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Isolation of potential new biocontrol strains from the blossom of Malus trilobata against the fire blight pathogen *Erwinia amylovora*

Elie Khoury[1], Antoine abou fayad[2,3,4], Dolla Karam Sarkis[5], Mireille kallassy awad[1*]

1 Biotechnology Laboratory, UR EGP, Saint-Joseph University, B.P. 11-514 Riad El Solh Beirut1107 2050, Lebanon

2 Department of Experimental Pathology, Immunology and Microbiology, American University of Beirut, Beirut, Lebanon

3 Center for Infectious Diseases Research (CIDR), American University of Beirut.

4 WHO Collaborating Center for Reference and Research on Bacterial Pathogens, American University of Beirut

5 Laboratory of Pathogens, School of Pharmacy, Saint-Joseph University, Beirut, Lebanone

***Corresponding author:**
Mireille Kallassy Awad

mireille.kallassy@usj.edu.lb

Faculty of Science, Biotechnology Laboratory, UR EGP, Saint- Joseph University, B.P. 11-514 Riad El Solh Beirut1107 2050, Lebanon

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Abstract

Background: Fire blight is a contagious disease that affects members of the *Rosaceae* family, caused by the bacteria *Erwinia amylovora*, which invades apple trees via their blossom.

Methods: In this study, using culture dependent methods, we isolated for the first time the microorganisms colonizing the blossom of the wild apple tree, *Malus trilobata*, in Lebanon. A total of 94 strains from the blossoms of trees originating from two regions, Ain Zhalta reserve and Dhour EL Choueir, were isolated. Genetic analysis of the strains revealed a wide and interesting variety of microorganisms quite different from those isolated from *Malus domestica* blossom. Direct and indirect inhibition assays of the isolates against *E. amylovora* were conducted.

Findings: While some strains belong to species known for their antibacterial activity, two new strains with interesting inhibitory activity against *E. amylovora*, have not been previously described. The first strain is a fungi, *Saccothecium sp*., inhibiting *E. amylovora* growth due to antimicrobial metabolite production and nutrient competition. As for the second strain, a bacterium, *Mycolicibacterium sp*., it inhibits *E. amylovora* growth via diffusible antimicrobial. In addition, novel strains potentially competing with *E. amylovora* for nicotinic acid and nicotinamide were identified such as *Filobasidium sp*., *Rhodotorula sp*. and *Acinetobacter sp*.

Conclusion: We report here two new isolates from the blossom *of Malus trilobata* with high potential of biocontrol activity against *E. amylovora* either via secondary metabolites production and/or by competition at nutrient levels

Keywords: Fire blight, *Erwinia amylovora*, *Malus trilobata*, antibacterial, *Mycolicibacterium*, *Saccothecium*

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Introduction

Fire blight disease of rosaceous plants, caused by *Erwinia amylovora*, affects mainly apple, pear trees and many other woody plants of *Rosaceae* family. This pathogen can be controlled via treatment with antibiotics, copper derivatives or with taking appropriate culture measures. However, certain countries, particularly in Europe, do not authorize the use of antibiotics i.e. streptomycin, to avoid several adverse effects, particularly development of antibiotic resistance in bacterial pathogens [1–3]. An alternative way to control *E. amylovora* is via the characterization of new biocontrol agents, preferably ones originating from the blossom ecosystem [4]. *E. amylovora* begins its infection cycle in the stigmas of blossom then, due to rain, it will be transferred to the hypanthium part and invade through the nectarthodes [2, 5]. At the hypanthium level, the pathogen finds the nutrients needed for its growth, which include Nicotinic Acid (NiAc) and Nicotinamide ($NiNH_2$). The 6-Hydroxynicotinic acid (6-HNiAc) has been shown as well to be required by *E. amylovora* as a growth factor at laboratory scale [6, 7]. A study showed that *Pseudomonas rhizosphaerae* can degrade both NiAc and $NiNH_2$ in a laboratory prepared medium and can colonize apples hypanthia leading to a strong suppression of *E. amylovora* [7]. Moreover, nicotinic acid and nicotinamide were found two times higher in pear than apples' hypanthium [6]. Also, the natural defense

against fire blight attacking the tree through the flower can be due to the flora that resides there competing for nutrients, for example the concentration of sugar on bean leaves decrease to the tenth upon colonization by *Pseudomonas fluorescens* A506 [8]. Since the phyllosphere is a reservoir for bacteria, fungi and yeasts, several studies focused on the leaves, the dominant surface of the epiphyte [9]. Epiphytic surfaces, as well as blossom stigmas and hypanthium, are considered to be a rich reservoir of diverse microorganisms [10, 11], potential biocontrol agents to fight against *E. amylovora* [2]. Although several ways to apply isolated biocontrol strains could be adopted, treatment with the antagonistic strain itself showed to be more efficient than its metabolites, since the latter remain on the surfaces of the treated crops whereas the strain will be spread with insects and other factors [5]. Moreover because of the lack of nutrient at epiphytic level, the colonizing strains compete between each other [4]. Therefore, from a biocontrol perspective, not only is the production of metabolites interesting but also the competition for space and nutrients. Hence the importance of searching for new biocontrol agents active against known pathogens such as *Erwinia amylovora*. Microbial agents can be distinguished by different modes of action, behavior, stability, colonizing capacity and consequently better antagonistic activity. For example, *Pseudomonas graminis* isolated from *Malus spp* phyllosphere was highly active against *E. amylovora* compared to different commercialized antibacterial agents such as: *Pantoea vagans* C9-1, the bioproducts BlightBan A506 *(Pseudomonas fluorescens* A506), the blossom protect (*Aureobasidium pullulans based*) and Hortocyna 18 SP (streptomycin). *Pseudomonas graminis* colonizes apple blossoms under different weather conditions and throughout the entire blooming period [12]. Other effective yeast biocontrol agents were isolated from *Malus pumila* blossoms such as *Cryptococcus* magnus and *Pichia guilliermondii* [2]. As *E. amylovora* is a serious plant pathogen for apple trees at the pre-harvest level, the aim of our study was to isolate the culturable microorganisms associated to blossom of the wild endemic apple tree, *Malus trilobata* (M. trilobata) and testing in vitro their ability to inhibit *E. amylovora* on plate, directly by metabolite productions or indirectly via nutrient or space competition in order to select new promising biocontrol agents against fire blight disease.

Materials and methods

Bacterial strain and culture condition

E. amylovora ATCC 271398 was kindly provided by Dr. Elia Choueiry from LARI (Lebanese Agricultural Research Institute) labs. The strain was routinely cultured on TSA (Biobasic) 28°C and conserved in 20% glycerol at – 80°C.

Sampling of blossom

Samples were collected from M. trilobata from two regions in Lebanon: 27 blossoms from Ain Zhalta cedars reserve (GPS 33.7480, 35.7232) and 40 blossoms taken from Dhour EL Choueir forest (GPS 33.9127, 35.7122), distant by about 19 Km, in mid-May 2017. Blossoms were sampled randomly from the periphery and the inside of each tree, 2 m above the ground, with yellow stigmas, fully opened blossom and falling petals when touched. These parameters are indicators of blossom maturity age of (4-5 days) where microorganisms are present in large quantity [13]. Collected blossoms were kept inside a sterile plastic container, transported on ice and analyzed 24 hours after sampling.

