Control the post harvest infection by *Aspergillus* spp. to Taify table grape using grape epiphytic bacteria

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Abstract: This study aimed to control the common fungi causing post-harvest black rot of Taify table grape (*Vitis vinefra* L.) and to induce resistance against black Aspergilli. Six fungal genera belonging to *Aspergillus*, *Penicillium*, *Trichoderma*, *Mucor*, *Rhizopus* and *Botrytis* were isolated from contaminated grape samples, with different frequencies. The predominant fungal species belonged to the genus *Aspergillus* and the isolation frequency ranged between 2.40-18.66%. The most predominant black Aspergilli were identified to the molecular level based on 18S- rRNA, ITS1 and 5.8S-rRNA gene sequence as *A. niger* BAVSH1, *A. parasiticus* BAVSH4 and *A. tubingensis* BAVSH5. Forty one bacterial isolates were obtained from soil, meswak and the surface of grape fruits, and screened for antagonistic activity. *In vitro* dual microbial culture showed variable % inhibition of fungal growth values. Out of the active antagonistic bacteria, five epiphytic from grape fruits and one from meswak, being the most antagonistic were identified to the molecular level based on 16S rRNA gene sequence as *Pseudomonas aeruginosa* EBMSH1, *P. aeruginosa* EBVSH13, *P. aeruginosa* EBVSH14, *P. aeruginosa* EBVHSH17, *Bacillus vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29. The ability of living cells or crude cell extracts of the best three latter antagonistic bacteria to induce the resistance in pre-wounded and intact- flooded grape fruits against black *Aspergillus* rot disease progress were demonstrated. Higher reduction or complete absence of disease incidence% and lesion diameter were obtained in intact, pre-wounded and living bacteria treated fruits than untreated control. This was clearer in flooding treatments with crude cell extract and living bacteria, compared to some of the wounded and induced by living bacteria and no treated control.

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1. Introduction

Grape (*Vitis vinifera* L.) represents 8.5 % of fruit crops planted area in Saudi Arabia. The most cultivated regions are Medinah and Tabouk which produce about 33.4 and 14.9 % of the total grape production, respectively (ARDA 2006). Taif area is also famous of fruits and grape is considered as one of the important summer fruits in this area. Significant portion of the grape is lost due to diseases caused by fungi and / or bacteria, physical injuries, over ripening and non-disease disorders (Boyette et al., 1992). If efficient steps are not taken to prevent the spread of these organisms and their toxins (Abrunhosa et al., 2001**;** Cabañes et al., 2002; Sage et al., 2002; Battilani et al., 2003; Medina et al., 2005 and Lui et al., 2010), it can infect all the products that are subsequently processed and pose a public health hazard (International programme on chemical safety**,** 1990; Majerus and Otteneder 1996; Heenan et al., 1998; Codex Alimentarius Commission, 1999; Abrunhosa et al., 2002 and Bayman et al., 2002). The contamination of grapes with fungi attracted the attention of some investigators (Sage et al., 2002; Battilani et al., 2003; Medina et al., 2005; Guzev et al., 2008; Allam et al., 2008 and Al-Qurashi, 2010).

Formation of fruit pathogens resistance to fungicides, contamination with chemical and detrimental effects of chemical control encouraged scientists to search for alternative control methods based on biological control of plant pathogens by means of non-pathogenic bacteria and fungi. However, considerable success has been achieved by utilizing antagonistic microorganisms for controlling plant diseases, post harvest diseases and mycotoxins (Pusey and Wilson, 1984; Spotts and Cervantes, 1986; Ferreira, 1990; El-Abyad et al., 1993,1996; El-Shanshoury, 1994; El-Ghaouth and Wilson, 1995,1998; El-Ghaouth et al., 2003; Gonzalez et al., 2005; Visconti et al., 2008; Abrunhosa et al., 2010; Lui et al., 2010 and Somma et al., 2012). In this

context, the epiphytic and endophytic microorganisms existing in the fruits were the object of studies conducted by many investigators (Wisniewski and Wilson, 1992; Sobiczewski and Bryk, 1995; He et al., 2003; Han et al., 2004; Bleve et al., 2006; El-Tarabily, 2006; Slavov, 2006; Chanchaichaovivata et al., 2007; Haıssam, 2011 and Parani et al., 2011). Recently, Somma et al. (2012) reviewed the prevention and control of black Aspergilli and Ponsone et al. (2011) demonstrated the efficacy of two yeast strains of *Kluyveromyces thermotolerans* for reducing the growth rate of ochratoxigenic fungi (from 11 to 82.5%), in the field.

The production of grapevine metabolites is highly conditioned by environment or pathogen attacks (Ali et al., 2010). Several abiotic and biotic agents have been reported to induce plant resistance to pathogens (Sticher et al., 1997; Sholberg and Conway, 2004; Nandeeshkumar et al., 2008; Yu et al., 2008 and Poiatti et al., 2009) in order to mobilize appropriate cellular defense responses before or upon pathogen attack (Sticher et al., 1997) triggered by non-pathogenic bacteria (Sholberg and Conway, 2004; Compant et al., 2005; Bakker et al., 2007; Magnin-Robert et al., 2007; Nandeeshkumar et al., 2008; Trotel-Aziz et al., 2008; Yu et al., 2008; Poiatti et al., 2009; Verhagen et al., 2010 and Baz et al., 2012).

The objectives of this study aimed to record the post harvest mycobiota of Taify table grape, to identify those causing black rot in different Taify farms to monitor their growth. This is in order to search for better management by using specific and preventive treatments. The potential of three biocontrol bacteria *Pseudomonas aeruginosa* EBVHSH17, *Bacillus vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29 to control *Aspergillus* black rot on Taify table grape and to enhance the resistance to subsequent infection with *A. niger* BAVSH1, *A. parasiticus* BAVSH4 and *A. tubingensis* BAVSH5 were explored.

2. Material and Methods

Samples

In the present study, a total of 30 Taify table grape samples (*Vitis vinifera* L.) were collected from six private farms at Taif city. Samples were harvested in July during the grape harvest. Grape bunches (each about 1 kg) were taken and placed in previously sterilized cardboard boxes, which were kept at 4°C until analysis.

Isolation and identification of fungi

Fungi were isolated from the infected grape fruits. Five weighted fruits were cut into small pieces and vigorously shaken in 100 ml of phosphate buffer (pH 6.5) for 20 min. The number of fungi was determined using serial dilution plate method on Sabouraud's agar medium containing the following ingredients 10 g l^{-1} meat peptone, 40 g l^{-1} dextrose and 15 g l^{-1} agar. Rose pengal (0.05 g l^{-1}) was added to the medium to suppress any bacterial growth. Colonies from fruits appeared on solid medium were chosen on the basis of their different visual characteristics. After 48-72 h of incubation at 28^0C, the number of CFU of filamentous fungi per gram of fruit was evaluated. The morphologically differing colonies of fungi were retransferred onto new plates for purification. Fungal colonies with different morphologies were maintained on Sabouraud's agar slants and stored at 4-8°C for further studies.

