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Diversity of *Aspergillus* section *Nigri* on the surface of *Vitis labrusca* and its hybrid grapes

Larissa de Souza Ferranti[b], Maria Helena P. Fungaro[b], Fernanda Pelisson Massi[b], Josué José da Silva[b], Rafael Elias Silva Penha[b], Jens Christian Frisvad[c], Marta Hiromi Taniwaki[a], Beatriz Thie Iamanaka[a,*]

[a] Instituto de Tecnologia de Alimentos - ITAL, P.O. Box 139, 13070-178 Campinas, SP, Brazil
[b] Universidade Estadual de Londrina, P.O. Box 6001, 6051-970 Londrina, PR, Brazil
[c] Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark

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ABSTRACT

This study investigated the presence of *Aspergillus* species belonging to *Aspergillus* section *Nigri* on *Vitis labrusca* and its hybrid grapes grown in Brazil. The ability of the fungi isolates to produce ochratoxin A (OTA) and fumonisin B_2 (FB_2) as well as the presence of these mycotoxins in the grapes were also studied. Eighty-eight samples were collected from the main grape producing states in Brazil: Rio Grande do Sul (n = 30), Pernambuco (n = 21), São Paulo (n = 21) and Paraná (n = 16). The highest average contamination level by *A.* section *Nigri* occurred on the grapes from Pernambuco (66.3%). A total of 2042 *A.* section *Nigri* isolates was analyzed and clustered in three groups according to morphology characterization: *A.* section *Nigri* uniseriate (79.3%), *A. niger* "aggregate" (18.3%) and *A. carbonarius* (2.4%). In order to precisely identify the *Aspergillus* species, two hundred and forty-eight strains were subjected to DNA sequencing. Among the *A.* section *Nigri* uniseriate group, the following species were found: *A. japonicus*, *A. uvarum*, *A. brunneoviolaceus*, *A. aculeatus* and *A. labruscus*. Within the *A. niger* "aggregate", the following species were found: *A.niger sensu stricto*, *A. welwitschiae* and *A. vadensis*. Regarding mycotoxin-production capacity, 3.2% of the total *A. section Nigri* isolates (2042) were positive for OTA production and from *A. niger* "aggregate" (373) tested, 42.1% were FB_2 producers. However, none of the 88 grape samples were contaminated with these mycotoxins.

1. Introduction

The *Vitis* species grape is one of the most economically important fruit species due to the numerous uses of its fruit in the production of wine, grape juice and other foods (Ali et al., 2010). Most of the world's vineyards grow European cultivars (*Vitis vinifera* L. cultivars) because of their high quality for use in wine production (Ferreira et al., 2014). However, in Brazil the majority of the vineyards grow American cultivars (*Vitis labrusca* L. or hybrid *V. vinifera* x *V. labrusca*), as they are less susceptible to fungal diseases and more adapted to the Brazilian environmental conditions. In 2015, a total of 703,271 t of grapes were harvested in Brazil, and approximately 90% of the total production was from *V. labrusca* (and hybrid) grapes (http://www.uvibra.com.br/dados_estatisticos.htm). These grapes are widely consumed "in natura" and used to produce juice, which is an excellent natural source of polyphenols with health benefits (Toaldo et al., 2015). As reviewed by Arcanjo et al. (2017) Brazilian grape juices are seen as one of the most promising export products of the food sector. Moreover, Brazil is one of the few countries that allow the commercialization of wine produced with *V. labrusca* grapes. The designations "table wine" and "fine wine" are used for those obtained from *V. labrusca* and *V. vinifera* grapes, respectively. In 2015, "table wine" production in Brazil reached 210 million liters (IBRAVIN, 2016).

Many fungi may occur in vineyards and especially at harvest time they can find conditions to infect the grape berries (Somma et al., 2012). *V. vinifera* grapes can be contaminated in the field mainly by the *Aspergillus*, *Alternaria*, *Botrytis*, *Cladosporium*, *Fusarium*, *Rhizopus* and *Penicillium* species (Abrunhosa et al., 2001; Bellí et al., 2004; Kizis et al., 2014; Somma et al., 2012).

