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International Journal of Food Microbiology 108 (2006) 204–209
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape

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Received 16 May 2005; received in revised form 18 November 2005; accepted 2 December 2005

Abstract

Antagonistic yeasts were isolated from the epiphytic flora associated with grape berries cv. Negroamaro and identified at species level using molecular methods. A total of 144 yeast isolates were tested in a preliminary screening on agar to select isolates showing a killer activity against *Aspergillus carbonarius* and *A. niger*, the main species responsible for the accumulation of ochratoxin A in grape. Twenty-eight yeast isolates were selected for their inhibitory effects on the above fungal species and assayed by an in vitro nutritional competition test for their antagonistic capacity towards three selected ochratoxigenic strains. Six yeast isolates belonging to five species, namely 2 isolates of *Issatchenkia orientalis* and one each of *Metschnikowia pulcherrima*, *Kluyveromyces thermotolerans*, *Issatchenkia terricola* and *Candida incommunis*, were finally selected and screened on wounded grape berries for their ability to inhibit infection by ochratoxigenic moulds. With the exception of the *K. thermotolerans* isolate, when inoculated at 10^9 CFU/wound, the other five challenger yeasts reduced the *A. carbonarius* and *A. niger* colonization on grape berry ($P<0.05$). In particular, the best antagonistic activity was shown by the two *I. orientalis* isolates. Results suggest that antagonist yeasts with the potential to control *A. carbonarius* and *A. niger* on grape can be found among the microflora associated with the berries.

Keywords: *Aspergillus*; Grape; Epiphytic yeast; Growth inhibition; Biocontrol agents

1. Introduction

Biological control is the use of living agents to control pests or plant pathogens. This approach is being increasingly considered by the scientific community as a reliable alternative to pesticide utilization in field and in post-harvest. This biological approach is highly desirable for controlling fungal growth on grapes, helping to reduce the amount of agrichemical residues in grapes, wine and related products (Cabras et al., 1999; Cabras and Angioni, 2000). Yeasts possess many features which make them eligible as biocontrol agents in fruits and other foods. They have (i) simple nutritional requirements, (ii) the capacity to grow in fermenters on inexpensive media, (iii) the ability to survive in a wide range of environmental conditions, (iv) no production of anthropotoxic compounds (Wilson and Wisniewski, 1989). Antagonistic yeasts were shown to reduce the growth of filamentous spoilage moulds both in vitro and in vivo (McGuire, 1994; Petersson and Schnürer, 1995). The antagonistic yeast *Pichia anomala*, for example, has been shown to reduce the in vitro fungal biomass of *Penicillium roquefortii* and *Aspergillus candidus* (Petersson and Schnürer, 1995). However, biocontrol microorganisms can inhibit the growth of the infecting fungi without reducing the metabolic activity of the active hyphae. Competition among microorganisms for essential environmental factors, such as nutrients and space, is expected to have a dramatic effect on the secondary metabolism of spoilage moulds. In particular, nutritional competition has been reported to play a fundamental role in yeast–mould interactions (Chalutz et al., 1988; Bjornberg and Schnürer, 1993; Chand-Goyal and Spotts, 1996). In fact, nutrient availability strongly affects secondary metabolism, including mycotoxin production (Luchese and Harrigan, 1993). Black aspergilli are good representatives of the mycobiota associated with grapes, and include a high percentage of ochratoxin-producing isolates particularly within the *Nigri* section (Cabanes et al., 2002; Battilani et al., 2003). The chlorinated isocoumarin compound, ochratoxin A (OTA), together with some related derivatives, is produced by *Penicillium*

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0168-1605/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2005.12.004

verrucosum and by several species of *Aspergillus*, including *A. carbonarius, A. ochraceus* and *A. niger*. Ochratoxin A is a potent nephrotoxic and carcinogenic compound, its potency varying markedly according to animal species and sex; it is also teratogenic and immunotoxic (Elling et al., 1985; IARC, 1993). The occurrence of OTA in raw agricultural products has been amply documented worldwide in a variety of plant products and has also been reported frequently in wine and grape juice (Zimmerli and Dick, 1996; Visconti et al., 1999; Pietri et al., 2001). It is almost impossible to eliminate the mycotoxin from the grape, mainly because it is highly stable and the producing fungi are an integral part of the microbiota associated with grape. Current research is aimed at developing natural biocontrol strategies, in order to reduce the presence of ochratoxigenic fungi in various commodities (Bhatnagar et al., 2002). The aim of the present work was to identify epiphytic yeasts which would show antagonist activity against *A. carbonarius* and *A. niger* occurring on grape berries, and which may have the potential to prevent the proliferation of ochratoxigenic fungi in grapes and derived products.

