Chapter 7
What Lies Beneath: Root-Associated Bacteria to Improve the Growth and Health of Olive Trees

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Abstract During the last decades we have witnessed growing public concern on the abuse/misuse of agrochemicals to control plant pathogens. The fact that some relevant phytopathogens (for instance, the soil-borne fungus *Verticillium dahliae* Kleb.) are very difficult to control by methods alternative to chemical-based products, has urged researchers to seek effective measures within integrated disease management frameworks. Biological control, alone or in combination with other approaches, emerges as one of the most promising alternatives to confront plant pathogens in a sustainable, environment-friendly strategy. Effectiveness of biological control agents (BCA) largely depends on colonization and persistence capabilities in the ecological niches (e.g. root and/or rhizosphere) where their benefits are expected to be deployed. As a consequence, due to BCA-host specificity (or co-adaptation) the search of potential BCAs in their target environments seems an appropriate strategy. This chapter describes the isolation, identification and characterization of indigenous antagonist bacteria from the olive rhizosphere that can be eventually exploited as BCA against relevant pathogens affecting this woody crop, with emphasis on *V. dahliae*. The approach here implemented could be of interest for other pathosystems involving trees and soil-borne pathogens.

Keywords *Pseudomonas* spp. • *Paenibacillus* spp. • Biological control • Plant growth promoting rhizobacteria • *Verticillium dahliae* • Olive pathogens

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7.1 Introduction

Olive (*Olea europaea* L.) is a woody cultivated species of major importance for many countries in the Mediterranean Basin, where constitutes a fundamental economical pillar. In fact, more than 90% of global olive oil and table olive production is concentrated in this area (FAOSTAT 2016). The soil-borne fungal phytopathogen *Verticillium dahliae* Kleb, the causal agent of Verticillium wilt of olive (VWO), is one of the main biotic constrains affecting olive. Nowadays, the disease is considered one of the most serious threats for this crop. It has been described in many areas where olive is relevant, and reported to have spread alarmingly during the last two decades (López-Escudero and Mercado-Blanco 2011). At present, a single effective control measure against VWO is not available (Bubici and Cirulli 2011), and the management of the disease is a very complicated task. This difficulty can be explained by a combination of several causes, including the current lack of effective fungicides able to access the pathogen during its parasitic phase (i.e. within the xylem vessels) (López-Escudero and Mercado-Blanco 2011) even when applied in the trunk (Bubici and Cirulli 2012). Furthermore, *V. dahliae* produces resistant, dormant structures (microsclerotia), under adverse conditions, able to endure in soils for a prolonged period of time. The wide range of plants that can be infected by the pathogen, its genetic diversity, or inadequate agronomic practices are other factors reducing the chance to control VWO successfully (López-Escudero and Mercado-Blanco 2011; Tsror 2011). Therefore, the development of new, sustainable and alternative disease control measures is crucial to confront this pathogen. Approaches that should be considered as complementary within an integrated disease management framework.

The public concern on the abuse of agrochemicals (i.e. chemically-based fertilizers, fungicides, insecticides, etc.) increased in recent years, due to relevant negative effects on the environment, the ecosystems biodiversity and human or animal health. Consequently, several research teams have focused their efforts on the development of alternative, environment-friendly inputs aiming to increase crops yields and to control biotic threats towards relevant agro-ecosystems (Ruano-Rosa and Mercado-Blanco 2015). However, the implementation of control strategies replacing the traditional use of chemical biocides can be difficult in many situations. This is the case of trees and woody plants, although examples of successful biological control of diseases affecting these hosts are available (Cazorla and Mercado-Blanco 2016; and references therein).

In the olive-*V. dahliae* pathosystem, the use of biological control tools has been proposed as a complementary approach within the above-mentioned integrated disease management strategy, mainly as a preventive measure (López-Escudero and Mercado-Blanco 2011; Tjamos 1993). Biological control usually involves the application of microorganisms with antagonistic and/or plant growth promotion (PGP) effects. For instance, fungi such as *Trichoderma* spp. (*e.g.* Aleandri et al. 2015; Carrero-Carrón et al. 2016; Ruano-Rosa et al. 2016) or several bacterial genera have been reported as effective (Sanei and Razavi 2011) and/or with potential (Aranda

et al. 2011) as biological control agents (BCA) against VWO. Regarding the use of bacteria to manage VWO, several studies showed that strains from *Pseudomonas fluorescens* and *Pseudomonas putida* (Maldonado-González et al. 2015b; Mercado-Blanco et al. 2004; Prieto et al. 2009), *Serratia plymuthica* (Müller et al. 2007) or *Paenibacillus alvei* (Markakis et al. 2016) performed well under different experimental conditions.

