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Use of Bacteria Isolated from Berry Rhizospheres as Biocontrol Agents for Charcoal Rot and Root-Knot Nematode Strawberry Diseases

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Abstract: Strawberry is a high-value crop in Spain, where fruits for fresh consumption are produced off-season and exported to central and northern European countries. Soil-borne pathogens (SBP), such as *Macrophomina phaseolina* and *Meloidogyne hapla,* are widely spread in strawberry crops in Spain. The prevalence of these pathogens is a significant barrier to producing strawberries of premium quality and at profitable yields. The current ban on fumigant pesticides drives the search for alternative SBP control methods that can simultaneously control both SBP. Twenty-nine bacterial strains were isolated and identified from strawberry and blueberry rhizospheres and screened for plant growth promotion activities as well as for biocontrol potential on *M. phaseolina* and *M. hapla*. Two of these bacterial strains (*Bacillus velezensis* FC37 and *Pseudomonas aeruginosa* AC17), together with *Brevibacterium frigoritolerans* Hvs8 coming from IFAPA's bacterial collection, were selected and evaluated in planta for their biocontrol potential on strawberry SBP diseases caused by *M. phaseolina* and *M. hapla*. The three tested bacterial strains reduced charcoal rot disease severity, crown incidence and severity, and petiole colonization by *M. phaseolina*. Root-knot nematode (RKN) symptoms were reduced by *P. aeruginosa* AC17 and *B. velezensis* FC37, but RKN reproduction was only reduced in plants inoculated with *P. aeruginosa* AC17. *Pseudomonas aeruginosa* AC17 showed the greatest potential as a biocontrol agent candidate to be included in integrated disease management programs to control the two most prevalent soil-borne pathogens of strawberry in Spain.

Keywords: biocontrol; *Macrophomina*; *Meloidogyne*; *Pseudomonas*; strawberry

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1. Introduction

Strawberry is a high-value crop in Spain, where fruits for fresh consumption are produced off-season and exported to northern European countries. Up to 93% of all strawberries produced in Spain are grown in the southwestern region (Huelva province), where 6867 ha produce 377,596 tons with a market value of EUR 392 million [1]. Spain produces between 25 and 33 percent of all strawberries grown in Europe. In intensive monoculture systems, strawberries are produced in yearly crop cycles from October to May under temporary plastic high tunnels. The fields are left fallow from July to September, while the harvest season runs from January to late May. Because of the extended strawberry cropping cycles and high farm productivity and profitability, crop rotation with other crops is not practical.

Due to monoculture, soil-borne pathogens (SBP), such as fungi *Macrophomina phaseolina* and *Fusarium* spp. [2–5], as well as nematodes, such as *Meloidogyne hapla*, *Pratylenchus penetrans*, and *Hemicycliophora* spp. [6], are widespread in strawberry crops in Spain, with

prevalence ranging from 60 to 80%. Strawberry yield losses due to SBP have been estimated at 10% in case of fungi and 6% for nematodes. As such, they are a significant barrier to producing strawberries of top quality with commercially sustainable yields [7]. There is no resistance reported in strawberry against these pathogens, and soil disinfestation with fumigant agrochemicals (1,3-dichloropropene, chloropicrin, metam-Na, or metam-K) has been the main strategy for SBP control in the area [8]. The continued use of soil chemical fumigants has a negative impact on soil biodiversity, accumulates residue with a significant risk of contaminating groundwater, and causes environmental issues. Therefore, the majority of soil fumigants are currently illegal or subject to tight regulations both inside and outside of the European Union (Directive 2009/128/CE). For the management of soil-borne diseases in vegetables, extensive research has been performed on different chemical and non-chemical SBP control strategies, such as solarization, biofumigation, biosolarization, and steaming. Nevertheless, none of these techniques are as effective as soil fumigation at preventing SBP, and they have not been shown to be reliable enough to be utilized in intensive horticulture production. [9]. As result, under the current regulatory framework, and with the exception of temporary authorizations, farmers do not have a soil disinfestation option effective enough to reduce high soil infestations by SBP, which seriously affect the productivity of these intensive strawberry crops. Thus, the search for environmentally friendly alternatives, among which the use of microorganisms stands out, is increasingly crucial.

The use of autochthonous biocontrol organisms (BCO) as a strategy for controlling pests and diseases is an established technique for controlling pests but less frequently used in controlling SBP. Given the need to avoid the introduction of allochthonous microorganisms that could become an ecological problem as an invasive species, biopesticides should be based on microbial strains identified locally. After isolation, some microorganisms are selected for their plant growth-promoting properties or for their biocontrol potential in vitro. They can function as homologous of the pathogen, release substances against them, or even induce a resistance response to the pathogen in the plant, helping in combination with other control methods within an integrated disease management strategy. The best biocontrol candidates are also characterized by 16sRNA sequencing to know the species (or genera) at which they are assigned in order to avoid species that could be of any risk to humans, animals, and/or plants.

