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Stepwise-Selected *Bacillus amyloliquefaciens* and *B. subtilis* Strains from Composted Aromatic Plant Waste Able to Control Soil-Borne Diseases

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Abstract: In the present study, 133 bacterial isolates from 11 composted aromatic plant wastes were selected for their ability to inhibit the mycelial growth of the soil-borne phytopathogenic fungi *Sclerotinia minor* and *Rhizoctonia solani*. Successively, a subset of 35 from them were further characterized for their ability to control, in vivo, rocket damping-off caused by the two fungi. Moreover, the isolates were characterized for morphology of the colonies, Gram reaction, siderophore production, P-solubilization and for the presence of antimicrobial lipopeptide genes in the genome. The screening for the in vitro antagonisms showed a mycelial growth reduction ranging between 31.7% and 56.1% for *R. solani* and 34.4% and 59.4% for *S. minor*. All the isolates were not able to produce siderophores and some of them were able to solubilize P. The isolates contained two or more of the five lipoproteins coding genes investigated in this study. The most promising isolates were identified at species level by 16S-rRNA partial gene sequence analysis and were grouped in two main clusters related to *Bacillus subtilis* and *Bacillus amyloliquefaciens* reference strains. Results indicated that *Bacillus* isolates from compost are good candidates for application in the biocontrol of cultivated plants.

Keywords: antagonists; biological control; compost; lipopedtide genes; sustainable agriculture

1. Introduction

Plant fungal diseases are responsible for the emergence of different symptoms, such as wilting, scabs, moldy coatings, rusts, blotches and rotted tissue, on a wide variety of crops causing heavy economic losses [1]. In recent decades, many control strategies, such as chemical-based tools, plant breeding and agronomic techniques (crop rotations, soil tillage, solarization, weed control, organic amendment, etc.), have been developed in order to reduce deleterious disease effects. Among them, synthetic fungicides still remain the most adopted means for reliability reasons [1]. However, ecofriendly alternatives are needed because of increasing public request for organic and chemical-free vegetables and the enactment of restrictive regulations about sustainable fungicide use, aimed at reducing risks for the environment and human health. Furthermore, the application of chemicals over the years may contribute to the development of resistance in pathogens, thereby impacting the real effectiveness of the control solution [2]. In the last few decades, many efforts have been made by scientists to find alternative tools, paying attention particularly to antagonistic microorganisms, such as bacteria belonging

to *Pseudomonas* and *Bacillus* genera [3–5], as potential biological control agents (BCAs). Several authors have described the ability of many *Bacillus* rhizosphere-competent strains to inhibit the growth of a plethora of fungal plant pathogens through different direct mechanisms [6–12]. It was demonstrated that some antagonistic *Bacillus* strains can, moreover, elicit systemic resistance by increasing the level of PR-proteins [13] or salicilic acid [14]. *Bacillus* spp. may exert antagonistic activity through the release of antifungal lipopeptides, such as surfactin, iturin, fengycin and other bioactive molecules with surfactant-like properties. These molecules are implicated both in the biocontrol of diseases and in the promotion of plant growth [15,16]. *Bacillus* spp. can produce endospores: such structures confer resistance to desiccation, have a threshold temperature of 90 °C and have extreme pH and osmotic tolerances. So, these bacterial cells are particularly suitable for commercial purposes for their potential to adapt to different environmental situations [17].

Composting is a biochemical process that involves a large variety of mesophilic and thermophilic aerobic microorganisms, including bacteria, actinomycetes, yeasts and fungi, in transforming low-value undecomposed materials into a high-value humified products [18]. A wide range of biowaste can be composted, including materials generated by the agriculture, food and wood processing, sewage treatment, industry and municipal waste [19,20]. Microorganisms such as bacteria, actinomycetes and fungi, play a fundamental role in the organic matter decomposing processes that takes place during composting, and moreover, confer specific biological characteristics to the compost, such as suppressiveness [21]. Several bacterial strains, belonging to species of *Bacillus*, *Micrococcus*, *Clostridium*, *Staphylococcus*, *Citrobacter*, *Serratia*, *Klebsiella*, *Pseudomonas*, *Enterobacter* and *Escherichia*, were isolated from composts [22]. Among the aerobic prokaryotes isolated from compost, *Bacillus* spp. are among the most important potential biocontrol agents. Spore-forming bacteria are abundant in the compost and are promising for the suppression of soil-borne phytopathogens, especially through the production of antifungal proteins, antibiotics and lipopeptides [23].

