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Control of *Aspergillus carbonarius* in grape berries by *Lactobacillus plantarum*: A phenotypic and gene transcription study

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ABSTRACT

The *in vitro* and *in situ* antifungal activity of *Lactobacillus plantarum* against the ochratoxigenic fungus *Aspergillus carbonarius* was investigated in this study. Four different fungal isolates from grape berries were co-cultured with four different strains of *L. plantarum* on Malt Extract Agar (MEA) plates at 30 °C. Bacterial strains inhibited fungal growth up to 88% and significantly reduced toxin production up to 100%. In addition, *L. plantarum* was evaluated as biocontrol agent against *A. carbonarius* growth and OTA production on table grapes. Temporal studies of bacterial antagonism were performed with two different grape cultivars. Artificially wounded and unwounded berries were pre-treated with 10^8 CFU/mL bacteria and inoculated with 10^6 spores/mL of *A. carbonarius* ochratoxigenic isolates. Biocontrol agents displayed high rate of colonization on grapes during 5 days of incubation at 30 °C. Scanning electron microscopy (SEM) also determined the presence of microorganisms on grape surface. Bacterial strains were effective in controlling fungal infection reaching up to 71% inhibition rates. However the presence of wounds on grape skin facilitated infection of berries by *A. carbonarius*, since unwounded berries showed lower levels of infection. Results also revealed significant reduction in mycotoxin production ranging between 32% and 92%. Transcriptome analysis following exposure to co-cultivation, exhibited differential expression for each gene studied of AcOTA*nrps* (*Aspergillus carbonarius* OTA nonribosomal), AcOT*Apks* (*Aspergillus carbonarius OTA polyketide synthase*) and *laeA*, emphasizing the significance of strain variability. The genes AcOTA*nrps* and *laeA* were most influenced by the presence of *L. plantarum*. This work is a contribution for the potential biocontrol of toxigenic fungi in table grapes by lactic acid bacteria (LAB). The above findings underline the significance of bacterial strain variability on the effectiveness of biopreservative features of *L. plantarum* strains.

1. Introduction

Table grapes (*Vitis vinifera* L.) are one of the most nutritionally and economically important food products in the world. According to estimates by the US Department of Agriculture (USDA, 2017), Greece is the second largest producer of table grapes in the EU-28, behind Italy. In 2017, EU production was estimated at 1.5 million tons with Greece producing 315.000 tons. It needs to be noted that 30–40% of post-harvest table grapes are lost every year due to inadequate handling and lack of proper methods to prevent decay and senescence (Hashem et al., 2013; Prusky, 2011). Fungal decay caused by *Aspergillus* spp. is hazardous to human health for direct consumption because of the production of mycotoxins. *A. carbonarius* is the main cause of black rot in table grapes (Ayoub et al., 2010; Guzev et al., 2008; Rooney-Latham et al., 2008) and its ability of ochratoxin A (OTA) production is a great food safety concern. Lasram et al. (2012) reported the presence of OTA in both wine and table grapes in a three year survey. Other surveys report also the presence of *A. carbonarius* on table grapes demonstrating that this fungus is ubiquitous on table grapes (Bellí et al., 2007). In another survey undertaken in Greece, Meletis et al. (2007) observed many varieties to be contaminated with OTA and reported that the incident of infected berries was low before veraison while at harvest the frequency was twice as high. Exposure to OTA through food consumption poses a health risk. OTA remains a challenge in the face of continuous efforts to produce quality table grapes meeting food safety standards. Control of fungal decay in table grapes has been achieved by application of sulfur dioxide gas (SO_2), either by frequent fumigation in storage rooms or by packing the grapes in polyethylene-lined boxes

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with SO_2 generator pads (Lichter et al., 2002). SO_2 is usually effective in preventing decay as long as levels are sufficiently high. However, high levels of SO_2 can injure grape berries, may cause an unpleasant aftertaste, and could be harmful to humans (Zoffoli et al., 2008).

The use of chemicals is still the main practice to control fungal infection. However, this has fostered fungi to develop resistance to the extensive use of preservatives and antibiotics. Therefore, over the last few decades the interest of scientists has been shifted on natural antimicrobials to control fungal growth on grapes and grape products, employing strategies with less chemical control measures. The approach of biological control agents (BCAs) is increasingly considered by the scientific community as an alternative application either in the field and/or in post-harvest operations and has been already reported on table grapes (Jiang et al., 2014; Sonker et al., 2016). From the available BCAs, lactic acid bacteria (LAB) can inhibit fungal growth and they have also the potential to interact with mycotoxins (Dalié et al., 2010). LAB are generally recognized as safe (GRAS) which usually compete with other microorganisms by secreting antagonistic compounds and modifying the surrounding microenvironment producing several metabolites that are able to inhibit fungal growth (Hassan et al., 2015; Wang et al., 2012).

A number of putative pathways for OTA biosynthesis have been proposed and the role of two genes namely NRPSs and PKSs, has been revealed as key enzymes in OTA biosynthetic pathway (Gallo et al., 2009, 2012, 2014). Therefore, they have been employed as targets to detect and quantify OTA producing molds by molecular techniques. Moreover, *laeA* gene has been correlated with the regulation of OTA biosynthesis in *A. carbonarius* as a global transcriptional factor (Crespo-Sempere et al., 2013). In general, there is a limited number of studies elucidating the expression of toxigenic related genes under different environmental and nutritional conditions. Only recently, Al-Saad et al. (2016) used relative gene expression as an indicator of the efficacy of bacterial antagonists against an aflatoxigenic strain of *A. flavus*. Also Samsudin et al. (2017) examined two bacterial antagonists for potential contamination of FUM1 in maize through gene expression studies.