Several beneficial bacteria have been shown to be effective against SBP, although the most frequently reported strains come from the genera *Bacillus* and *Pseudomonas* [10]. Their bioactive secondary metabolites act through several mechanisms, including plant growth promotion, induction of systemic resistance, changes in root exudates, and production of phytohormones, antibiotic substances, and volatile organic compounds [11]. The most frequently explored rhizobacterial metabolites with inhibitory activity against various SBP are extracellular enzymes, such as proteases and chitinases. The lipid layer required for the growth and preservation of nematode eggs was discovered to be destroyed by those compounds acting together, which had an effect on both nematode development and hatching, suggesting that they could act as biocontrol agents for soil pathogenic fungi and phytoparasitic nematodes at the same time [10].

Furthermore, some *Bacillus* and *Pseudomonas* strains are good BCO candidates because of their easy production, transport, and application [12], and because they have been recently proposed as good candidates for BCO for *Meloidogyne* [13,14] and *M. phaseolina* [15]. Most reports indicate biocontrol potential against a single SBP but reports of bacterial strains active in biocontrol of two or more SBP are scarce. Because many of the biocontrol mechanisms of these BCO are active against different SBP, screening for BCO active simultaneously against several SBP present in a region is of particular interest.

The purpose of this study was to identify possible BCO from berry rhizospheres in the southwest Spain strawberry growing region and to test their efficacy as biocontrol agents against *M. hapla* and *M. phaseolina* under in vitro and in planta conditions.

2. Materials and Methods

2.1. In Vitro Experiments for Biocontrol of M. phaseolina

2.1.1. Bacterial Strain Isolation

Bacterial strains occurring in strawberry and blueberry rhizospheres at the IFAPA experimental station "El Cebollar" (Moguer, Huelva, Spain) (37°14′ N–06°48′ W) were isolated and maintained at the IFAPA Las Torres bacterial collection. For strain isolation, serial ten-fold dilutions of 1 g of rhizosphere soil homogenized in 99 mL of mineral salt were achieved. The dilutions were spread on Plate Count Agar (PCA, Difco®, Madrid, Spain) supplemented with cycloheximide (100 mg/L, Sigma-Aldrich®, St. Louis, MO, USA) and incubated at 28 °C for 24–48 h until the appearance of individual colonies. For obtaining pure strains, colonies with different morphotypes were further streaked on individual plates. Strains were further cryo-preserved at −80 °C in 15% glycerol-0.5% peptone.

2.1.2. Bacterial Strain Identification

Bacillus velezensis FC37 and *Brevibacterium frigoritolerans* Hvs8 had been previously identified and characterized [16]. For identification of the remaining strains, the 16S rRNA coding gene was amplified by PCR using universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTTACG ACT T-3') [17]. Amplification was carried out in a thermicycler MyiQ™ (Bio-rad, Dubai, United Arab Emirates) as follows: denaturation at 95 °C for 5 min followed by 30 s at 94 °C, 30 s at 50 °C, 1 min at 52 °C, and 1 min at 72 °C, with a final extension at 72 °C for 7 min at the end of 35 cycles. Amplification and sequencing of purified amplicons was conducted by Universidad de Leon (Spain). To compare similarities with known sequences, the EzBioCloud database (https://www.ezbiocloud.net accessed on 16 January 2023) was used [18]. The 16S rRNA gene sequences were deposited in the DDBK/EMBL/GenBank database under the accession numbers OQ255851- OQ255878 (strains AC11.2, AC14, AC15, AC17, AC9.1, ACH10, ACH14, ACH15, ACH16, ACH2, ACH25, ACH29, FC10.1, FC10.2, FC14, FC15, FC17, FC18, FC20, FC21.2, FC25, FC28, FC32, FC34, FC35, FC36, FC39, FC5.2, respectively).

2.1.3. In Vitro Assays for Plant Growth-Promoting Activities

Strains were evaluated in vitro for plant growth-promoting abilities. Indolacetic (IAA) production was assessed in TSB (tryptic soy broth) supplemented with L-tryptophan (100 mg/L) and was measured in the supernatant after incubation at 28 °C with continuous shaking for 72 h [19]. Siderophore production was assessed in CAS plates following the protocol of Alexander and Zuberer [20], and phosphate solubilization was determinate in PVK plates following the procedure described by de Freitas et al. [21]. All assays were repeated twice. Activities were assessed visually from no activity (-) to very strong activity (++++). For graphical representation, a strain showing very strong activity (++++) in all the three measured properties will reach 100% (33% for each maximum activity). The other data were normalised accordingly.

2.1.4. In Vitro Assays for Biocontrol Enzymatic Activities

Strains were evaluated in vitro for the biocontrol enzymatic activities of cellulase, protease, amylase, β-glucosidase, and chitinase, as described by Viejobueno et al. [15]. The presence of volatile compounds (HCN) was estimated qualitatively by the sulfocyanate colorimetric method [15]. All assays were repeated twice. Graphical representation was performed as described before.

