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Journal of Plant Pathology

ISSN 1125-4653
Volume 100
Number 2

J Plant Pathol (2018) 100:179-190
DOI 10.1007/s42161-018-0042-x

Springer

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https://doi.org/10.1007/s42161-018-0042-x

ORIGINAL ARTICLE

Characterization of antagonistic microorganisms against *Aspergillus* spp. from grapevine leaf and berry surfaces

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Published online: 12 April 2018

Abstract
This study aimed at controlling the common fungi causing postharvest *Aspergillus* rot of cv. Thompson seedless table grape (*Vitis vinifera* L.) via application of epiphytic biocontrol agents. Antagonistic yeasts and bacteria were isolated from the epiphytic flora associated with grape berries and leaves from five vineyards in Iran. A total of 130 yeast and bacterial isolates from grapevine surfaces were screened for antagonism against *Aspergillus flavus*, *A. niger* and *A. ochraceus*, the main species responsible for the accumulation of aflatoxin and ochratoxin A in grape berries. Seven yeast and bacterial isolates were selected based on their inhibitory effects on *Aspergillus* spp. and assayed by an in vitro nutritional competition test for their antagonistic capability. These isolates showed obvious antifungal activity against three different species of *Aspergillus*. Five yeast isolates were identified based on ITS region sequences as *Candida membranifasciens* (isolates Ka15 and Kh69) and *Meyerozyma guilliermondii* (Ka21, Kh59 and Kh60). Two bacterial isolates were identified based on the 16S rRNA gene sequences as *Bacillus* sp. (Ka3 and A10). Finally, the effect of antagonistic isolates on inoculated grape berries for their ability to inhibit infection by *Aspergillus* spp. was also investigated. All isolates showed antagonistic properties against the pathogens assayed at 25 °C and significantly reduced the disease progress on grape berries. Our data demonstrated that application of antagonistic microorganisms could be a promising alternative to fungicide treatments for controlling postharvest diseases of grapevine.

Keywords *Aspergillus* spp. · Biocontrol agents · Epiphytic flora · Grapevine · Mold

Introduction

Postharvest losses of fruits, including pome fruits, stone fruits, small fruits and citrus, can be quite significant if handling, processing, and storage conditions are not optimal. Losses of up to 25% of the total production in industrialized countries and more than 50% in developing countries have been reported (Nunes 2012). High levels of decay caused by fungal pathogens can directly be attributable to the large amount of nutrients and water that these fruits contain, the low pH, and the decrease in their intrinsic decay resistance after harvest (Droby et al. 1992). The grapevine (*Vitis vinifera* L.) is a widespread crop producing fruits (grapes) with a relatively low rate of physiological activity that are among the most therapeutically and economically important in the world. However, 30–96% of table grapes are lost every year due to non-suitable handling and the lack of proper methods to prevent decay and senescence (Prusky 2011; Thomidis et al. 2016). Even in cold storage (0 °C), grapes are affected by blue molds (*Penicillium* spp.), black molds (*Aspergillus* spp.), gray molds (*Botrytis cinerea*), Alternaria rot (*Alternaria alternata*), and Rhizopus rot (*Rhizopus stolonifer*) (Karabulut et al. 2003; Romanazzi et al. 2012; Senthil et al. 2011).

Aspergillus rot is among the most important post-harvest disease of grapes, which has received much attention because of the contamination by fungi of the genus *Aspergillus*, i.e. the predominant fungal species infecting grapes worldwide (Madden et al. 2017; Serra et al. 2005), cause grape rot and produce several mycotoxins (Allam et al. 2008, 2012).

Postharvest diseases often account for a major part of the losses and their control requires the use of a large amount of fungicides (Wilson and Wisniewski 1994). Traditionally, postharvest diseases are controlled by the application of synthetic fungicides (Eckert and Ogawa 1988) such as fenhexamid, iprodione, cyprodinil, boscalid, pyrimethanil which are generally used in different phenological growth stages of the

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grapevine for the management of fungal decay incidence (Diguta et al. 2010; Gabriolotto et al. 2009). Although the use of these chemicals is the principal method for controlling postharvest diseases and the rots they cause, stricter regulatory policies are being imposed on their employment. However, due to problems related to fungicide toxicity, development of resistant strains of pathogens, and potential harmful effects on the environment and human health, alternative control methods based on biocontrol of phytopathogens have been proposed (Zahavi et al. 2000; Jiang et al. 2014).

In recent decades, a number of different microorganisms including bacteria, filamentous fungi, and yeasts have been isolated and shown to protect fruits against postharvest pathogens; some of them have been utilized to develop commercial products such as Aspire, Bio-Save 10 and 11, Yield Plus, and Serenade Max (Droby et al. 2000; Thomidis et al. 2016). Considerable success has been achieved by applying antagonistic microorganisms to control plant diseases, postharvest diseases and mycotoxins production (Visconti et al. 2008; Abrunhosa et al. 2010; Zhimo et al. 2016). The use of yeast or bacterial strains to control postharvest fungal decay of several fruits has extensively been studied, and several examples of successful disease control exist (Kim et al. 1997; Raspor et al. 2010; Jiang et al. 2014). Numerous studies have been focused on isolation and identification of antagonistic yeasts and yeast-like fungi such as *Aureobasidium pullulans* (Schena et al. 2003; Raspor et al. 2010), *Pichia guilliermondii* (Raspor et al. 2010; Chalutz et al. 1988), *Metschnikowia fructicola* (Karabulut et al. 2003), *Metschnikowia pulcherrima* (Raspor et al. 2010; Csutak et al. 2013), *Saccharomyces cerevisiae* (Raspor et al. 2010; Nally et al. 2013), *Cryptococcus laurentii* (Liu et al. 2002) *Issatchenkia terricola*, (Bleve et al. 2006) *Candida incommunis* (Bleve et al. 2006), and *Kluyveromyces thermotolerans* (Bleve et al. 2006). Yeasts can be effective biocontrol agents competing for space and nutrients with other microorganisms on colonized fruit surfaces (Filonow et al. 1996). Additionally, the production of metabolites inhibiting fungal growth and parasitism has also been described (Pimenta et al. 2009).

In recent decades, there has been continued and rigorous research worldwide with a greater focus on exploring a wide range of bacteria possessing antagonistic properties. They are also known to affect life cycles of different plant pathogens or pests by diverse mechanisms including production of extracellular metabolites and intracellular toxins. Some bacterial isolates can inhibit the growth of many pathogens (Chen et al. 2008; Leelasuphakul et al. 2008), including *Aspergillus* spp. (Foldes et al. 2000).