Aspergillus species belonging to *A.* section *Nigri*, commonly known as black aspergilli, have been detected in *V. vinifera* grapes around the world (Chiotta et al., 2013; García-Cela et al., 2015; Mikušová et al., 2014; Somma et al., 2012). Special attention has been given to this group of fungi because some species can produce mycotoxins, mainly

* Corresponding author.
E-mail address: beatriz@ital.gov.sp.br (B.T. Iamanaka).

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ochratoxin A (OTA) and fumonisin B_2 (FB_2). Both these toxins are classified as a possible human carcinogen, Group 2B (IARC, 1993). In 2011 the maximum limit of OTA (2 μg/Kg) was established for grape juice and wine by Brazilian authorities (Anvisa, 2011). For fumonisins in grape products, the maximum tolerable limit has not yet been established.

The taxonomy of *A*. section *Nigri* has evolved constantly, but some species are difficult or not possible to identify based solely on their phenotypic characters (Varga et al., 2011). Recently, Samson et al. (2014) provided an updated accepted species list for the genus *Aspergillus*, and 26 species are included in *A*. section *Nigri*. These species include biseriate and uniseriate species. Some morphologically indistinguishable species were treated as *A. niger* "aggregate" and together with *A. carbonarius* they are the biseriate species prevalent on grapes. Among the uniseriate species, the most frequently found on grapes are *A. aculeatus*, *A. japonicus* and *A. uvarum* (Chiotta et al., 2013; García-Cela et al., 2015; Mikušová et al., 2014; Spadaro et al., 2012).

Uniseriate species are not considered to be OTA producers (Perrone et al., 2007). However, the biseriate species *A. carbonarius*, *A. welwitschiae* and *A. niger,* the latter two belonging to the *A. niger* "aggregate", are the main species for the presence of OTA in *V. vinifera* grapes and derived products (Bau et al., 2005; Bellí et al., 2005; Chiotta et al., 2013; García-Cela et al., 2015; Magnoli et al., 2003; Mikušová et al., 2014; Sage et al., 2004; Spadaro et al., 2012; Visconti et al., 2008).

A. niger and *A. welwitschiae* can also produce FB_2, FB_4 and FB_6. Some studies have correlated the presence of fumonisins in *V. vinifera* grapes with the presence of these species (Abrunhosa et al., 2011; Mogensen et al., 2010b; Susca et al., 2010).

Based on the limited data concerning OTA and FB_2 in *V. labrusca* grapes, a survey of the black *Aspergillus* on the surface of this fruit obtained from the four main producing regions in Brazil was conducted. The presence of these mycotoxins in the grape samples was also evaluated.

2. Materials and methods

2.1. Sampling

A total of 88 grape samples from the states of Rio Grande do Sul (n = 30), São Paulo (n = 21) and Paraná (n = 16), which are located in the South and Southeast, and Pernambuco (n = 21) in the Northeast of Brazil, were analyzed.

The samples were collected during harvesting, according to Serra et al. (2003). Ten plants were selected randomly along two diagonal transects of the vineyard and two bunches of grapes were harvested from each plant. Therefore each sample (total of 88) was made up of 20 bunches. The varieties of *V. labrusca* and hybrid grapes analyzed were "Bordo", "Cora", "Concord", "Isabel", "Violeta", "Coder", "Rudder" and "Niagara".

The water activity was determined in all grape samples (peels) using AquaLab, Series 3TE equipment (Decagon, USA) at 25 ± 0.1 °C, in triplicate.

2.2. Fungal isolation

One hundred grape peels were plated directly onto Petri dishes containing Dichloran Rose Bengal Chloramphenicol medium agar (DRBC agar). The grape pulp was discarded. Plates were incubated at 25 °C for 7 days (Pitt and Hocking, 2009).

