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RESEARCH ARTICLE

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Identification of epiphytic yeasts and bacteria with potential for biocontrol of grey mold disease on table grapes caused by *Botrytis cinerea*

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Abstract

The objective of this study was to identify grapevine epiphytic yeasts and bacteria for biocontrol of *Botrytis cinerea* on grapes. Antagonistic yeasts and bacteria were isolated from the epiphytic flora associated with grape berries and leaves cv. 'Thompson seedless' from vineyards in Iran and identified by sequencing the conserved genomic regions. A total of 130 yeast and bacterial isolates from the surface of grapevine were screened *in vitro* for determining their antagonistic effect against *B. cinerea* and used to control postharvest gray mold. Among the 130 isolates, five yeasts and four bacterial isolates showed the greatest antagonistic activity *in vitro* against *B. cinerea*. Two yeasts species including *Meyerozyma guilliermondii* and *Candida membranifaciens* had high antagonistic capability against the pathogen. Also, 4 bacterial isolates belonging to *Bacillus* sp. and *Ralstonia* sp. showed significant biocontrol effect against *B. cinerea.* The isolates were capable of producing volatile and non-volatile substances, which suppressed the pathogen growth. The antagonistic activity of selected yeasts and bacteria against the pathogen was investigated on wounded berries of 'Thompson seedless'. On small clusters with intact berries, all of the antagonistic isolates considerably reduced the decay on grape berries and inhibition of gray mold incidence on fruits treated by these isolates was less than 50%, except for the isolate N1, which had higher capability in inhibiting the disease incidence. These results suggest that antagonist yeasts and bacteria with potential to control *B. cinerea* on grape can be found in the microflora of grape berries and leaves.

Additional keywords: antagonistic yeasts and bacteria; biological control; *Vitis vinifera.*

Abbreviations used: GII % (germination inhibition index); NA (nutrient agar); PDA (potato dextrose agar); PDB (potato dextrose broth); R (radial hyphal growth).

Authors' contributions: Conceived and designed the work: PT; performed the experiments and analyzed the data; KK; Wrote and revised the paper: KK and PT; Contributed reagents/materials/analysis tools: BJ and ST. All authors read and approved the final manuscript.

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Introduction

Table grapes (*Vitis vinifera* L.) are one of the most therapeutically and economically important fruits in the world. However, 30-40% of table grapes are lost every year owing to inadequate handling and the lack of proper methods to prevent decay and senescence (Prusky, 2011; Hashem *et al*., 2013). 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; Senthil *et al*., 2011; Romanize *et al*., 2012). Thakur & Saharan (2008) estimated that postharvest losses in grapes are about 39% of the yield, and 30% of the value.

The necrotrophic pathogen *Botrytis cinerea* Pers. is a filamentous fungus belonging to the Sclerotiniaceae family (Holz *et al*., 2004). *B. cinerea* (teleomorph: *Botryotinia fuckeliana*), the causal agent of gray mold, is an airborne plant pathogen that affects over 200 plant species worldwide in temperate and subtropical regions. This disease is considered as a limiting factor for storage and exporting table grapes (Elad *et al*., 2007). In grapevines it is responsible for botrytis bunch rot or grey mold, the effects of which are intensified by

its vigorous growth rate and ability to spread throughout clusters (Mlikota Gabler & Smilanick, 2001). It is the most important fungal disease that affects grape production in many temperate regions pre- and postharvest (Elmer & Reglinski, 2006). *B. cinerea* affects the vine's non-lignified aerial organs, such as leaves, buds, rachis, and flowers, causing tissue necrosis and soft rot of the berries (Elad *et al*., 2007; Williamson *et al*., 2007). This fungus generates abundant mycelia and produces a great quantity of conidia at the end of branched conidiophores. In adverse conditions also generates survival structures, known as sclerotia. Furthermore, it can survive as a saprophyte on plant residues during the winter. Conidia can persist as latent inoculum in floral residues, such as stamens and calyptrae causing postharvest rot (Viret *et al*., 2004).

Postharvest diseases often account for a major part of the losses and their control requires use of a large amount of fungicides (Wilson & Wisniewski, 1994). Control of this disease and other fungal diseases of grapevine is mainly performed by using chemical fungicides. Widespread use of chemical fungicides have certainly decreased the incidence of fungal diseases, but at the same time have contributed to the appearance of fungicide-resistant strains of the pathogens. Public demand to reduce fungicide application, stimulated by greater awareness of environmental and health issues, as well as development of resistance in some of the pathogens to the fungicides, limits the application of chemicals on agricultural products (Thind, 2012). There is an increasing demand to develop alternative environmentally safe methods for disease control (Elad *et al*., 1992). In recent years, several researches were focused on developing novel and effective control methods against pre- and postharvest decay in grapes as well as other agricultural commodities (Leibinger *et al*., 1997; Zahavi *et al*., 2000; Raspor *et al*., 2010). The use of biofungicides obtained from beneficial microorganisms appears as potential non-hazardous alternatives to fungicide application for controlling *B. cinerea* (Elmer & Reglinski, 2006; Chanchaichaovivat *et al*., 2007; Sharma *et al*., 2009). Biological control is defined as 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 fungicide utilization in the field and postharvest. This biological approach is highly desirable for controlling fungal growth on grapes, helping to reduce the amount of agrochemical residues in grapes and related products (Cabras *et al*., 1999; Cabras & Angioni, 2000). In recent years, a number of different microorganisms including bacteria, filamentous fungi, and yeasts have been isolated and shown to protect grape fruit against postharvest pathogens (Heydari & Pessarakli, 2010).

The interactions between yeasts, fungi and bacteria may play a key role in the natural process of biocontrol, although the molecular mechanisms involved are still largely unknown. Secretion of cell wall degrading enzymes (Masih *et al*., 2001), competition for nutrients (Filonow, 1998), predation (Lachance & Pang, 1997), production of syringotoxins, syringomycins (Woo *et al*., 2002) and killer toxins (Walker *et al*., 1995) are possible mechanisms of biocontrol. Several studies have demonstrated an efficient antagonistic activity of yeasts against *B. cinerea* (Saligkarias *et al*., 2002; Santos *et al*., 2004; Elmer & Reglinski, 2006; Dal Bello *et al*., 2008). *Candida oleophila* is an effective yeast against *B. cinerea* and has been used to protect apples after harvest (Jijakli & Lepoivre, 1998). Other yeasts are reported to be antagonists of a diverse group of phytopathogens: *Debaryomyces hansenii* against *Penicillium digitatum* on grapefruit, *Pichia guilliermondii* (syn: *Meyerozyma guilliermondii*) (anamorph: *Candida guilliermondii*) against Botrytis, Rhizopus, and Alternaria rots of tomato fruits, *Cryptococcus laurentii* and *C. albidus* against Mucor rot of pear and *Candida sake* against major postharvest pathogens of apple including *B. cinerea* and *Rhizopus nigricans* (Masih *et al*., 2000).