The aim of this research was to isolate and identify grapevine epiphytic yeasts and bacteria and to evaluate their effectiveness against *Aspergillus* species causing decay of grape berries both in vitro and in vivo.

Materials and methods

Isolation of antagonistic yeasts and bacteria from grapes and inoculum preparation One hundred and thirty samples were obtained from berries and leaves of the grapevine cv. Thompson seedless in the most important raisin-producing regions of eastern Iran. Ten plants were sampled along two major diagonals of each vineyard. Three bunches and leaves were collected from the central part of vines without any signs of infection. The samples were kept in paper bags and stored in portable refrigerators during transfer to the laboratory for isolation of yeasts and bacteria. From each vineyard, 10 different bunches were randomly selected and from each bunch 10 berries were collected and transferred to sterile distilled water (SDW) containing 0.02% Tween 20. Leaves were also collected and soaked in SDW. Epiphytic microorganisms were isolated by shaking berries and leaves in 100 ml of SDW for 1 h at 100 rpm on a rotary shaker (Peng and Sutton 1991). The wash was serially diluted and 1 ml of each dilution was spread on potato dextrose agar (PDA) and nutrient agar (NA) media. The Petri dishes were incubated at 28 °C for 4 days and colonies were selected randomly according to the color and morphological characteristics, were removed with a sterile loop and transferred to fresh PDA and NA plates to obtain pure cultures. Finally, the isolates were kept in tubes containing SDW at 4 °C for short-term storage, and in 15% glycerol at −80 °C for long-term storage in the culture collection of Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran. For long term storage many yeast and bacterial cells from culture plates were transferred into microtubes containing 1 ml of 15% glycerol. Then, the cells were suspended by shaking (or vortexing if necessary) and stored at −80 °C (Sherman et al. 1986). In this study, a standard *Trichoderma harzianum* TBI isolate obtained from the culture collection of Ferdowsi University of Mashhad was used as a positive control.

For inoculum production, yeast and bacterial isolates were activated from stored stock cultures by transferring them to PDA and NA media, respectively. Yeast and bacterial suspensions were prepared by suspending 3 full transfer loops of the culture in 5 ml of SDW. Suspensions were adjusted to the desired concentration (1×10^7 cells ml^{-1}).

Preparation of the pathogens for inoculation In this study, *A. flavus*, *A. niger* and *A. ochraceus* isolates (accession Nos. KY695466, KY695465 and KY695464, respectively) that had been recovered from grape berries with mold symptoms were obtained from culture collection of Ferdowsi University of Mashhad. Fungal cultures were grown on PDA at 4 °C. Spore suspensions of *Aspergillus species* were prepared by collecting spores from 5-day-old colonies in SDW additioned with 0.02% Tween 20 to facilitate dispersal of conidia. Spore

concentration (1×10^5 cells ml^{-1}) was determined with a hemocytometer.

Inhibition of *Aspergillus* spp. by epiphytic yeasts and bacteria on PDA A total of 130 isolates of bacteria and yeasts were obtained from the surface of table grape berries and leaves and tested using a four-point test to determine the inhibitory effect on the growth of pathogenic fungi. Seven isolates (including 5 yeasts and 2 bacterial isolates) reduced pathogen growth and created an inhibition zone were selected for the next steps.

Dual culture assay The seven isolates mentioned above were tested in an in vitro preliminary screening to select those showing antagonism against *Aspergillus* spp. For this purpose, a loop of yeast and bacterial cells from 3-day-old cultures was streaked on a PDA plate (9 cm diameter) at ca. 2 cm distance from the rim of the plate. Then, a 5 mm disk of each *Aspergillus* species from a 5-day-old PDA culture was placed at the distance of 5 cm from the yeast or bacterium. Plates only with the *Aspergillus* species under test were used as control. Petri dishes were incubated at 28 °C for seven days and were daily observed for assessing the mycelial growth of the pathogen. Each yeast and bacterial isolate was tested three times in an experiment. Colony diameter was determined seven days post inoculation and the percentage of inhibition was calculated using the following formula (Mari et al. 1996):

$$R(\%) = (R_1 - R_2)/R_1 \times 100\%$$

where R is the percentage of inhibiting radial hyphal growth, R_1 is the hyphal growth of the control, and R_2 the hyphal growth in the Petri dish inoculated with yeast or bacterial isolates.

Volatile compounds assay The inhibitory effect of volatile metabolites produced by antagonistic isolates was evaluated using the method described by Arrebola et al. (2010). Briefly, yeast and bacterial cell suspensions (1 ml) were spread on PDA and NA media, respectively, and incubated at 28 °C for 24 h. A fungal disk of each pathogen was located at the center of PDA plates. Then two "sandwhich" Petri dishes were made by superimposing face up a dish with the culture of the antagonistic isolate with a dish of the pathogen face down. The sandwiched plates were sealed using parafilm and incubated at 28 °C. PDA plates containing each pathogen cultured under the same conditions were used as control. The hyphal growth (mm) of *Aspergillus* spp. was measured daily for seven days post inoculation using a Vernier caliper. The percentage of inhibiting radial growth (R) was calculated using the equation mentioned before.

Non-volatile compounds *assay* Production of non-volatile substances by antagonistic isolates against *A. flavus, A. niger* and *A. ochraceus* was studied using the modified method described by Kraus and Lopper (1990). Briefly, yeast and bacterial cell suspensions (1 ml) were spread on PDA and NA media, respectively, and incubated at 28 °C for 72 h. Then, yeast and bacterial colonies were cleaned from the surface of the media by sterile cotton under a laminar hood. Petri dishes were then cleaned with cotton soaked in chloroform and placed under UV light for 0.5 h. Then, the dishes were placed half open for 1.5 h for evacuating the chloroform vapours. Finally, the dishes were inoculated with a mycelial plug of each pathogen. The control was not inoculated with any of the antagonistic isolates. The dishes were incubated at 28 °C until the colony of control reached the edge of plate. Then, the percentage of inhibition was calculated as above.

Inhibitory effect of yeasts and bacteria on spore germination of *Aspergillus spp* Yeasts and bacterial isolates were assayed for their capability to inhibit spore germination of the pathogens at 25 °C according to the method described by Droby et al. (1997). A volume of 100 μl of suspended yeast or bacteria (1×10^7 cells ml^{-1}) and 100 μl of suspended pathogen conidia (1×10^5 conidia ml^{-1}) were added to an Eppendorf tube containing 800 μl potato dextrose broth (PDB). For the control, 100 μl of sterile distilled water without antagonist was added to a tube. The tubes were incubated for three days at 25 °C. Two 50 μl drops from each tube were placed on a microscopic slide and the number of germinated conidia was determined after one and three days. The spore germination inhibition index (GII) was calculated according to the formula described by Manici et al. (1997), where GII (%) = (spore germinated in control minus spore germinated in treatment) × 100/spore germinated in control.