2.3. Fungal characterization

The fungal population was isolated on Czapek Yeast Extract Autolysate agar (CYA agar) and incubated at 25 °C for 7 days (Klich and Pitt, 1988). After incubation, the plates were examined and all *A.* section *Nigri* were first isolated in Petri plates containing CYA agar. After the incubation period, the morphological aspects of the *Aspergillus* strains, including color and size of the colony, size and characteristics of the conidia and conidia head, the species were classified into three groups: *A.* section *Nigri* uniseriate, *A. niger* "aggregate" and *A. carbonarius* (Klich and Pitt, 1988; Samson et al., 2004).

2.4. Test for ochratoxin A production by A. section Nigri

All strains of *A.* section *Nigri* (n = 2042) were tested for OTA. The capacity of each isolate to produce OTA was analyzed according to the method described by Filtenborg et al. (1983). The strains were one-point inoculated into Yeast Extract Sucrose agar (YES agar) and incubated at 25 °C for 7 days. One plug was removed from the center of the colony, the OTA extracted with methanol: chloroform (1:1 *v*/v) and the plug placed on a silica plate (Merck, Germany) to perform thin layer chromatography. The mobile phase used consisted of toluene: ethyl acetate: 90% formic acid: chloroform (7:5:2:5, v/v/v/v). The OTA was visualized under UV light at 365 and 256 nm. The OTA standard (Sigma, St. Louis, USA) was placed on the TLC plate so that the fluorescence and retention factors could be compared with the samples.

2.5. Test for fumonisin B_2 production by A. section Nigri

Three hundred and ninety-one strains belonging to *A.* section *Nigri* were tested for their ability to produce FB_2. Each strain was inoculated onto Czapek Yeast Extract 20% Sucrose agar (CY20S agar) and incubated at 25 °C for 7 days, following the methodology in Frisvad et al. (2007). Five small pieces of mycelium were removed (plugs) from the central portion of the colony and the toxin extracted with 1 mL of methanol in a vortex for 3 min. The extract was filtered in a 0.45 μm and 0.22 μm Millex membrane. Then 55 μL of the extract were transferred to an HPLC vial, adding 55 μL of ortho-phthaldialdehyde reagent (OPA), according to the method in Visconti et al. (2001). The extract was mixed using vortex for 30 s. Chromatography was performed using a Shimadzu LC-10VP (Shimadzu, Japan) HPLC system, with a fluorescence detector set at 335 nm excitation and emission at 440 nm. A YMC column - Pack ODS-A (5 μm, 4.6 × 150 mm) (YMC Co., Ltd., Japan) was used with the following mobile phase: acetonitrile: water: acetic acid (51:47:02 *v*/v/v) and mobile phase flow rate at 1.0 mL/min. The oven temperature was 40 °C and volume injection 20 μL.

2.6. Molecular analysis

A total of 248 strains, representatives of each group distinguished by morphological characteristics (59 isolates of *A. niger* "aggregate" and 189 isolates of *A.* section *Nigri* uniseriate group), were subjected to Calmodulin (*CaM*) gene sequence analysis. After growing in liquid Complete Medium (Pontecorvo et al., 1953), the mycelia were collected, frozen in liquid nitrogen and ground to a fine powder. Nucleic acids were extracted using the BioPur Mini Spin Extraction Kit (Biometrix, Brazil) according to the manufacturer's instructions. Amplification of a *CaM* gene region was performed using the primers cmd5 (5′ CCG AGT ACA AGG AGG CCT TC 3′) and cmd6 (5′ CCG ATA GAG GTC ATA ACG TGG 3′) described in Hong et al. (2006). The PCR products were cleaned up using ExoProStar™ 1-Step (GE Healthcare Life Sciences, UK) and directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI 3500XL Genetic Analyzer (Applied Biosystems, USA). The partial *CaM* sequences were subsequently aligned with those from *A.* section *Nigri* available in the GenBank database and a phylogeny reconstruction was performed using a distance-based Neighbor-Joining method (Saitou and Nei, 1987). The tree was drawn using MEGA7 (Kumar et al., 2016).