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Modelling the influence of temperature, water activity and sodium metabisulphite on the growth and OTA production of *Aspergillus carbonarius* isolated from Greek wine grapes

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ABSTRACT

The purpose of the present study was to develop a modelling approach to quantify the effect of temperature (15–38 °C), a_w (0.88–0.98) and sodium metabisulphite (NaMBS) concentration (0–200 mg L^{-1}) on the growth and OTA production of *Aspergillus carbonarius* on a Grape Juice based Medium (GJM). Growth responses of the fungus were recorded over time in terms of colony diameter changes, and fitted to the primary model of Baranyi and the estimated maximum growth rates (μ_{max}) and lag phases (λ) were subsequently modelled as a function of temperature, a_w and NaMBS concentration using the cardinal values model with inflection (CMI). Moreover, OTA production was measured during fungal growth and modelled as a function of the same parameters through a quadratic polynomial model. Results showed that NaMBS increased the lag phase of *A. carbonarius*, particularly at 38 °C/0.98 a_w and 38 °C/0.96 a_w, as well as at lower a_w levels regardless of temperature. In the lowest NaMBS concentration (50 mg L^{-1}) there was no inhibitory effect, while at higher concentrations (100 and 150 mg L^{-1}) fungal growth was delayed. No growth was observed at 200 mg L^{-1} of NaMBS irrespective of temperature and a_w levels. The optimum values for growth were found in the range 30–35 °C and 0.96 a_w, while for OTA production at 20 °C and 0.98 a_w. The developed models were subjected to internal and external validation and presented satisfactory performance as inferred by graphical plots and statistical indices (bias and accuracy factors). The present study will complement the findings on the ecophysiology of *A. carbonarius* using NaMBS as an inhibitory agent.

1. Introduction

A number of certain filamentous fungi are able to cause food spoilage with considerable economic losses in the food chain. In the last years, the focus on these fungi has been shifted on their ability to produce secondary metabolites, such as mycotoxins, which can cause deleterious effects on animals and humans. Ochratoxin A (OTA) is a mycotoxin of a major concern which can be produced by several species of *Aspergillus* section *Nigri* (black aspergilli), *Circumdati*, and *Penicillium* genera (Pitt and Hocking, 2009). Black aspergilli can be isolated from a wide range of products, including cereals, cocoa, coffee, dried fruits, dried grapes, must, wine, etc. (Bucheli and Taniwaki, 2002; Abarca et al., 2003; Leong et al., 2004; Magnoli et al., 2004; Varga et al., 2004; Valero et al., 2005; Bellí et al., 2006a). In particular, *Aspergillus carbonarius* is the most distinct member of this section and it has been reported as the main source of OTA contamination in grapes and related products (Cabañes et al., 2002; Counil et al., 2005; Anli and Bayram, 2009). OTA has been accused for teratogenic, mutagenic, carcinogenic, and immunosuppressive effects to humans and animals (IARC, 1993; Castegnaro et al., 1998), while it is also associated with the Balkan Endemic Nephropathy (Abouzied et al., 2002). Concerning the European Union legislation for OTA, authorities have set up a limit of 2.0 μg kg^{-1} for wine, must or grape juice and 10 μg kg^{-1} for dried vine products (European Commission, 2006).

The most powerful strategy to control OTA contamination in grapes and their products is the prevention of mycotoxigenic fungal growth before crop harvest (Magan and Aldred, 2007; Ponsone

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et al., 2012). Specifically, the incidence of *A. carbonarius* ochratoxigenic strains reduction in vineyards could be successfully achieved by chemical control measures which focus on the use of appropriate fungicide applications in the field (Bellí et al., 2006b). Fungicide formulations such as Azoxystrobin, Dinocap, Chorus and Switch have been tested for their efficacy to prevent fungal growth of black aspergilli in the field and consequently suppress OTA accumulation in grapes and wine (Lo Curto et al., 2004; Tjamos et al., 2004; Bellí et al., 2006b). Results obtained by the aforementioned researchers showed that the use of these fungicides could influence either positively or negatively the accumulation of OTA. In addition, toxigenic fungi can produce toxins not only in the field, but also post-harvest, during processing and storage of grape products. Post-harvest control of toxigenic fungi could be achieved with the application of NaMBS, a salt of sulphurous acid with known antimicrobial action, extensively used in foods and beverages for many decades (Jay, 2000). NaMBS is often used as a post-harvest antifungal agent for table grapes' storage, during processing of raisins and grape juice, and during grape crushing for wine production (Prabhakar and Reddy, 2000; Magan and Aldred, 2007). Moreover, Pateraki et al. (2007) have examined the antifungal efficacy of NaMBS and reported that an effective control against fungal growth and OTA production of *A. carbonarius* could be achieved at high NaMBS concentrations. Interestingly, Jiang et al. (2014) demonstrated that the residual OTA in the musts treated with SO_2 increased with the addition of *A. carbonarius* spores in the must. Moreover, aforementioned researchers presented that absence of SO_2 resulted to lower residual OTA in the must compared with the control, and thus application of NaMBS may have a key role in OTA accumulation during winemaking.