Isolation of microorganisms from the blossom

To isolate microorganisms from the blossom, we followed the protocol as previously described by Lawrence et al. 2009 [2] with some modifications. At the laboratory, the blossoms were placed inside a plastic container on ice. The petals were removed using sterile gloves and sterile forceps. Hereafter, every 20 flowers were assembled for dissection. The stigmas of each flower were cut along with a portion of its supporting style and placed in 1 mL phosphate buffer 10 mM, pH 7.0. Dissected stigmas from two separate tubes from each region were mixed. Two mL of stigmas extract were used for microorganism isolation. The mix was vortexed for one minute, sonicated for one minute and from a serial dilution 10^{-1}, 10^{-2} aliquot were spread onto TSA supplemented with cycloheximide 100 µg/mL for bacteria isolation and onto PDA supplemented with chloramphenicol 100 µg/mL for yeast and fungi isolation. From the remaining part of blossoms, the corolla, calyx and pedicle were removed with sterile gloves in order to reach the hypanthium. The recovered hypanthium was placed in a separate microcentrifuge tube containing one mL phosphate buffer 10 mM, pH 7.0. Approximately 10 hypanthia were distributed per tube, vortexed briefly and placed in a sonication bath for 60 seconds, vortexed again, diluted to 10^{-1}, 10^{-2} and 100 µL of each tube were spread on TSA supplemented with cycloheximide 100 µg/mL for bacteria isolation and PDA supplemented with chloramphenicol 100 µg/mL for yeast and fungi isolation. The incubation at 27 °C for three days followed for all the culture plates.

Sequencing and identification of the strains

Strains were initially observed under an optical microscope. Bacterial isolates were separated from yeast and fungi. The genetic identity of each was revealed after sequencing partial 16S rDNA with the set of primers 27f (5'-AGA GTT TGA TCM TGG CTC AG -3') and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3') which produce an amplicon of ~1500 bp for bacteria [14] and a second set of primers for yeast and fungi with ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') which produce an amplicon of ~500 bp [15]. One colony of each bacterial isolate was resuspended in 10 µL sterile water, of which 1 µL was added to the PCR tube. The 25 µL PCR master mix for each one consisted of: 5 µL 5 × high fidelity buffer, 0.5 µL of 10 mM dNTP, 2.5 µL of 10 µM of each primer 1492R and 27F [14] for bacteria identification, 0.25 µL Taq polymerase (0.5 U/µL) and 13.25 µL sterile nuclease-free water. The PCR reaction was performed out in a thermal cycler with an initial denaturing step of 98 for 30 seconds, 30 cycles of denaturing at 98 for 10 s, annealing at 60 for 30 s and an elongation at 72 for 1 minutes and a final cycle of extra elongation at 72 for 10 minutes. The same procedure was carried out for yeast and fungi except that used primers were ITS4 and ITS5 [15] and the elongation time was 40 s. Samples were separated on 0.8% agarose gel. Only forward (ITS5) for fungi and yeast, and forward/reverse PCR products for bacteria were

sent for sequencing at 1st Base Sequencing-Apical Scientific in Malaysia (http://www.base-asia.com/dna-sequencing-services, March 2020). Chromatograms for sequence quality were checked with Chromas version 2.4.4 and sequences were assembled using the online tool Benchling (https://www.benchling.com, January (2019). Sequences were then searched by nucleotide BLAST (default parameters) against the 16S rRNA database for bacteria and complete non-redundant nucleotide collection for fungi and yeast. Genera identification was based on hits having a minimum of 92% identity and 97% query cover.

Direct *in vitro* antagonistic activity

Antibacterial effect of the isolated strains against *E. amylovora*, the perpendicular streak method was adopted [16]. Bacteria strains were plated on two different media, Trypticase Soy Agar (TSA) and Mueller-Hinton agar (MH), while yeast and fungi isolates were screened on Potato Dextrose Agar (PDA) and Mueller-Hinton (MH) media. After incubation for several days at 28°C until a good growth was observed, *E. amylovora* ATCC 271398 was streaked perpendicular to the antagonistic strain. The plates were then incubated at 28°C for 2 days and the percentage of inhibition was evaluated as follow: inhibition%=(control-test)/control, where control is the growth of *E. amylovora* on the plate without any other strain and test is the growth of *E. amylovora* on the plate in the presence of the potential antagonist on the same plate [16].

Indirect antagonism: Growth on media supplemented with nicotinic acid and nicotinamide

E. amylovora uses Nicotinic Acid (NiAc) and Nicotinamide ($NiNH_2$) as growth factors when infecting the blossoms [6,7]. Indirect antagonism was conducted by testing competition of strains with *E. amylovora* on Nicotinic Acid (NiAc) or Nicotinamide ($NiNH_2$) supplemented DF salt medium pH6, in which the only N-source added was NiAc or $NiNH_2$ at 2 mM [7,17]. The strains spread on the culture media were incubated for 10 days, then inspected for good growth or no growth based on colony growth size and density.

Deferred growth inhibition assay

Two strains showing interesting antibacterial activities were selected for further analysis, in order to invest in the type of antagonism, using a method adapted from the one described by Moran et al., the strain BH8S23 identified as *Saccothecium*, was cultured on PDA pH 7.3 for 7 days at 28 and the strain BS2S3 identified as *Mycolicibacterium*, cultured on MH agar for 2 days at 28 [17]. Saccothecium was then grown in 10 mL PDB pH 7.3 for 4 days and Mycolicibacterium in 10 mL TSB for 2 days at 28. Afterwards, 25 µL of each culture were spotted on PDA pH 7.3 and MH, respectively and left to dry. The plates were then incubated for 5 days for the Saccothecium strain, and 2 days for Mycolicibacterium at 28. Hereafter, a bacterial suspension of 4.106 CFU of E. amylovora ATCC 271398 liquid culture in TSB were sprayed 3 times on each culture plate and incubated for 2 more days at 28 to record the zone of inhibition along with the inhibition score. The inhibition zone is calculated after subtracting the diameter of the observed inhibition zone (X) from the diameter of the inhibitor strain (Y). The clarity score of inhibition ranged from: 0 when the inhibition is complete (very clear inhibition zone surrounding the colony of inhibitor strain), 1 for almost complete inhibition, 2 describes a zone of reduced growth by > 50%, 3 corresponds to minimal growth inhibition where the growth is reduced by less than 50%, 4 no visible growth inhibition, 5 to describe that the growth of the competitor strain was increased by the proximity of the inhibitor strain [17]. The two selected strains were tested 4 times and the test was repeated twice.

Statistical analysis

Experiments were performed three times with three experimental plates for each experiment which provides a mean result for each test and a standard deviation (+/-).

