International Journal of Food Microbiology 144 (2011) 432–439

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Is intraspecific variability of growth and mycotoxin production dependent on environmental conditions? A study with *Aspergillus carbonarius* isolates

Daiana Garcia, Antonio J. Ramos, Vicente Sanchis, Sonia Marín *

Food Technology Department, Lleida University, XaRTA-TPV, Rovira Roure 191, 25198 Lleida, Spain

ARTICLE INFO

Article history:
Received 6 August 2010
Received in revised form 21 October 2010
Accepted 24 October 2010

Keywords:
Intraspecific variability
Aspergillus carbonarius
Growth mould
Mycotoxin productin

ABSTRACT

The aim of this study was to assess the impact of suboptimal environmental conditions on the intraspecific variability of *A. carbonarius* growth and OTA production using thirty isolates of *A. carbonarius*.

Three a_w/temperature conditions were tested, one optimal (0.98a_w/25 °C) and two suboptimal: 0.90a_w/25 °C and 0.98a_w/37 °C as suboptimal water activity and temperature, respectively, which might take place through over ripening and dehydration of grapes. For each condition, 12 Petri dishes were inoculated, and colony growth and OTA production were measured over time.

ANOVA revealed significant differences among μ and λ within the 30 assayed isolates. Coefficients of variation (CV%) revealed a wider dispersion of growth rates at 0.90a_w/25 °C compared to 0.98a_w/25 °C, and a more than 4-fold higher CV at 0.98a_w/37 °C compared to 0.98a_w/25 °C. However, dispersion of lag phases was similar at 0.98a_w/25 °C and 0.90a_w/25 °C and wider at 0.98a_w/37 °C.

There were significant differences ($p<0.05$) among OTA levels (ng/mm^2) for the different conditions, values being lower under marginal conditions, and particularly at 0.98a_w/37 °C. Coefficients of variation (CV%) revealed a wider dispersion of OTA production at 0.90a_w/25 °C compared to 0.98a_w/25 °C, while CV at 0.98a_w/37 °C was similar to that at 0.98a_w/25 °C.

In order to address the strain variability in growth initiation and prove the well-established notion of reducing OTA in foods by preventing fungal growth, a greater number of strains should be included when developing models for conditions that are suboptimal both for a_w for OTA production and temperature levels for growth.

1. Introduction

Mycotoxin contamination of food products is an important health hazard of growing interest around the world. *Aspergillus carbonarius* has been considered the main species responsible for ochratoxin A (OTA) accumulation in grapes, wine, dried vine fruits and probably in coffee (Cabañes et al., 2002, Abarca et al., 2003). OTA is a toxic fungal secondary metabolite which poses a risk for the human and animal health. The toxicological profile includes teratogenesis, nephrotoxicity and immunotoxicity (Kanisawa, 1984; Bendele et al., 1985; Krogh, 1987; Patel et al., 1997) and it is classified as a possible human renal carcinogen (group 2B) (International Agency for Research on Cancer, 1993).

A. carbonarius has been found in a great variety of substrates for human and animal consumption in regions with warm and tropical climates. Strains of this mould have been isolated and identified in several Mediterranean wine producing countries, including Spain, Italy, Israel, Portugal and Greece (Serra et al., 2003; Battilani et al., 2004; Bellí et al., 2004a; Mitchell et al., 2004; Tjamos et al., 2004, 2006).

There are many factors that influence mould growth and mycotoxin contamination such as temperature, substrate aeration, water activity, inoculum concentration, microbial interactions, physiological state of mould, etc. Many studies have been published on the effects of some of these factors on growth of one to eleven mycotoxigenic strains. Some authors reported a high intraspecific variability on mould growth and mycotoxin production when several strains were included in the studies (Bellí et al., 2004b; Parra and Magan, 2004; Pardo et al., 2004, 2005a; Arroyo et al., 2005; Astoreca et al., 2007, 2010; Romero et al., 2007; Tassou et al., 2009). Others did not find differences among isolates of the same species (Bellí et al., 2004b; Pardo et al., 2005b).