2.1.5. In Vitro Assays for Biocontrol Activity against *M. phaseolina*

In vitro assays were performed in 6 cm diameter Petri plates containing TSA medium, which is TSB medium plus 16 g/L of bacteriological agar (Difco®, Madrid, Spain). Four droplets of inoculum (10 µL) of each bacterial strain were distributed at the four perpendicular directions at the periphery of the plate and incubated at 28 °C for 24 h. Then, mycelium discs of *M. phaseolina* isolates TOR-102 or TOR-872 were placed in the center of

the plate and incubated for 7 more days at 28 °C. Plates without bacteria inoculum were used as negative controls. Percentage of inhibition of pathogen development compared to the control was assessed at seven days after confrontation and was calculated using Formula (1):

$$\text{Percentage of inhibition (\%)} = [(Rc - Ri)/Rc] \times 100 \quad (1)$$

where Rc is the radial growth of the fungal pathogen in the control plates (mm) and Ri is the radial growth of the fungal pathogen in the test plates (mm). Experiments were conducted in triplicates.

2.2. In Planta Experiments for Biocontrol

The effects of both bacterial inoculation on the development of strawberry (*Fragaria* × *ananassa* Duch., 'Rociera') and the control of strawberry soil-borne diseases were evaluated in pot tests conducted in a growing chamber. Three antagonist biocontrol bacteria (BCO) were selected for their biocontrol potential in vitro against *M. phaseolina* and their plant growth-promoting properties. *Bacillus velezensis* FC37, *Brevibacterium frigoritolerans* Hvs8, and *Pseudomonas aeruginosa* AC17 were tested in planta against the two most prevalent SBP in the strawberry growing area of southwestern Spain: *M. phaseolina* and *M. hapla*.

2.2.1. Experimental Design

Twelve treatments were used in each pot test, divided into two components with three and four levels, respectively: SBP (*M. hapla*/*M. phaseolina*/non-SBP) and BCO (*B. velezensis*/*B. frigoritolerans*/*P. aeruginosa*/non-BCO) (Table 1). In the growing chamber, pots were placed in a completely randomized design, with each treatment being replicated eight times. Two experiments were conducted.

Table 1. Treatments evaluated in pot trials for biocontrol of strawberry soil-borne diseases.

Treatment Code	BCO	SBP
Ø-Ø	Ø	Ø
Ø-FC37	*B. velezensis* FC37	Ø
Ø-Hvs8	*B. frigoritolerans* Hvs8	Ø
Ø-AC17	*P. aeruginosa* AC17	Ø
MH-Ø	Ø	*M. hapla*
MH-FC37	*B. velezensis* FC37	*M. hapla*
MH-Hvs8	*B. frigoritolerans* Hvs8	*M. hapla*
MH-AC17	*P. aeruginosa* AC17	*M. hapla*
MP-Ø	Ø	*M. phaseolina*
MP-FC37	*B. velezensis* FC37	*M. phaseolina*
MP-HVS8	*B. frigoritolerans* Hvs8	*M. phaseolina*
MP-AC17	*P. aeruginosa* AC17	*M. phaseolina*

BCO: Biocontrol organism. SBP: Soil-borne pathogen.

2.2.2. Plant Material

Strawberry ('Rociera') transplants were obtained from high-altitude nurseries and stored at 4 °C until the establishment of the pot trials. Twenty-four hours before planting, the roots of all plants were cut and immersed in 3.5% sodium hypochlorite solution for 5 min, washed under running tap water, dried in absorbent paper, and put back into storage at 4 °C until planting in the pots.

2.2.3. Bacterial Inoculation

A final concentration of 10^9 CFU/mL was achieved by growing the selected bacterial strains in tubes containing 5 mL of Trypto-casein Soy Broth (TSB) (Scharlap®, Barcelona, Spain) that were incubated at 28 °C for 72 h while being continuously stirred (180 rpm). For inoculation on plants, cultures were centrifuged at 12,000 rpm for 5 min and the cells were resuspended in sterile 0.03 M magnesium sulfate buffer to a concentration of 10^8 CFU/mL.

Strawberry seedlings were inoculated at planting by root immersion in a solution of each bacteria strain (10^8 bacteria/mL for 1 h). Under the same conditions, control plants were submerged in sterile 0.03 M magnesium sulfate buffer. Seven days after planting, a reminder dose with bacteria (10 mL of a 10^8 bacteria/mL solution) was applied by irrigation onto the soil surface of each pot.

2.2.4. Fungal Inoculation

Eight days after planting, strawberry plants were infected with *M. phaseolina* isolate TOR-102 from pure cultures from the IFAPA's fungal collection. To recover the virulence of *M. phaseolina* TOR-102, it was grown on soybean (*Glycine max* 'Osumi 2004') seeds from the collection of the IFAPA Center Las Torres [22]. Each pot received 50 mL of a suspension containing 2×10^3 sclerotia of *M. phaseolina* TOR-102 per milliliter [23,24].

2.2.5. Nematode Inoculation

A population of *Meloidogyne hapla* isolated from infected strawberry roots was used for the growing chamber tests. To promote nematode proliferation, tomato plants of the 'Roma' variety (*Solanum lycopersicum*) were infected with the nematode and kept for 8 weeks in a growth chamber at 25 °C. The diseased tomato roots were stirred in a 0.5% sodium hypochlorite solution to remove nematode eggs [25]. To obtain second-stage juveniles (J2), the egg suspension was concentrated on a 20 µm filter and deposited on Baermann funnels [26]. Only juveniles hatched within 24–48 h were used as inoculum, whereas J2 hatchings within the first 24 h were eliminated. Nematode inoculation was carried out eight days after planting by pouring an aqueous suspension of 1500 J2 of *M. hapla* per pot through three 5 cm deep holes around the strawberry plant.