Taxonomic identification of all fungal isolates was achieved through macroscopic and microscopic observation with the aid of guidelines published for each genus or general guidelines (Barnett and Hunter, 1972; Pitt and Ailsa, 1985 and Tuite and Cantone, 1990). Molecular identification was also applied on the selected black Aspergillii on the basis of determination of restriction patterns of PCR-amplified rRNA gene products. Fungal DNA was isolated according to the method described by Lee and Taylor (1990). The ITS1-5.8S-rRNA gene-ITS2 region was amplified by PCR. Two oligonucleotide fungal primers (ITS1 and ITS2) described by White et al. (1990) were used for amplification. Amplified products were digested overnight at 37°C with restriction endonuclease RsaI (Boehringer Mannheim). To perform DNA sequencing, PCR products were cleaned with the Gene Clean II Purification kit (Bio 101, La Jolla, Calif.). Then, PCR products were sequenced using the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Falmer, Brighton, United Kingdom) and an Applied Biosystems automated DNA sequencer (model 3130xI) according to the manufacturer's instructions. The universal primers for ITS1 and ITS2 were used to obtain the sequence of both strands. The National Center for Biotechnology Information (NCBI) Nucleotide Database was used to compare nucleotide sequences and aligned using CLUSTAL W (1.81) Multiple Sequence Alignment generating phylogenetic tree. The sequences of fungal isolates reported here were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under the accession numbers: AB761050 (*Aspergillus niger* BAVSH1), AB761051 (*Aspergillus parasiticus* BAVSH4), AB761052 (*Aspergillus tubingensis* BAVSH5) and AB761053 (*Rhizopus oryzae* BRVSH7).

Isolation of antagonistic bacteria

Naturally occurring epiphytic bacteria were isolated from the surface of Taify table grape at harvest and periods of storage according to the

methods of Assis and Mariano (1999) and Yrjälä et al. (2010), with some modifications. Five grapes were picked from bunches, rinsed in 100 ml of sterile distilled water in Erlenmeyer flask, mixed with 1 g sterilized glass beads (0.2 cm diameter) and shaken at 150 rpm and at 28°C for 20 minutes. Another five grapes were picked up for isolation of rare epiphytic bacteria, rinsed in 100 ml of sterile distilled water in Erlenmeyer flask, boiled for 20 minutes, mixed with 1 g sterilized glass beads (0.2 cm diameter) and shaken at 150 rpm and at 28°C for 20 minutes. One gram of soil was also dispensed in 100 ml sterile distilled water and shaken for 20 minutes. The rinsing waters resulted from the grape and soils were subjected to decimal dilutions under sterile conditions. An aliquot of 1 ml of each dilution was placed in plates containing nutrient yeast dextrose agar (NYDA) medium (containing 8 g of nutrient broth, 5 g of yeast extract, 10 g of glucose, and 20 g of agar in 1 liter of distilled water). Colonies appeared were chosen on the basis of their different visual characteristics. After 24-48h of incubation, the morphologically differing colonies of the isolates were re-streaked on the corresponding medium to obtain pure cultures. These pure cultures were maintained on slants containing the corresponding medium and stored at 4°C until further study.

Identification of antagonistic bacteria

Morphological, physiological and biochemical confirmatory tests in Butler (1986) were followed for characterization of the antagonistic bacteria. The results on morphological and physiological characteristics were used, in addition to 16S rRNA sequencing technique for the confirmation of bacteria identification.

Molecular characterization of bacterial isolates

Genomic DNA was extracted from bacterial isolates as described by Sambrook and Russell (2001) using GenEluteTm bacterial genomic DNA kit, Sigma Aldrich. The genomic DNA was resuspended in 50 ml of TE buffer (10 mM Tris, 1mM EDETA), pH 8.0 and stored at -20°C until used in PCR amplification. The 16S rRNA gene about 1.5 Kb long was PCR amplified using set of the universal primers; forward primer, 5'-AGAGTTTGATCCTGGTCAGAACGCT-3' and reverse primer, 5'TACGGCTACCTTGTTACGACTTCACCCC-3' (Yanagi and Yamasato, 1993), 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′AAGGAGGTGWTCCARCC-3′) (11, Lane) and forward: AGA GTT TGA TCC TGGCTC AG; reverse: ACG GCT ACC TTG TTA CGA CTT (Weisburg et al., 1991). The PCR reactions were carried out according to the method described by Chouari et al. (2005). The PCR products of 16S rRNA gene were purified using 3130XI genetic analysis (Applied Biosystems).

DNA sequencing and phylogenetic tree

The 1.5 Kb-PCR products of 16S rRNA genes were used for DNA sequencing. Sequence analysis of the DNA fragments was performed. The PCR products were sequenced using the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Falmer, Brighton, United Kingdom) and an Applied Biosystems automated DNA sequencer (model 3130xI) according to the manufacturer's instructions. Selected sequences of other microorganisms with greatest similarity to the 16S rRNA sequences of bacterial isolates were extracted from the nucleotide sequence databases in The National Center for Biotechnology Information (NCBI) Nucleotide Database and aligned using CLUSTAL W (1.81) Multiple Sequence Alignment generating phylogenetic tree. The 16S rRNA sequences of bacterial isolates reported here were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under the accession numbers: AB758242 (*Pseudomonas aeruginosa* EBMSH1), AB760555 (*Pseudomonas aeruginosa* EBVSH13), AB761046 (*Pseudomonas aeruginosa* EBVSH14), AB761047 (*Pseudomonas aeruginosa* EBVHVSH17), AB761048 (*Bacillus vallismortis* EBHVSH28) and AB761049 (*Bacillus amyloliquefaciens* EBHVSH29).

***In Vitro* antagonism tests**

The forty one isolated bacterial strains were tested *In Vitro*, using the dual culture method (modified procedure of Fuhrmann (1994). Plates (90 mm) diameters containing Sabouraud's agar medium were inoculated with bacteria, 48 hr prior to the fungal inoculation. Each isolate was placed on a Petri dish making uniformly crossed streaks equidistantly near the periphery of each plate. Agar plugs (5-mm diameter) were taken from a growing edge of a 5-day-old test fungal colony and transferred to the centre of the test agar plate surface (3 replicates). Cultures were incubated at 28°C and when growing edges of control fungi (without any antagonistic inoculums) were at the edge of the plates, the diameters of the test fungal colonies toward each bacterium were measured. Antagonism against the fungi was evaluated using percentage inhibition values (I).