Also, in recent decades, there has been continued and rigorous research worldwide with a greater impetus to explore a wide range of bacteria possessing antagonistic properties against *B. cinerea* (Elmer & Reglinski, 2006; Compant *et al*., 2013). However, in the majority of these studies, the efficacy of biocontrol agents was evaluated under controlled conditions, and the fact that most of them were not effective against the pathogen in the field is now widely known. Despite the large number of scientific papers published on this topic, the number of efficient bacteria commercialized for using as microbial fungicides against *B. cinerea* in the pre- and/or postharvest stages remains limited (Nicot *et al*., 2011; Romanazzi *et al*., 2016). Some of these products that inhibit *B. cinerea* contain bacteria such as *Bacillus* sp., as their active ingredient. For example, Serenade (AgraQuest, Davis, CA, USA), which is used to control Botrytis bunch rot, has *Bacillus subtilis* as its active ingredient. These bacteria have several advantages over gram negative bacteria, including the production of endospores that are tolerant to heat and desiccation and also production of secondary metabolites with broad-spectrum activities (Jock *et al*., 2002). Thus, *Bacillus* sp. offers biological solutions to commercial formulation problems as they can be included in a stable dry powder product (Emmert & Handelsman, 1999). Several bacterial biocontrol agents have been isolated from vineyards, including *Acinetobacter lwoffii* (Magnin Robert *et al*., 2007; Trotel-Aziz *et al*., 2008), *Pseudomonas fluorescens* (Magnin Robert

et al., 2007; Trotel-Aziz *et al*., 2008), *Pantoea agglomerans* (Magnin Robert *et al*., 2007; Trotel-Aziz *et al*., 2008), and *Bacillus subtilis* (Trotel-Aziz *et al*., 2008). However, some of the yeast and bacterial based commercial biocontrol products which are effective against *B. cinerea* are available, including Serenade ® (containing *B. subtilis*) (Chen *et al*., 2008), Shemer™ (*Metschnikowia fructicola*) (Karabulut *et al*., 2004), Candifruit™ (*Candida sake*) (Vinas *et al*., 1998) and Boni-Protect™ (the yeast-like fungus, *Aureobasidium pullulans*) (Schena *et al*., 2003).

Biocontrol effects of fungi such as Trichoderma and Gliocladium have been extensively studied (Elad *et al*., 1982). The *Trichoderma harzianum* (Rifai) is an extremely versatile biocontrol agents suppressing diseases caused by a number of airborne plant pathogens, including anthracnose and grey mold on strawberry (Freeman *et al*., 2004). The variety of controlling primary postharvest diseases caused by *R. stolonifer*, *B. cinerea* and *P. expansum* on a variety of fresh fruits was achieved with an invert emulsion formulation of *T. harzianum* (Rifai) (Batta, 2007).

Considering the importance of gray mold in Iran and the disease management problems, the objective of this study was to isolate and identify grapevine epiphytic yeasts and bacteria to evaluate their effectiveness against *B. cinerea* not only *in vitro*, but also *in vivo* on grape berries.

Material and methods

Isolation of antagonistic yeasts and bacteria from grapes and inoculum preparation

The antagonists were isolated from the samples without any signs of infection. These samples seemed to be healthy, whereas, botrytis attack was clearly observed on other grapes of the same vineyards. Therefore, it was supposed that the unaffected plants surviving out the *B. cinerea* attack might harbor bioprotecting agents against the pathogen. Grape (*Vitis vinifera*) berries and leaves belonging to the 'Thompson seedless' cultivar were sampled from the most important raisin-producing regions in the east of Iran. Ten healthy plants were sampled along two major diagonals of each vineyard. Three bunches and leaves were collected from the central part of each plant 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 ten different bunches were randomly selected and from each bunch, ten berries and ten leaves from each vineyard were collected and transferred to sterile distilled water containing 0.02% Tween-20. Epiphytic microorganisms were isolated by shaking the berries and leaves in 100 mL of sterile distilled water for 1 h at 100 rpm on a rotary shaker (Peng & Sutton, 1991). The wash was serially diluted and 1 mL of each dilution was dispersed 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, removed with a sterile loop and transferred to fresh PDA and NA plates to obtain pure cultures. Finally, isolates were kept in tubes containing sterile distilled water and stored at 4 ºC in the culture collection of the Department of Plant Protection, Ferdowsi University of Mashhad (Iran), for subsequent analysis. In this study, standard *T. harzianum* TBI isolate obtained from the culture collection of the Department of Plant Protection, Ferdowsi University of Mashhad, was used as a positive control.

For inoculum production, yeasts and bacterial isolates were activated from stored stock cultures by transferring them to plates containing PDA and NA media, respectively. After growing, they were transferred again to PDA and NA media, grown overnight, and the yeast and bacterial suspensions were prepared by suspending 3 full transfer loops of each culture in 5 mL of sterile tap water. Suspensions of the yeast and bacterial cells were adjusted to the desired concentration (1×10^7 cells/mL) with a hemocytometer.

Preparation of the pathogen for inoculation

In this study, *B. cinerea* BC81 isolate, previously isolated from grapes with gray mold symptoms, was used as pathogen. Spore suspensions of BC81 were prepared by collecting spores from 5-day-old colonies (grown on PDA at 25 °C) in sterile distilled water with addition of 0.02% Tween 20 to assist the dispersal of conidia. The spore concentration (1×10^5 cells/mL) was calculated with a hemocytometer.

Inhibition of *B. cinerea* on PDA by epiphytic yeasts and bacteria

Dual culture assay

All yeasts and bacterial isolates were tested in an *in vitro* preliminary screening to select isolates showing antagonism against *B. cinerea* mycelial growth. For this purpose, a loop of yeasts and bacterial cells from 3-day-old cultures was streaked on a PDA plate (9 cm diameter) at ~2 cm distance from the rim of each plate. Then, a fungal disk (5 mm diameter) of *B. cinerea* from a 5-day-old PDA culture was located at the distance of 5 cm from the yeast or bacterial line. Plates with

only *B. cinerea* were used as control. Petri dishes were incubated at 28 °C for 7 days and were daily observed for investigating mycelial growth of *B. cinerea* in each plate. Each yeast and bacterial isolate was tested three times and the whole experiment was repeated three times. Fungal growth inhibition was determined as the percent of colony diameter decrease compared to the control. Percent inhibition of radial hyphal growth (R) was calculated using the following equation (Mari *et al*., 1996):

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

where, R is percent inhibition of radial hyphal growth, R_1 is hyphal growth of the control, and R_2 is hyphal growth in the Petri dish inoculated with yeasts and/or bacterial isolates.