A reasonable strategy for BCA selection is to search for microorganisms residing in the same ecological niche in which they will be eventually applied (Knudsen et al. 1997; Ruano-Rosa and López-Herrera 2009). It is therefore expected that these BCA will be adapted to such a niche, being able to effectively colonize and endure in the target site (*e.g.* the roots) where they can deploy their beneficial effects (i.e. biological control activity). In this context, the aims of the present work were: (i) to generate a collection of cultivable bacteria originated from and adapted to the olive roots/rhizosphere; (ii) to screen for bacterial isolates showing antagonistic ability against *V. dahliae* and other phytopathogens; and (iii) to molecularly, biochemically and phenotypically characterize selected bacterial isolates showing traits traditionally associated with biological control and/or PGP. The final objective is to identify novel bacterial strains with potential to be used in future bioformulations effective against different olive diseases, with emphasis in VWO.

7.2 Materials and Methods

7.2.1 Sampling and Isolation of Cultivable Bacteria from Olive Roots

Root samples from one-year-old olive plants (cv. Picual) were collected from ten commercial nurseries located in Córdoba province (South Spain). Ten grams of fresh root tissue, thoroughly washed under tap water to remove most of the attached soil/substrate particles, and representative of the whole radical system of each plant (three per nursery), were cut and ground using a mortar and pestle with 10 mL of sterile distilled water. Aliquots (100 µl) of 10-fold serial dilutions of the macerates obtained from each sample were plated under aseptic conditions on different culturing media, namely potato dextrose agar (PDA; Oxoid, Basingstoke, UK), Luria-Bertani agar (LB, 1% Bacto-tryptone, 0.5% yeast extract and 1% NaCl) and nutrient agar (NA, Oxoid). Plates were incubated at 28 °C in the dark at least for 72 h. Pure cultures originating from single colonies grown in these media were carefully selected and isolated attending to distinctive morphological characteristics, and cryopreserved in 2-ml vials containing 33% glycerol at −80 °C, until use.

7.2.2 *Molecular Identification of Bacteria*

A selection of bacteria (189 strains) from the generated collection was molecularly identified (genus level) by partial sequencing of the *16S rDNA* ribosomal gene. Then, a subset of 40 strains selected according to their potential as BCA, were further characterized by partial sequencing of the *gyrB* genes (coding for DNA gyrase, subunit B). For this aim, genomic DNA was obtained from 1 mL of each bacterial isolate grown in LB medium during 16 hours (28°C in the dark; 200 rpm) by using 'JETFLEX Genomic DNA Purification Kit' (Genomed, Löhne, Germany) according to the manufacturer's instructions. Amplifications were performed in a total volume of 25 µl containing 2.5 µl of 10× PCR buffer (50 µM KCl, 10 mM Tris–HCl pH 9 [25 °C], 1% v/v Triton X- 100), 1.5 (*16S rDNA*) or 2 (*gyrB*) mM $MgCl_2$, 0.2 µM each primer, 0.2 mM each dNTP, 1.25 U of BioTaq DNA Polymerase (Bioline Ltd, London, UK) and 100 ng of bacterial DNA. The *16S rDNA* sequence was amplified by PCR as follows: denaturation for 4 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C and a final extension step of 10 min at 72 °C. The primers pair F27 (5′AGAGTTTATCMTGGCTCAG3′) and R1492 (5′GRTACCTTGTTACGACTT3′) was used for *16S rDNA* amplification (1,465 bp amplicon size). PCR conditions for *gyrB* were: denaturation for 3.30 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 53.8 °C and 2 min at 72 °C. The degenerated primers UP-1 (5′GAAGTCATCATGACCGTTCTGCAYGCNGGN GGNAARTTYGA3′) and UP-2r (5′AGCAGGGTACGGATGTGCGAGCCRTCN ACRTCNGCRTCNGTCAT3′) were used for amplification (amplicon size ≈ 1200 bp). Amplicons were purified using 'Favorgen GEL/PCR Purification Mini Kit' (Biotech Corp.; Pingtung, Taiwan) according to the manufacturer's specifications. DNA sequencing was performed by a commercial service (Sistemas Genómicos S.L., Valencia, Spain). For *gyrB,* primers UP-1S (5′GAAGTCATCA TGACCGTTCTGCA3′) and UP-2Sr (5′AGCAGGGTACGGATGTGCGAGCC3′) were used for sequencing as described by Yamamoto and Harayama (1995). DNA sequences were assembled in 'contigs' with CLC bio software (Aarhus, Denmark) and compared (http://www.ncbi.nlm. nih.gov/) with available databases to identify each bacterial isolate genus and, when possible, species.