I = radius of control fungus - radius of test fungus x100/ radius of control fungus

Antagonistic activity of the highly antagonistic strains was repeated on plates with a single individual antagonistic streak and checked after three and 10 days of co-cultivation. Each treatment (each isolate) was done in triplicate and each experiment was conducted three times. The

isolates that showed the highest inhibition or completely inhibited fungal growth called antagonistic isolates were used for further studies.

Nature of the interactions between antagonists and fungi

The morphology of test fungal mycelia adjacent to the bacteria growth was examined under compound microscope (400 x) for abnormal growth patterns and to ascertain whether or not there were direct contacts between bacterial and fungal colonies. The response of spores or mycelia plugs of the three susceptible fungi to form viable colonies was evaluated on agar blocks (5mmx5mm) cut from clear zones formed in dual culture plates.

Fruit material, antagonists and fungal pathogens

Taify table grape fruits (*Vitis vinifera* L.) were harvested at maturity and those having uniformity in size and ripeness, and lack of any apparent injuries or infection were selected. Fruit samples were surface-disinfected with sodium hypochlorite at 0.1% (v/v) for 1 min, rinsed with sterile tap water for three times, and allowed to air dry at room temperature (20^{o}C).

The best antagonistic bacteria *P. aeruginosa* EBVHSH17, *B. vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29 that were originally isolated from the surface of grape fruit as mentioned before, streaked on nutrient yeast dextrose agar (NYDA) and incubated at 28°C for 48 hr. The bacterial cells were cultured in nutrient yeast dextrose broth (NYDB) and incubated for 24 h at 28^{o}C on a rotatory shaker at 200 rpm. The cells were harvested by centrifuging at 4,000 rpm for 20 min and washed twice with sterile distilled water. The concentrations of inoculants of biocontrol agents were adjusted to 10^8 cells/ml colony forming unit (CFU) by dilution plating onto NYDA.

The pathogens *A. niger* BAVSH1, *A. parasiticus* BAVSH4 and *A. tubingensis* BAVSH5 that originally isolated from grape fruits and showing typical black rot were cultivated on Sabouraud's agar medium at 28^{o}C in the dark for seven days. Spore suspensions were prepared by gently rubbing the culture surface of sporulating cultures of the test fungi by glass rod after adding adequate amount of sterile distilled water contained 0.1% Tween 80. The spore concentration was adjusted to 10^4 spores/ml CFU by dilution plating on Sabouraud's agar.

***In vivo* biocontrol of black Aspergilli rot**

For postharvest disease assays, the surface-disinfected grape fruits were wounded in the middle with a sterile cylindrical tool (approximately 2 mm diameter and 3 mm deep) and the cut tissue was removed. Into each wound, 30 µl of sterile water or bacterial living cells suspension of the antagonistic bacteria *P. aeruginosa* EBVHSH17, *B. vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29, in sterile distilled water, adjusted at 1×10^8 cells/ml were pipetted and inoculated. Intact grape fruits were flooded and shaked for 20 min at 120 rpm with either the living bacterial cell suspension at 1×10^8 cells/ml or with crude cell extracts from freeze dried and thawed bacterial cell suspension at 1×10^8 cells/ml. After the suspensions were absorbed, the fruits were wounded after 72 hr. Then after, the fruits were challenged each with 30 µl containing 1×10^4 spores/ml CFU with the pathogens *A. niger* BAVSH1, *A. parasiticus* BAVSH4 or *A. tubingensis* BAVSH5. Wounds treated with sterile distilled water and with fungal spore suspension served as the inoculated positive disease controls. In each experiment, wounds treated with either sterile distilled water, or antagonistic bacteria suspension, without pathogen inoculation was used as negative controls. For all experiments, negative controls did not show disease symptoms. After air drying, the treated grapes were stored at 20 and 5^{o}C in pre-disinfected, covered plastic containers to maintain a high relative humidity. The number of infected fruits and their lesion diameters were examined daily and recorded every fourth day after fungal challenge, started after four and ten days for those incubated at 20^{o}C and 5^{o}C, respectively.

3. Results

Fungal contamination of grapes

Table 1 shows the contamination levels of the Taify table grape, collected from six different private farms at Taif by the fungal genera and the total counts. Umm El-Erad number 3 was the most contaminated farm with 3.25×10^2 CFU/ g. Mathnaa farm number 1 showed the lowest level of fungal contamination 0.79×10^2 CFU/g, followed with Umm El-Erad number 4 that contaminated with 1.58×10^2 CFU/ g. The remaining grape farms showed nearly similar contamination levels which were 2.56×10^2, 2.56×10^2 and 2.30×10^2, respectively. From the fungal infected samples, 11 species with variable frequencies from the grape samples were purified and identified. They were mainly assigned to six genera namely *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus*, *Trichoderma* and *Botrytis*. *Penicillium* was isolated from four farms but not found in the remaining farms. *Aspergillus fumigatus*, *Mucor* and *Botrytis* spp. were isolated from three farms but not found in the remaining farms, while *Rhizopous* sp. was isolated from only two farms and not found in the remaining farms. Occurrence of *Penicillium*, *Mucor*, *Rhizopus* and *Botrytis* was very low.

The genus *Aspergillus* was the most frequently isolated from all farms with average count

ranged between 2.6-43.7 CFU/ml and 2.4-18.66%, compared to the total count of fungi. The isolates

Table 1. Fungal contamination levels in Taify table grape (*Vitis vinefra* L.) collected from six different farms at Taif.

Fungus	Farms																			
	Um El-Erad 1			Um El-Erad 2			Um El-Erad 3			Um El-Erad 4			Mathnaa1			Mathnaa 2			Total (30)	
	(n)[a]	(CFU/g)[b]	(%)[c]	(n)[a]	(CFU/g)[b]	(%)[c]	(n)[a]	(CFU/g)[b]	(%)[c]	(n)[a]	(CFU/g)[b]	(%)[c]	(n)[a]	(CFU/g)[b]	(%)[c]	(n)[a]	(CFU/g)[b]	(%)[c]	(CFU/g)[b]	(%)[c]
A. niger BAVSH1	3	64.0	18.8	4	46.5	19.2	4	70.2	21.5	4	29.0	18.0	2	14.0	17.7	3	39.7	17.2	43.7	18.66
A. parasiticus BAVSH4	3	96.5	20.4	3	37.0	15.0	3	59.2	18.2	3	28.5	18.0	2	13.0	16.4	3	43.0	18.6	41.7	17.70
A. tubingensis BAVSH5	2	58.2	17.0	3	39.2	15.8	3	71.0	21.8	2	20.3	12. 8	1	8.3	10.9	2	30.0	13.0	37.8	15.20
A. sp.	2	52.7	15.5	3	33.3	13.4	3	40.3	12.4	2	33.2	21.0	1	19.0	24.0	2	41.5	13.6	36.6	16.65
A. flavus	3	71.5	21.0	4	56.5	20.2	4	59.5	18.3	3	42.0	15.1	3	8.5	10.9	2	30.0	18.3	37.6	15.60
A. fumigates	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	2	6.0	3.0	2	8.7	11.0	1	1.0	0.4	2.6	2.40
Trichoderma sp.	2	20.4	6.0	2	29.6	11.9	2	21.4	6.6	2	17.5	11.0	1	7.2	9.1	2	28.5	12.3	22.4	9.40
Penicillium sp.	1	2.0	0.6	1	3.0	1.2	1	1.0	0.3	0	0.0	0.0	0	0.0	0.0	1	1.0	0.4	1.2	0.42
Mucor sp.	1	1.0	0.3	0	0.0	0.0	1	1.0	0. 3	0	0.0	0.0	0	0.0	0.0	1	1.0	0.4	0.5	0.17
Rhizopus oryzae BRVSH7	0	0.0	0.0	1	2.0	0.8	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0	1	1.0	0.4	0.5	0.25
Botrytis sp.	1	1.0	0.3	0	0.0	0.0	1	1.0	0.3	0	0.0	0.0	0	0.0	0.0	1	1.0	0.4	0.5	0.17
	$2.56 \times 1o^2$			2.56×10^2			3.25×10^2			1.58×10^2			0.79×10^2			2.30×10^2		3.31×10^2	