Volatile compounds assay

The inhibitory effect of volatile products produced by antagonistic isolates was evaluated using the method reported by Arrebola *et al*. (2010). Briefly, yeasts and/or 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 *B. cinerea* (5 mm diameter) from a 5-day-old PDA culture was inoculated at the center of PDA plates. Then, a Petri dish "sandwich" was made, with the antagonist isolates culture on the bottom and the *B. cinerea* PDA culture on the top. The sandwiched plates were sealed using parafilm and incubated at 28 °C. The PDA plates containing *B. cinerea* cultured under the same conditions were used as control. The hyphal growth of *B. cinerea* was measured daily for 7 days post-inoculation using a Vernier caliper. Percent inhibition of radial hyphal growth (R) was calculated using the equation mentioned before. All treatments were performed in triplicate and the experiment was repeated three times.

Non-volatile compounds assay

The production of non-volatile substances by antagonistic isolates against *B. cinerea* was studied using the modified method described by Kraus & Lopper (1990). Briefly, yeasts and bacterial cell suspensions (1 mL) were spread on PDA and NA media, respectively, and incubated at 28 °C for 72 h. Then, the colonies were cleaned from the surface of culture media by sterile cotton in sterile conditions under the laminar hood. Afterward, petri dishes were cleaned with cotton soaked in chloroform and placed under UV light for 0.5 h. Then, petri dishes were placed half open for 1.5 h in order to evacuate the steams of chloroform. Finally, petri dishes were inoculated with mycelial plugs (5 mm diameter) of *B. cinerea* at the centres. The control petri dish was not inoculated with any of the antagonistic isolates. The petri dishes were incubated at 28 °C until the control colony reached the plate edge. Then, colony diameters and percentage inhibition were calculated in relation to the control by the formula mentioned before. There were three replicates for each treatment in an experiment and the whole assay had three repetitions.

Inhibitory effect of yeasts and bacteria on *B. cinerea* conidial germination

Yeasts and bacterial isolates were assayed for their capability to inhibit germination of *B. cinerea* conidia at 25 ºC according to the methodology described by Droby *et al*. (1997). A volume of 100 µL of suspended yeast and bacteria (1×10^7 cells/mL) and 100 µL of suspended pathogen conidia (1×10^5 conidia/mL) were added to an Eppendorf tube containing 800 µL potato dextrose broth (PDB) medium. For the control, 100 µL of sterile distilled water without antagonist was added to a tube. Three replicates were used for each treatment. Treatments were incubated for one and three days at 25°C. Two 50-µL drops from each tube were placed on a microscopic slide and 100 conidia per drop were examined. The number of germinated conidia were determined. The germination criterion considered that conidium was germinated when the length of the germination tube was more than twice the greatest spore diameter. The conidial germination inhibition index (GII %) was calculated from the results according to the formula described by Manici *et al*. (1997), where GII (%) = (conidia germinated in control – conidia germinated in treatment) × 100/conidia germinated in control.

In vivo yeasts and bacteria pathogenicity

To determine if yeasts and bacteria selected in the previous assay were pathogenic on grapes, 15 µL of suspended yeast (1×10^7 cells/mL) were inoculated in a micro-wound made on grape berries 'Thompson seedless' that were homogeneous in size. These berries were previously disinfected with a commercial sodium hypochlorite solution at 1% v/v for 1 min. They were placed in plastic boxes at 25 ºC for 7 days. It was determined that the yeast was pathogenic when an alteration of the berry tissue was observed (Vargas *et al*., 2012).

Inhibition of *B. cinerea* by epiphytic yeasts and bacteria on wounded berries

Biocontrol activity of the antagonistic isolates was evaluated against *B. cinerea* on grape fruit as described by Poppe *et al*. (2003). Clusters of cv. 'Thompson

seedless' were divided into smaller clusters with 10-15 berries. The grape berries were homogenous in size and color, without any visible damage or mold. The berries were surface disinfected by dipping each bunch for 1 min in 1% (v/v) sodium hypochlorite and rinsed twice with distilled water. The berries were punctured with a cylindrical tool to produce a wound with 3 mm diameter and 3 mm depth. A 15 µL drop of each yeast and bacterium suspension in water (1×10^7 CFU/mL) was pipetted into each wound, and left to dry for 1–2 h. Then, 15 µL of the conidial suspension of *B. cinerea* (1×10^5 CFU/mL) were injected into the same sites. Treated grapes were air-dried and placed in plastic bags with wet paper towels to maintain high humidity. The fruits were incubated at 25 °C for 7 days. Each treatment consisted of three replicates of four bunches. 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 *B. cinerea* suspension (1×10^5 CFU/mL).

The percentage of fungal growth inhibition was determined 7 days after B. cinerea inoculation, using the following formula (Pantelides *et al*., 2015):

100 – [(diameter of fungal colony growth on berry treated with yeasts and bacteria/without yeasts and bacteria) × 100].

Total genomic DNA of the selected yeast isolates (with higher biocontrol effect against *B. cinerea*) was extracted by the hexadecyl-trimethylammonium bromide method according to Zolan & Pukkila (1986). Total DNA was dissolved in 50 to 200 µL of Tris-EDTA (TE) buffer depending on the size of DNA pellet. Dissolved DNA was stored at –20°C until used.

For DNA extraction from the bacteria showing greater antagonistic activity against *B. cinerea*, the samples were prepared from 1–3 mL of a liquid culture grown in LB-broth overnight. Bacteria were centrifuged at 13,000g for 2 min and pellets were resuspended in 1 mL sterile H_2O. The samples were centrifuged again for 2 min, resuspended in 200 µL sterile H_2O and heated at 100 ºC 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 & Russel, 2001).

Identification by rDNA sequence analysis

Yeast identification was carried out by a molecular procedure based on PCR amplification of the 5.8 S ribosomal RNA gene using universal primers for fungi, including ITS1 (5` TCC GTA GGT GAA CCT GCG G 3`) and ITS4 (5` TCC TCC GCT TAT TGA TAT GC 3`) as previously described (Drik, 2000). The amplification reaction was performed in a final volume of 25 µL containing 50 pmol of each primer (ITS-1 and ITS-4), 200 µM of each dNTP, 0.5 units Taq DNA polymerase and 3 µL of DNA sample in 1x Taq polymerase buffer (Invitrogen) (White *et al*., 1990). The mixture was first denatured at 94°C for 7 min. Then, 35 cycles of PCR were performed with by denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min. At the end of the last cycle, the mixture was incubated at 72°C for 10 min.