The aim of this work was to isolate and characterize spore-forming bacteria from a set of composted aromatic plant residues for the in vitro and in vivo ability to control *Sclerotinia minor* and *Rhizoctonia solani* on rocket. Our investigation was directed to a stepwise antagonistic screening program as a general criterion to recruit new *Bacillus* biocontrol agents for agricultural applications by using composts as suitable sources of beneficial microbes.

2. Materials and Methods

2.1. Composts

Eleven different composts (P1–11), have been produced on a static pile, manually turned system [24] using different aromatic plants and vegetables waste. Among these, composts P2, P3, P5, P7, P9 and P11 were produced using defatted feedstock, from which essential oils were previously extracted by distillation; the remaining originated by raw feedstock. The composition of each compost was the following (percentages are expressed as dry matter): P1: 40% wood chips, 30% escarole (*Cichorium endivia* L.) and 30% a mixture of sage, basil, mint and parsley; P2: 40% wood chips, 30% escarole and 30% by a mixture of essential oil-free sage (*Salvia officinalis* L.), basil (*Ocimum basilicum* L.) and rosemary (*Rosmarinus officinalis* L.); P3: 20% wood chips, and a mixture of essential oil-free parsley (*Petroselinum crispum* (Mill.) Fuss) 50.5%, basil 29.9%, mint (*Mentha x piperita* L.) 1.6%, thyme (*Thymus vulgaris* L.) 6.2%, laurel (*Laurus nobilis* L.) 2%, red radish (*Raphanus sativus* L.) 3.6% and rocket salad (*Diplotaxis tenuifolia* L.) 6.2%; P4, basil 100%; P5: essential oil-free basil 100%; P6: wood chips 20%, and a mixture of mint 5.5%, thyme 28%, parsley 50%, oregano (*Origanum vulgare* L.) 3.8%, rosemary 8.1%, tarragon (*Artemisia dracunculus* L.) 0.7%, sage 2.5%, basil 0.5% and laurel 0.9%; P7: wood chips 20%, and a mixture of mint 6.9%, parsley 17.6%, rosemary 19.7%, sage 16.5% and basil 39.3%; P8: rosemary 100%; P9: essential oil-free rosemary 100%; P10: 100% sage; P11: essential oil-free sage 100%.

2.2. Isolation of Spore-Forming Bacteria

Five grams (fresh weight) of each compost was used to collect spore-forming bacteria, from a vital environment, by serial dilution technique. Each compost sample was 5 mm sieved and added to 45 mL of Na-phosphate buffer 0.1 M pH 7.6; the suspension was placed on an orbital shaker at 120 rpm for 60 min at room temperature. After 10 min of decantation, 1 mL of suspension was heated at 90 °C for 10 min to select spore-forming bacteria by killing microbial vegetative cells. The suspension was serially diluted until to 10^{-8} and 100 µL of this dilution was spread on nutrient agar (NA, Oxoid), and the plate was incubated at 30 °C overnight [25]. Spore-forming colonies grown on agar plates were picked-up and further purified by streaking on agar plates. The isolates were maintained on NA slants at 4 °C.

2.3. Characterization of Isolated Bacteria for In Vitro Antibiosis Activity

All spore-forming bacterial isolates were assayed for their ability to inhibit growth of *S. minor* and *R. solani* by dual-culture method [26] on potato dextrose agar (PDA, Oxoid). Fungal pathogens used in this study were isolated from cabbage and lettuce respectively, and maintained on PDA [23]. The bacterial isolates were streaked at the edges of Petri dish, while a mycelial plug (diameter 5 mm) was deposited at the center of the plate. The inoculated plates were incubated at 25 °C for 5 days. The fungal growth inhibition (I) was calculated as the percentage of reduction of mycelium colony expansion compared to control plates without bacteria by the formula: $I\ (\%) = (R - r) \times 100/R$, where R is the colony diameter of the fungus in the control plate, and r is the diameter of the colony in the treated plate. Results are reported as the averages of three independent trials.

2.4. Characterization of the Potential Antagonistic Bacteria

Bacteria that showed inhibitive growth rates higher than the general average were further characterized. Selected isolates were characterized for Ryu test and for colony morphology, siderophore production and phosphorus solubilization. The Ryu test was performed using 3% KOH: Gram-negative bacteria become viscous, while gram-positive bacteria do not [27,28]. The colony morphology was evaluated by visual observation of bacteria grown on NA plates. The potential production of siderophores was detected growing bacteria on an iron-free substrate (per liter: sucrose (20 g), L-asparagine (2 g), K_2HPO_4 (1 g), $MgSO_4$ (0.5 g) and agar (18 g)) and the release of siderophores was highlighted by a halo formation around the colony 3–4 days after incubation [29]. To assess the phosphorus solubilization activity, the selected isolates were grown on National Botanical Research Institute Phosphate medium (NBRIP-medium) [30] and the presence of a translucent halo was evaluated by visual observation after 3–4 days of incubation at 28 °C. Other features evaluated by visual observations were shape, thickness, edge and pigmentation [31]. All isolates were stored on slants at 4 °C and in glycerol stocks (20% v/v) at −80 °C. Tests were carried-out in triplicate.