2. Materials and methods

2.1. Isolation and identification of yeast isolates

Grape (*Vitis vinifera*) berries belonging to the "Negroamaro" cultivar were sampled in the Copertino and Galatina areas, two of the most representative areas of the Salento region, an important wine-producing area in Southern Italy. Epiphytic yeasts were isolated from the grapes by washing berries (10 for each of the two samples) in 50 ml of sterile water on a rotary shaker at 200 rpm for 30 min. The sample was centrifuged at 5000 ×*g* for 10 min and the sediment was recovered and suspended in 1 ml of Yeast Peptone Dextrose medium (YPD; Sigma-Aldrich, St. Louis, USA). Sample dilutions from 10^{-1} to 10^{-4} were prepared on YPD with 2% agar added. After incubation at 28 °C for 48 h, the agar plates carrying the higher sample dilutions (10^{-2} to 10^{-4}) were used to isolate yeast colonies which were submitted to molecular methods for identification. The isolates were identified according to the polymorphisms of the rDNA region spanning the 5.8S rRNA gene and flanking the internal transcribed spacers 1 and 2 (5.8-ITS; White et al., 1990). The 5.8S-ITS region was amplified by polymerase chain reaction (PCR) using ITS1 (5′ TCCGTAGGTGAAC CTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) primers, previously described by White et al. (1990). The PCR conditions were identical to those described by Esteve-Zarzoso et al. (1999). The amplified DNAs (0.5–10 μg) were digested without further purification with the restriction endonucleases *Hinf*I and *Hae*III (Roche Molecular Biochemicals, Mannheim, Germany) according to the supplier's instructions. The PCR products and their restriction fragments were separated on 1% and 3% agarose gels, respectively, with 1X TAE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8). After electrophoresis, gels were stained with ethidium bromide (5 μg/ml) and visualized under UV light (300 nm). A 100-bp DNA ladder marker (Invitrogen, Carlsbad, USA) served as the standard size.

2.2. DNA sequencing

In order to obtain a DNA template suitable for direct sequencing, the PCR products were purified by the PCR Purification Spin Kit (Genomed, Löhne, Germany) and quantified by agarose gel analysis. The PCR sequencing mix (final volume, 20 μl) contained 1× Ready mix (Applied Biosystems, Foster City, USA), 1 μl of 3.2 μM ITS1 primer and 300 ng amplicon DNA. Reactions were run using a PCR Express System (Hybaid, Franklin, U.S. A.), for an initial denaturation at 96 °C for 2.5 min and for 25 cycles of 10 s at 96 °C, 10 s at 56 °C, and 4 min at 60 °C. After PCR reactions, the sample was purified and then sequenced by the ABI PRISM 310 sequencer (Applied Biosystems, Foster City, USA). Data output were analysed by the Chromas program version 1.45 and sequences were identified by a database similarity search in the GENBANK Collection using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. Fungal strains and preparation of the inocula

Two strains of *Aspergillus carbonarius* (ITEM 5010 and ITEM 5012) and one strain of *A. niger* (ITEM 7096), included in the ITEM Collection (http://www.ispa.cnr.it/Collection/), were selected for this work based on their high virulence and OTA production level (G. Perrone, unpublished data). Fungal cultures were maintained on slants of potato dextrose agar (PDA; Sigma-Aldrich, St. Louis, USA) at 25 °C. Spore suspensions of both *A. carbonarius* and *A. niger* were prepared by collecting spores from 5-day-old colonies (grown on PDA at 25 °C) in peptone-water (2 g/l) with 0.015% Tween 80 added to assist the dispersal of conidia. The spore concentration was enumerated by a hemocytometer. Yeast cultures were maintained on slants of YPD agar. Suspensions were prepared by inoculating 100 ml of YPD with a loopful of cells and incubation on a rotary shaker (180 rpm) at 28 °C for 24 h. The density of yeast cultures was determined spectrophotometrically by measuring their optical density (OD) at 600 nm.