For identification of bacteria, 16S rDNA was amplified from genomic DNA with the primers 27F (5` AGA GTT TGA TCM TGG CTC AG 3`) and 1492R (5` TAC GGY TAC CTT GTT ACG ACT T 3`) as previously described (Yashiro *et al*., 2011). PCR amplification was performed as follows: 4 min at 95 ºC, 35 cycles of 95 ºC for 1 min, 1 min at 55 ºC, 90 s at 72 ºC, and a final elongation step at 72 ºC for 5 min, 4 ◦C save. For each reaction, a negative control missing DNA template was included. The PCR products were separated in a 1% agarose gel in parallel with PCR 100 bp Low DNA ladder (Sigma-Aldrich) as molecular size standard. After electrophoresis, the gel was visualized under UV light. The PCR products were sequenced by Macrogen Inc. (Seoul, Korea). Sequence similarity searches were performed using National Center for Biotechnology Information (NCBI) databases with the Basic Local Alignment Search Tool (BLAST) program. The nucleotide sequences were registered in GenBank.

Experimental design and statistical analysis

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

Results

Isolation of microorganisms and selection of antagonists

A total of 130 isolates of bacteria and yeasts were isolated from the surface of table grape berries and leaves. The selection process was carried out *in vitro*. First, 5 yeasts and 4 bacterial isolates inhibiting *B. cinerea* growth were selected among all of the isolates,

which represent 6.9% of the total isolates, and they exhibited obviously antimicrobial activity *in vitro*. This effect was considered as indicative of the fungal 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 with higher than 10 mm diameter around the pathogen in dual cultures. Finally, isolates were kept in tubes containing sterile distilled water and were placed in the culture collection of the Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad and kept at 4 ºC. For long term storage of yeast and bacterial isolates, many yeast and bacterial cells from the plate were transferred to 1 mL sterile 15% glycerol. The cells were suspended by shaking (or vortex if necessary) and stored at –80 ºC (Sherman *et al*., 1986).

Effect of antagonistic isolates on mycelial growth of *B. cinerea in vitro*

Results of the dual culture experiments showed that 9 of the isolates tested were able to significantly inhibit mycelial growth of *B. cinerea.* In the dual culture treatments formation of inhibitory zones between colonies of yeasts and/or bacteria with *B. cinerea* was observed after 7 days incubation. Although the mycelial growth was not fully inhibited by the yeast and bacterial isolates, in some treatments the mycelial growth was confined compared to the control plates and a zone with spore production inhibition was observed between the yeasts or bacteria and the pathogen (Fig. 1). Comparison of the data obtained from the dual culture revealed that all 9 antagonistic isolates inhibited the mycelial growth of *B. cinerea* from 28.3% to 50%. *T. harzianum* TBI isolate was the most effective, suppressing 88.3% of *B. cinerea* mycelial growth. *Bacillus* sp. isolate (Ka3) was the next most effective antagonist, suppressing 50% of *B. cinerea* mycelial growth (Table 1).

All these isolates, which showed high levels of inhibitory effect on the pathogen growth in the dual culture test, were used for determining the capability of producing volatile and non-volatile metabolites. The results indicated that antagonistic isolates apparently produced volatile and non-volatile substances that suppressed the pathogen growth (Figs. 2 and 3). Data presented in Table 2 clearly indicate that volatile substances of *Bacillus* sp. isolate A10 caused maximum inhibitory effect (80.7%) on the mycelial growth of *B. cinerea.* Inhibition of the pathogen mycelial growth by volatile metabolites of *M. guilliermondii* kh60, *C. membranifaciens* kh69, *Ralstonia* sp. N1 and *T. harzianum* TBI isolates was 75.4%.

Significant differences were observed among the antagonistic isolates for the effect of non-volatile metabolites against *B. cinerea* and all of their inhibition rates were more than 50% (Table 2). Non-volatiles of *T. harzianum* showed the highest inhibitory effect on mycelial growth of *B. cinerea* (100%), followed by *Bacillus* sp. kh26 (70.2%), *Bacillus* sp. Ka3 and *Bacillus* sp. A10 (64.9%). The lowest level of inhibition via non-volatiles against this pathogen was observed for *C. membranifaciens* Ka15 (50.9%).

Inhibitory effect of yeasts and bacteria on *B. cinerea* conidial germination

All 9 antagonistic isolates inhibiting *B. cinerea* growth were investigated for their effect on *B. cinerea* conidial germination. The highest level of conidial germination inhibition against *B. cinerea* was obtained using *M. guilliermondii* Kh59 and *C. membranifaciens* Ka15 isolates with 99% and 98% inhibitory effects, respectively, after 1 day and *C. membranifaciens* Kh69 and *M. guilliermondii* Ka21 isolates with 99%

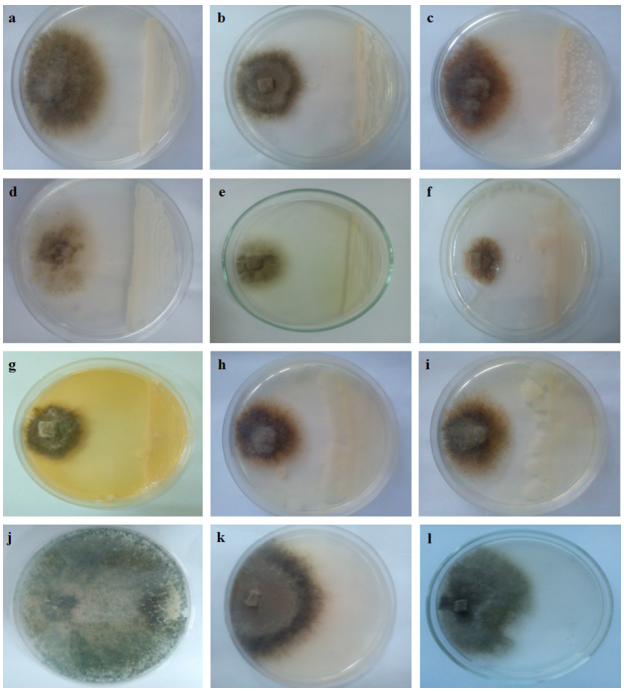

Figure 1. Antagonistic effect of yeasts and bacteria against *Botrytis cinerea* using the dual culture technique on PDA plates. Pictures are taken 7 days after challenging the antagonists with the pathogen. Inhibition was clearly discerned by limited growth of fungal mycelium and inhibition of spore production in the zone surrounding the yeast and bacterial colony. *Meyerozyma guilliermondii* Ka21 (a), *Meyerozyma guilliermondii* Kh59 (b), *Meyerozyma guilliermondii* Kh60 (c), *Candida membranifaciens* Ka15 (d), *Candida membranifaciens* Kh69 (e), *Bacillus* sp. Ka3 (f), *Bacillus* sp. A10 (g), *Bacillus* sp. Kh26 (h), *Ralstonia* sp. N1 (i), *Trichoderma harzianum* TBI (j) and control (k and l).

