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Formulation of maize- and peanut-based semi-synthetic growth media for the ecophysiological studies of aflatoxigenic *Aspergillus flavus* in maize and peanut agro-ecosystems

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ABSTRACT

In studying the ecophysiology of fungal phytopathogens, several stages are involved (*in vitro*, greenhouse, *in planta*). Most *in vitro* studies extensively utilise the general growth media such as Potato Dextrose Agar and Malt Extract Agar. Although the crop components in these media serve as excellent carbon sources and yield luxuriant growth, they are not naturally contaminated with *Aspergillus flavus* and thus might result in under- or over-estimation of its actual toxigenic potentials. Empirical data on the formulation of semi-synthetic growth medium mimicking the natural crop commonly contaminated by *A. flavus* for the ecophysiological studies *in vitro* are scarce. The present work was aimed at investigating the ecophysiology of *A. flavus* on commercial growth media (PDA, MEA); formulating maize- and peanut-based semi-synthetic growth media using two methods of raw material preparation (milling, hot water extraction) at different concentrations (1, 3, 5, 7, 9% *w/v*), and comparing the ecophysiological parameters between commercial and formulated growth media. Growth rates were obtained by computing the hyphal expansion data into $y = mx + c$ equation. AFB_1 was quantified using high performance liquid chromatography with fluorescence detector. Formulated media were found to yield significantly higher growth rates when compared to commercial media. However, commercial media yielded significantly higher AFB_1 when compared to all formulated media. Between the two formulations, milling yielded significantly higher growth rates and AFB_1 when compared to hot water extraction. Although *in vitro* data cannot directly extrapolate *in planta* performance, results obtained in the present work can be used to gauge the actual toxigenic potential of *A. flavus* in maize and peanut agro-ecosystems.

1. Introduction

Maize and peanut are globally-produced and widely-consumed crops. Both maize and peanut typically contain carbohydrates, proteins, fats/oils, vitamins and minerals. A wide variety of maize and peanut products have been produced for human consumption as major dietary sources, and also as ingredients in animal feed (Chaudhary et al., 2014; Zhao et al., 2012). In the field, maize and peanut plantations are naturally threatened by a variety of plant diseases, pest infestations and microbial infections especially by the microfungi. This is further exacerbated by the interacting environmental factors which will undoubtedly contribute to lower yields and quality and subsequently, huge economic losses (Rosenzweig et al., 2001).

Of the many maize and peanut fungal contaminants studied worldwide (Mohale et al., 2013a; Sultan and Magan, 2010), *Aspergillus flavus* is the most relevant species in Malaysian climate as it can grow over a wide range of temperature in the field (Magan et al., 2004; Sanchis and Magan, 2004) making the subtropical and tropical countries an excellent breeding ground. As a result, maize and peanut have been reported as the most susceptible crops to aflatoxin contamination (Fountain et al., 2015). In these crops, *A. flavus* are present in the rhizosphere as conidia and sclerotia, and in the phyllosphere as mycelia (Amaike and Keller, 2011), and able to inflict different diseases on different parts of the crops and at different developmental stages (Pitt et al., 2013). As the proliferation of *A. flavus* and the subsequent aflatoxin contamination are hugely affected by the environmental conditions, it is therefore important to understand the impacts of different ecophysiological conditions on these parameters (Samapundo et al., 2007).

In studying the ecophysiology of fungal contaminants with respect

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to plant pathology several stages are normally involved namely laboratory trial (*in vitro*), greenhouse trial and ultimately field trial (*in situ* or *in planta*). At present, most *in vitro* studies extensively utilise the general growth media such as Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA). Although the natural crop components in these growth media serve as excellent carbon sources and therefore will yield luxuriant fungal growth, none of them are contaminated with *A. flavus* in nature and thus might result in under– or overestimation of the actual toxigenic potentials of the pathogen. To the best of our knowledge, no work has been documented thus far on the formulation and optimization of semi-synthetic growth media mimicking the natural commodities (maize, peanut) commonly contaminated by *A. flavus* for *in vitro* studies.

Therefore, the present work was aimed at (i) investigating the ecophysiology of *A. flavus* (growth rates, mm/d; aflatoxin B_1 production, μg/g) on commercial growth media (PDA, MEA), (ii) formulating maize– and peanut–based semi-synthetic growth media using two types of raw material preparation (milling, hot water extraction), and (iii) comparing the ecophysiological parameters between commercial and formulated growth media.

2. Materials and methods

2.1. Aspergillus flavus strain

A commercial aflatoxigenic type strain of *Aspergillus flavus* NRRL3357 (New South Wales, Australia) was obtained and maintained axenically on Potato Dextrose Agar (PDA; Oxoid, UK) at 30 °C until sporulation, and refrigerated at 4 °C until further analysis.

2.2. Chemicals and reagents

All solvents and reagents used in the present work were of analytical and HPLC grade unless otherwise stated. Acetonitrile and methanol were purchased from Merck (Germany) and the ultrapure water was obtained from Elga Purelab Classic UV MK2 (UK). Aflatoxin B_1 (AFB_1) standard was purchased from Supelco (USA). Malt Extract Agar (MEA) was purchased from Hardy Diagnostics (USA).