2.4. Biotypization of yeast isolates

The sensitivity level revealed by the two *A. carbonarius* and the *A. niger* strains to the action (killer activity) of yeast was used as a discriminative parameter in a preliminary in vitro screening and characterization of the 144 yeast isolates. This experiment was carried out according to the procedures of Polonelli et al. (1987). Briefly, one ml of a 10^6CFU/ml conidial suspension was mixed with 20 ml of Sabouraud agar (Sigma-Aldrich, St. Louis, USA), buffered at pH 4.5 with methylene blue (0.003%), maintained at 45 °C and poured into a Petri dish. A heavy loopful of each yeast grown on YPD agar (for 48 h at 28 °C) was streaked onto the surface of the agar. The plates were then incubated at 25 °C for 7 days. The killer effect was considered positive when a clear zone of inhibition surrounded the streak of the challenger yeast. A numeric code, arranged into triplets, was used to record the inhibitory effect induced by each yeast isolate to the growth of the three fungal strains (Table 1). The pattern of the fungal sensitivity was recorded for each yeast isolate adopting a

conventional code (Table 1), ranging from the value 7 (all three fungal isolates revealed susceptibility to the yeast action) to the value 0 (no yeast effect on fungal growth).

2.5. Inhibition of *A. carbonarius* and *A. niger* by epiphytic yeast isolates on agar plates

Yeast and mould spore suspensions were prepared as described above. Inhibition experiments were performed on YPD agar medium. A top agar was prepared by mixing 6 ml of YPD with 0.7% agar and 1 ml of yeast suspension containing 10^6 cells. The agar–yeast suspension was poured into Petri dishes that contained 15 ml of the YPD agar medium. Once the top agar had set, three 10-μl portions of a mould suspension (10^5 CFU/ml) were separately spotted on each plate. Three replicate experiments for each fungal strain were performed. Moulds were spotted to make it possible to measure the radial extension rates of the colonies. Plates not inoculated with yeasts were used as control. Fungal growth inhibition was determined as the percent of colony diameter decrease compared to the control.

2.6. Inhibition of *A. niger* and *A. carbonarius* by epiphytic yeast isolates on wounded berries

Mature grape berries of the "Negroamaro" cultivar, obtained from a local market in Apulia, Southern Italy, were surface disinfected with 1% sodium hypochlorite for 2 min and rinsed twice with distilled water. A calibrated wound (about 2 mm diameter) was made with a sterile needle on each fruit and a water suspension (20 μl) of antagonist yeast (1×10^9 CFU/ml) was inoculated into the wound, followed by inoculation of 20 μl of an aqueous suspension of fungal conidia (5×10^4 CFU/ml). Each sample, constituted by 9 berries and reproduced with three replicates for each yeast isolate, was incubated for 6 days at 25 °C in a plastic box under high relative humidity (100%). The results obtained are the mean of three independent experiments. A positive control was performed with berries inoculated only with sterile water and then with *A. niger* or *A. carbonarius* suspension (5×10^4 CFU/ml). After incubation, each single experimental sample (9 berries) was homogenized and decimal dilutions of the slurry thus obtained, corresponding to 1g of infected tissue, were spread onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar plates. CFUs were counted after 3 days' incubation at 25 °C.

Table 1
Coding of the antagonistic effect of the selected yeast isolates towards the fungal testing strains of *Aspergillus carbonarius* (ITEM 5012 and ITEM 5010) and *A. niger* (ITEM 7096)

Code	*A. carbonarius*		A. niger
	5012	5010	7096
7	+	+	+
6	−	+	+
5	+	−	+
4	+	+	−
3	−	−	+
2	−	+	−
1	+	−	−
0	−	−	−

The pattern of fungal sensitivity was indicated for each yeast isolate by a conventional code, ranging from the value 7 (all three fungal isolates revealed susceptibility to the yeast action) to the value 0 (no yeast effect on fungal growth).