Table 1. *In vitro* screening of antagonistic isolates against *Botrytis cinerea* by dual culture test at 7 days post inoculation

Antagonistic isolates	Species	Dual culture	
		Mean radial growth (mm)	Inhibition[a] (%)
Yeasts			
Ka21	*Meyerozyma guilliermondii*	28.7±1.7	28.3 e
Kh59	*Meyerozyma guilliermondii*	23.7±1.3	40.8 d
Kh60	*Meyerozyma guilliermondii*	23±2	42.5 cd
Ka15	*Candida membranifaciens*	20.7±2.3	48.3 bc
Kh69	*Candida membranifaciens*	24.3±1.3	39.16 d
Bacteria			
Ka3	*Bacillus* sp.	20±2	50 b
A10	*Bacillus* sp.	20.36±1.3	49.1 bc
Kh26	*Bacillus* sp.	25±2	37.5 d
N1	*Ralstonia* sp.	23.7±1.3	40.8 d
Positive control			
TBI	*Trichoderma harzianum*	4.7±1.3	88.3 a
Negative control		40±0.3	0 f

Three replicates were used for each treatment. [a] Means with the same letter do not have significant difference according to Duncan's multiple range test at $p < 0.05$

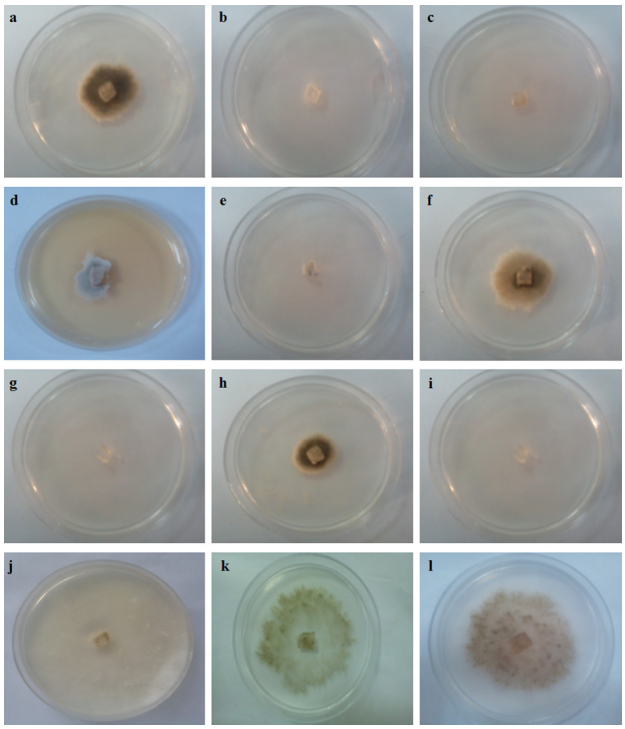

Figure 2. *In vitro* test of antagonism of yeasts and bacteria against *Botrytis cinerea* using the volatile metabolites technique on PDA plates. Pictures were taken 7 days after challenging of antagonist isolates with the pathogen. *Meyerozyma guilliermondii* Ka21 (a), *Meyerozyma guilliermondii* Kh59 (b), *Meyerozyma guilliermondii* Kh60 (c), *Candida membranifaciens* Ka15 (d), *Candida membranifaciens* Kh69 (e), *Bacillus* sp. Ka3 (f), *Bacillus* sp. A10 (g), *Bacillus* sp. Kh26 (h), *Ralstonia* sp. N1 (i), *Trichoderma harzianum* TBI (j) and control (k and l).

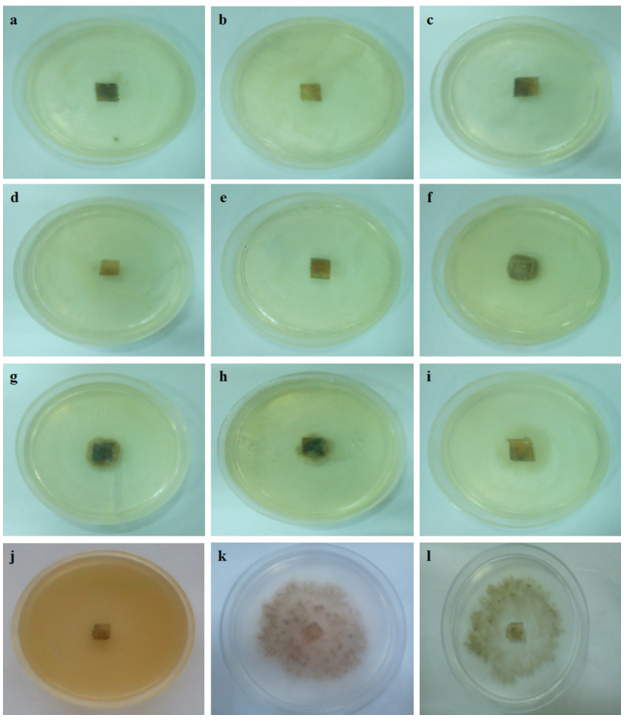

Figure 3. *In vitro* test of antagonism of yeasts and bacteria against *Botrytis cinerea* using the non-volatile metabolites technique on PDA plates. Pictures were taken 7 days after challenging of antagonist isolates with the pathogen. *Meyerozyma guilliermondii* Ka21 (a), *Meyerozyma guilliermondii* Kh59 (b), *Meyerozyma guilliermondii* Kh60 (c), *Candida membranifaciens* Ka15 (d), *Candida membranifaciens* Kh69 (e), *Bacillus* sp. Ka3 (f), *Bacillus* sp. A10 (g), *Bacillus* sp. Kh26 (h), *Ralstonia* sp. N1 (i), *Trichoderma harzianum* TBI (j) and control (k and l).