7.2.3 In vitro *Antagonism Assays Against* V. dahliae *and Other Plant Pathogens*

The antagonistic activity of bacteria isolated from olive roots was assessed by *in vitro* dual culture assays in two stages. An initial screening of the whole collection (327 isolates) was performed attending to the antagonistic activity against V-937I, a highly-virulent, defoliating (D) isolate of *V. dahliae* previously characterized at the genetic, molecular and pathogenic level (Collado-Romero et al. 2006; Prieto et al. 2009; Maldonado-González et al. 2015a, b) (Table 7.1). A second screening round against a group of additional plant pathogens (Table 7.1) was then performed, using

Table 7.1 Plant pathogens used for *in vitro* assays to test the antagonistic activity of rhizobacteria isolated from olive roots

Specie	Isolate/strain	Reference/Source
Alternaria alternata	CN191, CN194	Dr. Longxian Ran, Agricultural University of Hebei, P.R. China
Colletotrichum nymphaeae	Col. 114	Moral et al. (2012)
Colletotrichum godetiae	Col. 516	Dr. A. Trapero Casas, Universidad de Córdoba, Córdoba, Spain
Fusarium oxysporum f. sp. *dianthi*	Fod 113, Fod108	Gómez-Lama Cabanás et al. (2012)
Phytophthora cinnamomi	CH1100	Dr. C. J. López-Herrera, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain
Pseudomonas savastanoi pv. savastanoi	PSS-3	Culture collection of the Plant-Microorganism Interactions Laboratory, IAS-CSIC
P. savastanoi pv. savastanoi	NCPPB 3335	Pérez-Martínez et al. (2007)
Rosellinia necatrix	Rn 320, Rn 400	López-Herrera and Zea-Bonilla (2007)
Verticillium dahliae (D)[a]	V-937I	Collado-Romero et al. (2006)
V. dahliae (D)[a]	Lebrija 1	Culture collection of the Plant-Microorganism Interactions Laboratory, IAS-CSIC
V. dahliae (ND)[a]	V-249I	Collado-Romero et al. (2006)

[a]*D* defoliating pathotype, *ND* non-defoliating pathotype

only those isolates that already showed positive antagonism against V-937I in the first screening (189 isolates). Assays for fungal pathogens were performed by placing combinations of the target pathogen (5-mm diameter mycelial agar plugs from 7-day-old cultures)/potential BCA (inoculated with a sterilized toothpick) at 2.5 cm distance on PDA (for fungal pathogens) and NA (for bacterial pathogens). Plates were incubated (25 °C in the dark) until the pathogen covered the distance separating both microorganisms in control plates (i.e. without antagonist) (Fig. 7.1a; upper left plate). For assays involving bacterial pathogens, potential antagonists were grown as described above over a pathogen lawn (OD_{600}: 0.1) on PDA and NA plates (28 °C in the dark) during 48 hours. Antagonistic activity (i.e. production of haloes and/or inhibition zones) was then scored. These experiments were repeated at least once for each phytopathogen/antagonist combination.

