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ScienceDirect
Postharvest Biology and Technology 44 (2007) 141–149
Postharvest Biology and Technology
www.elsevier.com/locate/postharvbio

Influence of sulphur dioxide, controlled atmospheres and water availability on in vitro germination, growth and ochratoxin A production by strains of *Aspergillus carbonarius* isolated from grapes

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Received 6 April 2006; accepted 21 November 2006

Abstract

The potential for control of germination, growth and ochratoxin A (OTA) production by five strains of *Aspergillus carbonarius* isolated from grapes (*Vitis vinifera*) using sodium metabisulphite (NaMBS, mg L^{-1}) or controlled atmospheres (25 and 50% CO_2) at different water activity levels (0.985, 0.965 and 0.93a_w) on grape juice-based media at 25 °C was determined. The efficacy of NaMBS against the five strains was relatively similar. Generally, germination was inhibited by >500 mg L^{-1} of NaMBS. However, mycelial growth was stimulated by low NaMBS concentrations (100 and 250 mg L^{-1}). Up to 1000 mg L^{-1} was required for complete inhibition of growth. The production of OTA was inhibited by up to 750 mg L^{-1} NaBMS. However, at lowered a_w (0.93) OTA production was inhibited by 500 mg L^{-1}. The ED_{50} and ED_{90} ranges were determined for both growth and OTA production. The efficacy of controlled atmospheres × a_w showed that there was very little effect on spore germination, even by 50% CO_2, regardless of a_w treatment. However, 50% CO_2 inhibited growth after 5 days exposure, although after 10 days growth was not as effectively controlled. OTA production by *A. carbonarius* strains was influenced predominantly by the a_w treatment and less so by the controlled atmospheres used.

Keywords: *Aspergillus*; Germination; Growth; Ochratoxin; Sulphur dioxide; Sulphur; Sulphite; Controlled atmospheres; Environmental factors; Raisins; Grapes

1. Introduction

Vine fruit (currants/sultanas) are usually dried in the sunshine in the open-air and quality is thus dependent on consistent weather conditions and turning to ensure an even drying of the grapes. However, when intermittent sunshine and rain episodes occur then drying can be slowed down and this can lead to colonisation by *Aspergillus* section *nigri* species, especially the ochratoxigenic species such as *Aspergillus carbonarius* (Dekanea, 2005; Magan and Aldred, 2005). This can lead to OTA contamination of vine fruit above the legislative limits resulting in consignments being rejected. Thus, appropriate additives are being sought to either reduce or prevent *A. carbonarius* becoming established on such grape-based products.

Sulphur dioxide (SO_2) is one of the oldest food additives and it has a long history as a disinfectant by the burning of elemental sulphur and the use of the resultant flames. After the development of inorganic chemistry, SO_2 and its salts became commonly used as preservatives, particularly of food and beverages (Magan, 1993a). It is commonly used as a fungal inhibitory treatment of grapes and sometimes raisins. For example, it is used for table grape storage in order to prevent growth of *Botrytis cinerea*. It is also used in the process of wine making and it is a necessity for the storage and preservation of white wines. During processing, golden raisins are often treated with SO_2 in order to prevent the enzymatic browning and additionally act as an antimicrobial to prevent growth of moulds, yeasts and bacteria.

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doi:10.1016/j.postharvbio.2006.11.016

SO_2 can be highly toxic to microorganisms as it has mutagenic effects, and thus inactivates mRNA and reacts with disulphide linkages in proteins, enzyme cofactors, aldehyde and ketone structures of five and six carbon sugars; it deaminates cytosine derivatives to uracil compound, and it has deleterious effects on membranes (Babich and Stotzky, 1980). In contrast, small quantities of SO_2 may stimulate growth as the sulphur is an essential element for the growth.

There is very little detailed information on the actual tolerance and sensitivity of OTA-producing fungi to SO_2. Previously, Magan (1993b) examined the effect of different concentrations of SO_2 in solution on the growth of *Penicillium* and *Aspergillus* spp. and showed that interactions with environmental factors such as water activity has a significant and differential effect on germination and mycelial growth. Furthermore, Majumber et al. (1973) reported the inhibition of both fungal growth and mycotoxin production. However, no studies have examined the effect of SO_2 alone or interactions with water activity on germination, germ tube extension and OTA production by isolates of *A. carbonarius*.