Results

Sampling and isolation

In order to isolate the culturable microbiota associated to *M. trilobata* blossom (Fig 1a), sampling from two different regions of Lebanon, Ain Zhalta and Dhour EL Choueir, was conducted. After dissection of several blossom from each region, the culturable microbiota of two parts of the blossom: the stigmas and hypanthium (Fig 1b) were put on culture separately. In total, 94 strains distributed between bacteria, fungi and yeast were obtained from all dissected blossom. From Ain Zhalta, we isolated 18 strains from stigmas and 25 strains from hypanthium (the attributed codes for the isolates are respectively: BS2S# and BH2S#). As for the sample from Dhour EL Choueir, we isolated 26 strains from stigmas and 25 others from hypanthium (the attributed codes for these isolates are respectively: BS8S# and BH8S#).

Sequencing and identification of the strains

Genetic identity of all the strains was identified following sequencing of 16S rDNA using primers 27F and 1492R for bacteria and ITS 4 and 5 for yeast and fungi. The obtained DNA sequences were searched by nucleotide BLAST using default parameters, after which each the genus to which each strain belong was pinpointed. In order to observe more closely the detailed distribution of the identified strains, we compared the genera varieties and distributions among phylum between the two studied sample type and region. Our results show that the most representative phylum of the isolates for two studied samples types and regions is Ascomycota. Regarding the global genera distribution between the two regions, we noticed that in Ain Zhalta the dominant genera were Aureobasidium (2 isolates from stigmas and 6 from hypanthium), *Cladosporium* (4 isolates from stigmas and 1 from hypanthium), Bacillus (1 isolates from stigmas and 7 from hypanthium) and Pseudomonas (4 isolates from stigmas and 1 from hypanthium). As for Dhour EL Choueir the most dominant genera were *Cladosporium* (3 isolates from stigmas and 8 from hypanthium), *Penicillium* (6 isolates from stigmas and 5 from hypanthium) and Erwinia (4 isolates from stigmas and 2 from hypanthium), as shown in Fig 2 and supplementary (Table S1-S4). Moreover, similar genera were isolated from these two regions. The Venn diagram showed that in Ain Zhalta samples,

Pseudomonas, Bacillus and Dothioraceae were commonly found in stigmas and hypanthium. In addition, in Dhour EL Choueir samples, *Beauveria*, *Alternaria* and Erwinia were commonly found in both stigmas and hypanthium (Figure 3). Filobasidium was a common genus between Ain Zhlata and Dhour EL Choueir stigmas, whereas Aspergillus and Rosenbergiella were commonly found in both Ain Zhalta hypanthium and Dhour EL Choueir stigmas (Figure 3). In contrast, *Cladosporium*, *Aureobasidium* and *Penicillium* were common genera in all the samples from the two regions.

Direct and indirect antagonism results

Our results regarding the direct antagonism on plate showed that among the variety of strains isolated from Ain Zhalta and Dhour El Choueir blossom, isolates that are very-well known for their antibacterial activity were identified. From Ain Zhalta samples, Pseudomonas (BS8S1), and Penicillium (BS8S15) strains from stigmas, showed inhibition of 63 and 100%respectively and *Penicillium* (BH8S17, BH8S23) strains from hypanthium showed inhibition of 50% and 100% towards *E. amylovora*. From Dhour EL Choueir, strains from stigmas Erwinia (BS2S5, BS2S6, BS2S7) and *Penicillium* (BS2S24) showing inhibition of 60 and 83% towards *E. amylovora*, respectively, were identified (Table S1-S4). In total, strains showing antagonistic activity of over 50% are as follow: from Ain Zhalta, 2 isolates out of 18 from stigmas and 2 of 25 from hypanthium, and from Dhour El Choueir 4 of 26 from stigmas and 0 of 25 from hypanthium. In view of the results on genetic identity of the strains and tests of antagonisms, 2 strains have particularly attracted our attention among the variety of the isolates from Ain Zhalta and Dhour El Choueir blossom: one identified fungi unknown for its antibacterial activity (Saccothecium BH8S23, Ain Zhalta) showing 93% inhibition effect against *E. amylovora* (Fig 1S) and another bacterial strain unknown for its antibacterial activity (Mycolicibacterium BS2S3, Dhour EL Choueir), showing 18% of inhibition against E. amylovora (Fig 1S). All results were confirmed three times (Table 2) Since nicotinic acid (NiAc) and nicotinamide (NiNH2) are present in the hypanthium [6] and *E. amylovora* requires one of those two compounds as a growth factor [6, 7], an antagonistic test was conducted by culturing all the isolates on DF salt media supplemented with nicotinic acid or nicotinamide at 2 mM for each. The results showed that out of 94 screened strains, 63 strains (68%) grew well on nicotinic acid supplemented media, 67 strains (72%) have good growth on nicotinamide supplemented media. Moreover, sixty-three strains grow commonly on these two media and four strains grow only on one of the two. Particularly the strain *Saccothecium* (BH8S23) did grow on nicotinamide-based media (Table 2).

Deferred growth inhibition assay

Among all the isolates, two were selected for further analysis, since they have not been previously described for their antibacterial activity: *Saccothecium* (BH8S23, Ain Zhalta) and *Mycolicibacterium* (BS2S3, Dhour EL Choueir), even though the latter showed low activity. In order to decipher the mode of inhibition of these two strains towards *E. amylovora* and to understand if the activity is due to secondary metabolites production or to nutrient competition, a deferred test was conducted by spraying *E. amylovora* on top of a grown culture of the inhibitor strain. Tested plates were incubated for 2 days at 28, following which the inhibition zones were calculated for each strain (Fig 4 a and b), by measuring and subtracting the inhibition diameter X to X from Y to Y, as shown in Fig 4. The zone of inhibition for *Mycolicibacterium* was 16 mm and the one of *Saccothecium* was 7 mm. Whether a defined strain produces an antibiotic or acts via nutrient competition was determined by raising the clarity score for zones of inhibition. For *Saccothecium* the score is equal to 2 since we observed a visible zone of reduced growth of the competitor strain and for *Mycolicibacterium* equal to 0 since the zone of inhibition is very clear.

Table 1: *In vitro* assay confirmation of *Saccothecium*, BH8S23. Strain was cultured for 5 days on PDA and streaked against *E. amylovora*

	Saccothecium, BH8S23	*Mycolicibacterium*, BS2S3
Inhibition %	100	23
	80	15
	100	15
Total	93% +/- 11.547	18% +/- 4.441

Table 2: Number of strains showing good growth on nicotinic acid (NiAC) media and nicotinamide ($NiNH_2$) media.