The objective of this study was to: (a) investigate the *in vitro* and *in situ* antiochratoxigenic efficacy of different *L. plantarum* strains on *Aspergillus carbonarius* isolated from grape berries, (b) assess of antiochratoxigenic activity on gene transcription level, and (c) confirm bacterial/fungal co-existence on grapes by means of Scanning Electron Microscopy (SEM) images.

2. Materials and methods

2.1. Microorganisms and inocula preparation

All the microorganisms used in the present study are indicated in Table 1. They consisted of four bacterial strains of *L. plantarum* (T571, 345, 195, and 1645), three *A. carbonarius* wild isolates (Ac29, Ac33, and A47) originated from grape berries and one reference strain of *A. carbonarius* (ITEM 5010). All strains were maintained in 20% (v/v) glycerol at −22 °C.

Lactobacillus plantarum strains were grown in de Man-Rogosa-Sharpe (MRS, Biolife, Italy) broth for 24 h at 30 °C and sub-cultured in the same medium for 18 h at 30 °C. Cells were harvested by centrifugation at 5000 ×*g* for 10 min at 4 °C. The supernatants were discarded and the cells were washed twice with Ringer solution and resuspended in the same medium to obtain a final concentration of 10^5 CFU/mL (confirmed by plating on MRS agar) for use as inoculum.

Spore suspensions of each *A. carbonarius* isolate were prepared by collecting spores from 7-day old colonies grown on Malt Extract Agar (MEA: malt extract, 20 g; peptone, 1 g; glucose, 20 g; bacteriological agar, 20 g; distilled water, *ca.* 1000 mL) at 25 °C in the dark to induce sporulation. Conidia were harvested from sub-cultures by adding 10 mL of sterile distilled water containing 0.01% Tween 80 (Merck, Schuchardt, Germany) and scraping the surface of the mycelium with a sterile glass rod. The suspensions were filtered through a four layer cleaning tissue to remove any mycelia fragments and left still for 5 min to allow spore settling. Supernatants were discarded and the spores were resuspended in Tween 80 to remove any nutrients from the medium. Conidia concentration was adjusted to a final volume of 10^5 spores/mL assessed by a Neubauer counting chamber (Brand, Wertheim, Germany).

Table 1
Microorganisms used in the present study.

Microorganism	Strain number	Origin	Country
Aspergillus carbonarius	Ac29[a]	Grape berries	Greece
	Ac33[a]	Grape berries	Greece
	Ac47[a]	Grape berries	Greece
	ITEM 5010[b]	Grape berries	Italy
Lactobacillus plantarum	T571[c]	Feta cheese	Greece
	1645[d]	Fermented cauliflower	Greece
	345[a]	Black olive	Greece
	195[a]	Grape berries	Greece

[a] Food Microbiology Culture Collection (FMCC), Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens.
[b] Provided by Dr. D. Tsitsigiannis, Laboratory of Phytopathology, Agricultural University of Athens.
[c] Provided by the Institute of Technology of Agricultural Products, Hellenic Agricultural Organization "DEMETER".
[d] Provided by Dr. E. Drosinos, Laboratory of Food Quality Control & Hygiene, Agricultural University of Athens.

2.2. In vitro experimental settings

The *in vitro* experiments of bacterial/fungal co-cultures were performed in MRS agar for optimal growth of LAB. Specifically, 0.5 mL suspension of each *L. plantarum* isolate was poured into 9 mL of MRS medium (in 6-cm Petri plates) in a final concentration of *ca.* 10^6 CFU/mL. Following media solidification, permeable cellulose membranes were placed onto the agar surface and *A. carbonarius* was centrally spot inoculated at a concentration of 10^6 spores/mL. Plates not inoculated with bacteria were used as control. Considering the importance of temperature as a modulator factor in fungus-bacteria interaction systems, co-cultures were incubated at 30 °C for optimal growth and metabolic activity of fungi and bacteria for three days. The same volume of growth medium (10 mL) was added in the plates to avoid any variation that could contribute to differences in the results among co-cultures. All experiments were undertaken with three replicates per treatment. The percentage of fungal growth inhibition was determined 3 days after *A. carbonarius* inoculation using the formula:

$$\text{Fungal inhibition } (\%) = 100 - \left[\frac{\text{Biomass of fungal colony on agar treated with LAB}}{\text{Biomass of fungal colony on agar without LAB}} \times 100\right]$$

2.3. In situ antagonistic assay

Mature healthy table grapes from two seasonally different and widely consumed grape cultivars, namely Victoria from the area of Tirnavos (white cultivar) and Attika from the area of Korinthos (red cultivar) were purchased from a local retail market. Homogenous bunches were selected according to size, shape, color, weight and absence of mechanical damage and fungal infection. Grape berries were surface disinfected with 70% ethanol for 10 min (Tryfinopoulou et al., 2015), rinsed twice with sterile distilled water and allowed to air dry on absorbent paper in a laminar flow bench until inoculation.

Berries were separated into two groups. In the first group, a calibrated wound of about 2 mm in diameter was made on each berry by means of a sterile needle (wounded berries) (De Curtis et al., 2012), whereas in the second group no such treatment was given and hence the berries remained intact (unwounded berries). Disinfected unwounded