Pathogenicity tests of the yeasts and bacteria on grape berries To determine if yeasts and bacteria selected in the previous assays were pathogenic to grapes, the berries were punctured with a cylindrical tool to produce a cut 3 mm in diameter and 3 mm deep. Then, 15 μl of suspended yeasts or bacteria (1×10^7 cells ml^{-1}) were inoculated in the wound made on grape berries that had been previously disinfected with 1% sodium hypochlorite solution for 1 min. The inoculated berries were placed in plastic boxes at 25 °C for seven days. The yeast or bacterial isolate was retained as pathogenic when an alteration of the berry tissue was observed (Vargas et al. 2012).

Inhibition of *Aspergillus* decay on wounded berries by epiphytic yeasts and bacteria All the five yeasts and two bacterial isolates, which showed the highest inhibitory effect on the pathogens in the previously described assays, were tested for their antagonistic activity against *Aspergillus* spp. in a

wounded berry test as described by Poppe et al. (2003). Clusters of cv. Thompson seedless were divided into smaller bunches with 10–15 berries that were homogenous in size and color, and showed no visible damage or mold. The berries were surface-disinfected by dipping in 1% sodium hypochlorite for 1 min, rinsed twice with SDW. and wounded as described before. Fifteen μl of an antagonist suspension (1×10^7 CFU/ml) were pipetted into each wound and left to dry for 1–2 h. The control was treated with the same volume of SDW under the same conditions. Then, each wound was inoculated with 15 μl of the spore suspension prepared from cultures of *A. flavus, A. niger* or *A. ochraceus* (1×10^5 spore/ml), individually. Inoculated bunches were air-dried, placed in covered plastic containers to maintain high humidity and were kept at 25 °C for seven days. The percentage of fungal growth inhibition was determined seven days after *Aspergillus* spp. inoculation, using the following formula:

$$[1-(\text{diameter of fungal colony growth on berry treated with yeasts and bacteria}/\text{without yeasts and bacteria})] \times 100$$

DNA extraction Total genomic DNA of yeasts was extracted by the hexadecyl trimethyl ammonium bromide method according to Zolan and Pukkila (1986). The DNA samples were stored at −20 °C until used. To identify bacteria showing greater antagonistic activity against pathogens, PCR templates were prepared from 1 to 3 ml of a liquid culture grown in lysogeny broth (LB) overnight. Bacteria were centrifuged at 13,000 *g* for 2 min and pellets were resuspended in 1 ml sterile H_2O. Samples were centrifuged again for 2 min, and resuspended again in 200 μl sterile H_2O and heated at 100 °C for 10 min. After cooling, the solution was centrifuged at 8000 rpm for 3 min and the supernatant was either directly used for PCR or, if necessary, for DNA purification by phenol/chloroform-extraction and precipitation in ethanol (Sambrook and Russel 2001).

Identification of antagonist isolates by rDNA sequence analysis Yeast identification was carried out by a molecular procedure based on PCR amplification of the 5.8 S rDNA using universal primers for fungi, including ITS1 (5` TCCGTAGG TGAACCTGCG G 3`) and ITS4 (5` TCCTCCGCTTATTG ATATGC 3`) as previously described (Drik 2000). For identification of bacteria, 16S rDNA was amplified from genomic DNA with the primers 27F (5` AGAGTTTG ATCMTGGCTCAG 3`) and 1492R (5` TACGGYTACCTTGTTACGACTT 3`) as described by Yashiro et al. (2011). The PCR products were separated in a 1% agarose gel in parallel with 100 bp Low DNA ladder (Sigma-Aldrich, USA) as a molecular size standard. After electrophoresis, the gel was visualized under UV light. The PCR products were sequenced by Macrogen Inc. (Korea) and the sequences were aligned with sequences in the BLAST databases of the NCBI site. The nucleotide sequences were deposited in GenBank under the accession numbers reported in Table 2.

Experimental design and statistical analysis The experiments were conducted in a complete randomized design with three replicates. All data obtained from the antagonistic activity experiments were analyzed by one-way ANOVA and the means were separated by Duncan test at 0.05 significance level. Statistical software SPSS 23 was used for data analysis.

Results

Isolation and selection of yeasts and bacteria Seven isolates (five yeasts and two bacterial isolates) which showed highest inhibitory effect on *Aspergillus* spp. were selected, that represented 5.4% of total epiphytic isolates obtained in this study. These isolates exhibited antimicrobial activity in vitro. This effect was considered as an indication of the pathogen sensitivity to the action of a yeast or bacterial isolate in the same biological niche. Also, these isolates were able to generate an inhibitory halo around the pathogen in dual cultures.

Effect of antagonistic isolates on mycelial growth of *Aspergillus* spp. in vitro Results of the dual culture experiments showed that all seven isolates tested were able to considerably inhibit mycelial growth of *Aspergillus* spp. in vitro. In the dual culture tests, formation of inhibition zones between colonies of the yeasts and bacteria with *A. flavus, A. niger* and/or *A. ochraceus* was observed after seven days incubation. Although the mycelial growth was not fully inhibited by the yeasts and bacterial isolates, in some treatments the mycelial growth was limited compared to the controls and a zone with spore production inhibition was evident between the yeasts and/or bacteria with the pathogen (Fig. 1).

In several researches, *T. harzianum* has been reported as an effective antagonistic agent, which prevents growth of pathogenic fungi. Therefore, the antagonistic activity of bacteria and yeasts isolated in this study was compared with that of *T. harzianum*. Comparison of the data obtained from the dual culture revealed that the highest level of inhibition on mycelial growth of *Aspergillus* spp. was induced by *T. harzianum*, which was used as a positive control. This was followed by *C. membranifaciens* Ka15 whereas the lowest inhibition level level was given by *M. guilliermondii* Ka21 (Table 1).

