well without disturbing mycelium development. Thus, the opportunity is given to precisely deliver to the medium any compound to be tested, according to the AF biosynthetic phase.

Nevertheless, to improve the sensitivity of the procedure, compounds that were reported to enhance UV-induced fluorescence of AF molecules (Vasquez *et al.* 1992; Dall'Asta *et al.* 2003; Galaverna *et al.* 2008) were included, in the medium formulation. However, the different cyclodextrins tested here did not dramatically increase the sensitivity of the procedure to low concentrations of toxin (the lower limit of AF detection was 100 $\mu g\ l^{-1}$ in the presence of α-cyclodextrin). This is particularly important if the overall costs of the procedure are to be taken in consideration: α-cyclodextrin, the most effective among the cyclodextrins tested, is also the most expensive.

Oxidative stress and AF biosynthesis are strictly related events (Fanelli *et al.* 2004; Reverberi *et al.* 2010). This observation and similar results obtained in different my-

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cotoxin producer fungi (Reverberi *et al.* 2010) have aroused interest in testing the effect of antioxidants and mixtures of antioxidants in preventing mycotoxin production by fungi during either pre- or postharvest contamination. Here, again the availability of a simple and 'in laboratory' procedure to assess the efficacy and the time-course effect of antioxidants (or multiple combination of antioxidants) on mycotoxin accumulation is particularly interesting. A clear evidence of this is given: LA efficacy in preventing AF biosynthesis is limited to a time interval spanning from 0 to 65 h, in our experimental conditions. Addition of LA to mycelium cultures that have already started to produce AF is ineffective in blocking mycotoxin accumulation. However, the strength of this effect is probably strain dependent. LA, when delivered in the late phase of toxin accumulation, recovers its capacity of blocking AF biosynthesis. Similarly, pro-oxidants may be tested for their effect in stimulating mycotoxin production. Besides the interest of these data for correctly designing a possible strategy for AF containment, the above-described approach may also be used to perform comparative profiling experiments to uncover the regulatory networks controlling AF biosynthesis (Reese *et al.* 2011) and identify enzymes and genes involved in the response to modifications of oxidants/antioxidants ratio of the cell.

Diverse inhibitors of AF biosynthesis (Holmes *et al.* 2008) may similarly be tested: for example, plant-derived compounds capable of contrasting mycotoxin accumulation by the colonizing fungus. This, in turn, may help implement a selection programme for the identification of crop cultivars that produce the relevant compound(s) and, consequently, do not elicit or antagonize AF biosynthesis by the infecting fungus.

Moreover, new mechanisms of 'antagonistic' activity on toxin production may also be discovered. In fact, both yeasts and bacteria grow in the coconut-derived media here described, thus giving the opportunity to perform experiments in which (i) the effect of interspecific biocompetition on mycotoxin production may be assessed and (ii) isolation or analysis of mycotoxin-degrading micro-organisms may be conducted.

The possibility to empower the procedure by performing the assay on 384-well microplates was also tested (data not shown) giving evidence that this can be done reasonably provided that CCM and the 'direct reading' mode are used. However, manually loading such a crowded set of wells is a cumbersome task prone to error. To realize the full potential of the above-reported modification of the procedure, some kind of automation would be required.

It would also be interesting to verify whether the procedure may be adapted to the detection of other mycotoxins, such as ochratoxin A and zearalenone, that naturally fluoresce under UV excitation and are produced by fungal species that represent a serious threat to many important crops.

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References

Abbas, H.K., Shier, W.T., Horn, B.W. and Weaver, M.A. (2004) Cultural methods for aflatoxin detection. *J Toxicol Toxin Rev* **23**, 295–315.

Abbas, H.K., Zablotowicz, R.M., Bruns, H.A. and Abel, C.A. (2006) Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. *Biocontrol Sci Technol* **16**, 437–449.

Arseculeratne, S.N., de Silva, L.M., Wijesundera, S. and Bandunatha, C.H.S.R. (1969) Coconut as a medium for the experimental production of aflatoxin. *Appl Environ Microbiol* **18**, 88–94.

Chiavaro, E., Dall'Asta, C., Galaverna, G., Biancardi, A., Gambarelli, E., Dossena, A. and Marchelli, M. (2001) New reversed-phase liquid chromatographic method to detect aflatoxins in food and feed with cyclodextrins as fluorescence enhancers added to the eluent. *J Chromatogr A* **937**, 31–40.