		Nicotinic Acid	Nicotinamide	Nicotinic acid	Nicotinamide
		Stigmas		**Hypanthium**	
Ain Zhalta	Active	12	14	14	14
	Total Assayed	18	25	18	25
Dhour EL Choueir	Active	17	17	20	22
	Total Assayed	26	25	26	25

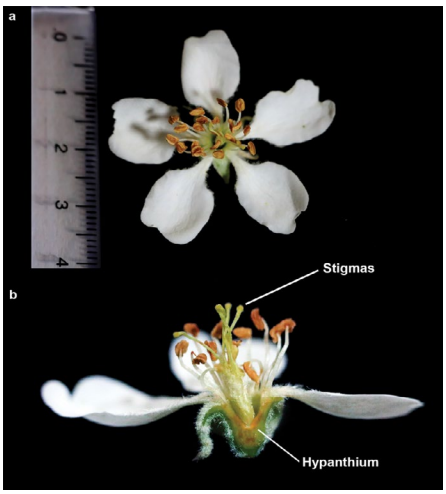

Figure 1 The morphology of *M. trilobata* blossom: (a) mature blossom (b) the hypanthium and stigmas part of dissected blossoms

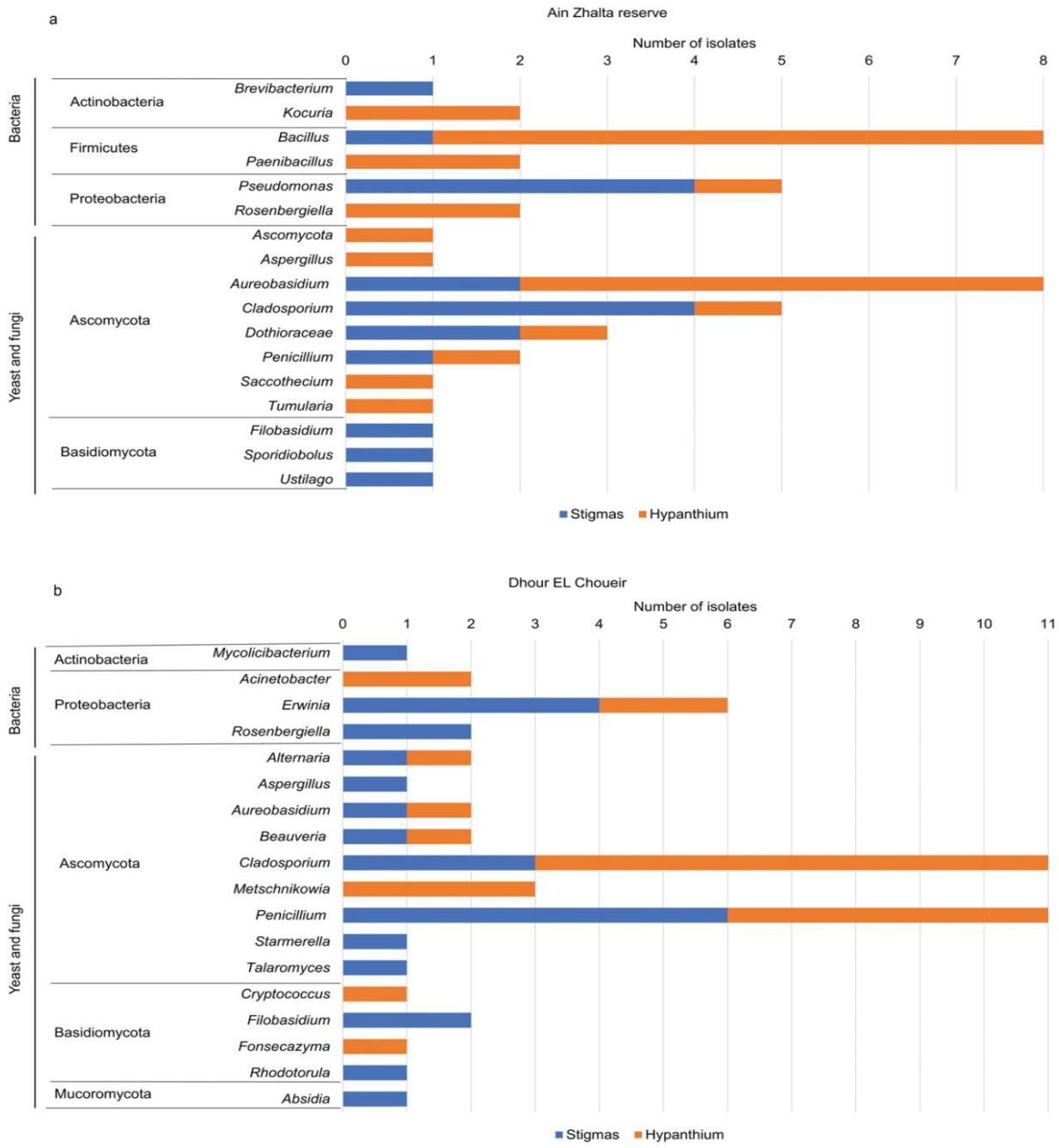

Figure 2 Chart of genera and phylum distribution. Distribution of genera of isolated microorganisms in samples collected from (a) Ain Zhalta and (b) Dhour EL Choueir

Table S1: Description of the isolates. Results from BLASTn and analysis of 16S rDNA and ITS sequences are shown along with antibacterial activity on two different media and the growth capacity of the isolates on media supplemented with nicotinamide or with nicotinic acid.

								Inhibition %						
Stigmas Dhour Choueir	Microscopic	NCBI Best annotation	Accession No.	Phylum	% ID	% Query Coverage	Sequence Length	TSA	MH	NiAC		$NiNH_2$		
BS2S1	Bacteria	*Rosenbergiella epipactidis strain 2.1A*	NR_126303.1	*Proteobacteria*	100	99	1346	0	0	No	No	No	No	No	No
BS2S2	Bacteria	*Rosenbergiella EPIPACTIDIS strain 2.1A*	NR_126303.1	*Proteobacteria*	99	100	1357	0	11	No	No	No	No	No	No