7.2.4 *Phenotypic Characterization of Indigenous Bacteria from Olive Roots*

To determine the presence of phenotypes associated to antagonism and/or PGP among isolates selected after the first screening, several activities were assessed, namely protease (Naik et al. 2013), catalase (Holt et al. 1994), phosphatase (Katznelson and Bose 1959), chitinase (Murthy and Bleakley 2012), siderophore production (Schwyn and Neilands 1987) and 2,3-butanediol (Methyl Red Voges Proskauer [MRVP] medium, according to manufacturer's specifications; Micro

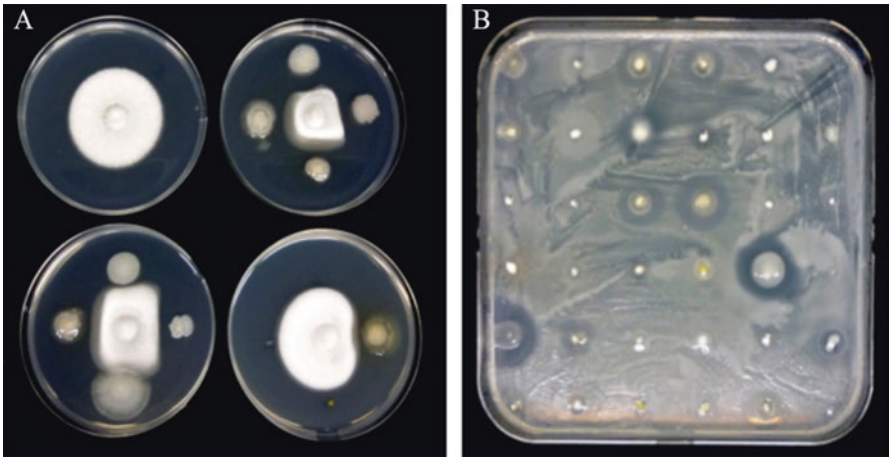

Fig. 7.1 *In vitro* antagonism of olive rhizosphere bacteria against two olive pathogens, on PDA. (**a**) antagonistic activity displayed by rhizobacteria against *V. dahliae* (D pathotype) (7-days-after inoculation). A comparison plate shows normal, full growth of *V. dahliae* in absence of bacteria (control, upper left plate). (**b**) antagonistic activity revealed by inhibition haloes around some bacterial colonies against *Pseudomonas savastanoi* pv. savastanoi (2-days-after inoculation)

Media, Nebotrade Ltd.; Budapest, Hungary). Additionally, in order to perform an in-depth evaluation of the nutritional requirements of a selected group of isolates (44, selected based on their antagonistic capability, phenotypic traits and suitability to be produced as BCA), we assessed their capability to metabolize 71 C sources and their sensibility to 23 chemical substances by using GEN III MicroPlate™ (Biolog; Hayward, CA). Evaluation was performed according to the manufacturer's specifications. All assays were repeated at least once.

7.3 Results

7.3.1 Generation of a Collection of Culturable Bacteria from Olive Roots and First In Vitro Antagonism Screening

A collection of 327 culturable bacterial isolates, natural inhabitants of roots from olive (cv. Picual) plants propagated under conditions usually implemented in nurseries at Southern Spain, was obtained following the sampling/isolation scheme described. Bacteria selected were representative of all colony morphologies found in the culturing media used. Overall, similar types of bacterial colonies were found regardless the media used (i.e. PDA, LB or NA). No major difference (i.e. prevalence of any colony morphology) was observed among nurseries either. After two rounds of growth to check for possible contaminants, all isolates selected were individually cryopreserved at -80°C until further use, and a code was assigned to each of them.

The first screening based on the antagonistic activity against *V. dahliae* V-937I (D pathotype) resulted in the selection of 121 isolates. Additionally, 68 isolates were included in the final selection according to different colony morphology, irrespective of their antagonistic capability against the D pathotype of *V. dahliae*. In the final selection, representative bacteria originating from all sampled nurseries were present.

7.3.2 Molecular Characterization of Cultivable Bacteria from Olive Roots

Sequencing of the *16S rDNA* (amplicon size 1,465bp) and *gyrB* (amplicon size ≈ 1,200bp) genes revealed the prevalence, in the collection, of taxa representative of the Proteobacteria (54.5%) followed by *Firmicutes* (34.4%) and *Actinobacteria* (9.5%) (Table 7.2). The genera distribution showed *Bacillus* spp. as the most abundant (33.3%), followed by isolates identified as *Pseudomonas* spp. (32.3%). Some isolates (1.57%) could not be unequivocally identified and were thus catalogued as unclassified (Table 7.2).