2.5. Biocontrol Assays against Sclerotinia Minor and Rhizoctonia Solani on Rocket

Isolates that showed an in vitro inhibition rate above 50% were chosen for assessing in vivo diseases suppression caused by *S. minor* and *R. solani* on rocket (*Diplotaxis tenuifolia* L.). The fungal inoculum was prepared by infecting 100 g of wetted millet (*Panicum miliaceum* L.) contained in 1 L flask previously hydrated with 100 mL of distilled water and autoclaved at 120 °C for 21 min. Flasks inoculated with 10 mycelial plugs (diameter 5 mm), obtained from an actively growing colony, were incubated at 25 °C for 10 days. The bioassays were performed on sterile peat inoculated with 1% (w/w) of infected millet and dispensed in a nursery-polystyrene try-container (170 holes). Antagonistic bacteria were grown on NA plates, recovered and suspended into sterile water at two concentrations, 10^7 and 10^8 CFU ml^{-1}. Treatments are represented by the different antagonistic bacterial suspensions added (5 mL) to pathogen inoculated holes. Infected holes treated with water only, and not-infected and not-treated ones, were used as controls. Five holes (replicates) per treatment were sowed, each with

30 seeds of rocket. Seed homogeneity and health were both verified before starting the biocontrol tests. The container was kept in climatic chamber at 25 °C with 70% of humidity under a photoperiod of 16 h of light (≈8000 Lux) and 8 h of dark. The number of healthy plants per replicate was recorded at 7-days post inoculation, and then, damping-off percentages were calculated according to the formula of Yang et al. [32]. The experiment was carried out twice.

2.6. Identification of Antagonists/PGPR Isolates and Phylogentic Analisys

Bacterial antagonists were identified by partial sequencing of 16S rRNA gene [33] by using 16S primers (Table 1). DNA was extracted using the Sigma's GenElute™ Bacterial Genomic DNA kit (Promega), following the manufacturer's instructions, and quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA samples were analyzed by running on 1.2% agarose gel electrophoresis in 1 × Tris-borate EDTA (TBE) (89 mM tris pH 7.6, 89 mM boric acid, 2 mM EDTA) and visualized by Sybr Safe DNA Gel Stain (Invitrogen) staining, to determine DNA size and to assess RNA contamination. PCR reactions were carried out in a total volume of 50 µl with 1 × PCR Green Buffer, 0.2 mM dNTPs, 0.2 µM of each primer and 1.0 U of DreamTaq DNA Polymerase (Thermo Scientific), using the following conditions: 95 °C for 3 min, 35 cycles at 94 °C for 1 min, annealing temperature at 55 °C for 1 min and extension at 72 °C for 1 min; a final extension step at 72 °C for 3 min was followed by a 4 °C step for to preserve samples until electrophoresis. The amplicons were loaded in 1.2% agarose gel in 1 × TBE with 1 kb Opti-DNA Marker (abm), run for 60 min at 100 V and viewed, after staining, in a Gel Doc 2000 Visualizer (Biorad). The obtained amplicons were excised from the gel, purified by Wizard® SV Gel and PCR Clean-Up System (Promega) and directly sequenced by Sanger method. The sequences were analyzed by Serial Cloner Software 2.6.1 and aligned by the free tool MUSCLE available at this link: http://www.ebi.ac.uk/Tools/msa/muscle/ [34]. Database search was carried out for similar nucleotide sequences with the BLAST search database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Partial length 16S rRNA gene sequences of the strains closely related to the isolates were retrieved from NCBI for further analysis (accession numbers: CP015004, AY162126, NZ_CP011151, KF911350.1, NZ_CP011534, NZ_CP010052, KF001839, KM051086, NR_074540, NZ_CP015589, JF899265, NR_075005, JN107751, AB682190, AB682188, AB681490, JF899254). For describing their phylogenetic relationship, the 16S rRNA gene partial sequences were aligned using MEGA version 6 software and a phylogenetic tree was constructed by means of neighbor-joining method [35]. The nucleotide sequences of 16S rRNA were obtained and deposited in the GenBank database (EMBL, Cambridge, UK).