								Inhibition %						
BS2S3	Bacteria	*Mycolicibacterium pyrenivorans strain 17A317A3*	NR_028970.1	*Actinobacteria*	98	100	1330	37	30	No	No	No	No	No	No
BS2S4	Bacteria	*Erwinia billingiae strain Billing E63*	NR_104932.1	*Proteobacteria*	99	100	1408	38	46	No	No	No	No	No	No
BS2S5	Bacteria	*Erwinia billingiae strain Billing E63*	NR_104932.1	*Proteobacteria*	99	100	1394	62	67	No	No	No	No	No	No
BS2S6	Bacteria	*Erwinia billingiae str. LMG 2613*	NR_118431.1	*Proteobacteria*	99	100	1394	62	44	No	No	No	No	No	No
BS2S7	Bacteria	*Erwinia billingiae str. Billing E63*	NR_104932.1	*Proteobacteria*	99	100	1374	60	61	No	No	No	No	No	No
BS2S8	Filamentous fungi	*Beauveria pseudobassiana isolate C5*	MK142275.1	*Ascomycota*	100	100	535	NG	0	No	No	No	No	No	No
BS2S9	Filamentous fungi	*Filobasidium oeirense Strain DK-2b*	MF062224.1	*Basidiomycota*	99	99	597	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S10	Filamentous fungi	*Cladosporium ramotenellum isolate D1*	MG548565.1	*Ascomycota*	100	99	529	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S11	Filamentous fungi	*Cladosporium cladosporioides*	LN834358.1	*Ascomycota*	100	99	527	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S12	Yeast	*Filobasidium oeirense Strain DK-2b*	MF062224.1	*Basidiomycota*	99	100	610	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S13	Filamentous fungi	*Alternaria alternata isolate COL-25*	MH879767.1	*Ascomycota*	100	99	561	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S14	Filamentous fungi	*Aureobasidium pullulans strain RMEQr41*	MF497401.1	*Ascomycota*	99	99	559	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S15	Filamentous fungi	*Penicillium glabrum strain CBS 130049*	MH865723.1	*Ascomycota*	100	100	552	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S16	Filamentous fungi	*Absidia repens isolate A-327*	JQ683219.1	*Mucoromycota*	93	99	600	NG	0	No	No	No	No	No	No
BS2S17	Filamentous fungi	*Penicillium glabrum isolate 136*	KU847873.1	*Ascomycota*	100	99	557	NG	19	Yes	Yes	Yes	Yes	Yes	Yes
BS2S18	Filamentous fungi	*Talaromyces purpureogenus isolate 38_NO.ST86.TLOM1*	KY977598.1	*Ascomycota*	99	99	563	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S21	Filamentous fungi	*Penicillium miczynskii FRR 1077*	NR_077156.1	*Ascomycota*	99	99	576	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S22	Filamentous fungi	*Penicillium glabrum isolate 136*	KU847873.1	*Ascomycota*	99	99	566	NG	30	Yes	Yes	Yes	Yes	Yes	Yes
BS2S23	Filamentous fungi	*Penicillium spinulosum*	KF588647.1	*Ascomycota*	99	98	568	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S24	Filamentous fungi	*Penicillium carneum isolate G2*	KX243324.1	*Ascomycota*	99	99	560	NG	83	Yes	Yes	Yes	Yes	Yes	Yes
BS2S25	Filamentous fungi	*Aspergillus terreus strain MBL1414*	KM924436.1	*Ascomycota*	100	100	585	NG	18	Yes	Yes	Yes	Yes	Yes	Yes
BS2S26	Yeast	*Rhodotorula bacarum strain P34A003*	JX188221.1	*Basidiomycota*	99	98	680	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S27	Fungi			*Ascomycota*					0						
BS2S28	Filamentous fungi	*Cladosporium ramotenellum isolate D1*	MG548565.1	*Ascomycota*	100	97	541	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS2S30	Yeast	*Starmerella bombicola strain CBS 9710*	HQ111046.1	*Ascomycota*	99	99	455	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
NG: non growth															
Inhibition % = (control-test)/ control															

Table S2 : Description of the isolates. Results from BLASTn and analysis of 16S rDNA and ITS sequences are shown along with antibacterial activity on two different media and the growth capacity of the isolates on media supplemented with nicotinamide or with nicotinic acid

								Inhibition %						
Hypanthium Dhour Choueir	**Microscopic**	**NCBI Best Annotation**	**Accession No.**	**Phylum**	**% ID**	**% Query Coverage**	**Sequence Length**	**TSA**	**MH**	**NiAC**			**$NiNH_2$**		
BH2S2	Bacteria	*Acinetobacter nectaris strain SAP 763.2*	NR_118408.1	*Proteobacteria*	99	100	1401	0	0	No	No	No	No	Yes	Yes
BH2S3	Bacteria	*Erwinia billingiae strain Billing E63*	NR_104932.1	*Proteobacteria*	99	100	1110	0	41	No	No	No	No	No	No

								Inhibition %							
BH2S4	Bacteria	*Acinetobacter nectaris strain SAP 763.2*	NR_118408.1	*Proteobacteria*	99	100	1407	0	0	No	No	No	No	Yes	Yes
BH2S5	Bacteria	*Erwinia billingiae str. LMG 2613*	NR_118431.1	*Proteobacteria*	99	100	1389	32	47	No	No	No	No	No	No
BH2S6	Yeast	*Metschnikowia reukaufii*	MH047200.1	*Ascomycota*	98	58	499	NG	0	Yes	No	No	Yes	Yes	Yes
BH2S8	Filamentous fungi	*Cryptococcus dimennae strain PD1511*	KF981862.1	*Basidiomycota*	99	97	498	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S9	Filamentous fungi	*Cladosporium ramotenellum*	LN834386.1	*Ascomycota*	99	99	538	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S10	Filamentous fungi	*Aureobasidium pullulans strain RMEQr41*	MF497401.1	*Ascomycota*	99	99	559	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S11	Yeast	*Metschnikowia reukaufii*	MH047200.1	*Ascomycota*	99	99	375	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S12	Yeast	*Metschnikowia reukaufii*	MH047200.1	*Ascomycota*	99	99	377	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S14	Filamentous fungi	*Cladosporium ramotenellum isolate D1*	MG548565.1	*Ascomycota*	99	100	525	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S15	Filamentous fungi	*Cladosporium limoniforme*	MF473139.1	*Ascomycota*	99	99	543	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S16	Filamentous fungi	*Penicillium glabrum isolate 136*	KU847873.1	*Ascomycota*	99	99	568	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S17	Filamentous fungi	*Penicillium fagi strain CBS 689.77*	MH861113.1	*Ascomycota*	99	99	562	NG	17	Yes	Yes	Yes	Yes	Yes	Yes
BH2S18	Filamentous fungi	*Cladosporium cladosporioides*	LT603043.1	*Ascomycota*	99	99	541	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S19	Filamentous fungi	*Penicillium glabrum isolate 136*	KU847873.1	*Ascomycota*	99	99	565	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S20	Filamentous fungi	*Cladosporium cladosporioides strain VGVR-06*	KX639814.1	*Ascomycota*	99	99	541	NG	0	Yes	Yes	Yes	No	No	No
BH2S21	Filamentous fungi	*Cladosporium sp. Strain HBUM07193*	MF662392.1	*Ascomycota*	99	99	539	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S22	Filamentous fungi	*Penicillium novae-zeelandiae strain DTO 184-13*	KP016830.1	*Ascomycota*	99	99	572	NG	27	Yes	Yes	Yes	Yes	Yes	Yes
BH2S23	Filamentous fungi	*Alternaria alternariae strain CBS 126988*	MH864320.1	*Ascomycota*	100	99	577	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S24	Filamentous fungi	*Beauveria pseudobassiana isolate C5*	MK142275.1	*Ascomycota*	100	100	544	NG	0	No	No	No	No	No	No
BH2S25	Filamentous fungi	*Cladosporium ramotenellum isplate D1*	MG548565.1	*Ascomycota*	100	99	528	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S26	Yeast	*Fonsecazyma mujuensis CBS 10308*	NR_137814.1	*Basidiomycota*	92	97	503	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S28	Filamentous fungi	*Cladosporium sp. isolate D2*	MG548566.1	*Ascomycota*	100	99	524	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BH2S29	Filamentous fungi	*Penicillium patens strain CBS 260.87*	MH862075.1	*Ascomycota*	99	99	556	NG	0	Yes	Yes	Yes	Yes	Yes	Yes

Table S3: Description of the isolates. Results from BLASTn and analysis of 16S rDNA and ITS sequences are shown along with antibacterial activity on two different media and the growth capacity of the isolates on media supplemented with nicotinamide or with nicotinic acid.