An innovative and promising approach to assess fungal responses and therefore mycotoxin contamination in food products, in relation to key controlling parameters in the food environment, is based on predictive mycology (Dantigny et al., 2005; Garcia et al., 2009; Dagnas and Membré, 2013). Until now, many studies have been focused in modelling fungal growth under diverse environmental conditions (Dantigny et al., 2005; Samapundo et al., 2005; Tassou et al., 2007; Marín et al., 2008; Gougouli and Koutsoumanis, 2010; Panagou et al., 2010), but few have taken into account the possibility to model toxin production (Medina et al., 2007; Giorni et al., 2011; Garcia et al., 2013). Due to the high variability in mycotoxin potential by different fungal strains, modelling of toxin formation could be difficult to deal with (Marín et al., 2006; Kapetanakou et al., 2011) and hence the use of mathematical models for mycotoxin quantification should receive more attention to enhance food safety.

The purpose of the present work was to quantify the effect of temperature, a_w and NaMBS on growth and OTA production of an *A. carbonarius* isolate from Greek vineyards on a grape juice based medium, and develop a modelling approach that would allow prediction of growth and OTA production under the studied factors. The developed models were validated with independent data from other similar studies on *A. carbonarius* as well as with data from additional experiments undertaken in our laboratory.

2. Materials and methods

2.1. Fungal isolates

Two isolates of *A. carbonarius* (Ac-57 and ATHUM 5659) with high ochratoxigenic potential, 745 and 4.900 ng OTA g^{-1} CYA (Czapek Yeast Agar medium; 7 days at 25 °C), respectively, isolated from Greek vineyards were selected in this study. The former was isolated from table grapes of cv. Calmerian from Corinth region of the Peloponnese and was used in model development, while the latter from wine grapes of cv. Rhoditis of Achaia region of the Peloponnese and was used in model validation. The isolates were stored at 4 °C in the fungal collection of the Laboratory of Microbiology and Biotechnology of Foods (LMBF) of the Agricultural University of Athens.

2.2. Media preparation and sodium metabisulphite formulation

A Grape Juice based Medium (GJM) was used as the basal medium in this study by mixing 50% (v/v) organic pasteurized white grape juice (composition per 100 mL: fat, 0.02 g; proteins, 0.2 g; carbohydrates, 15.8 g; energy, 64 Kcal; Vitamin C, 0.2 mg; data provided on the nutritional label of the product) and 1.5% agar (LabM, Agar No. 1 Bacteriological, UK) in distilled water. The a_w of this medium was 0.985, measured by an AquaLab LITE (Degacon, USA) water activity meter at 25 °C. Different a_w levels were achieved by adding the required amounts of glycerol (99% purity, Lach-Ner, Czech Republic) to the basic medium and adjust to 0.88, 0.90, 0.93 and 0.96. Low levels of a_w were selected in order to take into account the possibility of fungal growth during sun-drying dehydration process taking place for production of raisins and sweet wines (Valero et al., 2007). The pH of the medium was adjusted to 3.5 using 2 M KOH. A stock solution of 20% (w/v) sodium metabisulphite ($Na_2O_5S_2$; Sigma–Aldrich, Germany) was prepared from which appropriate volumes were added to the substrate to obtain 50, 100, 150 and 200 mg L^{-1}. Petri plates with no NaMBS were also prepared and served as control treatment. The adjustment of pH and incorporation of NaMBS to the medium occurred after autoclaving at 121 °C for 15 min and cooling to approximately 50 °C, in order to assure medium solidification at low pH and antifungal activity of NaMBS, respectively.

2.3. Inoculation and incubation conditions

Inocula were prepared from sub-cultures grown on malt extract agar (MEA; Biolab, Hungary) for 7 days at 25 °C to obtain sporulating cultures. Spore suspension of each isolate was prepared by flooding 10 mL of sterile distilled water containing 0.05% Tween 80 (Merck, Schuchardt, Germany) and gently scratching the colony surface of the media using a sterile spatula. The final concentration of the spore suspension was adjusted to 10^5 spores mL^{-1} using a haemocytometer slide (Brand, Wertheim, Germany). Petri dishes (9 cm diameter) containing *ca.* 20 mL of the solidified growth medium were centrally inoculated with 5 μL of the spore suspension and incubated at 15, 20, 25, 30, 35, and 38 °C in high precision (±0.2 °C) incubators (MIR-153, Sanyo Electric Co., Osaka, Japan). Plates of the same water activity were enclosed in polyethylene bags to minimize desiccation. The effect of temperature, a_w and NaMBS on fungal growth and OTA production was examined by means of a full factorial design. Each treatment was carried out in triplicate and the whole experiment was repeated twice ($n = 6$).

2.4. Model development

2.4.1. Modelling of growth responses

A two-step modelling approach was followed including both primary and secondary model development to determine the effect of temperature, a_w and NaMBS concentration on fungal growth responses. Specifically, fungal growth was quantified by daily diametric measurements of the mycelium of growing colonies at right angles. Measurements were preceded for a period of 40 days or until Petri dishes were fully colonized by fungal mycelium depending on the combination of the environmental conditions assayed. Changes in colony diameter were plotted against time and the maximum growth rate (μ_{max}) and lag phase duration (λ) were