Table 1. List of the oligonucleotides used in this study to detect antibiotic genes by PCR screening and to amplify the 16S rDNA gene in the genomes of the selected antagonists. Annealing temperature and lengths of the amplicons are indicated.

Gene	Primer	Sequence	Annealing T (°C)	Amplicon Length (bp)
srfAA	SRFAF SRFAR	TCGGGACAGGAAGACATCAT CCACTCAAACGGATAATCCTGA	60	201
bacA	BACF BACR	CAGCTCATGGGAATGCTTTT CTCGGTCCTGAAGGGACAAG	60	498
ituC	ITUCF ITUCR	GGCTGCTGCAGATGCTTTAT TCGCAGATAATCGCAGTGAG	60	423
bmyB	BMYBF BMYBR	GAATCCCGTTGTTCTCCAAA GCGGGTATTGAATGCTTGTT	60	370
fenD	FENDF FENDR	GGCCCGTTCTCTAAATCCAT GTCATGCTGACGAGAGCAAA	60	269
16S	fD1 rD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC	63	1600

2.7. Detection of Antimicrobial Lipopeptides Genes

Selected isolates were further characterized for the presence in the genome of five antimicrobial lipopeptides genes: surfactin (*srfA*), iturin (*ituC*), fengycin (*fenD*), bacillomycin (*bmyB*) and bacilysin

(*bacA*). The oligonucleotides used for this aim [36] and the corresponding melting temperatures are listed in Table 1. PCR and electrophoretic conditions used were the same as described above.

2.8. Statistical Analysis

All data were analyzed by one-way ANOVA, with type of bacterial isolate as the independent variable and Student's *t*-test for simple pair-wise comparisons. Variables were arcsin transformed, if necessary. Means of damping-off percentages were separated by Fisher's LSD test.

3. Results

3.1. Isolation and Characterization for Antibiosis Activities of Spore-Forming Bacteria

Over 300 different spore-forming bacterial colonies were picked-up from primary plate cultures of eluates from the eleven composts. Based on their size, color and margin shape features, 133 colonies were visually chosen among them; they were transferred in pure culture and submitted to the preliminary qualitative in vitro assays, in order to discriminate antagonistic properties against *S. minor* and *R. solani*. At this step, the number of isolates per compost ranged from seven to 20 and the largest numbers of individuals were recruited from composts P5, P8 and P11, while the lowest numbers were from composts P7 and P10. The in vitro qualitative experiments (Table 2) allowed us to individuate eight isolates able to only inhibit mycelial growth of *S. minor*, eight isolates able to only inhibit mycelial growth of *R. solani* and 104 isolates able to inhibit the mycelial growth of both pathogens. It is interesting to note that from the composts P4, P5, P6, P8, P9, P10 and P11—all obtained by a single feedstock with the exception only for P6—the highest number of antagonistic isolates were obtained.

Table 2. General report on the in vitro antagonistic activity of the isolated bacterial colonies from the composts (P1–11) against the target pathogens (*Sclerotinia minor* and *Rhizoctonia solani*).

Compost	N° of Isolates				
	All	without *in-Vitro* Antibiosis	with *in-Vitro* Antibiosis Against *Sclerotinia Minor*	with *in-Vitro* Antibiosis Against *Rhizoctonia Solani*	with *in-Vitro* Antibiosis Against both Pathogens
P1	10	2	3	0	5
P2	10	3	2	0	5
P3	11	1	0	6	4
P4	13	0	0	0	13
P5	16	4	0	2	10
P6	11	0	0	0	11
P7	9	3	3	0	3
P8	17	0	0	0	17
P9	9	0	0	0	9
P10	7	0	0	0	7
P11	20	0	0	0	20
Total	133	13	8	8	104

Then, the 104 bacterial isolates exhibiting antagonistic behavior against both phytopathogenic fungi, were characterized for a quantitative in vitro assay in order to assess the fungal growth inhibition rate (Table 3). The assayed isolates showed percentages of fungal growth inhibition accounting in the range 10.0–56.1% for *R. solani* and in the range 13.9–59.4% for *S. minor*. The in vitro antibiosis performances allowed us to select a panel of 35 promising antagonistic isolates on the basis of a higher multiple pathogen inhibition criteria. The highest numbers of antagonistic isolates originated from the composts in the rank order P9, P11, P7 and P8; whereas, from the composts P1 and P3, only one isolate each was selected. None were taken from the composts P3, P4, P5 and P6. The selected isolates were subsequently used in the disease biocontrol assays.