Controlled atmosphere technology is well established for insect control especially as there is intense pressure to phase out methyl bromide as a fumigant. Changing the proportion of atmospheric gases in an environment can affect fungal development. The ratio of elevated CO_2 to reduced O_2 also has an influence on fungal development (Magan and Lacey, 1984; Magan et al., 2003). The effects of modified atmospheres on mycotoxigenic fungi has been studied but none have examined the black *Aspergilli* and OTA production. Paster et al. (1983) demonstrated that atmospheres containing 30% CO_2 or more completely inhibited OTA production by *Aspergillus ochraceus* regardless of the O_2 concentration, although growth was inhibited only with CO_2 concentrations >60% and was stopped by 80% CO_2. Furthermore, T-2 toxin production was reduced by 80% or 50% CO_2 and 20% O_2 but the growth of *Fusarium sporotrichioides* was not affected by <60% CO_2 (Paster et al., 1986). Recently, Cairns-Fuller et al. (2005) showed that growth and OTA production by strains of *Penicillium verrucosum* were only slightly affected by up to 50% CO_2, regardless of water activity, both in vitro and in wheat grain.

The objectives of this study were to examine the efficacy of interacting conditions of: (a) the food grade preservative, sodium metabisulphite, (b) controlled atmospheres and (c) water activity on germination, growth and OTA production in vitro by isolates of *A. carbonarius* obtained from dry raisons and wine grapes.

2. Materials and methods

2.1. Fungal strains

Five isolates of *A. carbonarius* were used in this study. Two were from Greek Corinth currants (*Vitis* species; isolates GR119 and GR127), two from sultana raisins (isolates GR105 and GR117) and for comparison an Italian isolate from red wine grapes (isolate IMI388653). All isolates were confirmed as *A. carbonarius* by comparison with type isolates (Mitchell et al., 2004; CABI BioSciences, Egham, Surrey). They were also all demonstrated to produce OTA using coconut cream agar (Heenan et al., 1998) and by HPLC analyses.

2.2. Inoculation, measurement of germination, germ tubes extension and mycelial growth

Inocula were prepared by growing the strains on malt extract agar (Oxoid) at 25 °C for 7 days, to obtain heavily sporulating cultures. Spore suspensions of each isolate were prepared by harvesting spores from these cultures and suspending them in sterile distilled water containing 0.005% of a wetting agent (Tween 80) to assist dispersion of the spores. The final concentration of the spore suspension was assessed by using a haemocytometer slide, and was adjusted to approximately 10^6 spores mL^{-1}.

The Petri plates of each treatment were centrally inoculated with a small loop of spore suspension (Pitt and Hocking, 1997) for mycelial growth studies. For germination studies 0.2 mL of the spore suspension was spread on the surface of the plates using a sterile glass spreader (Magan, 1988). Plates of the same a_w were enclosed in polyethylene bags to ensure no fluctuation in a_w. All experiments were carried out with four replicates per treatment, incubated at 25 °C and carried out twice.

Spore germination was evaluated in both experiments (a_w × NaMBS, a_w × CO_2). Three agar plugs from each replicate plate were cut out with a sterile cork borer (1.5 cm) and placed on a slide. Plugs were removed after 6, 12, 24 and 36 h of incubation. The disks were stained with lactophenol blue solution (Fluka) and examined microscopically. A total of 50 spores per plug (150 per agar plate) were counted. Spores were considered to have germinated when the germ-tube length was equal to or greater than the diameter of the spore.

Mycelial growth rates were determined by daily measurement of the colony diameter in two directions at right angles to each other. Measurements were carried out for 4–10 days. Linear regression of the colony radius against time (days) was used to obtain the growth rates (mm day^{-1}) under each set of growth conditions. In the case of a_w × CO_2 experiment the mycelium diameter was measured after 2, 5 and 10 days.

2.3. Effect of a_w and sodium metabisulphite (NaMBS) on germination, growth and ochratoxin production

Grape juice agar medium (GJM) was used as the basal medium for all studies. The medium was prepared by mixing 25% (v/v) red or white grape juice and 2.5% agar (Oxoid, UK Technical Agar No. 3) in distilled water. The medium after the water activity and NaMBS adjustment was sterilized at 121 °C for 15 min, cooled to approximately 50 °C and