[a]Number of samples showing fungal contamination.
[b]CFU/g of grape (average of positive samples).
[c]Percentage of CFU of each fungus/g of grape with respect to the total CFU/g count in each farm.

belonged to the genus were identified in most samples at harvest and it survived through cold storage. Storage of table grapes for 7 days at 5 and 20°C resulted in occasional increase in the number of isolates recovered from the grape fruits. The highest contaminants to Taify table grape were *A. niger* BAVSH1, *A. parasiticus* BAVSH4, *A. tubingensis* BAVSH5 and *A. flavus*. *A. niger* was the most dominant species (18.66%) compared to other isolated *Aspergillus* species. However, *A. fumigatus* was isolated at a low level (2.6 CFU/ml) and in only 3 of the 6 grape farms. The three former isolates were then selected and subjected to *In vitro* control by dual culture antagonism with the most antagonistic bacteria.

Molecular characterization of *Aspergillus* isolates

Identification of isolates of *Aspergillus* was based on PCR amplification of the ITS1-5.8S-rRNA gene-ITS2 region, followed by the subsequent digestion of PCR products with restriction endonuclease RsaI. The product size was about 600 bp (596 to 600 bp). The ribosomal ITS1-5.8S-rRNA gene-ITS2 region was amplified using two oligonucleotide fungal primers (ITS1 and ITS2), sequenced and compared with the database library using analysis soft ware. The program Blast (http://www.ncbi.nlm.nih.gov/BLAST/) was used to assess the DNA similarities. Phylogenetic tree was constructed by aligning sequences of different fungi taken from NCIB and the sequences of ITS1-5.8S-rRNA gene-ITS2 of the most contaminating *Aspergillus* isolates to Taify table grape. Phylogenetic identification indicated that the selected isolates were confirmed to be: *Aspergillus niger* BAVSH1 that shares high similarity to *Aspergillus niger* strain A-3207 (100%), *Aspergillus parasiticus* BAVSH4 that shares similarity to *Aspergillus parasiticus* strain MTCC 2796 (77%), *Aspergillus tubingensis* BAVSH5 that shares similarity to *Aspergillus tubingensis* strain T. TT. A2 (89%) and *Rhizopus oryzae* BRVSH7 that shares similarity to *Rhizopus oryzae* isolate 1289 (93%). Comparative ITS1-5.8S-rRNA gene-ITS2 region sequence analysis of these isolates showed that *Aspergillus* isolates could be allocated in one phylogenetic cluster (Fig. 1) and *Rhizopous* isolate in another cluster. These similarities are sufficient to deduce that the highest contaminants Aspergilli to Taify table grape are belonging to *A. niger* BAVSH1, *A. parasiticus* BAVSH4, *A. tubingensis* BAVSH5 and *Rhizopus oryzae* BRVSH7.

Isolation and identification of antagonistic bacteria

Forty one pure bacterial isolates (10 from grape fruit surface, 19 from boiled grape fruit surface, 6 from soil samples, 6 from meswak) were obtained and stored at -20°C in 50% glycerol for further study.

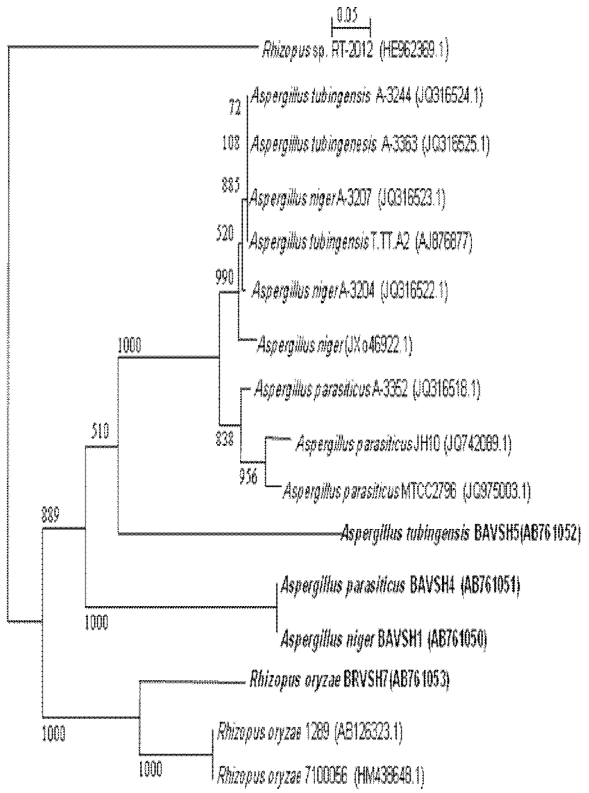

Figure 1. Neighbor-joining polygenetic tree constructed using partial 18S rRNA gene sequences of fungi producing significant alignments to closely related strains. Fungal isolates in each phylogenetic cluster occurring on grapes are highlighted in bold. The geneBank accession numbers are shown in parentheses.

Among the best antagonistic isolates, 5 epiphytic bacterial isolates of grape fruit and one from meswak were selected for morphological, physiological and biochemical characterization. These isolates were further subjected to PCR amplification and identified using 16S rRNA gene sequences. The 1500 bp of 16S rRNA gene sequences of the isolates were amplified using the universal primers for the 16S rRNA gene, sequenced and compared with the database library using analysis soft ware. The program Blast (http://www.ncbi.nlm.nih.gov/BLAST/) was used to assess the DNA similarities. Phylogenetic tree was constructed by aligning 16S rRNA gene sequences of different bacteria taken from NCIB and the sequence analysis of these isolates showed that they could be allocated in two phylogenetically different clusters (Fig. 2). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain Esp-1(T) formed a cluster together with *Pseudomonas*

knackmussii DSM 6978 (T) (98.9% sequence similarity), *Pseudomonas delhiensis* MTCC 7601 (T) (98.5%), *Pseudomonas nitroreducens* DSM 14399 (T) (98.5%), *Pseudomonas citronellolis* DSM 50332 (T) (98.7%), *Pseudomonas multiresinivorans* ATCC 700690 (T) (98.9%) and *Pseudomonas jinjuensis* DSM 16612 (T) (97.8%).