2.7. Statistical analysis

Statistical software SPSS 8.0 (SPSS Inc., Chicago, U.S.A.) was used for data analysis. Before performing ANOVA and the Duncan's test ($P<0.05$), the CFU/g values were log transformed.

3. Results and discussion

The Negroamaro grape cultivar has been shown to be sensitive to infection caused by ochratoxin A-producing fungi (Battilani et al., 2003). In order to study the epiphytic yeast population associated with this grape variety, the microflora was directly collected from the berry surface and 144 yeast isolates were taken at random. The epiphytic yeast isolates were identified by PCR amplification of the 5.8-ITS region, which had proven to be highly discriminative for yeast identification (White et al., 1990). The PCR products obtained, ranging from 400 to 750 bp, were separately digested with *Hinf*I and *Hae*III restriction enzymes. Coupled digestion analyses for *Hinf*I/*Hae*III revealed six different profiles which were identified by comparison with those previously described for several yeast species (Esteve-Zarzoso et al., 1999). At species level, the yeast isolates belonged to six taxonomically distinct groups: *Metschnikowia pulcherrima* (58% of the total population identified), *Hanseniaspora uvarum* (36%), *Issatchenkia orientalis* (2%), *Issatchenkia terricola* (2%), *Candida incommunis* (1.3%) and *Kluyveromyces thermotolerans* (0.7%). Three amplicons were selected from each group, deriving from three isolates representative of the group, and submitted to direct sequencing to confirm the species identification.

The epiphytic population identified was exclusively composed of non-*Saccharomyces* yeast species, which had been previously shown as the typical microflora associated with grape in the Mediterranean regions (Versavaud et al., 1995; Granchi et al., 1999; Pramateftaki et al., 2000; Povhe Jemec et al., 2001; Clemente-Jimenez et al., 2004). A preliminary biotyping of the 144 identified yeast isolates was obtained by evaluating the antagonistic effect against *A. carbonarius* and *A. niger* by an in vitro co-inoculation (fungus–yeast) assay performed on agar plates. This effect was considered as indicative of the fungal sensitivity to the action of a yeast isolate present in the same biological niche. We selected OTA-producing isolates belonging to *A. carbonarius* species, because they play a significant role as the source of OTA contamination in wine (Cabanes et al., 2002). An *A. niger* isolate was also considered in this study since this species is associated with bunch rot disease complex and some of its strains have been reported to be OTA producers (Battilani et al., 2003).

As shown in Table 2, 28 isolates, representing 20% of the analyzed population, showed a pattern ranging from 7 to 1 (isolates scoring 0 were not reported). Among the six species

identified, all *H. uvarum* isolates failed to induce toxic effects on the challenger fungal strains. Two yeast isolates (16C2 and 17C2), belonging to the *I. orientalis* group, were active both against the two *A. carbonarius* (ITEM 5012 and ITEM 5010) and against the *A. niger* (ITEM 7096) isolates (Code 7), whereas isolates belonging to *I. terricola*, *C. incommunis* and *K. thermotolerans* induced inhibitory effects on two of the three fungal strains assayed (Codes 4, 5 and 6). A high number of *M. pulcherrima* isolates, namely 25 out of the 83 identified, were active against challenger moulds, and 5 of them induced susceptibility in two of the three fungal strains. This finding is consistent with a similar in vitro behavior described for this yeast species against *Penicillium glabrum* (Sinigallia et al., 1998) and *Botrytis cinerea* (Guinebretiere et al., 2000).

Thirteen yeast isolates with scores of between 4 and 7 were selected and further characterized by using a nutritional competition assay. This test was proposed in order to select yeast strains able to overwhelm the co-inoculated fungi when colonizing a common ecological niche.