									Inhibition %						
Stigmas Ain Zhlata reserve	Microscopic	NCBI Best Annotation	Accession No.	Phylum	% ID	% Query Coverage	Sequence Length	TSA	MH	NiAC			$NiNH_2$		
BS8S1	Bacteria	*Pseudomonas tremae strain TO1*	NR_025549.1	*Proteobacteria*	99	100	1385	0	63	Yes	Yes	Yes	Yes	Yes	Yes
BS8S2	Bacteria	*Pseudomonas lutea strain OK2*	NR_029103.1	*Proteobacteria*	99	100	1403	0	0	No	No	No	No	No	No
BS8S3	Bacteria	*[Brevibacterium] frigoritolerans strain DSM8801*	NR_117474.1	*Actinobacteria*	99	100	1403	0	0	No	No	No	Yes	Yes	Yes

					Inhibition %										
BS8S4	Bacteria	*Bacillus safensis strain NBRC 100820*	NR_113945.1	*Firmicutes*	100	100	1422	4	20	No	No	No	No	No	No
BS8S5	Bacteria	*Pseudomonas syringae strain ICMP 30223*	NR_117820.1	*Proteobacteria*	99	100	1403	4	17	No	No	No	Yes	Yes	Yes
BS8S6	Bacteria	*Pseudomonas rhizosphaerae strain IH5*	NR_029063.1	*Proteobacteria*	99	100	1377	0	0	No	No	No	No	No	No
BS8S7	Yeast	*Cladosporium sp. 13 NK-2011*	HQ846579.1	*Ascomycota*	100	100	530	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BS8S8	Filamentous fungi	*Aureobasidium pullulans strain RMEQr41*	MF497401.1	*Ascomycota*	99	99	559	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BS8S9	Yeast	*Dothioraceae sp. SN-2008*	EU755002.1	*Ascomycota*	100	99	557	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BS8S10	Yeast	*Dothioraceae sp. SN-2008*	EU755002.1	*Ascomycota*	100	99	558	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BS8S11	Yeast	*Filobasidium chernovii culture CBS:8679*	KY103413.1	*Basidiomycota*	99	99	619	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BS8S12	Filamentous fungi	*Ustilago hordei strain CBS 131470*	MH877396.1	*Basidiomycota*	99	100	296	NG	NG	No	No	No	No	No	No
BS8S13	Yeast	*Aureobasidium pullulans strain CNRMA6.840*	KP131644.1	*Ascomycota*	99	99	559	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BS8S15	Filamentous fungi	*Penicillium commune isolate MC-9-L*	KU527787.2	*Ascomycota*	99	100	586	100	55	Yes	Yes	Yes	Yes	Yes	Yes
BS8S16	Filamentous fungi	*Cladosporium sp. Isolate R97206*	MK268136.1	*Ascomycota*	99	100	526	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BS8S17	Yeast	*Sporidiobolus metaroseus culture CBS:5541*	KY105476.1	*Basidiomycota*	99	99	582	NG	0	Yes	Yes	Yes	Yes	Yes	Yes
BS8S18	Filamentous fungi	*Cladosporium ramotenellum isolate E22285*	MK267741.1	*Ascomycota*	100	99	531	0	13	Yes	Yes	Yes	Yes	Yes	Yes
BS8S19	Filamentous fungi	*Cladosporium ramotenellum isolate E20346*	MK267417.1	*Ascomycota*	100	100	514	0	0	Yes	Yes	Yes	Yes	Yes	Yes
NG: no growth Inhibition % = (control-test)/ control															

Table S4: Description of the isolates. Results from BLASTn and analysis of 16S rDNA and ITS sequences are shown along with antibacterial activity on two different media and the growth capacity of the isolates on media supplemented with nicotinamide or with nicotinic acid.

					Inhibition %										
Hypantium Ain Zhlata reserve	**Microscopic**	**NCBI Best Annotation**	**Accession No.**	**Phylum**	**% ID**	**% Query Coverage**	**Sequence Length**	**TSA**	**MH**	**NiAC**			**$NiNH_2$**		
BH8S1	Bacteria	*Rosenbergiella epipactidis strain 2.1 A*	NR_126303.1	*Proteobacteria*	99	100	1366	0	0	No	No	No	No	No	No
BH8S2	Bacteria	*Kocuria turfanensis strain HO-9042*	NR_043899.1	*Actinobacteria*	99	100	1393	0	0	No	No	No	No	No	No
BH8S3	Bacteria	*Bacillus proteolyticus strain MCCC1A00365*	NR_157735.1	*Firmicutes*	99	100	1419	0	0	Yes	Yes	Yes	No	No	No
BH8S4	Bacteria	*Paenibacillus tritici strain RTAE36*	NR_157638.1	*Firmicutes*	99	98	1413	0	0	No	No	No	No	No	No
BH8S5	Bacteria	*Bacillus safensis strain NBRC100820*	NR_113945.1	*Firmicutes*	100	100	1418	20	38	No	No	No	No	No	No

Inhibition %															
BH8S7	Bacteria	*Kocuria rosea strain DSM20447*	NR_044871.1	*Actinobacteria*	99	100	1318	0	0	No	No	No	No	No	No
BH8S8	Bacteria	*Paenibacillus tritici strain RTAE36*	NR_157638.1	*Firmicutes*	99	98	1410	0	0	No	No	No	No	No	No
BH8S9	Bacteria	*Pseudomonas rhizosphaerae strain IH5*	NR_029063.1	*Proteobacteria*	99	100	1386	0	0	Yes	Yes	Yes	No	No	No
BH8S10	Bacteria	*Bacillus aryabhattai strain B8W22*	NR_115953.1	*Firmicutes*	100	100	1312	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S11	Bacteria	*Bacillus salsus strain A24*	NR_109135.1	*Firmicutes*	97	99	1424	0	0	No	No	No	No	No	No
BH8S12	Bacteria	*Rosenbergiella epipactidis strain 2.1A*	NR_126303.1	*Proteobacteria*	99	100	1359	0	0	No	No	No	No	No	No
BH8S13	Bacteria	*Bacillus megaterium strain NBRC15308*	NR_112636.1	*Firmicutes*	99	100	1342	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S14	Yeast	*Dothioraceae sp. SN-2008 clone o17*	EU755002.1	*Ascomycota*	100	99	558	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S15	Filamentous fungi	*Aureobasidium pullulans strain RMEQr41*	MF497401.1	*Ascomycota*	99	99	559	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S16	Filamentous fungi	*Aureobasidium pullulans Strain RMEQr41*	MF497401.1	*Ascomycota*	99	99	562	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S17	Filamentous fungi	*Penicillium olsonii strain 1.17*	KM265447.1	*Ascomycota*	99	100	558	50	45	Yes	Yes	Yes	Yes	Yes	Yes
BH8S18	Filamentous fungi	*Cladosporium sp. Strain FKL2*	MF281313.2	*Ascomycota*	100	100	517	0	35	Yes	Yes	Yes	Yes	Yes	Yes
BH8S19	Yeast	*Ascomycota sp. AU61*	KP403976.1	*Ascomycota*	100	88	577	0	0	Yes	Yes	Yes	Yes	Yes	
BH8S20	Filamentous fungi	*Aureobasidium pullulans strain RMEQr41*	MF497401.1	*Ascomycota*	99	99	560	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S21	Filamentous fungi	*Aureobasidium pullulans strain RMEQr41*	MF497401.1	*Ascomycota*	99	99	559	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S23	Yeast	*Saccothecium sepincola strain CBS 749.71*	MH860331.1	*Ascomycota*	97	99	980	100	0	No	No	No	Yes	Yes	Yes
BH8S24	Filamentous fungi	*Tumularia aquatic*	FJ000399.1	*Ascomycota*	99	99	571	NG	NG	No	No	No	No	No	No
BH8S25	Filamentous fungi	*Aureobasidium pullulans isolate UTFC-AP58-9*	KY767023.1	*Ascomycota*	100	98	561	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S26	Filamentous fungi	*Aureobasidium pullulans strain RMEQr41*	MF497401.1	*Ascomycota*	99	99	559	0	0	Yes	Yes	Yes	Yes	Yes	Yes
BH8S27	Filamentous fungi	*Aspergillus tubingensis isolate 132*	KU847852.1	*Ascomycota*	100	99	577	NG	0	No	No	No	Yes	Yes	Yes
NG: no growth Inhibition % = (control-test)/ control															