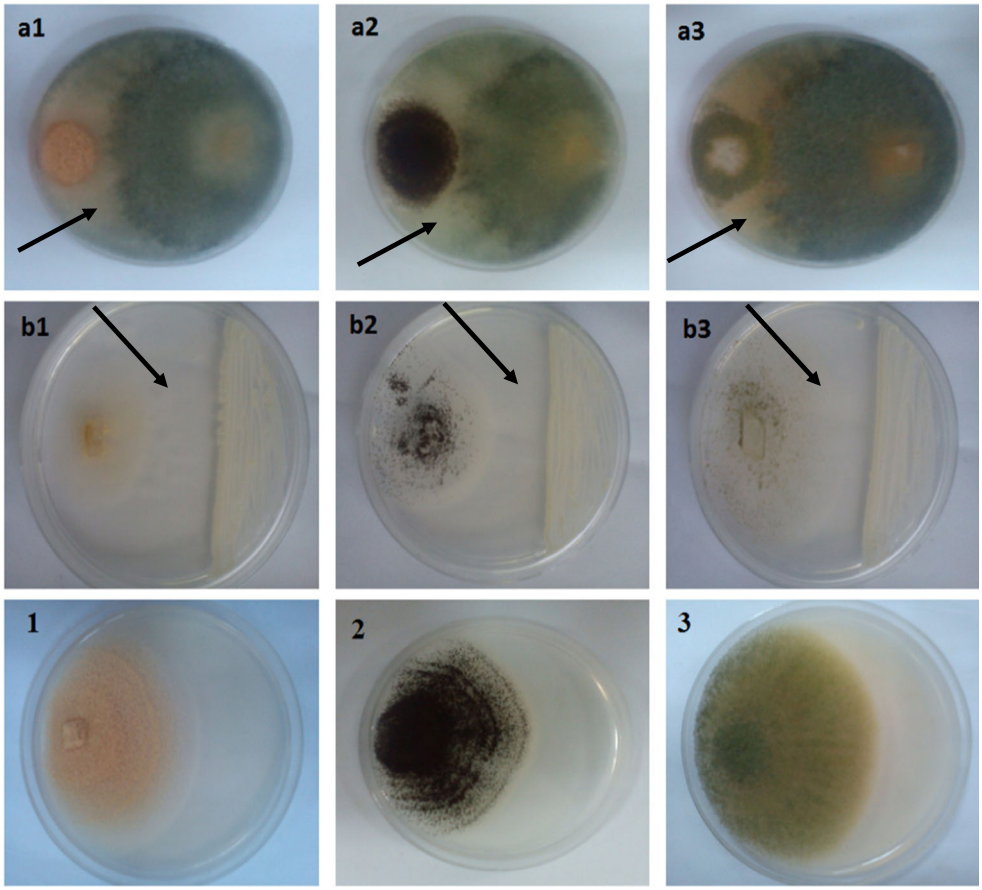

Fig. 1 The most effective antagonistic isolates against *Aspergillus ochraceus* (1), *Aspergillus niger* (2) and *Aspergillus flavus* (3) using the dual culture technique on PDA plates. Pictures are taken 7 days after challenging the antagonists with the pathogens. Inhibition was clearly distinguished by limited growth of fungal mycelium and inhibition of spore production in the zone surrounded by the yeast and/or bacterial colony. *Trichoderma harzianum* TBI (**a**) and *Candida membranifaciens* isolate Ka15 (**b**). Arrows indicate inhibition zones

All isolates under study were checked for determining the capability of producing volatile and non-volatile metabolites. The results indicated that antagonistic isolates apparently produced volatile (Fig. 2) and non-volatile (Fig. 3) substances

Table 1 In vitro screening of antagonistic isolates against *Aspergillus flavus, A. niger* and *A. ochraceus* by dual culture test (7 days post inoculation). Isolates Ka21, Kh59, and Kh60: *Meyerozyma guilliermondii;* Ka15 and Kh69: *Candida membranifaciens*; Ka3 and A10: *Bacillus* sp.; and TBI: *Trichoderma harzianum*

Antagonistic isolates	*A. ochraceus*		*A. niger*		*A. flavus*	
	mean radial growth(mm)	Inhibition[a] (%)	mean radial growth(mm)	Inhibition[a] (%)	mean radial growth(mm)	Inhibition[a] (%)
Yeasts						
Ka21	30.66 ± 1.4	9.8 d	39.3 ± 1.3	14.5 e	44.7 ± 1.3	10.6 d
Kh59	24.3 ± 1.7	28.4 c	33.3 ± 1.3	27.5 d	40 ± 2	20 c
Kh60	24.3 ± 2.7	28.4 c	37 ± 2	19.5 e	39.3 ± 1.3	21.3 c
Ka15	19.3 ± 1.3	43.13 b	27.4 ± 1.4	40.5 b	31 ± 2	38 b
Kh69	25 ± 2	26.5 c	31 ± 2	32.6 cd	40.6 ± 1.4	18.7 c
Bacteria						
Ka3	24 ± 1	29.4 c	31.4 ± 1.4	31.8 d	41.3 ± 1.3	17.3 c
A10	22.7 ± 2.3	33.3 c	28.7 ± 1.3	37.6 bc	41.3 ± 1.3	17.3 c
Positive control						
TBI	5.7 ± 1.3	83.3 a	10.7 ± 1.3	76.8 a	10.7 ± 1.3	78.6 a
control	34 ± 1	0 e	46 ± 1	0 f	50 ± 1	0 e

Three replicates were used for each treatment

[a] the means with the same letter did not have significant difference according to Duncan's multiple range test at $p < 0.05$

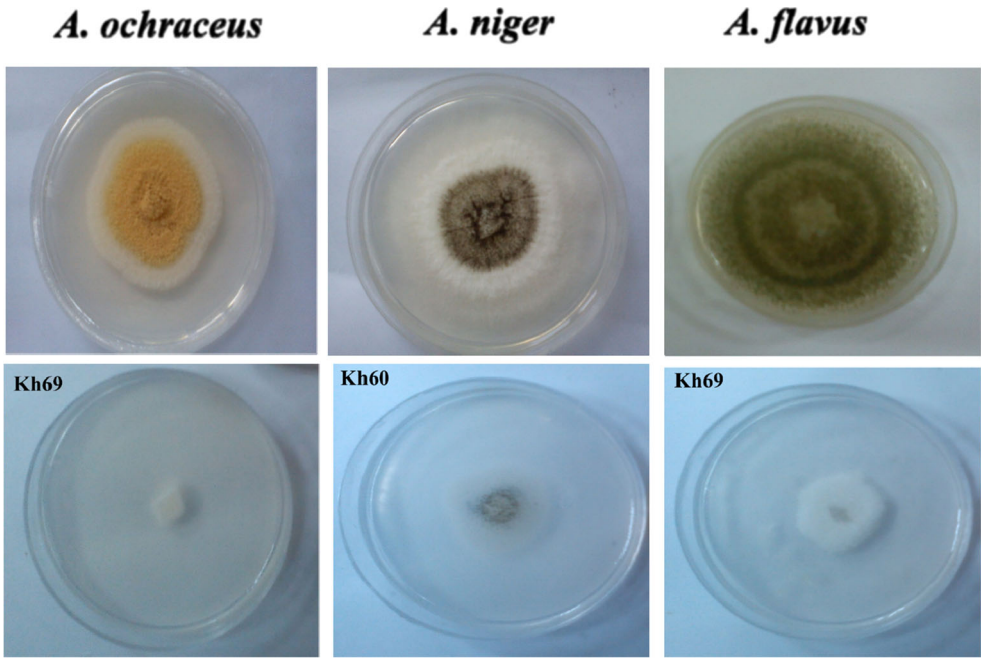

Fig. 2 The most effective antagonistic isolates against *Aspergillus ochraceus, Aspergillusniger* and *Aspergillusflavus* using volatile compounds on PDA plates. Pictures are taken 7 days after challenging the antagonists with the pathogen. Kh59 and Kh60: *Meyerozyma guilliermondii*, Ka15 and Kh69: *Candida membranifaciens*, A10: *Bacillus* sp. and TBI: *Trichoderma harzianum*. The first row is representing the controls