Table 3. In vitro inhibition percentage of *Sclerotinia minor* and *Rhizoctonia solani* mycelial growth in a dual culture assay of the 104 isolates obtained from the composts (P1–11), against the target pathogens. In bold are indicated the 35 isolates chosen for showing a growth inhibition above the average of all the isolates.

Compost	Isolate	Inhibition (%)		Compost	Isolate	Inhibition (%)	
		RS	SM			RS	SM
P1	1	26.1	35.6		3	**32.2**	**35.0**
	2	**33.3**	**35.0**		4	**32.8**	**36.1**
	3	27.8	40.6		5	**39.4**	**39.4**
	4	28.9	23.3	P8	1	32.8	32.8
P2	1	23.3	20.6		2	13.3	33.3
	2	21.7	21.1		3	12.8	35.6
	3	29.4	22.8		4	27.8	38.3
	4	28.3	38.9		5	**39.4**	**43.3**
	5	25.6	13.9		6	**41.7**	**45.0**
	6	25.6	13.9		7	**44.4**	**47.2**
	7	28.9	21.7		8	10.0	40.6
	8	12.8	38.9		9	29.4	38.9
P3	1	10.3	21.7		10	**41.1**	**50.6**
	2	**31.7**	**40.6**		11	16.7	39.4
P4	1	20.6	21.7		12	25.0	40.6
	2	26.1	34.4		13	11.7	39.4
	3	30.0	28.3		14	**38.9**	**35.0**
	4	28.9	28.9		15	11.1	41.1
	5	**31.7**	**36.1**	P9	1	**43.3**	**44.4**
	6	**34.4**	**37.2**		2	**50.0**	**55.0**
	7	25.6	31.1		3	22.2	38.9
	8	33.3	25.0		4	**45.0**	**57.2**
	9	28.3	26.7		5	**41.1**	**36.1**
	10	25.0	21.7		6	**40.6**	**43.3**
	11	30.6	26.7		7	45.6	31.7
	12	30.0	24.4		8	**38.3**	**35.0**
P5	1	32.8	32.2		9	**38.3**	**35.0**
	2	30.6	27.8	P10	1	11.1	35.6
	3	32.2	23.9		2	29.4	38.3
	4	35.0	27.8		3	13.3	36.7
	5	32.2	30.8		4	16.7	37.2
	6	29.4	27.8		5	**41.7**	**52.8**
	7	28.3	36.7		6	**45.0**	**51.7**
	8	32.2	25.8		7	**31.7**	**36.7**
	9	31.1	26.7	P11	1	32.2	31.1
	10	32.2	30.0		2	**43.3**	**46.7**
	11	19.4	26.7		3	29.4	48.9
	12	30.6	21.9		4	**33.9**	**39.4**
	13	34.4	34.7		5	**56.1**	**59.4**
P6	1	**41.7**	**41.7**		6	**45.0**	**34.4**
	2	38.3	32.8		7	40.6	31.7
	3	37.8	30.6		8	**36.7**	**42.8**
	4	**40.6**	**37.8**		9	28.9	32.2
	5	36.1	33.1		10	**38.3**	**36.1**
	6	36.1	31.1		11	30.6	41.1
	7	37.8	32.8		12	**32.2**	**48.3**
	8	34.4	32.2		13	**33.3**	**37.2**
	9	**34.4**	**37.2**		14	14.4	38.9
	10	**39.2**	**50.0**		15	36.7	29.4
	11	**38.3**	**41.1**		16	38.9	34.4
P7	1	25.3	26.7		17	21.1	26.7
	2	30.8	28.6		18	32.2	33.3

3.2. Characterization of the Selected Bacterial Isolates

The in vitro experiments allowed us to individuate a promising set of antagonists, including 35 isolates, as previously reported. An in-depth morpho-physiological characterization separated the isolates into seven classes (Table 4).

The selected isolates do not produce siderophores, and, except for PXI-4, P10-7 and P11-12, showed a weakly positive Gram reaction (Table 5). Twelve out of 35 bacterial isolates showed P-solubilizing activity (PSB) (Table 5, Figure 1), and all the isolates obtained from the raw sage compost (P10) had PSB. Interestingly, no isolates from the composts P1, P8 and P9 showed this activity (Table 5).

Table 4. Morphological descriptions of the 35 bacterial isolates obtained from the composts (P1–11).