2.2.6. Plant Growing Conditions

Strawberry plants were grown singly in polypropylene pots that were 11 cm tall, 12 cm in diameter at the top, and 10 cm in diameter at the bottom. The pots also contained 750 mL of peat substrate (Indalofertil premium ®), which had been previously sterilized in an autoclave twice on consecutive days at 120 °C and 1 atmosphere for 30 min. The plants were placed in the growing chamber at random and allowed to grow with an average daytime temperature of 28 °C, a nighttime temperature of 22 °C, and a photoperiod of 16 h of light. All pots were fertilized by adding 2 g of Osmocote® (15% N + 10% P_2O_5 + 12% K_2O + 2% MgO_2 + microelement, Scotts Company, Heerlen, the Netherlands) on the soil surface. The pots were watered according to requirements with 50–100 mL of water once or twice a week, maintaining a slight water stress. Plants were grown for 98 days in 2019–2020 and 159 days in 2020–2021 experiments.

2.2.7. Parameters Evaluation for Plant Growth and Disease

Weekly observations were employed to follow the progression of charcoal rot caused by *M. phaseolina*. The incidence of charcoal rot was calculated as the proportion of dead plants to the total. Disease severity was estimated using a scale from 0: healthy plant to 4: dead plant [27]. To confirm infection by the pathogen, isolations from symptomatic tissues were performed on culture medium potato dextrose agar, keeping the Petri dishes at 30 °C and in darkness for 7 days. The percentage of *M. phaseolina*-infected plants (roots or petioles), crown incidence, and severity of symptoms (scale 0 to 5) were estimated [28].

At harvest, strawberry plants were uprooted, washed to remove soil debris, and their fresh and dry weights were determined, including stolons, fruits, and roots.

At the end of each test, root-knot nematode disease was evaluated by assessing the severity of the nodulation symptoms in the roots using a gall index on a 0–10 grading scale (0 = no galls; 7 = 100% of the roots had galls; 10 = dead plant) [29], the final populations of nematodes per pot, and different growth parameters of the plant. Three grams of root subsamples were cut into pieces 1–2 cm long and macerated by blending in a 1% solution of sodium hypochlorite to determine the nematode population in the roots [25].

For gathering *M. hapla* eggs, juveniles, and adults, the suspension was poured into sieves with a 20-µm mesh. Nematodes were counted in counting plates and the total number of nematodes in the entire root system was obtained by multiplying the nematodes count per g of roots by the root fresh weight. Nematodes were isolated from 250 cm^3 of soil using the sieve and decanting method and then centrifuged in a solution of magnesium sulfate heptahydrate with a specific gravity of 1.18 g/L to determine the nematode population density in the soil [30]. For further counting under a microscope, the nematode suspension was concentrated in 2 mL of water. Final nematode population densities were calculated as the sum of nematodes extracted from roots plus those extracted from soil (750 mL) and were expressed as nematodes per pot.

2.3. Statistics

For in vitro growth inhibition of fungal strains, a Kruskal–Wallis test was carried out using the program Statistix 9.1 to assess the inhibition of the bacterial strains on the growth of *M. phaseolina*. Pairwise comparisons were performed using an alpha of 0.05.

All results concerning plant tests are expressed as mean ± standard error of the mean. The statistical program Statgraphics Centurion XVI® (Statpoint Technologies Inc., Warrenton, VA, USA) was used to analyze the data. Data were subjected to the Kolmogorov–Smirnov and Brown–Forsythe tests to determine whether the variances were normal and homoscedastic; if significant, data were arcsine-transformed and tested again. Data were analyzed using ANOVA when normality and homoscedasticity of variances could be assumed. The HSD Tukey's test ($p < 0.05$) was used to compare the means if the F values were significant. When the homoscedasticity of variances could not be assumed, Welch's ANOVA was used. The data were analyzed using Kruskal–Wallis non-parametric tests when normality was not achieved after transformation. If H values were significant, means were compared by Dunn's multiple comparison test ($p< 0.05$).

3. Results

3.1. Bacterial Identification

Twenty-nine bacterial strains were isolated from strawberry (FC codes) and blueberry (AC codes) rhizospheres from IFAPA El Cebollar berry crops and taxonomically identified using their 16S rRNA gene sequences (Table 2).

Table 2. Phylogenetic affiliation of bacterial strains isolated from strawberry (FC codes) and blueberry (AC codes) rhizospheres at IFAPA El Cebollar (Huelva) Spain.