that suppressed the pathogen growth. Fig. 4 clearly indicates that most of the antagonist isolate-derived volatile substances caused maximum inhibition of mycelial growth of *A. ochraceus*. Significant inhibition against *A. niger* was given by volatile metabolites of *M. guilliermondii* Kh60 and *T. harzianum* TBI, whereas the volatile metabolites of *M. guilliermondii* Kh60, *C. membranifaciens* Kh69 and Ka15 caused maximum inhibition on mycelial growth of *A. flavus* (Fig. 4).

The highest level of *A. ochraceus* inhibition was afforded by non-volatile metabolites of *C. membranifaciens* (Kh69 and Ka15) and *Bacillus* sp. A10 with significant differences among these treatments (Fig. 5). Strong inhibitory effects against *A. niger* were observed by three antagonist isolates, including *C. membranifaciens* (Ka15 and Kh69) and *T. harzianum* TBI. The highest level of inhibition against *A. flavus* was given by *C. membranifaciens* kh69 and the lowest level by *M. guilliermondii* ka21 and *Bacillus* sp. Ka3 (Fig. 5).

Inhibitory effect of yeasts and bacteria on spore germination of *Aspergillus* spp. Seven antagonistic isolates inhibiting *Aspergillus* spp. growth were investigated for their effect on conidial germination of the pathogens. All tested isolates inhibited spore germination of three species of the pathogen more than 90% after one day incubation at 28 °C. The highest

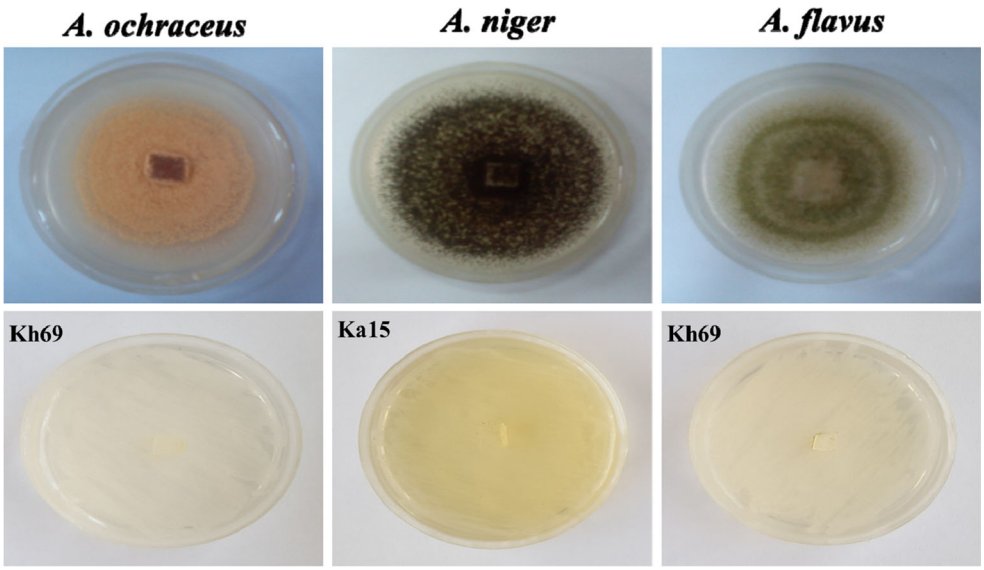

Fig. 3 The most effective antagonistic isolates against *Aspergillus ochraceus, Aspergillus niger* and *Aspergillus flavus* using non-volatile compounds on PDA plates. Pictures are taken 7 days after challenging the antagonists with the pathogen. Ka15 and Kh69: *Candida membranifaciens*, A10: *Bacillus* sp. and TBI: *Trichoderma harzianum*. The first row is representing the controls

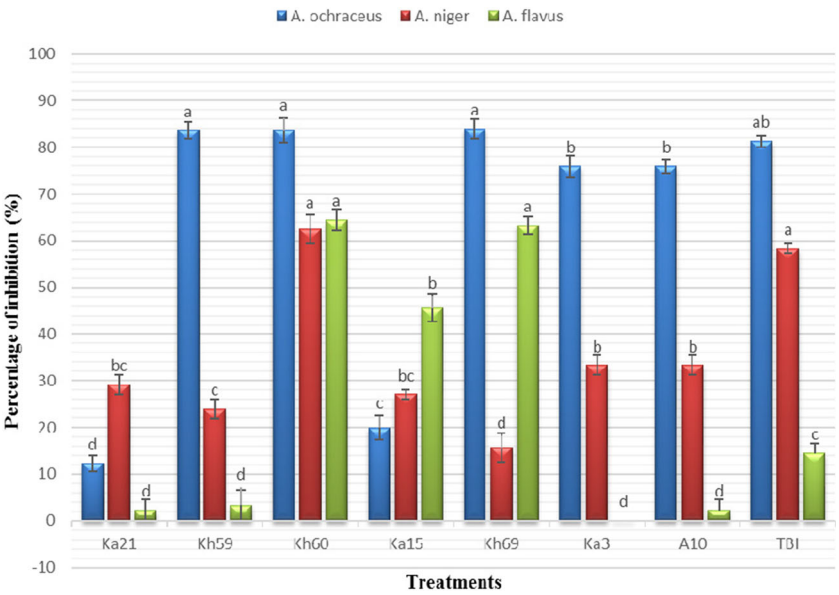

Fig. 4 Inhibition of fungal growth by antagonistic isolates against *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus ochraceus* by volatile compounds test (7 days post inoculation). *Meyerozyma guilliermondii* (Ka21), *Meyerozyma guilliermondii* (Kh59), *Meyerozyma guilliermondii* (Kh60), *Candida membranifaciens* (Ka15), *Candida membranifaciens* (Kh69), *Bacillus* sp. (Ka3), *Bacillus* sp. (A10) and *Trichoderma harzianum* (TBI)

level of inhibition was obtained using *C. membranifaciens* Ka15 and Kh69 isolates, which showed more than 90% inhibitory effect after three days incubation at 28 °C (Fig. 6).