2.3. Preparation of commercial growth media

The commercial growth media (PDA, 39 g/L dH_2O; MEA, 48 g/L dH_2O) were prepared according to the manufacturer's instruction, supplemented with the antibacterial chloramphenicol (Fisher, UK) and autoclaved (Hirayama, Japan) at 121 °C and 15 psi for 15 min. The sterilised media was left to cool for 20 min and poured into 90 mm Ø Petri plates. The solidified PDA and MEA plates were refrigerated at 4 °C until inoculation.

2.4. Preparation of maize– and peanut–based semi-synthetic growth media

Four semi-synthetic growth media namely milled-maize agar (MMA), milled-maize extract agar (MMEA), milled-peanut agar (MPA) and milled-peanut extract agar (MPEA) were formulated to simulate the natural crop commodity frequently contaminated by *A. flavus* in the field. The semi-synthetic media were prepared by separately oven-drying 500 g commercially-obtained food-grade maize and peanut kernels at 50 °C for 72 h. The dried kernels were milled to fine powder using Waring blender. For MMA and MPA, five different concentrations (1, 3, 5, 7, 9% *w/v*) of the milled powder were separately added to 1 L dH_2O and 15 g technical agar. For MMEA and MPEA, similar concentrations (1, 3, 5, 7, 9% *w/v*) were boiled in 1 L dH_2O (without technical agar) for 45 min and filtered through a double-layer muslin cloth. The filtrate was then top up to 1 L with dH_2O and added with 15 g technical agar (Barberis et al., 2009). The pH's of all media were adjusted to 5.6 with either 1 M NaOH or 1 M HCl since this pH has been reported to favour aflatoxin production (Buchanan and Ayres, 1975). All media were autoclaved at 121 °C and 15 psi for 15 min. The sterilised media was left to cool for 20 min and poured into 90 mm Ø Petri plates. The solidified plates were refrigerated at 4 °C until inoculation.

2.5. Inoculation and incubation of Aspergillus flavus NRRL3357 on commercial and formulated growth media

The inoculation of *A. flavus* NRRL3357 on commercial and semi-synthetic growth media were performed by aliquoting 20 μL spore suspension (from a heavily sporulating axenic culture) onto the centre of the agar plates. Uninoculated agar plate of each medium served as the negative control. Inoculated and uninoculated commercial media were incubated at 25, 30, 35 and 40 °C for 7 d, while the inoculated and uninoculated semi-synthetic media were incubated at 30 °C (to simulate the Malaysian climate) also for 7 d. Commercial media served as the positive control for the semi-synthetic media.

2.6. Diametric growth rates of Aspergillus flavus NRRL3357 on commercial and formulated growth media

The hyphal expansion of *A. flavus* NRRL3357 was recorded at days one, three, five and seven by measuring the fungal colony diameter at two directions perpendicular to each other (Samsudin and Magan, 2016). The diametric readings were averaged after which a $y = mx + c$ curve was plotted in which y was the colony diameter (mm), x was the incubation period (d), and m was the diametric growth rate (mm/d). c was set to 0 since at day 0, the colony diameter was 0.

2.7. Aflatoxin B_1 production by Aspergillus flavus NRRL3357 on commercial and formulated growth media

The AFB_1 extraction was done according to Bragulat et al. (2001) with a slight modification. The method involved transferring five circular hyphal plugs of *A. flavus* NRRL3357 into pre-weighed 2 mL microcentrifuge tubes (Eppendorf, Germany) and the weights were recorded. Next, 1000 μL of absolute methanol was added to the tubes, and the mixture was vortexed (LMS Co., Ltd., Japan) for 30 s. Then, the tubes were incubated for 30 min at room temperature before being centrifuged (Sartorius, Germany) at 10,000 rpm for 5 min. The resulting extract was filtered through a nylon filter (0.22 μm; Macherey-Nagel, Germany) into HPLC vials (Thermo Scientific, USA).

A calibration curve for AFB_1 (2, 4, 6, 8, 10, 25, 50, 100 ng/mL) was constructed to transform the luminescence unit (LU) areas obtained through HPLC analysis into actual concentration of AFB_1 from the hyphal plugs (μg/g). Four measurements ($n = 4$) were recorded for each calibrant solutions ($\Sigma = 32$) intermittently throughout the sample analysis to check for reproducibility and repeatability of quantification procedure. The goodness of fit (R^2) for the calibration curve was 0.9960. The limit of detection (LOD) and limit of quantification (LOQ) were estimated as prescribed by the International Conference on Harmonisation (ICH) by using the formulae; $LOD = 3\sigma/s$ and $LOQ = 10\sigma/s$, in which σ was the standard deviation of blank responses, and s was the slope of the calibration curve (Shrivastava and Gupta, 2011). The LOD and LOQ obtained for the present analysis were 3 ng/g and 9 ng/g. The retention time recorded from the AFB_1 calibrant solutions was 19.04 ± 0.42 min. Sample readings of ± 0.50 min were rejected.

The separation and detection of AFB_1 were performed based on Afsah-Hejri et al. (2011) with a slight modification. A reversed-phase HPLC instrument (Waters 600, USA) joined to a fluorescence detector (Waters 2475, USA) was used. Separation was done using C_{18} column (XBridge, 5 μm, 4.6 × 250 mm; Waters, USA) with a mobile phase of methanol:water:acetonitrile (35:55:10, *v*/v/v) at 40 °C and 0.6 mL/min flow rate. The injection volume was 20 μL. The excitation and emission wavelengths were 365 nm and 435 nm, respectively. The mobile phase