Strain Code	Related Species (blast/ncbl)	Accession Number	Similitude %
FC5.2	*Pseudomonas kribbensis*	OQ255878	99.59
FC10.1	*Xanthomonas translucens*	OQ255863	99.73
FC10.2	*Cupriavidus metalliduras*	OQ255864	100
FC14	*Cytobacillus firmus*	OQ255865	99.52
FC15	*Comamonas testosteroni*	OQ255866	100
FC17	*Arthrobacter pascens*	OQ255867	99.59
FC18	*Pararhizobium herbae*	OQ255868	99.7
FC20	*Pseudomonas brassicacearum* subsp. *neoaurantiaca*	OQ255869	99.93
FC21.2	*Agrobacterium arsenijevicii*	OQ255870	100
FC25	*Xanthomonas translucens*	OQ255871	99.73
FC28	*Pseudomonas flavescens*	OQ255872	98.83
FC32	*Bacillus albus*	OQ255873	100
FC34	*Arthrobacter humicola*	OQ255874	99.31
FC35	*Pseudomonas nitritireducens*	OQ255875	99.86
FC36	*Arthrobacter pascens*	OQ255876	99.45
FC37	*Bacillus velezensis*	OU487633	*

Table 2. *Cont.*

Strain Code	Related Species (blast/ncbl)	Accession Number	Similitude %
FC39	*Raoultella ornithinolytica*	OQ255877	99.86
AC9.1	*Priestia aryabhattai*	OQ255855	100
AC11.2	*Bacillus altitudinis*	OQ255851	100
AC14	*Mixta calida*	OQ255852	100
AC15	*Enterobacter quasiroggenkampii*	OQ255853	99.86
AC17	*Pseudomonas aeruginosa*	OQ255854	100
ACH2	*Chryseobacterium cucumelis*	OQ255860	99.93
ACH10	*Klebsiella variicola subsp. variicola*	OQ255856	99.73
ACH14	*Achromobacter veterisilvaer*	OQ255857	99.93
ACH15	*Microbacterium arborescens*	OQ255858	99.93
ACH16	*Bacillus velezensis*	OQ255859	99.93
ACH25	*Enterobacter kobei*	OQ255861	99.93
ACH29	*Curtobacterium citreum*	OQ255862	99.72
Hvs8	*Brevibacterium frigoritolerans*	OU487634	*

* Previously identified [16].

3.2. Plant Growth-Promoting Activities

Plant growth-promoting abilities in selected bacterial strains as synthesis of auxins, siderophores, and phosphate solubilization are shown in Figure 1. Two strains isolated from strawberry rhizosphere (*Pseudomonas kribbensis* FC5.2 and *Bacillus velezensis* FC37) and five from blueberry (*Mixta calida* AC14, *Enterobacter quasiroggenkampii* AC15, *Chryseobacterium cucumelis* ACH2, *Klebsiella variicola* ACH10, and *Enterobacter kobei* ACH25) displayed the three tested properties. *Klebsiella variicola* ACH10 showed the best results. It is noticeable that strains from blueberry rhizosphere showed more plant growth-promoting activities and with higher values than those from strawberry rhizosphere.

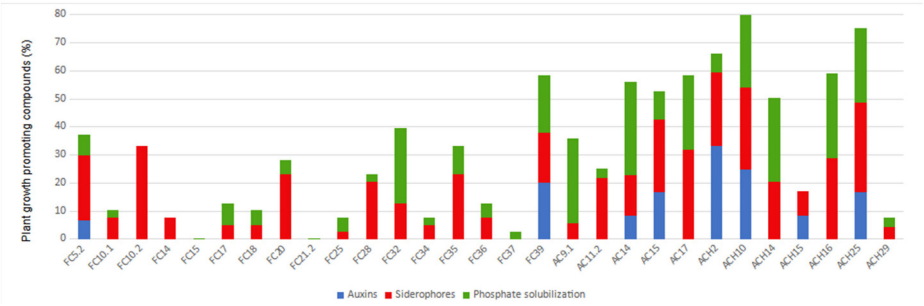

Figure 1. Plant growth-related activities, expressed as a percentage of the total plant growth activities, measured in bacterial strains isolated from strawberry and blueberry rhizospheres.

3.3. Biocontrol Enzymatic Activities

The biocontrol-related enzymatic activities (β-glucosidase, amylase, cellulase, chitinase, and casein) measured in the selected bacterial strains are shown in Figure 2. None of the isolated strains displayed all biocontrol activities or HCN production. The strain *Bacillus albus* FC32 was the only one that displayed chitinase activity. This strain, together with strains *Xanthomonas translucens* FC10.1, *B. velezensis* FC37, *Microbacterium arborescens* ACH15, and *B. velezensis* ACH16, showed four out of five biocontrol activities. Some strains from strawberry rhizospheres did not show any biocontrol activities.

