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Isolation, identification and *in vitro* screening of grapevine yeasts for the control of black aspergilli on grapes

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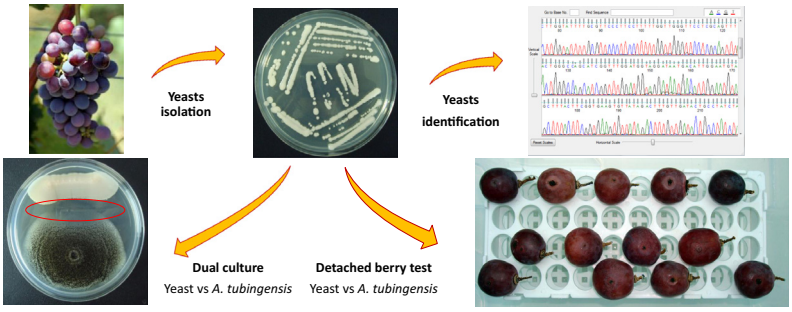
HIGHLIGHTS

- Antagonistic yeasts were isolated from grape surface in Cyprus vineyards.
- Yeasts were identified by molecular tools and found to belong to seven groups.
- 33 yeasts induced antisporulant activity on *Aspergillus tubingensis* on agar plates.
- 28 isolates inhibited infection of detached berries by *Aspergillus tubingensis*.
- *Aureobasidium pullulans* exhibited the highest antagonistic activity.

GRAPHICAL ABSTRACT



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ABSTRACT

Antagonistic yeasts were isolated from the surface of grape berries cv. "Cabernet sauvignon" and "Maratheftiko" from six vineyards in Cyprus and identified at species level using molecular methods. The identification revealed that the yeast isolates belonged to seven taxonomically distinct groups: *Aureobasidium pullulans*, *Cryptococcus magnus*, *Hanseniaspora uvarum*, *Candida zeylanoides*, *Candida sake*, *Rhodotorula mucilaginosa* and *Pseudozyma aphidis*. A total of 55 yeast isolates were evaluated in a preliminary screening test on agar to select isolates exhibiting inhibition against an ochratoxigenic strain of *Aspergillus tubingensis*. Thirty-three yeast isolates were selected for their antisporulant activity on *A. tubingensis* and their ability to reduce the growth of fungal mycelium. These isolates were assayed by a detached berry test for their ability to inhibit infection by the ochratoxigenic strain of *A. tubingensis*. Twenty-eight yeast isolates belonging to three species, namely 25 isolates of *A. pullulans*, 2 isolates of *C. magnus* and 1 isolate of *C. sake*, reduced the *A. tubingensis* colonization of grape berries. The highest antagonistic activity was shown by the *A. pullulans* isolates, with biocontrol efficacies ranging between 17.1% and 95.7%. The results of this study suggest that antagonist yeasts potentially effective for biological control of *A. tubingensis* on grape can be found among the microbiota associated with grape berries in Cyprus vineyards.

1. Introduction

Contamination of grapes and grape products by *Aspergillus* section *Nigri* is known to occur very widely. The fungal species *Aspergillus niger*, *Aspergillus tubingensis*, and *Aspergillus carbonarius* are included within this section and during their growth these

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fungi are able to produce mycotoxins including ochratoxin A (OTA) and fumonisin B2 (FB2) (reviewed by Somma et al. (2012)). OTA is the most common mycotoxin detected in grapes and grape derived products, such as grape juice, wine and dried vine fruits (Aksoy et al., 2007; Visconti et al., 2008; Zimmerli and Dick, 1996). In recent years OTA has been the subject of numerous studies worldwide due to its hazard to human and animal health (Mantle, 2002). It possesses very strong nephrotoxic, as well as carcinogenic, teratogenic and immunosuppressive properties because of which it is classified by the International Agency for Research on Cancer in Group 2B (IARC, 1993).

In order to reduce the risk of consumer exposure to this mycotoxin, research efforts have been primarily directed towards the development of control methods for restricting ochratoxigenic species that can colonize damaged parts of plants and thus contaminate the grape-producing chain. When permitted, the application of antifungal compounds is a very effective approach for preventing mycotoxin production. The antifungal compounds mepanipyrim, pyrimethanil, fluazinam, iprodione and cyprodinil/fludioxonil mixture were reported to reduce both the growth of ochratoxigenic fungi and levels of OTA in grape bunches (Visconti et al., 2008). The cyprodinil/fludioxonil mixture was the most effective treatment in several field trials carried out in the Mediterranean region, namely France, Spain, Greece and Italy (Bellí et al., 2007a; Kappes et al., 2005; Tjamos et al., 2004). However, the effect of fungicides on mycotoxin production needs to be considered, since it has been reported that some chemicals enhance OTA production (Lo Curto et al., 2004). Medina et al. (2007) observed that the application of carbendazim reduced fungal flora but stimulated OTA production. Although the application of fungicides remains one of the most powerful and cost-effective tools to reduce the incidence of fungal pathogens in most crops (Munimbazi et al., 1997), nowadays the European Union has established a strict legislation concerning their use, due to the development of resistant fungal strains and the negative effects of fungicides on human health and the environment (De Costa and Bezerra, 2009). Maximum residue levels of pesticides have been established for all foodstuffs intended for human or animal consumption in the European Union (European Commission, 2013). The above factors (development of resistance, increasing public concern over food safety and regulatory restrictions over pesticide residues on foods) highlight the need for replacing synthetic chemical compounds or complementing fungicide treatments with other methods to control toxigenic fungi at pre- and postharvest stages. Among alternative methods, biological control is one of the most promising strategies, and several effective microbial antagonists have already been tested on many fruits and vegetables (Sharma et al., 2009; Wilson and Wisniewski, 1989; Wisniewski and Wilson, 1992).