Pathogenicity of the yeasts and bacteria on table grape berries None of the selected yeasts and bacterial isolates which had significant antagonistic capability against *Aspergillus* spp., had a damaging effect on cv. Thompson seedless berries (data not shown).

Effect of antagonistic isolates on *Aspergillus* mold of wounded berries The isolates showing antagonistic activity in the agar plate tests were evaluated for their efficacy to inhibit the growth of *A. flavus, A. niger* and *A. ochraceus* on small grape bunches On the berries treated by the yeasts and bacteria before inoculation with the pathogens, the incidence of decay decreased compared to the controls (Fig. 7). The isolates were equally effective in reducing the decay caused by all three species of *Aspergillus*. The highest control level for *A. ochraceus* was given by *Bacillus* sp. isolate A10, which reduced disease incidence in grape bunches by 50%, followed by *M. guilliermondii* strains Kh59 (46.6%) and Kh60 (45%). Biocontrol activity of various isolates in reducing *A. niger* growth ranged between 27.3% and 45.4%. The two *M. guilliermondii* isolates (Kh59 and Kh60) showed a moderate inhibition effect (45.4%) on fungal growth. In particular, the antagonist isolates highly inhibited the decay caused by *A. flavus* on berries ranging between 27.7% and 55.5%

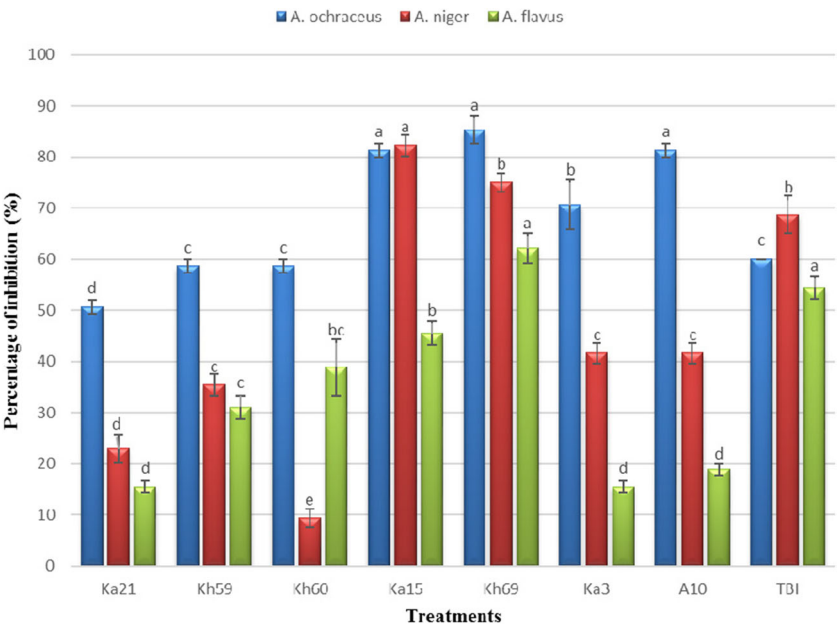

Fig. 5 Inhibition of fungal growth by antagonistic isolates against *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus ochraceus* by non-volatile compounds test (7 days post inoculation). *Meyerozyma guilliermondii* (Ka21), *Meyerozyma guilliermondii* (Kh59), *Meyerozyma guilliermondii* (Kh60), *Candida membranifaciens* (Ka15), *Candida membranifaciens* (Kh69), *Bacillus* sp. (Ka3), *Bacillus* sp. (A10) and *Trichoderma harzianum* (TBI)

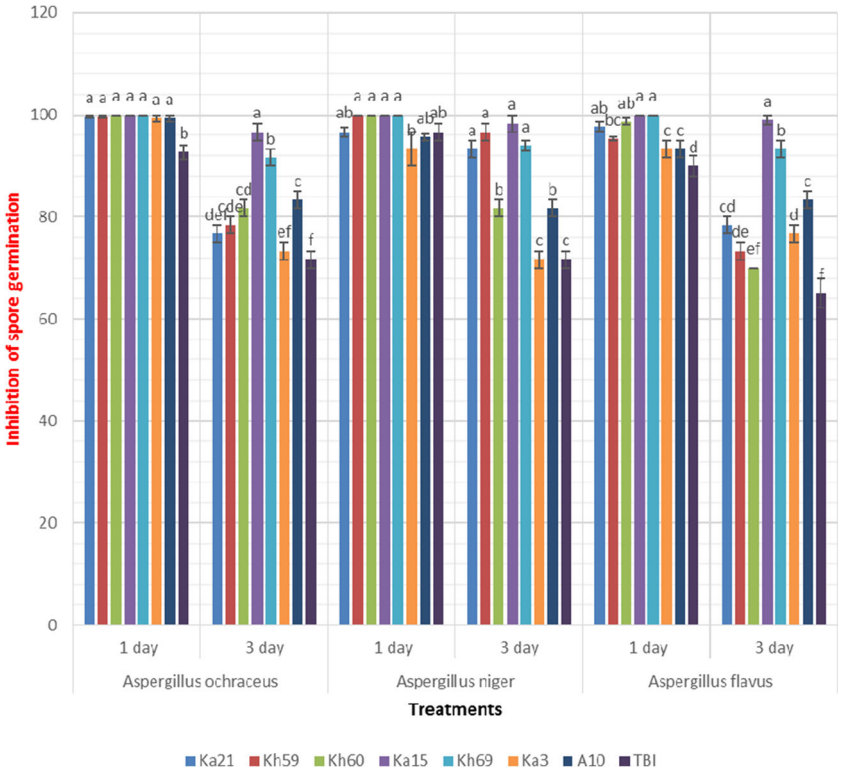

Fig. 6 Inhibition of spore germination of *Aspergillus flavus, Aspergillus niger* and *Aspergillus ochraceus* treated with different yeasts and bacteria isolates after one and three days at 28 °C. *Meyerozyma guilliermondii* (Ka21), *Meyerozyma guilliermondii* (Kh59), *Meyerozyma guilliermondii* (Kh60), *Candida membranifaciens* (Ka15), *Candida membranifaciens* (Kh69), *Bacillus* sp. (Ka3), *Bacillus* sp. (A10) and *Trichoderma harzianum* (TBI)

compared to the control. Results presented in Fig. 8 show that both isolates of *Bacillus* sp. (Ka3 and A10) were significantly effective (55.5% and 49.9%) in reducing the development of *A. flavus.* These were considered the best controllers of grape rots under the conditions of the present investigation. *T. harzianum* TBI showed the lowest mycelial growth inhibition of the pathogens. The results presented in Fig. 8 show that all seven antagonists were effective in reducing *Aspergillus* spp. development on grape berries.