The use of cocktail inocula of different isolates to minimize the variation that might be expected among different isolates of the same species has been proposed (e.g. Hocking and Miscamble, 1995; Patriarca et al., 2001; Pose et al., 2009; Romero et al., 2007, 2010). This concept was introduced for physiological studies on foodborne bacterial pathogens, particularly in acquisition of data for predictive modelling studies, as a way of determining the extremes of growth limits for particular species (Gibson et al., 1987; Buchanan et al., 1993).

Generally, foods and feeds are stored at marginal conditions for mould growth. Different environmental factors could influence the variability of responses of different isolates of the same species in their growth patterns and mycotoxin production, and this might be increased

* Corresponding author. Tel.: +34 973 702542; fax: +34 973 702596.
E-mail address: smarin@tecal.udl.cat (S. Marín).

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doi:10.1016/j.ijfoodmicro.2010.10.030

when marginal conditions exist. This point requires clarification for further development of predictive mycology.

The aim of this study was to assess the impact of suboptimal environmental conditions for growth on the intraspecific variability of *A. carbonarius* growth and OTA production using thirty isolates of *A. carbonarius*.

2. Materials and methods

2.1. Fungal isolates and preparation of inoculum

This work was carried out on thirty isolates of *A. carbonarius* previously isolated from grapes of Spain (Catalonia, Andalucía, La Rioja, Valencia). Isolates are maintained in the culture collection of the Food Technology Department of Lleida University. The isolates were sub-cultured on malt extract agar (MEA) plates and incubated at 25 °C for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from MEA plates and they were suspended in 5 ml of H_2O/glycerol solutions with two different water activity (a_w) levels: 0.98 and 0.90 (optimal and suboptimal conditions, respectively). After homogenizing, the suspensions were adjusted using a Thoma counting chamber and the final concentration was adjusted to $1\text{-}5\times10^5$ spores/ml.

2.2. Medium and water activity modification

Growth was determined on synthetic nutrient medium (SNM). This medium simulates the composition of grapes between veraison and ripeness (Delfini, 1982). The initial a_w was 0.99 and was modified to $0.98a_w$ and $0.90a_w$ by adding different amounts of glycerol (10 g/l and 510 g/l, respectively) and varying the sugar content: (90 g/l of D(+) glucose and 95 g/l of fructose for 0.98 a_w and 70 g/l of D(+) glucose and 30 g/l of fructose for $0.90a_w$ (Valero et al., 2005). Media were autoclaved and poured into 9 cm sterile Petri dishes. The a_w of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy of ±0.003, before, during and at the end of the experiment.

2.3. Inoculation, incubation and growth assessment

SNM plates were inoculated centrally with a needlepoint load. Previous repeated experiments showed that the number of spores inoculated through this technique was 10-100 spores: 23.8 CFU ± 14.5 colonies were counted when plated (mean ± standard deviation; n = 20). Petri dishes with the same a_w level were enclosed in polyethylene bags in order to maintain a constant water activity and were incubated at 25 °C for both a_w and also at 37 °C for 0.98 a_w condition. Thus three a_w/temperature conditions were tested, an optimal one ($0.98a_w$/25 °C), which is an optimal condition for the growth of this mould, and two suboptimal ones: $0.90a_w$/25 °C and $0.98a_w$/37 °C as suboptimal water activity and temperature, respectively, which might take place through over ripening and dehydration of grapes. For each condition, 12 Petri dishes were inoculated. Two perpendicular diameters of the growing colonies were measured daily until the colony reached the edge of the Petri dish. The diameters of the colonies were plotted against time.

2.4. OTA extraction from culture and quantification

OTA was extracted by a variation of Bragulat et al. (2001) method. Three agar plugs (diameter 5 mm) were removed along a diameter of the colonies when they reached 20 mm and from the inner, middle and outer part of the colonies when they reached 40, 60 and 80 mm. When colony diameter was 10 mm only one plug was removed. 1 ml of methanol was added, and the vials were shaken for 5 s. After 60 min, the extracts were shaken and filtered (Millex-HV 0.45 μm 25 mm, Millipore Corporation. Bedford, U.S.A.) into another vial and stored at 4 °C until analysis by HPLC (Waters, Mildford, MA, SA). Plug extraction was performed in duplicate.