Table 2. Effect of volatile and non-volatile metabolites of antagonistic isolates on mycelial growth of *Botrytis cinerea*

Antagonistic isolates	Species	Volatile metabolites		Non-volatile metabolites	
		Mean radial growth (mm)	Inhibition[a] (%)	Mean radial growth (mm)	Inhibition[a] (%)
Yeasts					
Ka21	*M. guilliermondii*	12.7±1.3	33.3 d	8.7±1.3	54.4 cd
Kh59	*M. guilliermondii*	6.7±1.3	64.9 b	7.3±1.3	61.4 bcd
Kh60	*M. guilliermondii*	4.7±1.3	75.4 ab	7.3±1.3	61.4 bcd
Ka15	*C. membranifaciens*	9.7±2.3	49.1 c	9.3±1.3	50.9 d
Kh69	*C. membranifaciens*	4.7±1.3	75.4 ab	7.3±1.3	61.4 bcd
Bacteria					
Ka3	*Bacillus* sp.	13.3±1.3	29.8 d	6.7±1.3	64.9 bc
A10	*Bacillus* sp.	3.7±1.3	80.7 a	6.7±1.3	64.9 bc
Kh26	*Bacillus* sp.	9.7±0.7	49.1 c	5.66±1.4	70.2 b
N1	*Ralstonia* sp.	4.7±1.3	75.4 ab	9±1	52.6 d
Positive control					
TBI	*T. harzianum*	4.68±0.7	75.36 ab	0±0	100 a
Negative control		19±0.3	0 e	19±0.3	0 e

Three replicates were used for each treatment. [a] Means with the same letter do not have significant difference according to Duncan's multiple range test at $p < 0.05$

inhibition after 3 days incubation at 28 °C (Fig. 4). In the control assay, germination was 100%.

Pathogenicity of the yeasts and bacteria on table grape berries

Among the selected yeasts and bacterial isolates, none of them exhibited a damaging effect when they were inoculated on table grape berries cv. 'Thompson seedless'.

Effect of antagonistic isolates on mycelial growth of *B. cinerea* on wounded berries

The isolates showing antagonistic activity in the agar plate tests were evaluated for their efficacy to inhibit the growth of *B. cinerea* on small bunches of grapes. In the berries treated with yeasts and bacteria before inoculation with the pathogen, the incidence of gray mold decreased compared to the control (Figs. 5 and 6). Biocontrol activity of different isolates on small grape bunches in reducing *B. cinerea* growth ranged from 23.8% to 54.7% compared to the fungal growth on control berries. The highest level of biocontrol was achieved by isolate N1 (*Ralstonia* sp.), which reduced the disease progress on grape bunches by 54.7%. Followed by *Bacillus* sp. kh26 (49.9%) and *M. guilliermondii* isolates Ka21 and Kh59 (47.6%). These were considered as the best controllers of gray mold on grape berries in this study. Results presented in Figure 5 showed that all antagonistic isolates obtained in this study were effective ($p<0.05$) in reducing the development of *B. cinerea.*

Identification of yeasts and bacteria with greater biocontrol activity

Our data revealed that the ITS and 16S rDNA genomic regions were discriminative for identification of yeasts and bacteria, respectively. The yeast isolates Ka21, Kh59 and Kh60, showed 99%, 100% and 99% homology, respectively, with the ITS rDNA sequences found in the GenBank database corresponding to *M. guilliermondii*. Furthermore, Ka15 and Kh69 isolates showed 99% and 100% homology, respectively, with the ITS sequences of *C. membranifaciens* in the GenBank. The bacterial isolates Ka3, A10 and Kh26 had 99% similarity to *Bacillus* sp. and the isolate N1 belonged to *Ralstonia* sp. with 99% similarity to this genus. These similarities are sufficient to deduce that our best antagonistic bacteria belonged to *Bacillus* sp. and *Ralstonia* sp. The nucleotide sequences were registered in GenBank and the accession numbers are presented in Table 3.

Discussion

Grey mold, caused by the phytopathogenic fungus *B. cinerea*, is one of the most important diseases on a large number of economically important agricultural and horticultural crops and it is considered as the

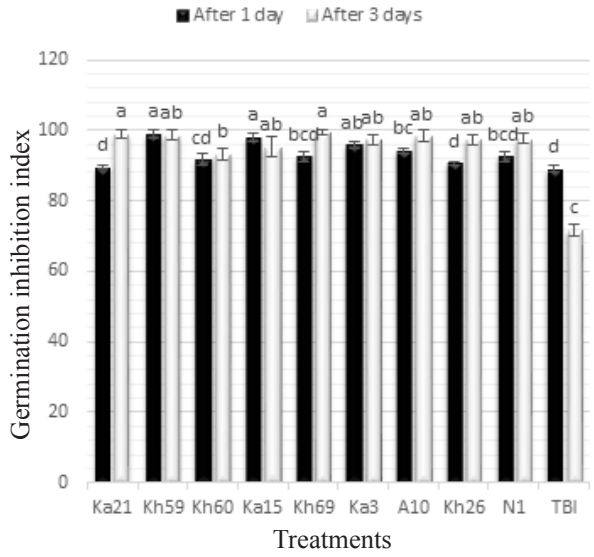

Figure 4. Germination inhibition index of *Botrytis cinerea* treated with different yeasts and bacteria isolates after one and three days at 28ºC. *Meyerozyma guilliermondii* (Ka21), *Meyerozyma guilliermondii* (Kh59), *Meyerozyma guilliermondii* (Kh60), *Candida membranifaciens* (Ka15), *Candida membranifaciens* (Kh69), *Bacillus* sp. (Ka3), *Bacillus* sp. (A10), *Bacillus* sp. (Kh26), *Ralstonia* sp. (N1) and *Trichoderma harzianum* (TBI).

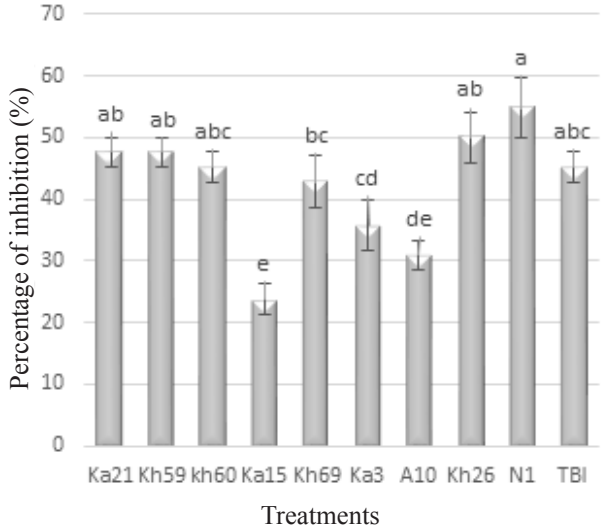

Figure 5. Percentage of disease progress inhibition 7 days after challenge inoculation on wounded berries treated with antagonistic isolates before challenging with *Botrytis cinerea*. *Meyerozyma guilliermondii* (Ka21), Meyerozyma guilliermondii (Kh59), *Meyerozyma guilliermondii* (Kh60), *Candida membranifaciens* (Ka15), *Candida membranifaciens* (Kh69), *Bacillus* sp. (Ka3), *Bacillus* sp. (A10), *Bacillus* sp. (Kh26), *Ralstonia* sp. (N1) and *Trichoderma harzianum* (TBI).

main postharvest decay of table grapes, because of the damage caused in the harvest season and during storage (Elad *et al*., 2015). The pathogen can also develop at low temperature, shortening the duration of storage and marketing.