The isolate 5C1 (*H. uvarum*), which did not induce sensitivity in any of the fungal strains, was also used as a supplementary negative control in this in vitro test. All thirteen isolates analyzed caused severe growth reduction in *A. carbonarius* (ITEM 5012 and ITEM 5010) and *A. niger* (ITEM 7096) (Table 3). This inhibitory effect was induced at a yeast concentration of 10^6 CFU/ml, which was previously shown to be effective against *P. glabrum* (Sinigallia et al., 1998), *P. roqueforti* (Petersson and Schnürer, 1995) and *P. verrucosum* (Petersson et al., 1998). In particular, the isolates 16C2 and 17C2 (*I. orientalis*) completely inhibited the growth of challenger moulds on agar plates, whereas the *H. uvarum* isolate (5C1) exhibited a low degree of inhibition of the fungal radial extension rate. Strong inhibitory effects were produced by six yeast isolates, namely *M. pulcherrima* 20C1, *I. orientalis* 2C2, *I. orientalis* 16C2, *K. thermotolerans* 26K1, *I. terricola* 12K2, and *C. incommunis* 24K2, by the in vitro nutritional competition assays on agar plates. The inhibitory effect was further proven on wounded grape berries by co-inoculation of the yeast isolates with *A. niger* and *A. carbonarius* test strains. Mould growth was quantified as the number of CFUs formed after spreading a sample deriving from a homogenate of the treated berries onto selective agar medium. As reported during the characterization of *Pichia anomala* strains as antagonists of *P. roqueforti* and *A. candidus* (Bjornberg and Schnürer, 1993), the determination of mould CFU per gram has proven to be as efficient a parameter as the measurement of hyphal length for evaluating the level of fungal growth. Indeed, these data were then used in the Duncan's test to reveal the significant differences existing between the mean CFU values produced by each mould grown in the presence of each selected epiphytic yeast, and the CFU values deriving from the positive control (containing moulds but no challenger yeast) (Fig. 1). When inoculated at 10^9 CFU/wound, five out of the six challenger yeasts reduced conidiophore production (Duncan test, $P<0.05$), with the exception of the isolate 26K1 (*K. thermotolerans*) which did not reduce the growth of the *A. carbonarius* strains. By contrast, the comparison of fungal growth levels in the absence of antagonist (ITEM 5012, Log CFU/g=6.7±0.1; ITEM 5010, LogCFU/g=6.7±0.1; ITEM 7096,

Table 2
Susceptibility of *Aspergillus carbonarius* (ITEM 5012 and ITEM 5010) and *A. niger* (ITEM 7096) to yeast isolates co-inoculated on agar plates

Yeast isolates		*A. carbonarius*		*A. niger*	Code
Nr.	Species	5012	5010	7096	
9C1	*Metschnikowia pulcherrima*	−	+	−	2
17C1	*Metschnikowia pulcherrima*	+	−	−	1
18C1	*Metschnikowia pulcherrima*	+	−	−	1
19C1	*Metschnikowia pulcherrima*	+	+	−	4
20C1	*Metschnikowia pulcherrima*	+	+	−	4
21C1	*Metschnikowia pulcherrima*	−	+	−	2
30C1	*Metschnikowia pulcherrima*	−	+	−	2
35C1	*Metschnikowia pulcherrima*	+	+	−	4
1C2	*Metschnikowia pulcherrima*	+	+	−	4
2C2	*Issatchenkia orientalis*	+	+	−	4
16C2	*Issatchenkia orientalis*	+	+	+	7
17C2	*Issatchenkia orientalis*	+	+	+	7
20C2	*Metschnikowia pulcherrima*	−	+	−	2
14C3	*Metschnikowia pulcherrima*	−	+	−	2
17C3	*Metschnikowia pulcherrima*	+	−	−	1
18C3	*Metschnikowia pulcherrima*	+	−	−	1
1K1	*Metschnikowia pulcherrima*	+	−	−	1
4K1	*Metschnikowia pulcherrima*	+	−	−	1
11K1	*Metschnikowia pulcherrima*	+	−	−	1
12K1	*Metschnikowia pulcherrima*	+	−	−	1
17K1	*Metschnikowia pulcherrima*	−	−	+	3
18K1	*Metschnikowia pulcherrima*	−	+	+	6
26K1	*Kluyveromyces thermotolerans*	+	−	+	5
5K2	*Metschnikowia pulcherrima*	+	−	−	1
6K2	*Metschnikowia pulcherrima*	−	+	+	6
12K2	*Issatchenkia terricola*	+	+	−	4
23K2	*Issatchenkia terricola*	−	+	−	1
24K2	*Candida incommunis*	−	+	+	6

+ antagonistic effect; − no effect.