Dall'Asta, C., Ingletto, G., Corradini, R., Galaverna, G. and Marchelli, R. (2003) Fluorescence enhancement of aflatoxins using native and substituted cyclodextrins. *J Incl Phenom Macro* **45**, 257–263.

Davis, N.D., Iyer, S.K. and Diener, U.L. (1987) Improved method of screening for aflatoxin with a coconut agar medium. *Appl Environ Microbiol* **53**, 1593–1595.

Degola, F., Berni, E., Dall'Asta, C., Spotti, E., Marchelli, R., Ferrero, I. and Restivo, F.M. (2007) A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. *J Appl Microbiol* **103**, 409–417.

Degola, F., Berni, E. and Restivo, F.M. (2011) Laboratory tests for assessing efficacy of atoxigenic *Aspergillus flavus* strains as biocontrol agents. *Int J Food Microbiol* **146**, 235–343.

Dorner, J.W. (2004) Biological control of aflatoxin contamination of crops. *J Toxicol Toxin Rev* **23**, 425–450.

van Egmond, H.P. and Jonker, M.A. (2004) Worldwide regulation on aflatoxins: the situation in 2002. *J Toxicol Toxin Rev* **23**, 273–293.

Fanelli, C., Ricelli, A., Reverberi, M. and Fabbri, A.A. (2004) Aflatoxins and ochratoxins in cereal grains: an open challenge. *Recent Res Dev Crop Sci* **1**, 295–317.

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Fente, C.M., Ordaz, J.J., Vazquez, B.I., Franco, C.M. and Cepeda, A. (2001) New additive for culture media for rapid identification of aflatoxin-producing *Aspergillus* strains. *Appl Environ Microbiol* **67**, 58–62.

Galaverna, G., Dall'Asta, C., Corradini, R., Dossena, A. and Marchelli, R. (2008) Cyclodextrins as selectors for mycotoxin recognition. *World Mycotox J* **1**, 397–406.

Holmes, R.A., Boston, R.S. and Payne, G. (2008) Diverse inhibitors of aflatoxin biosynthesis. *Appl Microbiol Biot* **78**, 559–572.

Lin, M.T. and Dianese, J.C. (1976) A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. *Phytopathology* **66**, 1466–1469.

Ordaz, J.J., Fente, C.A., Vàzquez, B.I., Franco, C.M. and Cepeda, A. (2003) Development of a method for visual determination of aflatoxin production by colonies of *Aspergillus flavus* group. *Int J Food Microbiol* **83**, 219–225.

Reese, B.N., Payne, G.A., Nielsen, D.M. and Woloshuk, C.P. (2011) Gene expression profile and response to maize kernels by *Aspergillus flavus*. *Phytopathology* **101**, 797–804.

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A.A. and Fanelli, C. (2010) Natural functions of mycotoxins and control of their biosynthesis in fungi. *Appl Microbiol Biotechnol* **87**, 899–911.

Rojas, T.R., Sampayo, C.A.F., Vazquez, B.I., Franco, C.M. and Cepeda, A. (2005) Study interferences by several metabolites from *Aspergillus* spp. in the detection of aflatoxigenic strains in media added with cyclodextrin. *Food Control* **16**, 445–450.

Rojas-Duràn, T.R., Fente, C.A., Vazquez, B.I., Franco, C.M., Sanz-Medel, A. and Cepeda, A. (2007) Study of a room temperature phosphorescence phenomenon to allow the detection of aflatoxigenic strains in culture media. *Food Control* **16**, 445–450.

Vasquez, M.L., Franco, C.M., Cepeda, A., Prognon, P. and Mahuzier, G. (1992) Liquid chromatographic study of the interaction between aflatoxins and β-cyclodextrin. *Anal Chim Acta* **269**, 239–247.

de Vogel, P., van Rhee, R. and Blanche Koelensmid, A.A. (1965) A rapid test for aflatoxin-synthesizing Aspergilli of the *flavus-oryzae* group. *J Appl Bacteriol* **28**, 213–220.

Yin, Y., Yan, L., Jiang, J. and Ma, Z. (2008) Biological control of aflatoxin contamination of crops. *J Zhejiang Univ Sci B* **9**, 787–792.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Time course of aflatoxin production by an *Aspergillus parasiticus* strain in CCM medium. *A. flavus* (Fri2; ○) and *A. parasiticus* (NRRL2999; □) conidia were inoculated in CCM at 25°C. Fluorescence readings are reported as arbitrary units (a.u.). Vertical error bars indicate standard deviation of mean, based on three samples.

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