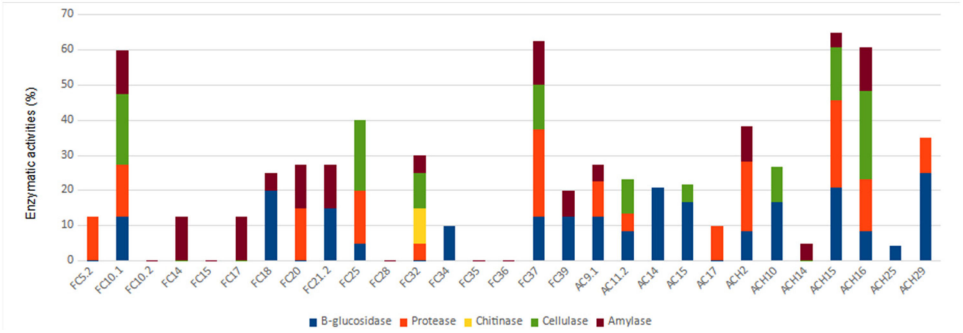

Figure 2. Biocontrol-related enzymatic activities, expressed as a percentage of the total enzymatic activities, measured in bacterial strains isolated from strawberry and blueberry rhizospheres.

3.4. In Vitro Antifungal Activities against M. phaseolina

M. phaseolina growth inhibition was assessed on TOR-102 and TOR 872 isolates. Most strains were able to inhibit *M. phaseolina* growth at some level, but *B. velezensis* FC37, *P. aeruginosa* AC17, and *B. velezensis* ACH16 totally inhibited the growth of both fungi isolates (Figure 3). We found a significant effect of the bacterial strains on the inhibition of *M. phaseolina* ($H(28) = 118.5$, $p < 0.001$).

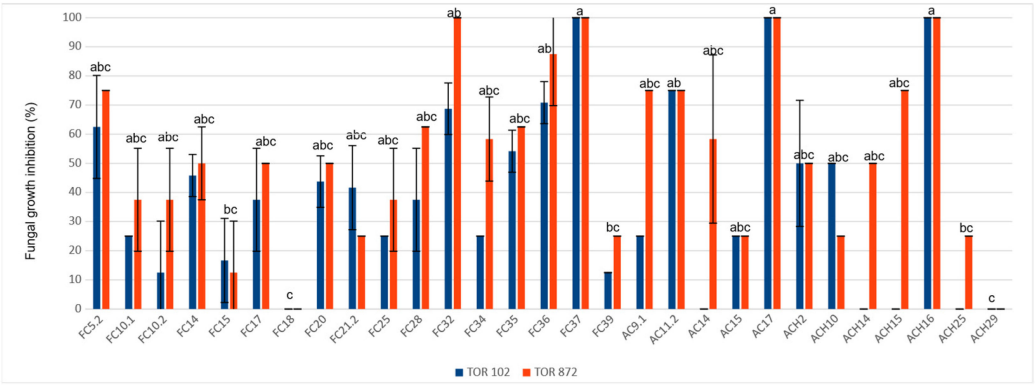

Figure 3. *M. phaseolina* growth inhibition (%) by bacterial strains isolated from strawberry and blueberry rhizospheres. Error bars correspond to standard deviations. Different letters indicate significant differences among bacterial strains from pairwise comparisons using an alpha of 0.05 after a Kruskal–Wallis test.

3.5. Strawberry Biomass

Plant biomass was reduced by 29.3% in strawberry plants infected with *M. phaseolina* (38.9 ± 2.2 g) when compared to non-SBP-inoculated plants (55.0 ± 2.8 g) ($p < 0.05$). Strawberry infection with *M. hapla* (52.0 ± 5.4 g) and bacterial inoculation with *B. frigoritolerans* Hvs8 (46.4 ± 3.6 g), *P. aeruginosa* AC17 (46.6 ± 2.3 g), and *B. velezensis* FC37 (43.3 ± 2.8 g) did not have any effect on plant biomass ($p > 0.05$).

3.6. M. phaseolina Disease Symptoms

Macrophomina phaseolina caused the death of 12.5% of the plants in the pot experiments when no BCO was inoculated, but no dead plants were found in *M. phaseolina*-infected strawberry plants if they were inoculated with *P. aeruginosa* AC17. The three tested bacterial strains reduced plant disease severity, crown incidence and severity, and petiole colonization by *M. phaseolina*. Root colonization by *M. phaseolina* was not affected by any BCO inoculation (Table 3).

Table 3. *Macrophomina phaseolina* symptoms in plants inoculated with BCO.

Treatment Code	Dead Plants(%)	Plant Severity Index	Crown Incidence (%)	Crown Severity Index	Petiole Colonization (%)	Root Colonization (%)
MP-Ø	12.5 ± 8.5 [a]	2.9 ± 0.2 [a]	75.0 ± 11.2 [a]	1.6 ± 0.3 [a]	75.0 ± 11.2 [a]	75.0 ± 11.2 [a]
MP-Hvs8	18.8 ± 10.0 [a]	2.0 ± 0.3 [b]	18.8 ± 10.0 [b]	0.8 ± 0.4 [ab]	12.5 ± 8.5 [b]	50.0 ± 12.9 [a]
MP-AC17	0.0 ± 0.0 [b]	1.5 ± 0.1 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	53.3 ± 13.3 [a]
MP-FC37	6.3 ± 6.1 [a]	1.9 ± 0.2 [b]	31.3 ± 11.9 [b]	0.6 ± 0.3 [b]	31.3 ± 11.9 [b]	43.8 ± 12.8 [a]

BCO: Biocontrol organism. SBP: Soil-borne pathogen. Data are the mean ± standard error of 16 replicates (two trials × eight replicated plots). Values followed by the same letter within a column do not differ significantly according to HSD Tukey's tests ($p < 0.05$).

