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Mycotoxin production and predictive modelling kinetics on the growth of *Aspergillus flavus* and *Aspergillus parasiticus* isolates in whole black peppercorns (*Piper nigrum* L)

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ABSTRACT

The growth and mycotoxin production of three *Aspergillus flavus* isolates and an *Aspergillus parasiticus* isolate were studied in whole black peppercorns (*Piper nigrum* L.) using a full factorial design with seven water activity (a_w) (0.826–0.984) levels and three temperatures (22, 30 and 37 °C). Growth rates and lag phases were estimated using linear regression. Diverse secondary models were assessed for their ability to describe the radial growth rate as a function of individual and combined effect of a_w and temperature. Optimum radial growth rate ranged from 0.75 ± 0.04 to 2.65 ± 0.02 mm/day for *A. flavus* and 1.77 ± 0.10 to 2.50 ± 0.10 mm/day for *A. parasiticus* based on the Rosso cardinal estimations. Despite the growth failure of some isolates at marginal conditions, all the studied models showed good performance to predict the growth rates. Validation of the models was performed on independently derived data. The bias factors (0.73–1.03), accuracy factors (0.97–1.36) and root mean square error (0.050–0.278) show that the examined models are conservative predictors of the colony growth rate of both fungal species in black peppers. The Rosso cardinal model can be recommended to describe the individual a_w effect while the extended Gibson model was the best model for describing the combined effect of a_w and temperature on the growth rate of both fungal species in peppercorns. Temperature optimum ranged from 30 to 33 °C, while a_w optimum was 0.87–0.92 as estimated by multi-factorial cardinal model for both species. The estimated minimum temperature and a_w for *A. flavus* and *A. parasiticus* for growth were 11–16 °C and 0.73–0.76, respectively, hence, achieving these conditions should be considered during storage to prevent the growth of these mycotoxigenic fungal species in black peppercorns. Following the growth study, production of mycotoxins (aflatoxins B1, B2, G1, G2, sterigmatocystin and O-methyl sterigmatocystin (OMST)) was quantified using LC–MS/MS. Very small quantities of AFB1 (<LOQ-9.1 μg/kg) were produced only by *A. parasiticus*. OMST was not produced in any growth conditions by both species. Sterigmatocystin (<LOQ-76.7 μg/kg) was the dominant mycotoxin found. High variability in mycotoxin production restricted the modelling of mycotoxin production in black pepper.

1. Introduction

Fungal spoilage of foodstuff is a worldwide problem because it causes large economic losses and a serious risk to public health. *Aspergillus* spp. is one of the most widely distributed fungal genera in nature, capable of spoiling agricultural products and producing toxic secondary metabolites (Klich et al., 2007). These secondary metabolites known as mycotoxins, cause a very broad range of health effects in humans and animals such as nephro-, neuro- and cytotoxic, carcinogenic, mutagenic, immunosuppressive and estrogenic effects (Bennet and Klich, 2003; Marroquín-Cardona et al., 2014). Among them aflatoxins are the most potent carcinogens produced predominantly by the two agronomically important fungal species of *Aspergillus* section *flavi*, *Aspergillus flavus* and *Aspergillus parasiticus*. *A. flavus* grows better in the warmer areas and they are the most common fungal species found in agricultural produce. They mainly produce aflatoxin B1 (AFB1) and B2 (AFB2) while *A. parasiticus* produces all the four aflatoxins, B1, B2, G1 and G2 (Schmidt-Heydt et al., 2010), with AFB1 being the most abundant mycotoxin and natural carcinogen known (IARC, 2002). Aflatoxins occur mostly in tropical regions with high humidity and temperature and accumulate post-harvest when food commodities are stored under conditions that promote fungal growth. Major food commodities affected by fungal growth and mycotoxins are cereals, nuts, dried fruits, coffee, spices, oil seeds, dried beans, peas and fruits, etc. (Turner et al., 2009).

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Black peppercorns (*Piper nigrum* L.), known as "king of spices", are one of the most widely consumed spices in the world. It is mainly cultivated in developing countries with tropical and/or semi tropical climates and exported worldwide (Santos et al., 2011). High temperature, high rainfall and relative humidity in these growing areas are highly conducive for fungal proliferation and mycotoxin production. Apart from the climatic conditions, lack of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) are of great concern in developing countries where black peppers are grown. With regard to mould contamination in black peppercorns, the most important moulds were *Aspergillus* spp., *A. flavus* and *A. parasiticus*, reported from Egypt (Abou Donia, 2008), Brazil (Gatti et al., 2003), Saudi Arabia (Hashem and Alamri, 2010), Bahrain (Mandeel, 2005), India (Geeta and Reddy, 1990) and Sri Lanka (Yogendrarajah et al., 2014) etc. In addition to the fungal infestation, black peppers have been frequently reported to be contaminated with aflatoxins and ochratoxin A (Aydin et al., 2007; Fazekas et al., 2005; Jalili et al., 2010; Santos et al., 2010; Yogendrarajah et al., 2014) at various concentrations. Thus, there is a need to control mould growth and consequently the mycotoxin contamination in black pepper.