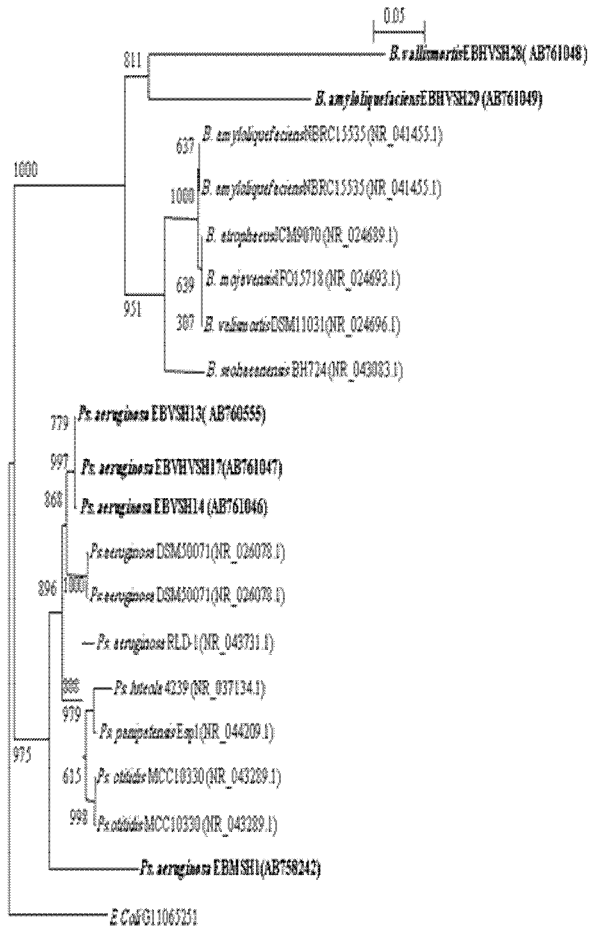

Figure 2. Neighbor-joining polygenetic tree constructed using partial 16S rRNA gene sequences of bacteria producing significant alignments to closely related strains. Bacterial isolates in each phylogenetic cluster occurring on Taify table grape are highlighted in bold. The gene Bank accession numbers are shown in parentheses.

Phylogenetic identification indicated that the selected isolates were confirmed to be: *Pseudomonas aeruginosa* EBMSH1 that shares similarity to *Pseudomonas aeruginosa* strain DSM 50071 (88%), *Pseudomonas aeruginosa* EBVSH13 that shares similarity to *Pseudomonas aeruginosa* strain DSM 50071 (98%), *Pseudomonas aeruginosa* EBVSH14 that shares similarity to *Pseudomonas aeruginosa* strain DSM 50071 (97%), *Pseudomonas aeruginosa* EBVHVSH17 that shares similarity to *Pseudomonas aeruginosa* strain DSM 50071 (96%), *Bacillus vallismortis* EBHVSH28 that shares similarity to *Bacillus vallismortis* strain DSM11031 (94%) and *Bacillus amyloliquefaciens* EBHVSH29 that shares similarity to *Bacillus amyloliquefaciens* strain NBRC 15535 (94%). These similarities are sufficient to deduce that our best antagonistic bacteria are belonging to *Pseudomonas aeruginosa* EBMSH1, *Pseudomonas aeruginosa* EBVSH13, *Pseudomonas aeruginosa* EBVSH14, *Pseudomonas aeruginosa* EBVHVSH17, *Bacillus vallismortis* EBHVSH28 and *Bacillus amyloliquefaciens* EBHVSH29.

***In Vitro* antagonistic activity**

In Vitro antagonistic study using dual culture method for antagonism toward three selected Aspergilli indicated that thirty of the isolated bacteria are antagonistic to some degree to the tested fungi. However, 28 bacteria isolates were more antagonistic to test fungi than others because they could inhibit the test fungi by more than 40% on the test medium (Table 2). Ten isolates; 1, 5, 13, 14, 17, 19, 21, 28, 29 and 40 were greater antagonistic, compared to others. They showed higher inhibition against the three test fungi. The antagonists showed more than 88% growth reduction against the three tested fungal strains (Table 2). Among these antagonists, the isolates 17, 28 and 29 that identified as the highest antagonistic activity being more than 99% growth reduction against the tested fungi. The growth of the test fungal colonies was highly or completely inhibited.

Nature of the interactions between antagonists and fungi

Visual and microscopic observations indicated that there were no direct contacts between the antagonists' colonies and the inhibited fungal colonies, indicating production of diffusible antifungal compounds. Colorless and sterile hyphae were observed on dual culture-agar blocks cut from the area surrounding colonies of highly antagonistic organisms. Germinating spores of test fungi showed aberrant germ tubes, colorless and sterile hyphae (data not shown). They did not form viable colonies and no visible growth of test fungi even at the end of the test period.

Impacts of biocontrol epiphytic bacteria on black Aspergillus rot development

When wounds on grape fruits were treated with *P. aeruginosa* EBVHSH17, *B. vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29, there was a significant reduction in both black *Aspergillus* rot incidence and lesion diameters that formed in response to subsequent challenge with *A. niger* BAVSH1, *A. parasiticus* BAVSH4 and *A. tubingensis* BAVSH5, especially during the periods of 28 and 50 days incubation period at 20 and 5^{o}C, after

4 days at 20°C and after 10 days at 5°C, respectively (Fig. 3 and Fig. 4). However, the disease incidence and lesion diameter started to appear after 4 and 10 days, when the fruits were incubated at 20 and 5°C, respectively.

Table 2. Inhibition levels of different bacterial isolates against *Aspergillus* spp. causing black rot of grape isolated from Taify table grape.