3.7. *M. hapla* Disease Symptoms

Root-knot nematode symptom severity was reduced by *P. aeruginosa* AC17 and *B. velezensis* FC37, but RKN reproduction was only reduced in plants inoculated with *P. aeruginosa* AC17 (Table 4).

Table 4. *Meloidogyne hapla* symptoms in plants inoculated with BCO.

Treatment Code	Galling Index	RKN Per Pot (750 mL)
MH-Ø	2.4 ± 0.1 [a]	3033 ± 172 [a]
MH-Hvs8	2.2 ± 0.1 [a]	2819 ± 214 [a]
MH-AC17	0.9 ± 0.9 [b]	1518 ± 130 [b]
MH-FC37	2.3 ± 0.2 [b]	2708 ± 121 [a]

BCO: Biocontrol organism. SBP: Soil-borne pathogen. Data are the mean ± standard error of 16 replicates (two trials × eight replicated plots). Values followed by the same letter within a column do not differ significantly according to HSD Tukey's or Dunn's tests ($p < 0.05$).

4. Discussion

Selection of biocontrol agents should include an isolation process from the ecological niche where they will be used, screening for their plant promotion interactions and biocontrol properties under in vitro conditions, and, finally, in vivo testing to assess the desirable properties for which they have been chosen. Furthermore, it is desirable that the selected strains can grow quickly and in a high number in synthetic media for an easy formulation. In this study, strains have been isolated from the rhizosphere of healthy blueberry and strawberry plants growing at Moguer (Huelva, Spain). All isolates have been characterized by their in vitro properties, and those able to control the growth of the pathogens under in vitro conditions have been chosen for further in vivo testing. All selected strains belong to species frequently used in bioinoculant formulations (*Bacillus* and *Pseudomonas*) for their easy growth and formulation capacity [31,32]. Intriguingly, strains isolated from blueberry rhizosphere displayed more plant growth-promoting properties and with higher values than those from strawberry. Similarly, some of the strains from strawberry rhizosphere did not show biocontrol properties at all. Blueberry is a perennial crop, and we chose healthy plants from an area with a high incidence of *M. phaseolina* for bacterial isolation. It could be that these rhizospheric bacteria were responsible for the better growth displayed by these plants.

Biocontrol of *M. phaseolina* by autochthonous bacteria has been reported for several crops. The use of biopesticides based on native *Bacillus* strains has proven effective in controlling charcoal rot in geranium [33] and strawberry [34,35]. Inoculation with *Azospirillum brasilense* REC3 strain induced a defense response in strawberry plants against virulent isolates of *M. phaseolina* and evidenced an increased tolerance to charcoal rot disease [36,37]. In the same studies, native *Bacillus* and *Pseudomonas* spp. have been reported as biocontrol agents of root-knot nematodes in several crops [13,38–40]. Although it is desirable to use autochthonous bacteria, use of bacteria isolated from similar and nearby ecosystems for biocontrol purposes has also been reported. Thus, some genera, mainly *Pseudomonas* and

Bacillus, have showed biocontrol activities against strawberry charcoal rot in southern Spain [15]

We report reductions in disease symptoms for both strawberry pathogens (*M. phaseolina* and *M. hapla*) by the *P. aeruginosa* AC17 strain. Strawberry petiole colonization by *M. phaseolina* and *M. hapla* reproduction was reduced in *P. aeruginosa* AC17-inoculated plants. *Pseudomonas aeruginosa* is a beneficial soil bacterium that promotes plant growth and development through production of a variety of regulatory compounds (IAA, ammonia, polysaccharides, and phosphate solubilization) directly in the rhizosphere and acts as a BCO directly by altering plant hormone levels or reducing the potential of SBP. *P. aeruginosa* also exhibited strong antagonistic properties against *M. phaseolina* in other crops, such as chir pine [41]. The most common molecules involved in this mechanism are, among others, siderophore, IAA, phenazines, sessilins, orfamides, chitinases, glucanases, and proteases [10]. *Pseudomonas aeruginosa* AC17 produces siderophores and proteases, while other isolated strains able to totally inhibit in vitro growth of *M phaseolina* (*B. velezensis* FC37 and *B. velezensis* ACH16) displayed much greater biocontrol capacities. It could be that *P. aeruginosa* AC17 produced secondary metabolites (i.e., antibiotics) not identified in this work that made this strain the best in vivo BCO against both pathogens.

Recent results obtained by Tian et al. (2022) [13] and by Yin et al. (2021) [42] show how different species of Bacillus (*B. velezensis* or *B. cereus*) could enhance resistance of cucumber to reduce *M. incognita* infection by activating several defense-responsive genes. Similarly, Egan and Kakouli-Duarte (2022) [43] investigated the potential role of some *Pseudomas* strains in *M. javanica* suppression in tomato plants through a split-root system and deduced that Induced Systemic Resistance (ISR) was the major nematode control mechanism. The mechanism of action of *P. aeruginosa* AC17 could be the production of one or more extracellular compounds with nematicide and fungicide properties or by triggering the ISR response in strawberry plants.