In order to improve the microbiological quality and safety of food, tools allowing the prediction of fungal growth are essential (Dagnas and Membré, 2013; Dantigny et al., 2005). Predictive modelling of filamentous fungi was not given the same importance as that of bacterial development due to the inherent difficulties in fungal growth assessment and gathering of sufficient, suitable and reproducible data (Dantigny et al., 2005; Garcia et al., 2009). The imperious need for characterizing the effects of factors that govern fungal growth during pre- or post-harvest stages triggered the interest in the application of mathematical approaches to describe and predict the fungal response to different growth factors. Several probability, mechanistic, semi-mechanistic and empirical models have been developed for a variety of toxigenic and spoilage fungal species (Garcia et al., 2009; Gibson et al., 1994; Rosso and Robinson, 2001; Sautour et al., 2002). A growing number of studies are now available dealing with the modelling approach to predict fungal growth and mycotoxin production in different food products. In the case of the potent aflatoxin producers, *A. flavus* and/or *A. parasiticus*, some studies are available reporting the effect of water activity (a_w) and temperature on fungal growth that has been performed directly on real food matrices like, yellow dent corn (Samapundo et al., 2007), maize grain (Garcia et al., 2013), pistachio nuts (Marín et al., 2012), polished and brown rice (Mousa et al., 2013). Though, it has been widely accepted that prevention of fungal growth will eventually prevent from the accumulation of mycotoxins, predictive mycology is still way behind. Studies are not yet available to predict these fungal growth and mycotoxin production in one of the economically significant spices, black pepper.

Hence, the aim of the present study was to (i) characterize the individual and combined effect of a_w and temperature on the radial growth rate and mycotoxin production of different strains of *A. flavus* and *A. parasiticus* in whole black peppercorns, (ii) to develop mathematical models describing the effect of a_w and temperature on growth kinetics and assess their performance on prediction, and (iii) to validate the models using independently derived data.

2. Materials and methods

2.1. Chemicals, reagents and mycotoxin standards

LC–MS grade absolute methanol (MeOH) and analytical grade acetonitrile (MeCN) were supplied by VWR International (Zaventem, Belgium). Formic acid ULC–MS grade (99%) was obtained from Bio Solve B.V. Ammonium formate (±99%) and anhydrous magnesium sulphate were purchased from Sigma-Aldrich, Steinheim, Germany. Formic acid analytical grade (98–100%) and sodium chloride (±99.5%) were obtained from Merck (Darmstadt, Germany) (±99%). Ultrafree®-MC centrifugal filter devices (0.22 μm) were purchased from Millipore (Bredford, MA, USA). Water was purified (18 MΩ) on a Milli-Q Plus apparatus (Millipore; Brussels, Belgium). All other chemicals and reagents used were of analytical grade.

The mycotoxin reference standards of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and sterigmatocystin (STERIG) were purchased from Sigma-Aldrich (Bornem, Belgium). O-methyl sterigmatocystin (OMST) was supplied by Chromadex (California, USA).

2.2. Fungal isolates and inoculum preparation

Three toxigenic *A. flavus* (UG AF06, UG AF35, UG AF60) and one *A. parasiticus* (UG AF542) strains were selected for this study. These strains were isolated from black pepper (*Piper nigrum* L.) samples collected from Sri Lanka (Yogendrarajah et al., 2014). Species level identification of these strains was confirmed at Mycothèque de l'Université Catholique de Louvain (MUCL, Louvain-la-Neuve, Belgium) using molecular techniques by comparison of DNA sequence. These isolates have been previously characterized to be heavy producers (ppm levels) of secondary metabolites in malt extract agar; more specifically *A. flavus* is known to produce AFB2, AFB1, STERIG and OMST and *A. parasiticus* is known to produce AFG2, AFG1, AFB2, AFB1, STERIG and OMST.

A spore suspension of each isolate was prepared at a concentration of 10^6 spores/mL. Tween 80 solution (0.1 g/100 mL water), PBS (1 tablet/100 mL water) and PBS + Tween80 solution (0.1 g Tween 80 and 1 tablet PBS per 100 mL water), cotton plugs and pipette tips were autoclaved for 15 min at 121 °C. To prepare the fungal inoculum, centrally inoculated MEA plates were incubated at 30 °C for 10 days to enable sporulation to take place. Five milliliters of Tween 80 solution (wetting agent) were spread on the agar plate containing sporulated mould culture. After gently spreading the solution and scrapping off the spores, this solution was pipetted out from the agar plates and transferred to a sterile falcon tube containing a cotton plug on top for filtering out debris and mycelium. This extraction process was performed a second time on the same plate. The extracts of three agar plates were used for one falcon tube. After removing the cotton plug, the falcon tubes were centrifuged at 8500 rpm for 15 min at 4 °C. Supernatant was discarded out and 20 mL of PBS + Tween 80 solution was added to the sedimented spores. After vortexing for 30 s, the spore suspension was centrifuged again at same conditions. After discarding the supernatant, 20 mL of the PBS solution was added to the sedimented spores and vortexed again. The spores were counted in a 16 cell thoma chamber using an inverse microscope (Olympus, IX81, Tokyo, Japan) and CellF imaging software. Appropriate dilution was made to obtain a standardized spore concentration of 10^6 spores/mL solution in PBS. This spore suspension was stored at 4 °C until further use. The spore suspension was renewed every month following the same procedure described.

2.3. Experimental design

The experimental design corresponded to a full factorial design with two main factors, temperature and water activity (a_w). The incubation temperatures studied were 22, 30 and 37 °C, while the seven a_w levels investigated were 0.826, 0.857, 0.892, 0.921, 0.936, 0.961 and 0.984. Six plates per condition, per isolate were prepared.