Identification of antagonists with greater biocontrol activity against *Aspergillus* spp. To identify the antagonistic yeasts, the ITS1-ITS4 region was amplified using two universal fungal primers (ITS1 and ITS4), sequenced and compared with the sequences deposited in GenBank. To identify bacterial isolates, 1500 bp of 16S rDNA sequences were amplified using the universal primers for the 16S rRNA gene (27F and 1492R). The amplified fragments were sequenced and compared with available DNA sequences by BLAST, which

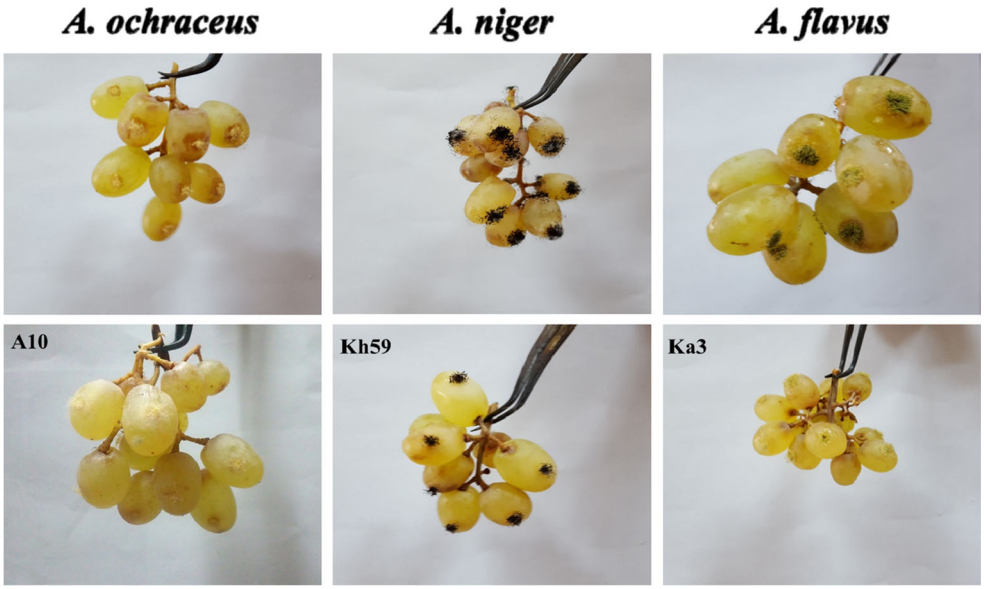

Fig. 7 Inhibition of *Aspergillus ochraceus, Aspergillus niger* and *Aspergillus flavus* by antagonistic isolates on grape berries cv. Thompson seedless. Grapes were treated with the isolates Kh59 and Kh60 belonging to *Meyerozyma guilliermondii*, isolate Ka15 of *Candida membranifaciens*, isolates Ka3 and A10 of *Bacillus* sp. The first row is representing the controls

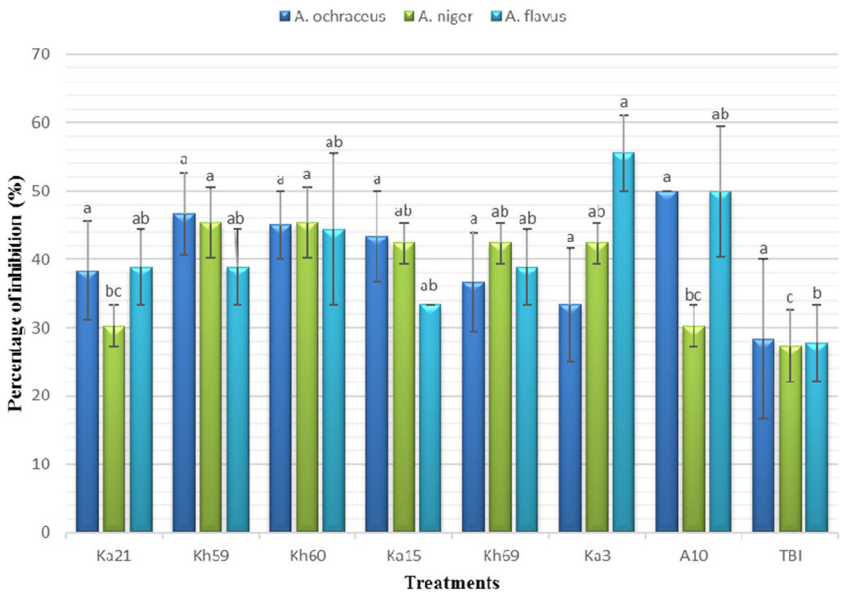

Fig. 8 Percentage of inhibition 7 days after challenge inoculation on wounded berries treated with antagonistic isolates before challenging with *Aspergillus flavus, Aspergillus niger* and *Aspergillus ochraceus* inoculation. *Meyerozyma guilliermondii* (Ka21), *Meyerozyma guilliermondii* (Kh59), *Meyerozyma guilliermondii* (Kh60), *Candida membranifaciens* (Ka15), *Candida membranifaciens* (Kh69), *Bacillus* sp. (Ka3), *Bacillus* sp. (A10) and *Trichoderma harzianum* (TBI)

revealed that the ITS and 16S rDNA genomic regions were discriminative for the identification of yeasts and bacteria, respectively.

The isolates Ka21, Kh59 and Kh60 showed 99%, 100% and 99% homology, respectively, with the ITS sequences from GenBank corresponding to *Meyerozyma guilliermondii*. Furthermore, the isolates Ka15 and Kh69 showed 99% and 100% homology, respectively, with the ITS sequences of *Candida membranifaciens*. Phylogenetic identification revealed that the bacterial isolates Ka3 and A10 had 99% similarity to *Bacillus* sp. The nucleotide sequences were registered in GenBank under the accession numbers listed in Table 2.

Discussion

Biological control is considered as a promising alternative to synthetic fungicides in the control of postharvest decay of fruits and vegetables (Wisniewski and Wilson 1992). The major objective of this work was to isolate and identify epiphytic yeasts and bacteria from grapes and to assess their ability for biocontrolling *Aspergillus* rots. The majority of organisms we isolated from grapevine berry and leaf surfaces showed some ability to reduce decay development. However, the natural epiphytic population isolated was very diverse in its capability to reduce decay caused by *Aspergillus* spp. and only a small percentage of the isolates was active to a significant level. The experimental data presented in this paper demonstrated that the best antagonistic yeasts belonged to *Candida membranifasciens* (Ka15 and Kh69) and *Meyerozyma guilliermondii* (Ka21, Kh59 and Kh60) whereas the antagonistic bacterial isolates belonged to *Bacillus* sp. (Ka3 and A10). Inhibition zones in the dual cultures could be due to the production of antibiotics, siderophores, toxic or antifungal metabolites and the size of the observed inhibition zones would represent the concentration and diffusivity of the inhibitory compounds secreted by each isolate (Swadling and Jeffries 1996). The antifungal potential shown on the culture medium does not represent the same potential in fruits.