Table 7.2 Molecular identification of 189 bacteria isolated from roots of olive plants (var. Picual) based on analysis of the *16S rDNA* and *gyrB* gene sequences

Phyla	Class	Genera	Frequency (%)
Actinobacteria	*Actinobacteria*	*Cellulomonas*	1.06
		Curtobacterium	0.53
		Microbacterium	2.65
		Micrococcus	0.53
		Streptomyces	4.76
Firmicutes	*Bacilli*	*Bacilllus*	33.33
		Paenibacillus	1.06
Proteobacteria	*α-Proteobacteria*	*Agrobacterium*	1.59
		Ensifer	0.53
		Rhizobium	3.17
		Roseomonas	0.53
		Sinorhizobium	0.53
		Sphingomonas	0.53
	β-Proteobacteria	*Achromobacter*	1.59
		Cupriavidus	2.12
		Massilia	1.06
	γ -Proteobacteria	*Cronobacter*	3.70
		Enterobacter	2.65
		Pseudomonas	32.28
		Pseudoxhantomonas	3.17
		Serratia	1.06
Unclassified*			1.57

*Sequencing of *16S rDNA* and *gyrB* genes did not unambiguously resolve the genus

7.3.3 In vitro *Antagonism of Indigenous Olive Rhizobacteria Against Selected Plant Pathogens*

The *in vitro* antagonism against a representative of the *V. dahliae* D pathotype determined in the first screening round was confirmed in a second assay (Table 7.3). In general, differences (presence/absence of antagonism) were observed depending on the media used (with the exception of *V. dahliae*) (Fig. 7.1a), highlighting the cases of *C. godetiae* and *P. savastanoi* pv. savastanoi in which antagonism events were more frequent in NA and PDA, respectively (Table 7.3). For some rhizobacteria, differences in the percentage of effective antagonism between the two isolates used were found (*viz. R. necatrix*, *F. oxysporum* f. sp. *dianthi* and *P. savastanoi* p. var. savastanoi). The most dramatic case was for strain *P. savastanoi* NCPPB 3335 in PDA, which was inhibited by a significant lower number of rhizobacteria compared to antagonism observed against strain PSS-3 (25.4% and 9.0%, respectively). On the contrary, similar percentages were observed for *V. dahliae* and *Colletotrichum* spp. representatives, even though isolates here used belonged to different pathotypes (*V. dahliae*) or species (*Colletotrichum* spp.). Overall, *Paenibacillus* spp., *Pseudomonas* spp., *Rhizobium* spp. and *Bacillus* spp. representatives displayed a broad-spectrum antagonism against phytopathogens assayed. Remarkably, the best results were achieved for one strain of *Paenibacillus polymyxa*, which was able to inhibit the growth of 92% (in PDA) and 100% (in NA) of the pathogens assayed.

7.3.4 *Phenotypic Characterization of Selected Olive Rhizobacteria with Potential as Biological Control Agents*

Some phenotypes traditionally associated with biological control and/or PGP activities were evaluated and results are summarized in Table 7.4. All bacteria tested showed catalase activity, while phosphatase activity was found only in 2% of

Table 7.3 Percent of tested olive rhizobacteria showing antagonistic activity against phytopathogens

Pathogen	Isolates (%)[a]	
	PDA	NA
Alternaria alternata[b]	43.4	37.0
Colletotrichum godetiae[b]	15.3	49.7
Colletotrichum nymphaeae[b]	18.0	32.8
Fusarium oxysporum f. sp. *dianthi*	13.8	15.1
Phytophthora cinnamomi	33.9	19.0
Pseudomonas savastanoi pv. savastanoi[b]	17.2	1.4
Rosellinia necatrix[b]	23.6	37.0
Verticillium dahliae[b]	62.0	60.6

[a]Means from two assays
[b]Pathogens affecting olive

Table 7.4 Percentage of olive rhizobacteria showing phenotypes associated with biological control and/or plant growth promotion activities

Phenotypic characterization	Isolates[a] (%)
2,3-butanediol	30
Protease	29
Phosphatase	2
Siderophore	49
Catalase	100
Chitinase	20