Table 3
Percentage of growth reduction of *Aspergillus carbonarius* (ITEM 5012 and ITEM 5010) and *A. niger* (ITEM 7096) caused by yeasts isolated from grape berries in a nutritional competition assay

Yeast isolates		*A. carbonarius*		*A. niger*
Nr.	Species	5012	5010	7096
PC	−	0	0	0
5C1	*H. uvarum*	57^+	60^+	44^+
19C1	*M. pulcherrima*	77^+	72^+	100
20C1	*M. pulcherrima*	100	77^-	100
35C1	*M. pulcherrima*	77^-	83^-	88
1C2	*M. pulcherrima*	83^+	77^+	100
2C2	*I. orientalis*	83^+	83^+	100
16C2	*I. orientalis*	100	100	100
17C2	*I. orientalis*	100	100	100
18K1	*M. pulcherrima*	86^+	77^+	78^+
26K1	*K. thermotolerans*	88^-	80^-	100
6K2	*M. pulcherrima*	83^-	71^-	100
12K2	*I. terricola*	100	74^-	100
24K2	*C. incomunis*	80^+	74^+	100

Percent reduction=(diameter of fungal colony growth on medium with yeast/without yeast)×100.
PC = positive control.
+ = presence of sporification.
− = absence of sporification.

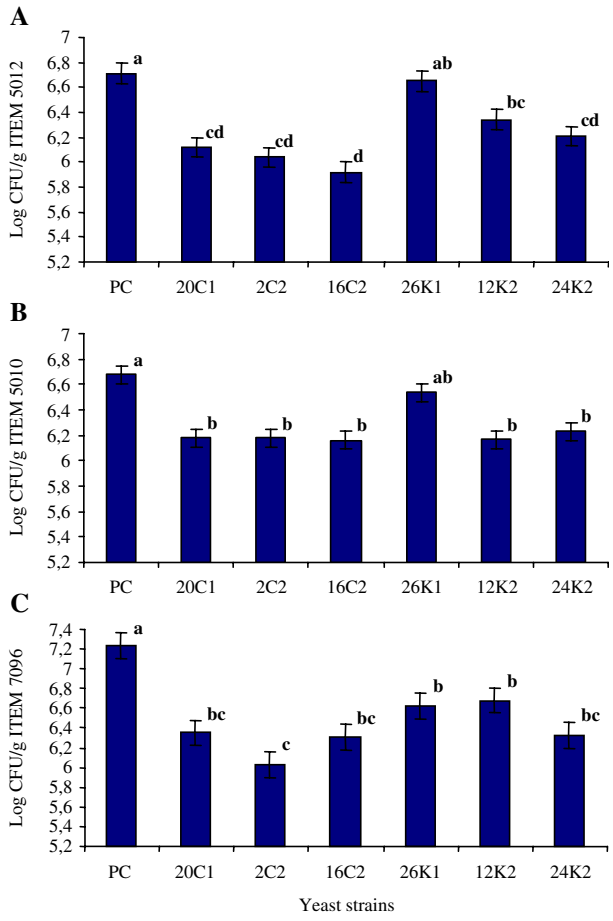

Fig.1. Effect on fungal growth of antagonistic yeasts co-inoculated with *Aspergillus carbonarius* ITEM 5012 (A) and ITEM 5010 (B) and *A. niger* ITEM 7096 (C) on grape berries. Bars represent the mean fungal concentrations (expressed as LogCFU/g) from three independent experiments (±standard deviation). Letters on the top of each bar indicate the results of the Duncan's test ($P<0.05$); values with shared letters in the same graph are not significantly different.