Spore germination of the pathogens was inhibited by all tested isolates more than 90% after one day and by *C. membranifaciens* Ka15 and Kh69 isolates more than 90% after three days incubation at 28 °C (Fig. 5). This inhibition could be due to different action mechanisms exerted by the yeasts and bacteria. One of them could be competition for nutrients, since it has been reported that *B. cinerea* conidial germination is dependent on the amount of nutrients obtained from the environment (Filonow et al. 1996). Another mechanism could be parasitism and/or production of enzymes such as glucanases that degrade the pathogen wall (Masih and Paul 2002).

The seven antagonists selected for further study (previously shown to inhibit fungal growth in vitro) were efficient in reducing *Aspergillus* spp. decay on wounded berries. Our data show that: (i) the biocontrol agents did not completely prevent infection of wounded berries, but decreased fruit rot (Fig. 6); (ii) *C. membranifaciens*, *M. guilliermondii* and *Bacillus* sp. are potent antagonists of *A. flavus, A. niger* and *A. ochraceus*; (iii) they are consistent with those of previous studies in which various isolates of *Candida* sp. were reported to be effective against several fungal pathogens (Bleve et al. 2006; Zahavi et al. 2000). In particular, Zahavi et al. (2000) reported that a *Candida guilliermondii* isolate from grapes was able to reduce decay caused by *Botrytis*, *Rhizopus* and *Aspergillus*, whereas Bleve et al. (2006) showed that two *Issatchenkia orientalis* isolates strongly reduced *A. carbonarius* and *A. niger* colonization of grape berry and

Table 2 The size of sequenced products and registered accession numbers in GenBank for the antagonistic yeasts and bacteria used in this study

strains	Specie	Sequenced product (DNA)	registered number in GenBank
Yeasts			
Ka21	*Meyerozyma guilliermondii*	582 pb	KY550351
Kh59	*Meyerozyma guilliermondii*	549 pb	KY550352
Kh60	*Meyerozyma guilliermondii*	566 pb	KY550353
Ka15	*Candida membranifaciens*	583 pb	KY550355
Kh69	*Candida membranifaciens*	538 pb	KY550354
Bacteria			
Ka3	*Bacillus* sp.	1028	KY617030
A10	*Bacillus* sp.	1067	KY617029

found that *Metschnikowia pulcherrima*, *Issatchenkia terricola* and *Candida incommunis* also inhibited infection of grape berries by *A. niger* and *A. carbonarius*. *Pichia guilliermondii* (syn: *Meyerozyma guilliermondii*), previously known as *Debaryomyces hansenii*, controls several fungi, such as *Penicillium digitatum* on grape (Droby et al. 1989), *Botrytis cinerea* and *P. expansum* on apples (Wisniewski et al. 1990), and *A. flavus* on soybeans (Paster et al. 1993).

Various mechanisms have been suggested to operate on biocontrol, including antibiosis (Janisiewicz and Roitman 1988), parasitism (Wisniewski et al. 1990), 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). Santos and Marquina (2004) described the effects of a killer toxins of *Pichia membranifaciens* in the biocontrol of *B. cinerea*. Competition for nutrients has a major role in the mode of action of *P. guilliermondii* against *P. digitatum* in citrus (Droby et al. 1992; Arras 1996); *D. hansenii* against *B. cinerea* (Chalutz et al. 1988); and *A. pullulans* against *P. expansum* in grapes (Castoria et al. 2001). *Bacillus subtilis* produced a peptidolipid that inhibited *A. ochraceus* (Klich et al. 1991). *B. thuringiensis*, which was used as a commercial insecticide during cultivation of grapes, inhibited the growth of *A. carbonarius* (Bae et al. 2004). Evidence has been found that the biocontrol activity of *Bacillus* isolate UYBC38 may be attributed to the production of antifungal substances capable of the in vitro inhibition of *B. cinerea*. Spore germination of *Aspergillus* spp. was completely inhibited by culture filtrates of UYBC38 (Rabosto et al. 2006).

Antagonistic *T. harzianum* was highly effective against *B. cinerea* on apple fruit (Batta 2004a), against *Alternaria alternata* on fig leaves (Batta 2000) and persimmon fruit (Batta 2001), and against *P. expansum* on apple fruit (Batta 2004b). According to Gachomo and Kotchoni (2008), *A. ochraceus* and *A. niger* were overgrown by *T. harzianum* when they were grown in paired cultures. *Trichoderma* spp. are plant symbionts and parasites of other fungi (Harman et al. 2004). They produce enzymes that degrade cell wall of other fungi (Benitez et al. 2004).

This study demonstrated the occurrence of native yeasts and bacteria on grapes in Iran, which were able to control growth of *A. flavus*, *A. niger* and *A. ochraceus* on wounded berries. Therefore, these isolates have biocontrol potential in post-harvest storage of table grapes. It has been shown that the efficacy of biocontrol agents can be variable and is dependent on population of pathogen and environmental conditions (O'Neill et al. 1996). Further studies should be done to evaluate the selected isolates in field trials and to investigate their mode of action in order to develop appropriate formulations and application methods.

Initial in vitro screening and wounded fruit assays are good methods for investigating antagonists, especially when the microorganisms are selected from epiphytic flora. However, a good performance in laboratory experiments does not necessarily correspond with a high antagonistic capacity in the field, where many factors can affect survival of the biocontrol agent (Stapleton and Grant 1992; Elad and Kirshner 1993), including climatic conditions and nutrient levels. This work is an initial step concerning the possible application of yeasts and bacteria for protecting grape against rot disease. It is necessary to evaluate culture conditions of the biocontrol agents at an industrial level, and implement field tests for the commercial use of these selected isolates, taking into account that *Aspergillus* rot develops at preharvest and postharvest conditions. In future studies, it would be important to evaluate the antifungal activity of selected yeasts and bacteria in mixed cultures, against phytopathogenic fungi isolated from rot damaged grapes and determine the mechanisms involved in biocontrol of grape pathogens.

Acknowledgements We thank Ferdowsi University of Mashhad, Iran, for financial support of this research with project number 3/39326 approved on 1/12/2015.

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