[a]Means from two replications, for a total of 189 isolates selected after the first screening

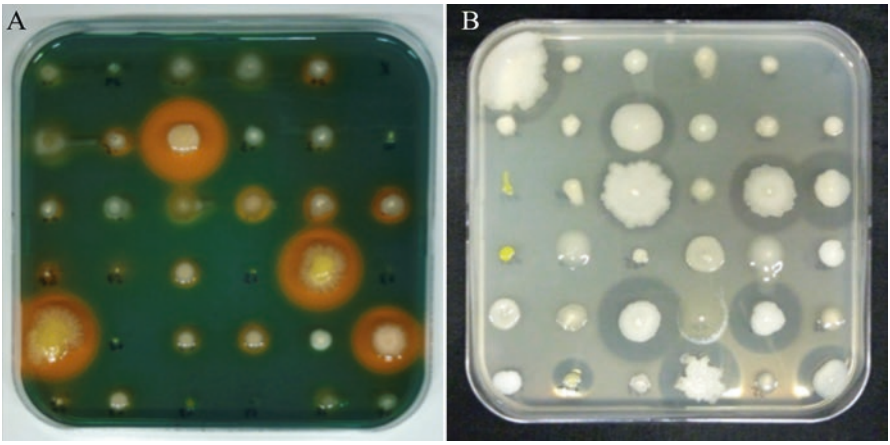

Fig. 7.2 Phenotypic characterization of olive rhizobacteria as potential biological control and plant-growth promotion agents. (**a**) siderophore production assay (according to Schwyn and Neilands 1987). (**b**) protease assay (according to Naik et al. 2013)

isolates (belonging to *Pseudomonas* and *Enterobacter*). A significant number of olive rhizobacteria displayed siderophore activity (49%), highlighting some *Pseudomonas* spp. isolates producing large halos when tested in CAS medium (Fig. 7.2a). The rest of activities tested showed a moderate-low presence. While catalase activity was present in representatives of all genera tested, production of 2,3-butanediol (30%), protease (29%) (Fig. 7.2b) and chitinase (20%) were associated almost exclusively to *Bacillus* spp. (Table 7.4).

GEN III MicroPlate™ assays provided a phenotypic fingerprinting of a selection of isolates (44). In general, high variability was observed among isolates regarding to the different substrates utilization, being *Proteobacteria* the most versatile phylum with some isolates able to metabolize up to 46 carbon sources (*Rhizobium* spp.). The capability to metabolize different C sources is summarized in Table 7.5. Regarding to chemical responsiveness, 98% of the assessed isolates grew in 1% NaCl and pH 6. It is worth mentioning the high percentage of isolates capable to grow in Na lactate 1% (93%), a well-known bactericidal used in alimentary industry, and the overall sensibility to minocycline, an antibiotic belonging to the tetracycline group (Table 7.6).

Table 7.5 Percentage of olive rhizobacteria showing the capability to metabolize different carbon sources according to the GEN III MicroPlate™ test

C source	Number of isolates (%)[a]	C source	Number of isolates (%)*
Acetic Acid	86	β-Hydroxy-D,L- Butyric Acid	35
α-D-Glucose	77	α-Keto-Glutaric Acid	30
Glycerol	70	D-Malic Acid	30
D-Fructose	67	Glycyl-L-Proline	26
Dextrin	65	N-Acetyl-D- Glucosamine	23
Pectin	65	myo-Inositol	23
L-Serine	58	Quinic Acid	21
Sucrose	53	D-Cellobiose	19
D-Fructose- 6-PO4	51	β-Methyl-D- Glucoside	19
L-Malic Acid	51	D-Mannose	19
Acetoacetic Acid	51	D-Salicin	16
L-Aspartic Acid	49	D-Galactose	16
L-Glutamic Acid	49	Glucuronamide	16
L-Histidine	49	Gentiobiose	14
Methyl Pyruvate	47	D-Turanose	14
D-Mannitol	44	D-Serine	14
L-Alanine	44	α-Keto-Butyric Acid	14
D-Gluconic Acid	44	L-Fucose	12
Citric Acid	44	Stachyose	9
D-Maltose	42	D-Raffinose	9
L-Pyroglutamic Acid	42	α-D-Lactose	9
D-Galacturonic Acid	42	D-Melibiose	9
L-Galactonic Acid Lactone	42	D-Sorbitol	9
Bromo-Succinic Acid	42	D-Fucose	7
Propionic Acid	42	L-Rhamnose	7
Gelatin	40	Inosine	7
L-Arginine	40	N-Acetyl-D- Galactosamine	5
D-Saccharic Acid	40	D-Arabitol	5
L-Lactic Acid	40	N-Acetyl-β-D- Mannosamine	2
γ-Amino-Butryric Acid	40	3-Methyl Glucose	2
Formic Acid	40	D-Aspartic Acid	2
D-Trehalose	37	p-Hydroxy- Phenylacetic Acid	2
D-Glucose- 6-PO4	37	D-Lactic Acid Methyl Ester	2
Mucic Acid	37	α-Hydroxy- Butyric Acid	2
Tween 40	37	N-Acetyl Neuraminic Acid	0
D-Glucuronic Acid	35		