Fungus [Percentage inhibition value (I)]			
Isolate No	*A. niger* BAVSH1	*A. parasiticus* BAVSH4	*A. tubingensis* BAVSH5
1	92.60	91.00	88.88
2	94.44	86.22	92.22
3	83.77	91.11	90.00
4	94.44	88.88	85.55
5	93.33	93.33	94.44
6	90.00	90.00	90.00
7	66.66	60.00	64.00
8	90.00	90.00	90.00
9	91.11	81.50	74.88
10	00.00	00.00	00.00
11	04.88	03.44	08.22
12	77.00	72.00	82.22
13	94.44	93.77	94.66
14	90.00	90.00	91.01
15	66.66	72.00	71.00
16	72.22	72.22	72.22
17	94.44	94.44	94.44
18	92.22	92.22	92.22
19	94.44	90.39	91.00
20	02.22	02.88	02.84
21	93.77	90.00	90.00
22	34.66	40.00	40.00
23	04.53	04.88	04.44
24	22.22	28.88	33.33
25	06.66	04.88	04.88
26	00.00	00.00	00.00
27	00,00	00.00	00.00
28	94.44	94.44	94.44
29	94.44	94.44	94.44
30	88.88	91.00	92.22
31	92.11	91.11	92.22
32	54.00	82.20	80.00
33	60.15	66.66	68.88
34	68.22	68.88	71.00
35	71.77	72.00	71.00
36	60.00	54.44	55.55
37	38.66	40.00	40.00
38	00.00	00.00	00.00
39	60.00	94.44	60.00
40	65.33	60.00	66.66
41	92.22	90.27	94.44

The incidence of disease and the average lesion diameter of *A. niger* BAVSH1, *A. parasiticus* BAVSH4 and *A. tubingensis* BAVSH5 were zero % and zero mm of those in the inoculated controls. This happened in case of flooding the intact grape fruits with living cells and crude cell extracts of all the antagonistic bacteria used (data not shown). The incidence of disease and the average lesion diameters were also zero% and zero mm of those in the inoculated controls. This happened in wounds treated with living cells of *P. aeruginosa*, *B. vallismortis* and *B. amyloliquefaciens*, and challenged with *Asp. niger* at 20°C (Fig. $3A_1$ and $4A_1$), wounds inoculated with living cells of *B. amyloliquefaciens* and challenged with *A. parasiticus* 20°C (Fig. $3B_1$ and $4B_1$), wounds inoculated with living cells of *P. aeruginosa* and *B. vallismortis* and challenged with *A. tubingensis* 20°C (Fig. $3C_1$ and $4C_1$) and wounds inoculated with living cells of *P. aeruginosa*, B. *vallismortis* and *B. amyloliquefaciens*, and challenged with *A. tubingensis* 5°C (Fig. $3C_2$ and $4C_2$). The effects of other treatments varied of those in the inoculated control according to the antagonistic bacteria and black rot fungus. The inhibitory effects were more pronounced at 20°C than at 5°C (Fig. 3 and 4). However, the efficacy of the antagonistic bacteria decreased with incubation time, although the mean values for lesion diameter in wounds inoculated with the antagonistic bacteria were lower than that in wounds of the fungal inoculated control ($P < 0.01$).

4. Discussion

Black Aspergillus rot is among one of the many rots occurring on grapes. The disease appears on the fruits as a black rot due to prolific fungal sporulation after it has invaded. This disease has received more attention since, in the last decade, it was associated with contamination of grapes and wine by ochratoxin A (OTA). This study demonstrates the mycoflora of Taify table grape and possible antagonistic activity of its microflora to control fungi causing postharvest diseases, with special emphasis to black Aspergillus rot. The objective of this study was to find out potential epiphytic bacteria of Taify table grape to antagonize and control the grape pathogens, causing the black Aspergillus rot disease.

The results found in this work about the occurrence of contaminant mycobiota in Taify table grape looks different from other grapes collected from other geographic locations reported by other authors (Abrunhosa et al., 2001; Cabañs et al., 2002; Medina et al., 2005 and Somma et al., 2012). It should be noted that despite the differences in geographic location, the varieties studied by the different authors were different as well, which could explain the disagreement of the results found among the samples. The fact that *Aspergillus* species was the most dominant species in table grape samples was reported by different investigators in different areas around the world (Magnoli et al., 2003; Medina et al., 2005; Samson et al., 2007; Guzev et al., 2008; Leong et al.,

2008; Allam et al., 2011 and Somma et al., 2012). However, more studies are needed to assure differences in grape varieties and geographic location with regard to susceptibility to fungal contamination. A deeper understanding of the species diversity of black Aspergilli, together with specific knowledge of their ecology and epidemiology, can help to predict their occurrences (Somma et al., 2012).