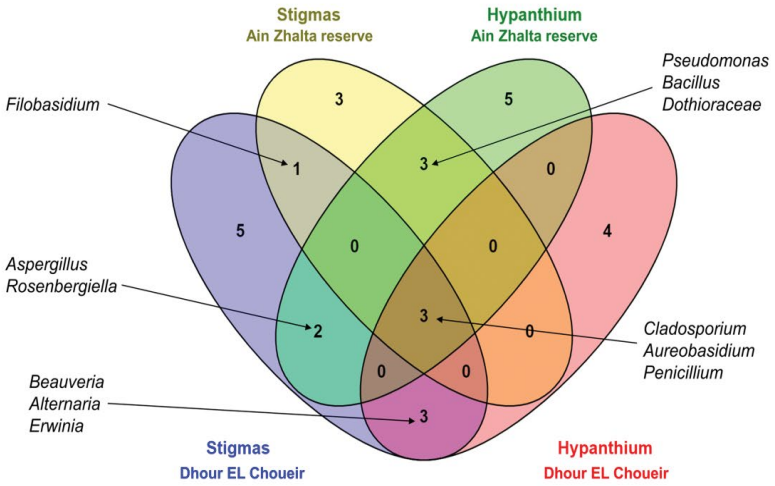

Figure 3 *ATAD3+/-*male mice circadian activity. A: Activimetry was measured individually by video recording of 9 WT and 9 *ATAD3+/-* males during a night period. Movements of each mouse were quantified by computer analysis and restituted as an activimetric curve; B: Movements of each mice restituted as accumulative activimetric curve; C: Cumulated inactivity (min/h) is represented as a histogram.

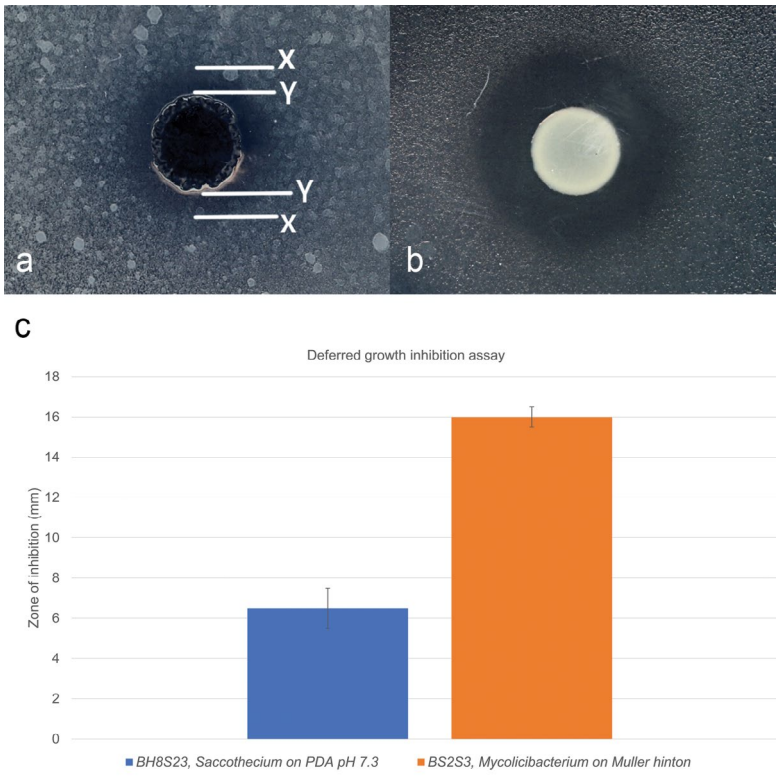

Figure 4 Deferred growth inhibition assay. (a) The inhibition zone of the inhibitor *Saccothecium*, BH8S23 face to the competitor *E. amylovora* with partial inhibition. (b) The inhibition zone of the inhibitor *Mycolicibaterium*, BS2S3, face to the competitor *E. amylovora* with a complete inhibition. (c) Quantitative measurement of the deferred growth assay. This graph represents the subtraction of Y from X. Y corresponds to the diameter of the competitor strain and X to the diameter of zone of inhibition

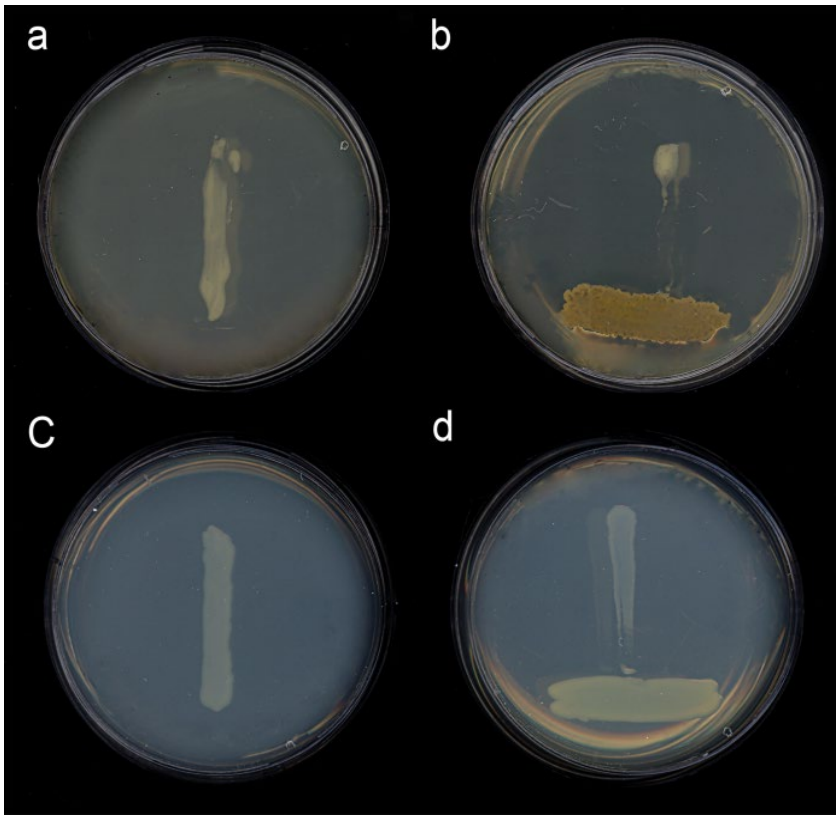

Figure1S Antibacterial perpendicular streak assay. (a) and (c) *E. amylovora* alone on the plate with no competitor strain (b) *Saccothecium*, BH8S23 against *E. amylovora* and (d) *Mycolicibacterium*, BS2S3 against *E. amylovora*