OTA production was detected and quantified by HPLC with fluorescence detection (λ_{exc} 330 nm; λ_{em} 460 nm) (Waters 2475), using a C_{18} column (5 μm Waters Spherisorb, 4.6×250 mm ODS2). The mobile phase (acetonitrile–water–acetic acid, 57:41:2) was pumped at 1 ml min^{-1}. The injection volume was 100 μl and the retention time was around 7 min. The detection limit of the analysis was about 0.21 ng OTA/g SNM or 0.0005 ng OTA/mm^2, based on a signal-to-noise ratio of 3:1. Quantification was achieved with a software integrator (Empower, Milford, MA, USA). OTA was quantified on the basis of the HPLC fluorimetric response compared with that of a range of OTA standards. OTA values were expressed per mm^2 of colony.

2.5. Statistical analyses

Diameters of growing colonies were plotted against time, and the Baranyi and Roberts (1994) model was used to estimate growth rate and lag phase for each growth condition ($0.90a_w$/25 °C, $0.98a_w$/25 °C, $0.98a_w$/37 °C) and isolate. Analysis of variance of growth rates and lag phases was used in order to assess significant differences due to isolates and growth conditions. OTA was expressed as ng per mm^2 of colony. For OTA results, analysis of variance was applied to assess the significance of growth conditions, colony diameter and intraspecific differences. In both cases, the Tukey test was used to establish the differences among mean values of the variables under the different levels of factors at $p<0.05$. Moreover, analysis of variance was carried out to test significance of time in OTA accumulation ($p<0.05$).

3. Results

3.1. Effect of marginal conditions on intraspecific growth differences

All isolates of *A. carbonarius* grew under the three conditions tested. Analysis of variance revealed significant differences in growth among isolates and among incubation conditions; moreover, differences among isolates depended on environmental conditions ($p<0.05$). Table 1 shows mean values of growth rate (μ, cm/day) and lag phase (λ, days) of all isolates for the conditions studied and their dispersion. As expected, there were significant differences ($p<0.05$) among estimated μ and λ levels for the different conditions, μ values being lower under marginal growth conditions; at $0.90a_w$/25 °C growth was inhibited to a higher extent than at $0.98a_w$/37 °C, in terms of μ. Regarding λ, it was longer at $0.90a_w$/25 °C, but it decreased at $0.98a_w$/37 °C compared to $0.98a_w$/25 °C. As a result the high temperature condition was not as suboptimal as expected, at least in terms of λ.

Moreover, ANOVA revealed significant differences among μ and λ within the 30 assayed isolates. Coefficients of variation (CV%) shown in Table 1, reveal a wider dispersion of growth rates at $0.90a_w$/25 °C compared to $0.98a_w$/25 °C, and a more than 4-fold higher CV at $0.98a_w$/37 °C compared to $0.98a_w$/25 °C. However, dispersion of lag phases was similar at $0.98a_w$/25 °C and $0.90a_w$/25 °C and wider at $0.98a_w$/37 °C. In particular, strain 8 showed a better adaptation to low a_w than the others, and strains 8, 9, 10, 14 and 29 grew better than average at 37 °C (Fig. 1a). In general, these isolates grew fast under suitable growth conditions, but not significantly faster than the rest. Isolates 3, 8, 11 and 14 initiated their growth significantly earlier than the others at 37 °C, while strains 3, 23 and 29 started growing early at 0.90 a_w; in this case isolates 3 and 8 showed also short lag phases at $0.98a_w$/25 °C (Fig. 1b).

3.2. Effect of marginal conditions on the growth variability within isolates

Twelve Petri dishes were inoculated per isolate. The minimum variability for μ was observed at $0.90a_w$/25 °C and the highest was at $0.98a_w$/37 °C (Table 2). However, the variability of lag phase values increased under marginal environmental conditions with the major