Among microorganisms considered for biological control, yeasts possess many features which make them particularly suitable as antagonistic agents. They have simple nutritional requirements and survive in a wide range of environmental conditions; they grow rapidly, colonizing fruit surfaces; they are tolerant of most agrochemicals and they do not produce anthropotoxic compounds (Richard and Prusky, 2002; Wilson and Wisniewski, 1989).

Yeasts can be effective biocontrol agents competing for space and nutrients with other microorganisms on colonized fruit surfaces (Filonow et al., 1996; Ippolito et al., 2000). This competition among microorganisms is expected to have a negative effect on mycotoxins production of spoilage fungi, since nutrient limitation inhibits secondary metabolism, including mycotoxin production (Luchese and Harrigan, 1993). Additionally, production of compounds inhibiting fungal growth and parasitism has also been described (El-Tarabily and Sivasithamparam, 2006; Pimenta et al., 2009).

In order to select a favourable biocontrol agent it is essential to isolate candidate microorganisms from ecological niches similar to those of the target pathogen (La Penna et al., 2004). In this context, several epiphytic yeasts isolated from the surface of grape berries have been reported as potential biocontrol agents against ochratoxigenic *Aspergillus* spp. (Bleve et al., 2006; Dimakopoulou et al., 2008; Ponsone et al., 2011; Zahavi et al., 2000). In particular, Zahavi et al. (2000) reported that one *Candida guilliermondii* isolate from grapes in Israel was able to reduce decay caused by *Botrytis*, *Rhizopus* and *Aspergillus*. Additionally, Bleve et al. (2006) showed that two *Issatchenkia orientalis* isolates strongly reduced *A. carbonarius* and *A. niger* colonization on grape berry; moreover they found that one each of *Metschnikowia pulcherrima*, *Issatchenkia terricola* and *Candida incommunis* also inhibited infection of grape berries by *A. niger* and *A. carbonarius*. Promising results were also obtained in another study by using one strain of *Aureobasidium pullulans* isolated from grapes in Greece against *A. carbonarius* (Dimakopoulou et al., 2008). It was demonstrated that the yeast isolate was able to reduce sour rot infection and inhibit the fungal growth on berries at harvest. Additionally, the yeast isolate was effective in reducing OTA contamination in must. More recently, it was demonstrated that two epiphytic strains of *Kluyveromyces thermotolerans* were able to control *A. carbonarius* and *A. niger* aggregate species growth in the field and reduce OTA accumulation (Ponsone et al., 2011).

The main objectives of this study were: (a) to isolate and identify epiphytic yeasts from grapes collected from Cyprus vineyards, (b) to evaluate yeast isolates for their efficacy in controlling infection by *A. tubingensis* derived from *Aspergillus* populations present in the winemaking regions of Limassol, Cyprus (unpublished data). The evaluation of the antagonists was performed *in vitro* by the dual culture technique in agar plates and the isolates exhibiting antagonistic characteristics were further evaluated for their ability to protect wine grape berries from *A. tubingensis* infection, using a detached berry test in laboratory-scale experiments. The positive results of several yeast isolates warrant further investigation for application in field conditions in order to identify strains that have negative effects on ochratoxigenic fungi proliferation in grapes and must products.

2. Materials and methods

2.1. Isolation of antagonistic yeasts from grapes

The isolation of yeasts from grapes was performed as described previously (Dimakopoulou et al., 2008) with small modifications. Grape berries belonging to cv. "Cabernet sauvignon" and "Maratheftiko" – a grape variety indigenous to Cyprus (Hvarleva et al., 2005) – were sampled from six vineyards in Koilani, Agios Amvrosios, Pachna and Arsos, representative of the major grape-growing areas of Limassol district, an important wine-producing area in Cyprus. Grape sampling was carried out in 2010 and epiphytic yeasts were recovered from the surface of grape berries. Ten plants were marked along two major diagonals of each vineyard. Three bunches were collected from the central part of each plant. The samples were kept in paper bags and stored in portable refrigerators during transfer to the laboratory for isolation of yeasts. From each vineyard ten different bunches were randomly selected and from each bunch ten berries were collected and transferred in sterile distilled water (SDW) containing 0.02% Tween-20. The berries were shaken at room temperature for 10 min at 150 rpm and then 10-fold dilutions were prepared in SDW. From each dilution 200 µl were plated in Petri dishes containing yeast malt agar (YMA, containing 3 g yeast extract, 3 g malt extract, 5 g bactopeptone, 10 g glucose, 15 g agar per lt). Petri dishes were