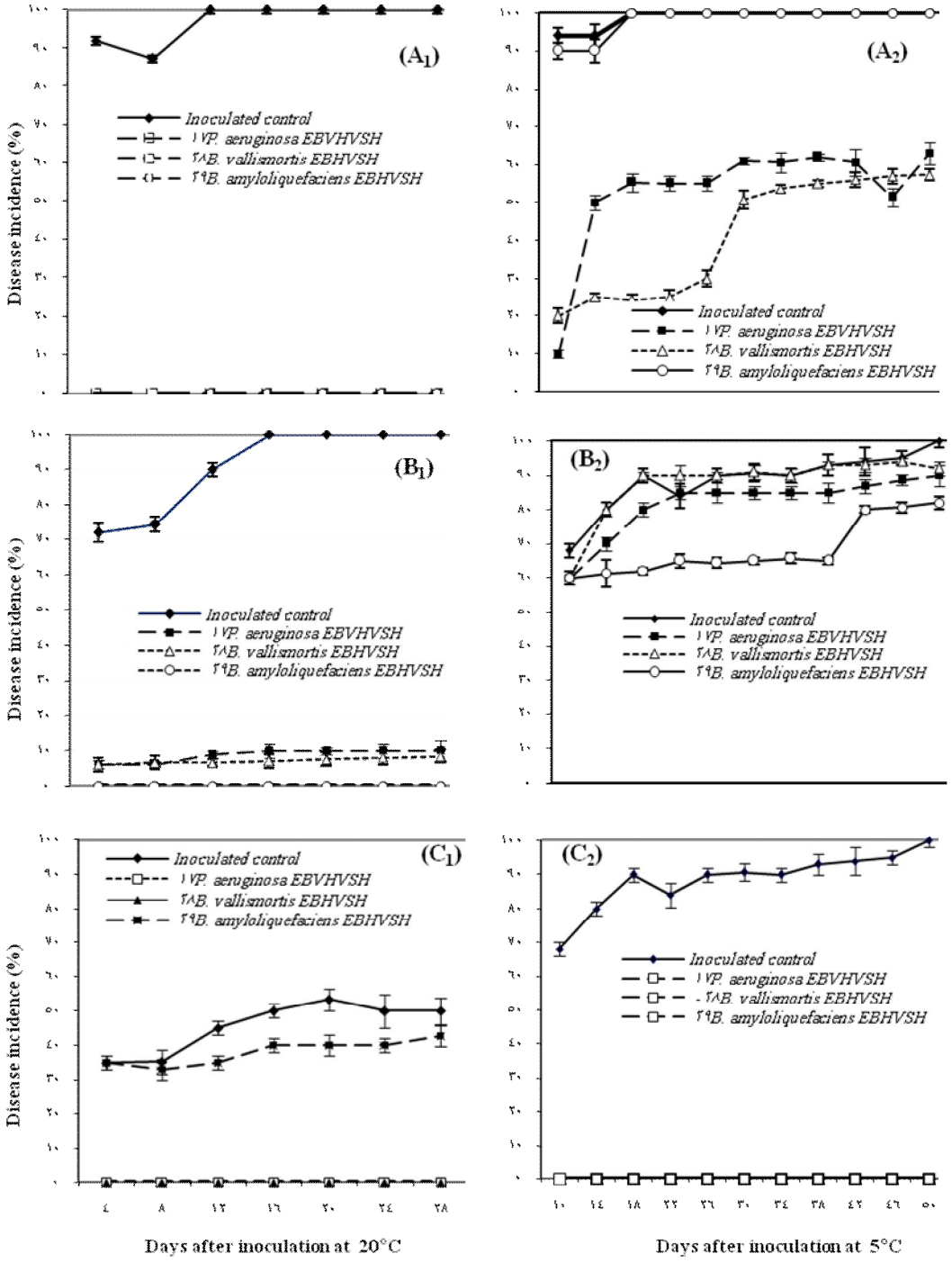

Fig. 3. Development of Aspergillus black rot (disease incidence) on Taify table grape fruit wounds treated with bacterial cells at $1x10^8$ cells/ml followed by challenge with *Aspergillus niger* BAVSH1(A_1, A_2), *A. parasiticus* BAVSH4 (B_1, B_2), and *A. tubingensis* BAVSH5 (C_1, C_2), at $1X10^4$ spores/ml after 72h at 20ºC and 5ºC, respectively.

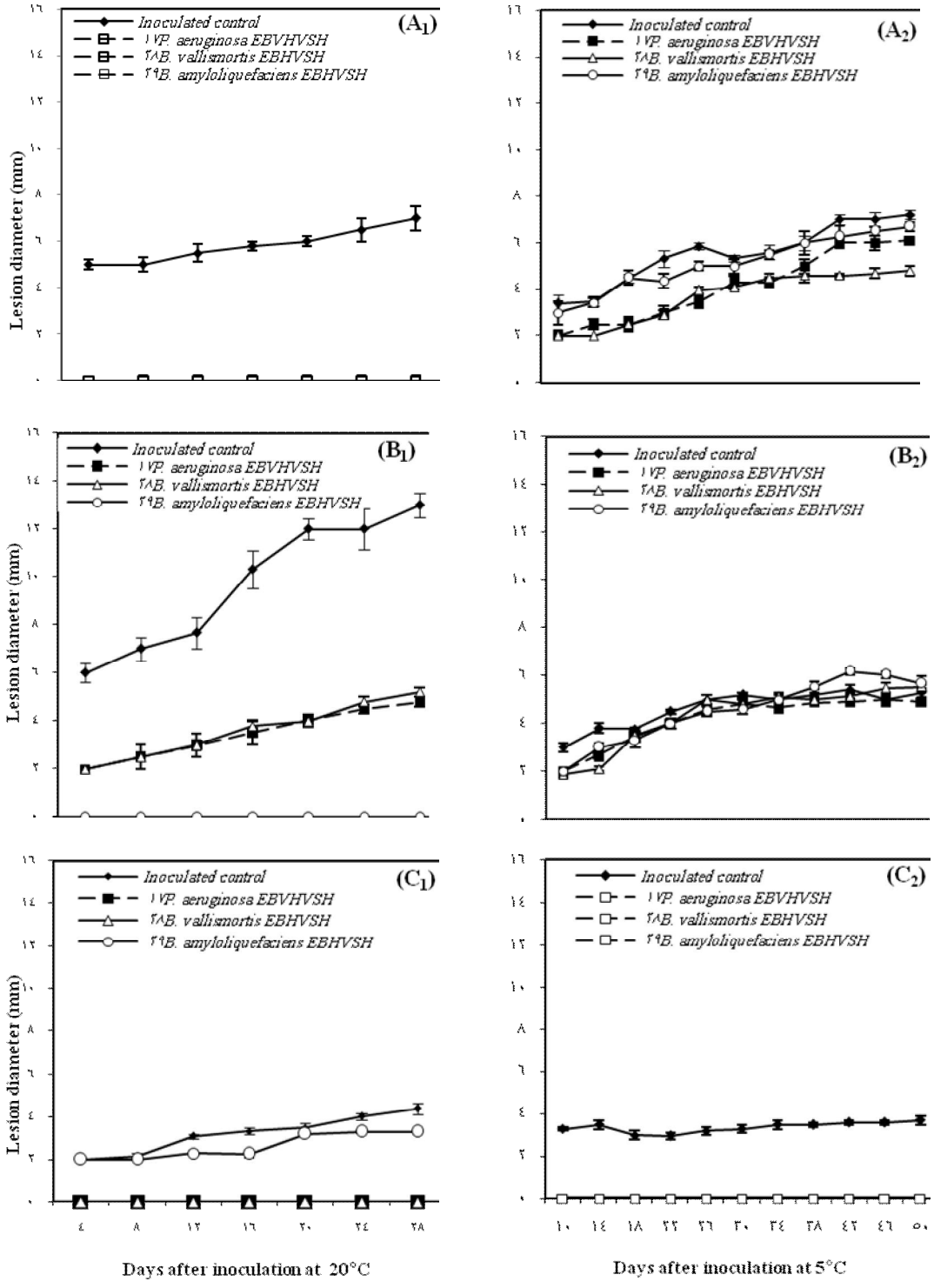

Fig. 4. Development of Aspergillus black rot (lesion diameter) on Taify table grape fruit wounds treated with living bacterial cells at $1x10^8$ cells/ml followed by challenge with *Aspergillus niger* BAVSH1(A_1, A_2), *A. parasiticus* BAVSH4 (B_1, B_2), and *A. tubingensis* BAVSH5 (C_1, C_2), at $1X10^4$ spores/ml after 72h at 20ºC and 5ºC, respectively.