Class of Morphology	Shape	Thickness	Edge	Pigmentation
A	Circular/Fried-egg	Umbonate	Regular	Glossy-white
B	Circular	Convex	Regular	Glossy-white
C	Circular	-	Regular	Matt-white
D	Circular	Cupuliform	Regular	Yellowish
E	Circular	Flat	Regular-full	Matt-white
F	Fried egg	Umbonate	Ondulate	White
G	Flat	Flat	Ondulate	Unpigmented

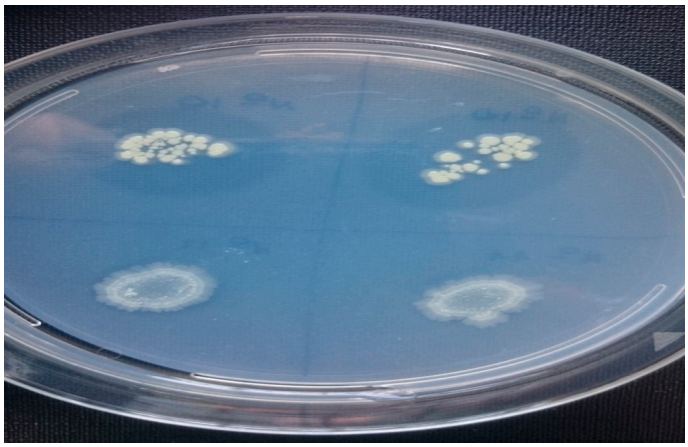

Figure 1. Phosphorous solubilization activity of bacterial isolates on the plate assay with the National Botanical Research Institute Phosphate-medium, in which translucent halos around the P-solubilizing colonies are visible.

Table 5. Class of morphology, Gram reaction and presence (+) or absence (-) of siderophore production and P-solubilization activity of the 35 selected bacterial isolates.

Isolate	Class of Morphology	Gram Reaction	Siderophore	P-Solubilization
P1-2	B	+	-	-
P3-2	F	+	-	+
P4-5	E	+	-	-
P4-6	C	+	-	+
P6-1	A	+	-	-
P6-4	F	+	-	-
P6-9	B	+	-	+
P6-10	B	+	-	+
P6-11	B	+	-	-
P7-3	B	+	-	-
P7-4	C	+	-	+
P7-5	E	+	-	-
P8-5	E	+	-	-
P8-6	E	+	-	-
P8-7	B	+	-	-
P8-10	B	+	-	-
P8-14	B	+	-	-
P9-1	B	+	-	-
P9-2	B	+	-	-
P9-4	C	+	-	-
P9-5	C	+	-	-
P9-6	A	+	-	-
P9-8	B	+	-	-
P9-9	A	+	-	-
P10-5	G	+	-	+
P10-6	B	+	-	+
P10-7	C	-	-	+
P11-2	C	+	-	+
P11-4	D	-	-	+
P11-5	B	+	-	-
P11-6	C	+	-	-
P11-8	C	+	-	-
P11-10	B	+	-	+
P11-12	B	-	-	+
P11-13	D	+	-	-

3.3. Biocontrol Assay, Identification of Bacterial Isolates and Detection of Lipopeptide Biosynthetic Gene Markers

Seven isolates chosen from the selected 35 on the basis of the in vitro best antagonistic activity results were used in the in vivo trials. The isolates P6-10, P8-10, P9-2, P9-4, P10-5, P10-6 and P11-5 displayed suppressiveness against *Sclerotinia* damping-off on rocket, whilst they did not show any control efficacy against *R. solani*. In general, bacterial concentration did not affect the magnitude of the antagonism. P9-2, P9-4 and P10-6 showed the highest *S. minor* biocontrol levels with a disease incidence reduction of about 50% at the largest concentration (Figure 2).

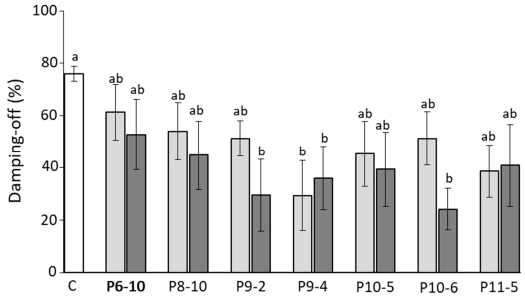

Figure 2. Control of *Sclerotinia* damping-off on rocket seedlings by the antagonistic spore-forming bacteria (x-axis) used in this study at two increasing concentrations, 10^7 (light grey bars) and 10^8 cfu mL^{-1} (dark grey bars) compared to the untreated control (white bar). Bars are the mean values ± standard error; different lower-case letters indicate statistically significant differences between bars, according to Fisher's LSD test ($p < 0.05$).

