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Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes

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Abstract

This study investigated the impact of skin damage on *Aspergillus carbonarius* colonization and ochratoxin A (OTA) production in grapes at different temperatures and relative humidity. Four ochratoxigenic *A. carbonarius* strains isolated from wine grapes were used to inoculate artificially damaged and undamaged table grapes. Grapes were stored at three levels of relative humidity (80%, 90% and 100%) and at two temperatures (20 and 30 °C). After seven days, the infection percentage of *A. carbonarius* was recorded and OTA accumulation in berries was analysed. Damaged grapes were more commonly infected and development of colonies was higher than in undamaged ones; consequently more OTA was detected in the former treatment. Temperature and relative humidity had significant influences on both infection and toxin content. The amount of OTA detected at 30 °C was higher than at 20 °C in most of the treatments. The highest relative humidity (100%) led to maximum amounts of OTA while no significant differences were found between 90% and 80% in the OTA content. The implementation of preventive measures in order to minimise berry damage in the field by controlling pathogenic fungi and insects during grape growing and removing visibly damaged grapes at harvest may significantly reduce OTA contamination in grapes.

Keywords: Ochratoxin A; *Aspergillus carbonarius*; Grapes; Skin damage; Temperature; Relative humidity

1. Introduction

Ochratoxin A (OTA) is a mycotoxin considered to be a possible carcinogen for humans (IARC, 1993). It has been shown to be nephrotoxic, carcinogenic, teratogenic and immunosuppressive in laboratory animals (Boorman, 1989; Dirheimer, 1998). It has been commonly found in cereals but it can also contaminate a variety of other plant and animal products. Grapes and wine contain important amounts of OTA, in particular, wine consumption could represent 15% of the total intake of this toxin (Codex Alimentarius Commission, 1998). Therefore, studies on these products aiming to reduce the consumer exposure to the toxin as much as possible are crucial.

OTA was originally isolated in 1965 as a metabolite from a strain of *Aspergillus ochraceus* (Van der Merwe, Steyn, Fourie, Scott, & Theron, 1965). Recently, OTA production has been reported from *Aspergillus* species belonging to the *Nigri* section, which are frequently isolated from grapes (Battilani et al., 2003; Bellí et al., 2004; Bellí, Mitchell et al., 2004; Sage, Krivobok, Delbos, Seigle-Murandi, & Creppy, 2002; Serra, Abrunhosa, Kozakiewicz, & Venancio, 2003; Tjamos et al., 2004). In this section, the reported OTA-producing species are *A. carbonarius* and those now included in the so-called *A. niger* aggregate (Abarca, Accensi, Bragulat, & Cabañes, 2001). *A. carbonarius* is less common than *A. niger*, but is the main species responsible for OTA in grapes because almost all strains are high OTA producers (Battilani et al., 2003; Bellí, Mitchell et al., 2004; Cabañes et al., 2002).

A preliminary study reported the ability of strains of *A. carbonarius* to colonise and penetrate intact and

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artificially damaged berries, finding OTA in the pulp, although berry skin was considered the major source of OTA in grapes (Battilani, Giorni, Bertuzzi, & Pietri, 2001). At the present time, the effect of temperature and water availability on *A. carbonarius* growth and OTA production has only been reported on synthetic medium (Bellí, Ramos, Coronas, Sanchis, & Marín, 2005; Mitchell, Parra, Aldred, & Magan, 2004). These studies highlighted the need to know more about the behaviour of these species on natural substrates. In the present work, table grapes were used as the nutrient source for *A. carbonarius*. The aim was to study the influence of skin damage on visible fungal growth and on OTA accumulation in berries at different temperatures and relative humidities (RH).

2. Materials and methods

2.1. Fungal isolates

Four *A. carbonarius* strains isolated from naturally infected grape berries from Italy (3.161), France (3.162), Portugal (3.166) and Spain (3.168) were used in this study. The same isolates were used in a previous study carried out by Bellí et al. (2005), coded as W9, W38, W89 and W120, respectively. All the isolates were previously found to be OTA producers and were supplied by the Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy; the Institut National Polytechnique de Toulouse, École Nationale Supérieure Agronomique de Toulouse, France; the Departamento Engenharia Biologica, Universidade do Minho, Braga, Portugal; the Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Univ. Autònoma de Barcelona, Spain. They are held in the culture collection of the Departament de Tecnologia d'Aliments, Escola Tècnica Superior d'Enginyeria Agrària, Universitat de Lleida, Spain.

2.2. Grape decontamination

Grape berries were separated from the bunches by cutting the stem with the aid of scissors at approximately 0.5 cm from each grape. Red table grapes (var. Red Globe) not physically damaged were used in this study. They were surface-sterilised by dipping them into a NaClO solution (0.1% Cl) for 1 min, followed by one more minute in ethanol (70%). Excess water was removed by placing berries in a laminar flow bench for 2 min until fungal inoculation.

2.3. Experimental design

A full factorial design with four isolates and a control, two temperatures (20 and 30 °C), three RH (80%, 90% and 100%) and two berry states (damaged and undamaged) was carried out. All treatments ($n = 60$) were made in triplicate.

2.4. Inoculation

Spore suspensions of each isolate (10^3 spores ml^{-1}) were prepared from colonies, previously grown on synthetic nutrient medium (SNM) (Bellí, Marín, Sanchis, & Ramos, 2004) for seven days at 25 °C, in distilled water containing Tween 80 (0.005%). Berries were dipped into 100 ml of the spore suspensions for 1 min. Control treatments were done in the same way, but sterile distilled water (0.005% Tween 80, no fungal spores) was used to simulate the inoculation step. Half of the berries were wounded before inoculation by puncturing them 3 mm deep approximately (diameter of lesion: 0.25 mm) with a sterile needle and later referred as *damaged* berries to differentiate them from the *undamaged* ones.

2.5. Incubation

For each treatment, 20 berries were placed on the top of a grate previously disinfected with ethanol (96%), preventing any contact among them. Grapes were placed into disinfected plastic boxes containing 300 ml of a glycerol–water solution to assure the RH of the treatment (101, 50 and 0 g glycerol/100 ml distilled water to produce 80%, 90% and 100% RH, respectively). Filled containers were hermetically closed and incubated at two temperatures (20 and 30 °C) for 7 days. Each treatment was performed in triplicate.

2.6. Percentage of infection

At the end of incubation, berries with visible growth were counted and were classified depending on the percentage of their surface colonised by *A. carbonarius* in order to estimate for each treatment the percentage of infection and the infection index (II), respectively. This index, which was proposed for this particular study, ranged from 0 (healthy berries) to 1 (the surface of the 20 berries completely colonised) and was calculated as follows:

$$\mathrm{II} = \frac{(a \cdot 0.25) + (b \cdot 0.5) + (c \cdot 0.75) + (d \cdot 1)}{20}$$

- a number of berries with less than one quarter of their surface colonised with *A. carbonarius*
- b number of berries with half of their surface colonised with *A. carbonarius*
- c number of berries with three quarters of their surface colonised with *A. carbonarius*
- d number of berries completely colonised with *A. carbonarius.*

2.7. OTA extraction and HPLC quantification

After seven days of incubation, grapes in each container were analysed for OTA (Bezzo, Maggiorotto, & Testa, 2000). The 20 berries were weighed and crushed with a hand blender machine (Opticlick Pro, Moulinex, France).