With regard to the different fungi encountered in grape in the present study, black Aspergilli were dominant and exhibited the highest relative abundance, accounting for 18.66 % of all fungi isolated. This predominance supports the findings of other investigators (El-Ghaouth and Wilson, 1995; Allam et al., 2008; Guzev et al., 2008 and Allam et al., 2012) where they found that Aspergilli are the most dominant filamentous fungi, the causative agents of black rot of grapes and the responsible for OTA and AFB1 production. The classification of this fungus was traditionally based on morphological identification (Accensi et al., 1999), which can lead to misidentification. Thus, different molecular tools reviewed by Samson et al. (2007) and Somma et al. (2012) have been used to differentiate taxa within section Nigri. On the basis of PCR with species-specific primers, based on internal transcribed spacer (ITS) regions used in this study and by Gherbawy and Hussein (2010), in addition to phylogenetic analysis based on 16S rRNA gene sequences, the results 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) Nucleotide Database. These similarities are sufficient to deduce that the most occurred fungi that are responsible for black rot in Taify table grape are belonging to *Aspergillus niger* BAVSH1, *Aspergillus parasiticus* BAVSH4 and *Aspergillus tubingensis* BAVSH5. Five epiphytic bacterial isolates of Taify table grape fruit and one from meswak showed the highest antagonistic activities against the previously identified black Aspergilli, and were selected out of 41 isolates for further studies. They were identified by partial sequencing of 16S rRNA gene using set of universal primers. The obtained sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database. The isolated strains are facultative aerobes and distributed into two phylogenetic clusters. On the basis of PCR with universal primers, based on 16S rRNA genes region, used in this study, and phylogenetic analysis based on 16S rRNA gene sequences that 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) Nucleotide Database are sufficient to deduce that our best antagonistic bacteria are belonging to *Pseudomonas aeruginosa* EBMSH1, *Pseudomonas aeruginosa* EBVSH13, *Pseudomonas aeruginosa* EBVSH14, *Pseudomonas aeruginosa* EBVHVSH17, *Bacillus vallismortis* EBHVSH28 and *Bacillus amyloliquefaciens* EBHVSH29.

Table grapes which could be subjected to fungal infection during storage, the infection could be reduced by several treatments. Among these treatments, the reduction of storage time and discarding visibly rotted bunches. In addition, the incidence of black Aspergilli in post-harvest could be reduced with sulphur dioxide in cold storage at 0°C (Lichter et al., 2005). However, attempts to reduce fungal colonization and mycotoxin content of grapes including agronomic practices, chemical and biological treatments have met with varying degrees of success, and the data obtained are sometimes controversial (Somma et al., 2012). Biocontrol as a strategy to reduce fruit diseases using biocontrol agents (BCAs) of fruit diseases, with special interest to grapes are reported elsewhere (Pitt and Ailsa, 1985; Janisiewicz et al., 2000; Janisiewicz and Korsten, 2002; He et al., 2003; Ruenwongsa and Panijpan, 2007 and Pusey et al., 2009). Most of the potential BCAs are yeasts followed by bacteria and then fungi. The results of the present study demonstrated that out of forty one bacteria isolated, the largest inhibitions were obtained with three grape epiphytic bacteria; *P. aeruginosa* EBVHVSH17, *B. vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29. In this context Zhao et al. (2013) used *B. amyloliquefaciens* for the control of post harvest soft rot caused by *Erwinia carotovora* of vegetables. The inhibition of fungal development by the antagonistic organisms suggests the diffusion of antifungal metabolites in agar blocks and the hyperparasitism demonstrated in viability tests. Antifungal production seems to be the major mode of action of most antagonists identified so far (Wilson and Wisniewski, 1989 and Ferreira, 1990). Some reports indicated that antibiotic substances induced malfunctions such as stunting, distortion, swelling, hyphal protuberances or highly branched appearance of fungal germ tubes (Gunji et al., 1983; Getha and Vikineswary, 2002). In some affected fungal colonies, colorless and sterile hyphae were observed in colony margins adjacent to the antagonistic colonies.

In this study, prophylactic treatment of Taify table grape fruits with isolates *P. aeruginosa* EBVHVSH17, *B. vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29, resulted in a dramatic reduction of Aspergillus black rot. The data indicated that the antagonistic bacteria interfered with rot initiation, even when the fruits were challenged with *A. niger* BAVSH1, *A. parasiticus* BAVSH4 and *A. tubingensis* BAVSH5 72 h, after they had been treated with the biocontrol bacteria.

The presence of any one of the three biocontrol agents, in the three applied forms, resulted in disappearance of lesions, smaller lesions and lower or zero disease incidence, compared to the control, when the biocontrol agents were introduced 3 days before the pathogen .This ability to suppress the disease continued to 50 days after the biocontrol agents were applied. This period is adequate for the biocontrol agents to synthesize significant amounts of antimicrobial compounds to effectively prevent the disease progress by the tested fungi. *Pseudomonas* species have been established as promoters of biofilms (Beattie and Marcell, 2002; Pimenta et al., 2003 and Citterio et al., 2004), and the ability of these bacteria to adhere to plant cells (Garrood et al., 2004) and fungi (Russo et al., 2003) has been reported. Carbohydrates and proteins have been implicated in the adhesion reaction (Mclean et al., 1997; Bodilis et al., 2004 and Ranjbariyan et al., 2011). The inhibition of black Aspergillus rot by *P. aeruginosa* EBVHVSH17 is the result of association of *P. aeruginosa* to the challenge fungi or to the plant cells at the wound site. The inhibitory compounds produced by *B. vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29 seems to be bactericidal rather than bacteriostatic in accordance with Ranjbariyan et al. (2011). Populations of many biocontrol agents that are introduced into artificial environments decline with time (Acea et al., 1988), thus the necessary quantities of antimicrobial agents may be lost with the resulting cessation in the synthesis of inhibitory compounds as observed in our study. The better response of black rot pathogens to flooding intact fruits with either living cells or cell-free extracts from the antagonistic bacteria, used in this study suggests that these biocontrol agents could be useful as a surface treatment during grape storage period to prevent the formation of black Aspergillus rot. Although the potential biocontrol agents isolated from *V. vinifera* were not successfully into grapevine (Bell et al., 1995), the bacteria used in this study appear capable of effectively re-introduced and have excellent antagonistic activity. If these bacteria persist on the fruit surface, it may provide additional benefit by preventing infection of grape fruits stored in contaminated habitats.

Conclusion

The microbiota of Taify table grape showed the predominance of filamentous black Aspergilli and epiphytic bacteria, some with antagonistic relationship that has been led to fungal growth reduction. Results of this study showed *In vitro* reduction to Taify table grape post harvest black rot disease causing organisms. This happened by isolates of bacteria from natural epiphytic microbiota of grape that could be of great interest for the control of quality of grape. Among these isolates, *Pseudomonas aeruginosa* EBVHSH17, *Bacillus vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29 induced the resistance of grape fruits against the black Aspergilli. This suggests the production of bacterial compounds serving as inducers of disease resistance. Thus, these isolates could be used *In vivo* for controlling the black rot disease. This is because these organisms can easily be grown in large quantities in simple media and in very short time. Depending on the flooding as potentially effective in inducing defense response, this highlights the importance of flooding grape fruits with living cells or crude cell extracts that induced grape fruits resistance by the bacteria *P. aeruginosa* EBVHSH17, *B. vallismortis* EBHVSH28 and *B. amyloliquefaciens* EBHVSH29 against black rot Aspergilli. Application of these organisms could be preferred and recommended over other antagonistic organisms for field level management, after doing more toxicity and safety tests.

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