The 16S rRNA gene partial sequences obtained by analyzing the seven isolates used for in vivo biocontrol experiments, were subjected to BLAST analysis. The isolates P6-10, P8-10, P9-2, P10-5 and P10-6, showed high similarity (>99%) to *Bacillus subtilis*, whereas the isolates P9-4 and P11-5, showed high similarity (>99%) to *Bacillus amyloliquefaciens*.

The 16S rRNA gene partial sequences have been deposited in GenBank under the following accession numbers: KY380056 (strain P6-10), KY380057 (strain P8-10), KY380058 (strain P9-2), KY380059 (strain P9-4), KY380060 (strain P10-5), KY380061 (strain P10-6) and KY380062 (P11-5). In Figure 3, their phylogenetic relationship is illustrated.

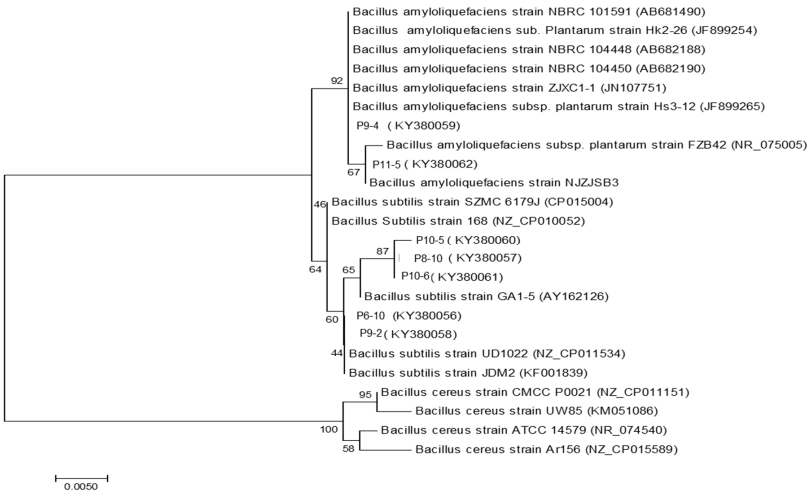

Figure 3. Phylogenetic tree of the seven *Bacillus* isolates (P6-10, P8-10, P9-2, P9-4, P10-5, P10-6 and P11-5) and closely related species based on 16S rDNA gene sequences, obtained from NCBI following BLAST analysis. The tree was constructed by the neighbor-joining method. The scales indicate the evolutionary distance of the nucleotide substitutions per site. Bootstrap values, derived from 1000 replicates, are indicated as percentage at all branches.

Genes *srfAA*, *bcaA*, *ituC*, *bmyB* and *fenD* belonging to the biosynthetic pathway of lipopeptides, were differently found in the genomes of the selected isolates. Indeed, all tested isolates contained two or more of the five lipoprotein coding genes (Table 6). Results indicate that all seven biocontrol isolates harbor biosynthetic lipopetide genes in their genome.

Table 6. Presence (+)/absence (-) of the biosynthetic genes *bacA*, *bmyB*, *fend*, *ituC* and *srfAA* in the selected biological control agent (BCA). strains.

BCA strain	Identified as	*bacA*	*bmyB*	*fenD*	*ituC*	*srfAA*
P6-10	BS	+	-	+	+	+
P8-10	BS	+	+	+	-	+
P9-2	BS	+	-	-	-	+
P9-4	BA	+	+	+	-	+
P10-5	BS	+	+	+	-	+
P10-6	BS	+	+	+	-	+
P11-5	BA	+	+	+	+	+

4. Discussion

Soil-borne fungal diseases are difficult to manage, and the causal pathogens are among the main threats which farmers must be face, due to their ability to survive in soil for a long time. Integrated approaches with the use of microbial biocontrol agents are welcome. In the last few decades, the use of eco-friendly control means of soil-borne pathogens has become a popular alternative to conventional chemical treatments in a lot of cropping systems [37]. In fact, several studies and reviews highlighted the possibility to isolate new potential BCAs and use them to reduce crop losses [12,38–41]. In this study, we carried out a step-by-step selection for the *S. minor* and *R. solani* controllability of spore-forming bacterial isolates, obtained from aromatic plant residue-based composts. A general assessment of the selecting program showed that composts obtained with defatted matrices, such as P9 and P11, respectively, oil-free rosemary and sage, gave the largest number of putative BCAs for the succeeding steps.