[a]Means from two assays, for a total of 44 isolates selected based on their antagonistic capability, phenotypic characteristics and suitability to be produced as BCA

Table 7.6 Olive rhizobacteria capable to grow under diverse chemical substances and conditions according to the GEN III MicroPlate™ test

Chemical responsiveness	Number of isolates (%)[a]	Chemical responsiveness	Number of isolates (%)[a]
pH 6	98	Troleandomycin	42
1% NaCl	98	Niaproof 4	42
1% Sodium Lactate	93	Vancomycin	42
Potassium Tellurite	88	Tetrazolium Violet	42
Guanidine HCl	86	Tetrazolium Blue	42
Aztreonam	86	Fusidic Acid	30
Lithium Chloride	70	pH 5	26
4% NaCl	65	Nalidixic Acid	26
Sodium Butyrate	58	8% NaCl	19
D-Serine	49	Sodium Bromate	12
Rifamycin SV	49	Minocycline	0
Lincomycin	44		

[a]Means from two assays, for a total of 44 isolates selected based on their antagonistic capability, phenotypic characteristics and suitability to be produced as BCA

7.4 Discussion

Rhizosphere microbial communities are exposed to several biotic and abiotic factors strongly determining their structure and composition, being one of them the host plant (Marschner et al. 2004). Considering this scenario, it is not surprising that when a given microorganism is used as a BCA, one of the main problems encountered is its low adaptation to the target environment. This may result in the lack of success or the inconsistency frequently reported when implementing biological control approaches (Handelsman and Stabb 1996). However, this obstacle can be somehow overcome by searching, identifying and characterizing potential BCA from the same ecological niche where its beneficial action will be required (Knudsen et al. 1997; Ruano-Rosa and López-Herrera 2009). Taking this premise as starting point, we designed a strategy in which the first objective was to generate a collection of indigenous olive rhizobacteria as potential BCA against *V. dahliae*.

The molecular analysis of members of this collection revealed a high diversity and prevalence of phyla *Proteobacteria* and *Firmicutes*. This finding is in agreement with results earlier reported for wild olives (Aranda et al. 2011).

The selection process of candidate BCA was primarily driven by checking effective *in vitro* antagonism against the D pathotype of *V. dahliae.* However, since a broad spectrum of biological control activity is a desirable characteristic for a BCA (Whipps and Davies 2000), a number of phytopathogens affecting olive trees and other crops were evaluated as potential targets of newly-isolated olive rhizobacteria. We found several bacteria showing *in vitro* antagonism against tested pathogens. Because of the intensity and spectrum of the inhibition displayed, representatives of *Paenibacillus* spp., *Pseudomonas* spp. and *Bacillus* spp. are worth mentioning.

Members of these genera are frequently reported as per their involvement on biological control and/or PGP (Borriss 2015; Lugtenberg and Kamilova 2009; Mercado-Blanco 2015; Hayat et al. 2012; Santoyo et al. 2012; Rybakova et al. 2016).