The natural presence of antagonistic microorganisms on grapes lends itself to the application of selected antagonistic bacteria and yeasts to manipulate these populations as a good strategy for biological control of pathogens. Currently, biological control is considered as a promising alternative to synthetic fungicides in controlling postharvest decay of fruits and vegetables (Wisniewski & Wilson, 1992), with special interest on grapes (He *et al*., 2003; Ruenwongsa & Panijpan, 2007; Pusey *et al*., 2009). Since grapes production is of high relevance in Iran, the objetives of this work were of promising importance and constituted a primacy of studies with Iranian grapes. The antagonist yeasts and bacteria found here with potential control of *B. cinerea* on grape berries justify this research.

The major objective of the present work was to isolate and identify epiphytic yeasts and bacteria from grapes and to assess their potential ability for biological control of botrytis rots. Application of microbial antagonists, which are naturally occurring on the surface of fruits and vegetables, is a basic approach for the biocontrol of plant diseases. Epiphytic yeasts and bacteria are the major components of the microbiota on the surface of plants and they are evolutionary adapted to these ecosystems (Andrews & Harris, 2000; Morris & Kinkel, 2002; Redford *et al*., 2010). The majority of organisms we isolated from grape berries and leaves showed some levels of efficacy for reducing decay development in the preliminary tests. However, the natural epiphytic population isolated was very diverse in its propensity to reduce decay by Botrytis rot and only a small percentage of the isolates tested reduced decay development to a level that could be considered significant.

The results of our work showed that there were some antagonistic yeasts and bacteria among the microbial community associated with grape berries and leaves which were able to control *B. cinerea*. They were identified by partial sequencing of ITS1-ITS4 region (for the yeasts) and 16S rRNA gene (for the bacteria) using the universal primers. The obtained sequences were deposited in the GenBank nucleotide sequence database. Molecular analysis based on ITS1-ITS4 region and 16S rRNA gene sequences showed high levels of sequence similarity of our isolates to closely related species in the nucleotide sequence databases in The National Center for Biotechnology Information (NCBI). The experimental data presented in this paper demonstrated that our best antagonistic yeasts belonged to *C. membranifasciens* (Ka15 and Kh69) and *M. guilliermondii* (Ka21, Kh59 and Kh60). The

Table 3. Sequenced product and accession numbers in GenBank for the antagonistic yeasts and bacteria obtained in this study.

Strains	Species	Sequenced DNA (bp)	Acc. No.
Yeasts			
Ka21	*Meyerozyma guilliermondii*	582	KY550351
Kh59	*Meyerozyma guilliermondii*	549	KY550352
Kh60	*Meyerozyma guilliermondii*	566	KY550353
Ka15	*Candida membranifaciens*	583	KY550355
Kh69	*Candida membranifaciens*	538	KY550354
Bacteria			
Ka3	*Bacillus* sp.	1028	KY617030
A10	*Bacillus* sp.	1067	KY617029
Kh26	*Bacillus* sp.	1074	KY617031
N1	*Ralstonia* sp.	1027	KY617032

antagonistic bacterial isolates belonged to *Bacillus* sp. (Ka3, A10 and Kh26) and *Ralstonia* sp. (N1). To our knowledge, this is the first report in which the epiphytic yeasts and bacteria were isolated from grape leaves and berries in Iran and assessed for their potential antagonistic ability against *B. cinerea*. The epiphytic yeasts and bacteria reduced growth of *B. cinerea* not only on agar plates, but also on grape berries. A total of 130 epiphytic yeasts and bacterial isolates were isolated and evaluated for their antagonistic effect against *B. cinerea* by an *in vitro* co-inoculation assay performed on agar plates and it was shown that 9 isolates (6.9% of the analyzed population) were able to inhibit fungal growth at a significant level (Table 1, Fig. 1). The effect of antagonists on fungal growth was considered as indicative of the pathogen sensitivity to the action of yeasts and bacteria obtained from the same biological niche. Inhibition zones in the dual cultures could be due to the production of antibiotics, siderophores, toxic or antifungal metabolites used by these organisms as biological control mechanisms, and the size of the observed inhibition zones would represent the concentration and diffusivity of the inhibitory compounds secreted by each isolate (Swadling & Jeffries, 1996). However, production of these compounds in the culture media is not indicative of its production in action sites on the fruits (Dal Bello *et al*., 2008). Significant differences were observed among assayed yeasts and bacteria in terms of inhibitory effects, with *T. harzianum* TBI being the most, and *M. guilliermondii* Ka21 the least effective in inhibiting *B. cinerea* growth (Table 1).

Spore germination of the pathogen was considerably inhibited using *M. guilliermondii* Kh59 and *C. membranifaciens* Ka15 isolates with 99 and 98%, respectively after 1 day, and by *C. membranifaciens* Kh69 and *M. guilliermondii* Ka21 isolates with 99% after 3 days incubation at 28 °C (Fig. 4). This inhibition could be due to different action mechanisms exerted by the yeasts and bacteria. One of them might 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 that degrade the pathogen wall, such as glucanases, which are responsible for degradation of glucans as the main polymers in the conidial wall structure (Masih & Paul, 2002).

The 9 antagonists selected for further study (which showed significant inhibitory effect on the pathogen growth *in vitro*) were efficient in reducing decay caused by *B. cinerea*, on clusters having intact wounded berries that were artificially inoculated after application of the antagonists. Our data showed that all 9 isolates not completely prevented infection of wounded berries throughout the cluster but decreased the pathogen mycelial growth and fruit rot (Fig. 6). These findings are similar to the report of Masih *et al*. (2001), who studied the effect of *C. membranifasciens* against *B. cinerea*. The obtained data are consistent with those of previous studies in which various isolates of *Candida* sp. were documented to be effective against several fungal pathogens (Guinebretiere *et al*., 2000; Zahavi *et al*., 2000; Bleve *et al*., 2006). Among yeasts, *Hanseniaspora uvarum* or *Kloeckera apiculata* (Suzzi *et al*., 1995; Karabulut & Baykal, 2003) and *Pichia* sp. (Fleet, 2003) have been reported as effective biocontrol agents against a wide range of fungal pathogens (Filonow *et al*., 1996). Similar results were reported by Raspor *et al*. (2010), who obtained a significant decrease in the degree of infection by *B. cinerea* on grapes treated with yeasts before inoculation with this pathogen compared to the grapes treated with