Phylogenetic analysis revealed that the best biocontrol agents of *Sclerotinia* damping-off selected here, are related to *Bacillus amyloliquefaciens* and *B. subtilis* species. Many reports indicate that microbiota present in compost can exert a suppressive effect on some phytopathogens [42–44]. *Bacillus* genus is a group of microorganisms widely present in soils and compost-amended soils [42,45], and well known for their beneficial effects exerted on plant growth, health and fitness [7]. Moreover, a consistent number of *Bacillus* species have been reported to behave both as direct antagonists of pathogens [46] and may function as elicitors of induced resistance mechanisms [47,48].

The in vitro evaluation through dual antagonist-pathogen assay, may indicate the potential of microorganisms to act as BCAs [49]. In the current study, to individuate the isolates with the best in vitro activities, a total of 133 candidates were found at beginning of this stepwise selection. This result suggests the possible production of diffusible metabolites in the media that are inhibitory for the in vitro development of the target pathogens, thereby indicating a possible antibiosis-like mechanism based on delivering antimicrobials outside the cells. Despite many *Bacillus* species having been shown to antagonize microbial pathogens that way [16,50,51], the contemporary presence of other types of interactions, such as competition for the space and/or resources, and predation, cannot be excluded. The percentage of the in vitro fungal growth reduction, as compared to untreated control, ranged from 10% to 56.1% for *R. solani* and was between 13.9% and 59.4% for *S. minor*. Thirty-five out of 104 BCAs candidates were able to inhibit mycelial growth of both pathogens. It is well established that *Bacillus* spp. can inhibit fungal pathogens under in vitro conditions by producing a plethora of active molecules, such as diffusible metabolites, volatile compounds and cyclic lipopeptides (LPs) belonging to different families [52]. LPs produced by many BCAs are responsible for the suppression of several phytopathogens belonging to different genera, such as *Sclerotium*, *Fusarium*, *Rhizoctonia* and *Aspergillus* [7]. In the present study, the most promising bacterial isolates harbor at least two of lipopeptide genes in the genome, as revealed by PCR investigation.

This agrees with several authors [9,11,53–55] who described the presence of multiple LP-genes in *Bacillus* strains exhibiting antagonistic activity. The antifungal properties of lipopeptides have been deeply investigated. For example, the activities of Iturin A and Surfactin rely on the surfactant properties of these molecules which induce the disruption of the pathogens' membranes by pore formation [56,57] in a dose-dependent manner, so as to lose cellular contents [9].

Among the seven most promising bacteria, two, namely, *B. subtilis* isolates (P9-2 and P10-6) and a *B. amyloliquefaciens* strain (P9-4), confirmed their biocontrol activity in the rocket/*S. minor* pathosystem. However, they failed in the *R. solani*/rocket experiments. The difficulty of controlling Rhizoctonia damping-off, probably, is linked to cruelty, high fitness and the development capability of the pathogen in the telluric environments.

For the selected strains, the LP screening revealed in all the isolates the contemporary presence of surfactin, bacylisin and fengycin genes, as previously described for other antagonists [58]. Instead, iturin and bacyllomicin genes were not always present. Furthermore, we noticed that the isolate P11-5 held all the detected genes, even if it did not show significant activity in in vivo assays in both pathosystems *R. solani*/rocket and *S. minor*/rocket. It must be considered that even if a BCA holds a set of genes for a function, it does not mean that the function itself is strictly correlated to the biocontrol ability [59]. In many cases of beneficial plant–microbe interactions, several mechanisms are involved at the same time [60] so that the disease suppression and the PGP mechanisms are difficult to differentiate, as are their relative importance, which can be different depending on the pathosystems [61].

In conclusion, seven spore-forming isolates obtained from aromatic plant waste composts, *B. amyloliquefaciens* strains P9-4 and P11-5, and *B. subtilis* strains P6-10, P8-10, P9-2, P10-5 and P10-6, could have the potential to exert biological control on soil-borne diseases. *B. subtilis* P6-10, and *B. amyloliquefaciens* P10-5 and P10-6 strains proved to be the most effective isolates. Findings confirm that compost is a suitable source and precious reservoir of beneficial microorganisms to be potentially advantageously applied not only for improving soil fertility, but also for increasing the sustainable management of plant diseases [62]. The production of antibiotics, secondary metabolites, volatile compounds or any other mechanisms, might be related to the biocontrol activities of these *Bacillus* isolates. However, other experiments and field evaluations of BCAs need to be done to elucidate the mechanisms elicited by the bacterial isolates that have protected rocket seedlings against *S. minor* in our study.

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