Accession number

The 16Sr DNA and ITS nucleotide sequences of the identified isolates were uploaded on GenBank under the following accession numbers: SUB7188877- MT254860-MT254888.

Discussion

The aim of the study was to isolate and identify the culturable microorganisms associated to M. trilobata blossom in order to study their potential use as biocontrol agents against the fire blight disease caused by *E. amylovora* by testing their ability to inhibit this bacterium directly via antibiotics and/or indirectly via nutrient competition. Therefore, two regions of Lebanon were selected to sample blossom which were dissected to separate stigmas from hypanthium and subsequently used to extract culture dependent microorganisms, since these two parts of the blossom are well described as the starting point of infection by *E. amylovora* in Malus species [2, 5, 18]. From these two regions, a total of 94 strains was isolated and genetically identified with a total of 17 and 18 different genera obtained from Ain Zhalta and Dhour EL Choueir, respectively. Strains isolated from *M. trilobata blossom, e.g. Mycolicibacterium*, *Beauveria*, *Filobasidium*, *Starmerella*, *Fonsecazyma*, *Ustilago*, *Sporidiobolus* and *Saccothecium*, are quite different from those obtained from *M. pumila* [2]. Only 3 genera, from Ain Zhalta reserve: *Pseudomonas*, *Aureobasidium* and *Kocuria*, and 2 genera from Dhour EL Choueir: Starmerella and Aureobasidium, are in common with isolates from *M. pumila* [2]. In addition, Aleklett et al. have shown that Metschnikowia, Cryptococcus, Pseudomonas and Acinetobacter are very commonly detected as consistent members of flowers across several plants [10] which was confirmed in our study with the isolates mainly from Dhour EL Choueir. As for the isolated strains' antibacterial activity, the in vitro direct antagonism analysis showed an interesting variety of very well-known microorganisms described for their antibacterial activity, belonging particularly to the following genera: *Aspergillus, Penicillium, Erwinia, Pseudomonas, Cladosporium* and *Bacillus*. Nonetheless, new interesting strains, not studied previously for their antibacterial activities against *E. amylovora*, were identified, including the fungi *Saccothecium* isolated from Ain Zhalta hypanthium showing very high activity (93%) and the bacteria *Mycolicibacterium* isolated from the stigmas of Dhour EL Choueir blossom showing 18% of inhibition effect within the perpendicular streak method. The first is an ascomycota pseudothecial, sexual genera, found in different areas across the globe and host of different plant species like *Aruncus sylvestris, Celtis planchoniana* and *Cornus sanguinea* (Hayova and Minter, 2011; Thambugala et al., 2014). Whereas the second is known for the degradation of the soil contaminant Polycyclic-Aromatic-Hydrocarbon (PAHs) [25, 26]. On another level, since *E. amylovora* requires small amounts of nicotinic acid or nicotinamide as growth factors, thus antibacterial effect through metabolites secretion is not the only way to inhibit it [6]. The conducted growth test on culture media containing nicotinic acid or nicotinamide as sole source of nitrogen, showed that 63 and 67 isolates respectively from both regions, grew on that media. Hypanthium in *Malus domestica* blossom contains (0.264-

0.761) µg hypanthium-1 of nicotinic acid and nicotinamide [27] which is (1.2-3.5) higher than the amount needed for pathogen to grow [27]. However, if competing microorganisms can degrade NiAc and $NicNH_2$ at the hypanthium level, they can reduce the availability of these compounds and thus control *E. amylovora* infection cycle [6, 27]. *Pseudomonas fluorescens* TN5 and *Pseudomonas rhizosphaerae* [7] are examples of microorganisms that degrade NiAc and NicNH2, hence control *E. amylovora*. In our study, some of the isolated strains from *Malus trilobata* trees belong to genera not described before for this nutrient competition activity. Their ability to grow on NiAc and $NiNH_2$ based media reduced the availability of these two compounds, thus could correspond to emerging biocontrol agents. A few examples are *Filobasidium*, *Rhodotorula*, *Starmerella* isolated from stigmas and *Acinetobacter* from hypanthium, both from Dhour EL Choueir and Saccothecium from hypanthium, Ain Zhlata. The latter corresponds to a potential biocontrol agent acting directly and indirectly against the pathogen *E. amylovora*. To test the ability of *Saccothecium* (BH8S23) and *Mycolicibacterium* (BS2S3) in inhibiting *E. amylovora* via nutrient or antibiotic production, we've resorted to deferred growth inhibition assay, a technique imitating what happens in nature, where one strain will first be established, so that any other strain will have to counteract any inhibition variables in order to invade the niche [17]. The related results regarding the inhibition zone and the clarity score showed that the *Saccothecium* strain inhibited *E. amylovora* via diffusible growth inhibitors and by reducing nutrient availability for the competitor strain. [17]. Interestingly, based on the deferred growth inhibition assay results, *Mycolicibacterium* presented very high and clear antagonistic activity against *E. amylovora* (16 mm inhibition and a score of 0) due to a diffusible antibiotic produced by the inhibitor strain. This study reports for the first time that *Saccothecium* and *Mycolicibacterium* can inhibit *E. amylovora* either via nutrient competition or via antibiotic production. In planta tests are perceived to better consolidate these observations. These two strains are potential novel biocontrol agent. A deep necessary chemical and genetical analysis is ongoing to study the structure of the biomolecules that they produce and their biosynthesis pathways.

Conclusion

In conclusion, our study describes the cultured microorganisms associated to the endemic wild apple *tree M. trilobata* blossom. A large variety of identified genera was shown to inhibit the fire blight causative bacterium *E. amylovora* via metabolite production, space and nutrient competition by degrading both nicotinic acid and nicotinamide. Two strains could be of big potential as biocontrol agent against the fire blight disease. Further chemical and genetical analysis are necessary to unravel the chemical structure of the biomolecules produced by these 2 strains, in addition to in vivo flower assay in order to test the power of the selected strain to survive/colonize on plant surfaces and to study its competitive ability against *E. amylovora* on stigmas in a time-dependant inoculation.

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