LogCFU/g=7.2±0.1) with those obtained by co-inoculation with 2C2 (ITEM 5012, LogCFU/g=6.0±0.3; ITEM 5010, LogCFU/g=6.2±0.3; ITEM 7096, LogCFU/g=6.0±0.2) and with 16C2 (ITEM 5012, LogCFU/g=5.9±0.1; ITEM 5010, LogCFU/g=6.2±0.2; ITEM 7096, LogCFU/g=6.3±0.3), showed that the *I. orientalis* isolates had the best antagonistic activity, thus confirming the very promising results obtained by inhibition experiments on agar plates.

The results of this study indicate that, among the microflora associated with grape berries, there are some antagonist yeasts which are eligible for the control of ochratoxin A-producing fungi on grape. Epiphytic yeasts are the major component of the microbial community on the surface of grape berries and they are evolutionarily adapted to this ecological niche (Beech and Davenport, 1970). Yeasts, indeed, may be an effective biocontrol tool because of their capacity to colonize grapes and compete for space and nutrients with other microorganisms (McLaughlin et al., 1990). Our findings showed that biocontrol activity is a strain-related characteristic and did not solely depend on species or genus, as observed by Suzzi et al. (1995) in a previous study on the antagonistic aptitudes of wine yeasts against plant pathogenic fungi. The screening of epiphytic yeasts showed, to our knowledge for the first time, that *I. orientalis* isolates have a strong antagonistic action against two ochratoxigenic species. Moreover, we were able to identify an isolate (20C1) of *M. pulcherrima* and an isolate (24K2) of *C. incommunis*, which also significantly reduced *A. niger* and *A. carbonarius* growth on agar plates as well as on grape berries. The antagonistic action exhibited by the above yeast isolates is consistent with a number of previous studies: *M. pulcherrima* has already shown the ability to control postharvest diseases in strawberry (Guinebretiere et al., 2000) and *Candida* spp. have been found to be highly effective against fungal pathogens (McLaughlin et al., 1990, 1992; Vinas et al., 1988; Guinebretiere et al., 2000). Several mechanisms have been suggested to operate on biocontrol, including antibiosis (Janisiewicz and Roitman, 1988), parasitism (Winiewski et al., 1991), induced resistance (El-Ghaouth et al., 1998) and competition for space and nutrients, which is often indicated as the way in which biocontrol agents act against pathogens in fruit orchards (Wilson and Wisniewski, 1989; Droby et al., 1989; Santos et al., 2000).

All the yeast strains selected in this study for the biocontrol of black aspergilli in grape (Table 3) showed a comparable growth rate when inoculated, in the absence of fungal spores as control, into grape wounds (G. Cozzi, personal communication). Because growth to similar biomass levels would require consumption of approximately equal amounts of nutrient, general nutrient competition in the grape berry is not in itself sufficient to explain yeast biocontrol activity. Nevertheless, this finding does not exclude the hypothesis that the antagonistic behaviour shown by the *I. orientalis* isolates could be the effect of competition for a specific growth limiting factor, e.g., a vitamin or another particular metabolite. It could be also hypothesized that the two *I. orientalis* isolates (2C2 and 16C2) might produce a secondary metabolite(s) with antifungal properties, as suggested by the data obtained through in vitro biotypization of yeast isolates, i.e. by the presence of a clear zone of inhibition surrounding the streak of 2C2 and 16C2 isolates. However, experiments are now under way to better ascertain the mechanism by which these *I. orientalis* isolates inhibit *A. carbonarius* and *A. niger*. Even though further investigations are needed to assess whether these yeast isolates have practical value in the control of ochratoxin A-producing fungi occurring on grapes, the data reported herein indicate that these yeasts, which occur naturally on grapes, are promising "ecological fungicides," which will not interfere with the equilibrium of the natural environment.

Acknowledgements

This research was supported by a grant from the Italian Ministry of Scientific and Technological Research (MURST) Project 12818 — SIVINA — "Individuazione di metodologie innovative prontamente trasferibili per migliorare la sicurezza dei vini rossi di qualità del Salento." The authors wish to thank Dr. Cosimo Vetrano for his valuable assistance in isolating yeast strains and Dr. Giancarlo Perrone (ISPA) for supplying

Aspergillus isolates. We would also like to thank Dr. Anthony Green for proofreading and providing valuable linguistic advice.

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