In the olive rhizobacteria collection generated in this study, two strains of *Paenibacillus* spp. highlighted due to their strong antagonistic capability, both in intensity and range of pathogens inhibited. Species of this genus have been described as BCA, plant growth-promoting rhizobacteria and microorganism with high biotechnological potential (Lal and Tabacchioni 2009; Bhattacharyya and Jha 2012). Some of them are present in several commercial products against a broad range of phytopathogens (Rybakova et al. 2016). For instance, *P. polymyxa* has been previously reported as an excellent BCA against *V. dahliae* in cotton (Yang et al. 2013) and other fungi, oomycetes or bacteria (Raza et al. 2015; Hong et al. 2016; Xu and Kim 2016). Likewise, a strain of *Paenibacillus alvei* showed effective against *V. dahliae* in olive trees (Markakis et al. 2016). Whether the two *Paenibacillus* spp. strains here characterized behave as true BCA against VWO and/or the other pathogens tested still needs confirmation by conducting *in planta* bioassays.

Strains belonging to other genera also showed *in vitro* antagonism against tested phytopathogens, although to a lesser extent. The genus *Pseudomonas* is one of the most studied regarding to biological control and PGP activities. Important features such as broad colonization ability, production of a wide diversity of secondary metabolites and versatility in using different molecules as C sources make beneficial strains of this genus excellent tools in biological control strategies (Mercado-Blanco and Bakker 2007). Regarding to biological control of VWO, different strains of *Pseudomonas* spp. have been previously reported as effective BCA (Mercado-Blanco et al. 2004; Sanei and Razavi 2011). Another renowned genus used in biological control approaches is *Bacillus*. Mechanisms ranging from production of antifungal compounds to induction of systemic resistance in the host plants along with high colonization and sporulation capabilities and advantageous competition for nutrients, make them excellent BCAs as well (Borriss 2015; Santoyo et al. 2012; Kumar et al. 2011; Zheng et al. 2013). Isolates of *Bacillus* spp. have shown good performance against Verticillium wilt of cotton. As for the *Paenibacillus* spp. strains mentioned above, suitable BCA candidates assigned to the genera *Pseudomonas* and *Bacillus* could be expected from the olive rhizobacteria collection here generated. The next step yet to be taken is *in planta* experiments to verify effectiveness against VWO.

Besides assessment of antagonist ability, the first step towards the identification of candidate BCA, aim of this study was to evaluate the presence of bacterial traits related to biological control and/or PGP in indigenous olive rhizobacteria. Among the phenotypes detected, it is worth mentioning the outstanding number of isolates displaying siderophore production. These low-molecular-weight, iron-chelating molecules (Höfte 1993), present in nearly 50% of the isolates in our collection, are produced under iron-limiting growth conditions and have been frequently described as involved in competition and disease suppression for a number of BCA, including beneficial *Pseudomonas* spp. (Mercado-Blanco 2015). Additionally, since chitin,

glucans and glycoproteins are the major components of the fungal cell wall (Bowman and Free 2006), it is also interesting to emphasize the presence of isolates displaying production of lytic enzymes such as chitinases and proteases. Both phenotypes are very appreciated during BCA selection processes, and pose relevance in industrial and medical biotechnology as well (Wang et al. 2009). While production of these exoenzymes is frequent in many rhizobacteria (Whipps 2001; Ajit et al. 2006), they were almost restricted to representatives of *Bacillus* spp. Although at low percentage, production of 2,3-butanediol was also found in our bacterial selection. This is an interesting volatile involved in growth stimulation (Ryu et al. 2005) and has been suggested to be implicated in induced systemic resistance responses (Han et al. 2006; Borriss 2015). Finally, substrate utilization and chemical responsiveness profiles obtained by the BIOLOG system provided relevant information for future formulations involving some of these bacteria, as well as to facilitate their identification (George et al. 2013).

7.5 Conclusions

A collection of bacteria originating from olive roots or rhizosphere of nursery-produced olive plants showing antagonism against several pathogens affecting olive trees, mainly *V. dahliae*, was generated. Furthermore, some of them displayed phenotypes related to biological control and/or PGP activities. Results indicate that the olive rhizosphere is actually a reservoir of candidate BCA against major olive diseases. These bacteria could therefore constitute the basis to develop bioformulations effective to control the pathogens evaluated in this work. Moreover, they offer the advantage to be adapted to the ecological niche where their benefits can be deployed. However, bioassays aiming at demonstrating their true biological control activity *in planta* are still needed, both under controlled and field conditions. This is now our current challenge.

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