yeasts and immediately inoculated with the pathogen (Filonow *et al*., 1996; Saligkarias *et al*., 2002). Qing & Shiping (2000) discovered that Rhizopus rot of nectarine was effectively controlled by the application of washed cells of *C. membranefaciens*. Wounded areas treated with *C. membranefaciens* showed no darkening or necrosis associated with application of a concentration of 5×10^8 CFU/mL to wounds. The yeast *Pichia guilliermondii* (syn: *Meyerozyma guilliermondii*), previously called *Debaryomyces hansenii,* controls a range of postharvest spoilage fungi, such as *Penicillium digitatum* on grapefruit (Droby *et al*., 1989), *B. cinerea* on apples (Wisniewski *et al*., 1991), and *Aspergillus flavus* on soybeans (Paster *et al*., 1993). Its adverse effects on *P. digitatum* and *B. cinerea* have been ascribed to competition for nutrients and secretion of cell wall-degrading enzymes (Droby *et al*., 1989). *P. guilliermondii* effectively controlled *P. italicum* in grapefruit and oranges. Also, it was effective in inhibiting the development of *Geotrichum candidum* in citrus fruit. *P. guilliermondii* was effective in reducing Rhizopus rot in both injured and non-injured grape berries (Wilson *et al*., 1991). Santos & Marquina (2004) described the effects of a killer toxin of *Pichia membranifaciens* in the biocontrol of *B. cinerea*. Nantawanit *et al*. (2010) reported that *M. guilliermondii*, strain R-13 induced resistance in peppers against infection by *Colletotrichum capsici.*

Our study showed that *C. membranifaciens, M. guilliermondii, Bacillus* sp. and *Ralstonia* sp. were potent antagonistic species against *B. cinerea* causing the grey mold disease of the grapevine. Evidence have been found that biocontrol activity of *Bacillus* isolate UYBC38 might be attributed to the production of antifungal substances capable of inhibiting *B. cinerea* growth *in vitro*. Spore germination of the pathogen was completely inhibited by culture filtrates of UYBC38 (Rabosto *et al*., 2006), which is in agreement with our findings.

It has also been reported that combined inoculations of *T. harzianum* and *B. cinerea* conidia, or inoculation of *T. harzianum* conidia only 8 h before inoculation with *B. cinerea* prevented wounded grape berries from becoming infected (O'Neill *et al*., 1996a). In other studies, antagonistic *T. harzianum* strain Th2 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).

This study demonstrated the presence of epiphytic yeasts and bacteria on Iranian grapes, which were able to control growth of *B. cinerea* not only *in vitro*, but also *in vivo* on grape berries. The effectiveness of selected yeasts and bacteria to inhibit fungal growth is promising but it is necessary to test these isolates under field conditions. It has been shown that the efficacy of biological control agents can be variable and is dependent on pathogen's inoculum level and environmental conditions (O'Neill *et al*., 1996b).

Based on the findings of this research, it could be concluded that initial *in vitro* screening and wounded fruit assays might be good methods for effective isolation of antagonists, especially when the microorganisms are selected from epiphytic flora. As the obtained results showed in Table 2 (in the case of volatile compounds which inhibit mycelial growth of the pathogen *in vitro*), Figure 4 (about spore germination inhibition) and Figure 5 (disease progress inhibition on grape berries), several antagonistic isolates had no significant differences with *T. harzianum*, as a positive control, in their biocontrol capabilities against *B. cinerea*. These data indicate finding of powerful epiphytic antagonists for controlling the pathogen in the present research. However, a good performance in laboratory

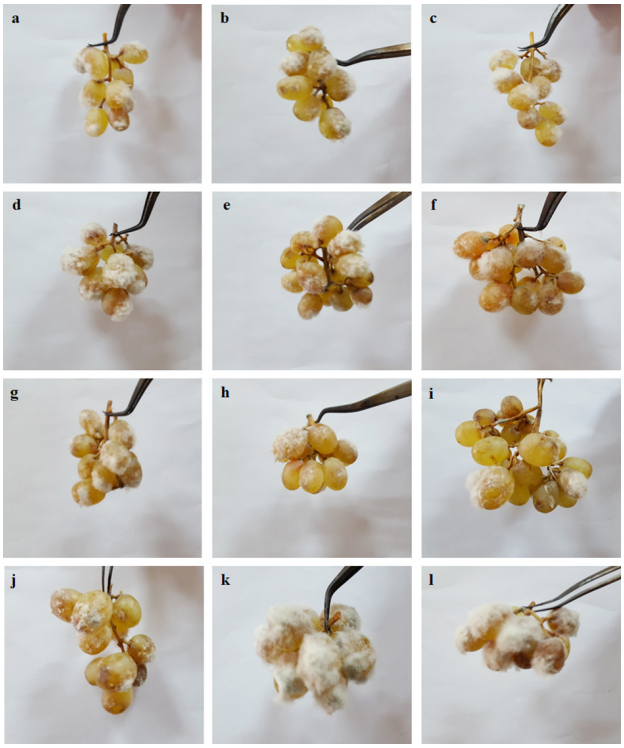

Figure 6. Inhibition of *Botrytis cinerea* by antagonistic isolates in Thompson Seedless grapes. Grapes were treated with *Meyerozyma guilliermondii* Ka21 (a), *Meyerozyma guilliermondii* Kh59 (b), *Meyerozyma guilliermondii* Kh60 (c), *Candida membranifaciens* Ka15 (d), *Candida membranifaciens* Kh69 (e), *Bacillus* sp. Ka3 (f), *Bacillus* sp. A10 (g), *Bacillus* sp. Kh26 (h), *Ralstonia* sp. N1 (i), *Trichoderma harzianum* TBI (j) and control (k and l). Photographs were taken at 7 days post-inoculation with the pathogen.

experiments does not necessarily correspond with a high antagonistic capacity in the field, where many factors can affect the survival of the biocontrol agents (Stapleton & Grant, 1992; Elad & Kirshner, 1993), including climatic conditions and nutrient levels, which affect colonization and development of populations.

Viticultural yeasts and bacteria isolated from table grapes were found effective as *in vivo* biocontrol agents against *B. cinerea*. The current study demonstrated that 5 yeasts and 4 bacterial isolates inhibited mycelial growth and spore germination of the pathogen. This work is an initial step concerning the possible application of the epiphytic yeasts and bacteria obtained from grape berries and leaves for botrytis rot disease prevention. It is necessary to evaluate culture conditions of the yeasts and bacteria at an industrial level, and implement some field tests for commercial use of these microbial antagonists, taking into account that gray mold develops at pre- and postharvest conditions. Since the antagonistic isolates coexist on grapevines, they might have synergistic effects in biocontrol of the pathogen and preventing disease development, which is necessary to be investigated in future researches. In future studies, it would be important to evaluate the antifungal activity of the 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.

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