Pseudomonas aeruginosa's ability to degrade many compounds that are recalcitrant to other bacterial species makes this strain useful for bioremediation and other industries; thus, many strains are formulated under commercial bioproducts [32]. The biocontrol activities showed by *P. aeruginosa* AC17 against both strawberry pathogens (*M. phaseolina* and *M. hapla*) suggest that this bacterial strain could be an optimal candidate for biocontrol of the two main SBP in the strawberry growing area of southern Spain.

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References

1. MAPAMA. *Anuario de Estadística. Avance 2021*; Ministerio de Agricultura, Pesca y Alimentación: Madrid, Spain, 2022.
2. Arroyo, F.T.; Llergo, Y.; Aguado, A.; Romero, F. First report of Fusarium wilt caused by *Fusarium oxysporum* on strawberry in Spain. *Plant Dis.* **2009**, *93*, 323. [CrossRef] [PubMed]
3. Borrero, C.; Capote, N.; Gallardo, M.A.; Avilés, M. First report of vascular wilt caused by *Fusarium proliferatum* on strawberry in Spain. *Plant Dis.* **2019**, *103*, 581. [CrossRef]
4. Avilés, M.; Castillo, S.; Bascón, J.; Zea-Bonilla, T.; Martín-Sánchez, P.M.; Pérez-Jiménez, R.M. First report of *Macrophomina phaseolina* causing crown and root rot of strawberry in Spain. *Plant Pathol.* **2008**, *57*, 382. [CrossRef]

5. Pastrana, A.M.; Capote, N.; de los Santos, B.; Romero, F.; Basallote-Ureba, M.J. First report of *Fusarium* solani causing crown and root rot on strawberry crops in Southwestern Spain. *Plant Dis.* **2014**, *98*, 161. [CrossRef]
6. Talavera, M.; Miranda, L.; Gómez-Mora, J.A.; Vela, M.D.; Verdejo-Lucas, S. Nematode management in the strawberry fields of Southern Spain. *Agronomy* **2019**, *9*, 252. [CrossRef]
7. Talavera, M.; Gómez-Mora, J.A.; de los Santos, B.; Miranda, L.; Vela, M.D.; Fernández-Plaza, M.; Medina-Mínguez, J.J.; Soria, C.; Verdejo-Lucas, S. Problemática Fitopatológica del cultivo de la fresa en Huelva. *Agricultura* **2018**, *1022*, 6–9.
8. López-Aranda, J.M.; Domínguez, P.; Miranda, L.; de Los Santos, B.; Talavera, M.; Daugovish, O.; Soria, C.; Chamorro, M.; Medina, J.J. Fumigant use for strawberry production in Europe: The current landscape and solutions. *Int. J. Fruit Sci.* **2016**, *16*, 1–15. [CrossRef]
9. Greco, N.; López-Aranda, J.M.; Saporiti, M.; Maccarini, C.; de Tommaso, N.; Myrta, A. Sustainability of European vegetable and strawberry production in relation to fumigation practices in the EU. *Acta Hortic.* **2020**, *1270*, 203–210. [CrossRef]
10. Dimkić, I.; Janakiev, T.; Petrović, M.; Degrassi, G.; Fira, D. Plant-associated *Bacillus* and *Pseudomonas* antimicrobial activities in plant disease suppression via biological control mechanisms—A review. *Physiol. Mol. Plant Pathol.* **2022**, *117*, 101754. [CrossRef]
11. Zhai, Y.; Shao, Z.; Cai, M.; Zheng, L.; Li, G.; Huang, D.; Cheng, W.; Thomashow, L.S.; Weller, D.M.; Yu, Z.; et al. Multiple modes of nematode control by volatiles of *Pseudomonas putida* 1A00316 from Antarctic soil against *Meloidogyne incognita*. *Front. Microbiol.* **2018**, *9*, 253. [CrossRef]
12. Lalloo, R.; Maharajh, D.; Görgens, J.; Gardiner, N. A downstream process for production of a viable and stable *Bacillus cereus* aquaculture biological agent. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 499–508. [CrossRef] [PubMed]
13. Tian, X.-L.; Zhao, X.-M.; Zhao, S.-Y.; Zhao, J.-L.; Mao, Z.-C. The biocontrol functions of *Bacillus velezensis* strain Bv-25 against *Meloidogyne incognita*. *Front. Microbiol.* **2022**, *13*, 843041. [CrossRef] [PubMed]
14. Dehghanian, S.Z.; Abdollahi, M.; Charehgani, H.; Niazi, A. Combined of salicylic acid and *Pseudomonas fluorescens* CHA0 on the expression of PR1 gene and control of *Meloidogyne javanica* in tomato. *Biol. Control* **2020**, *141*, 104134. [CrossRef]
15. Viejobueno, J.; Rodríguez-Berbel, N.; Miranda, L.; de los Santos, B.; Camacho, M. Potential bacterial antagonists for the control of charcoal rot (*Macrophomina phaseolina*) in strawberry. *Horticulturae* **2021**, *7*, 457. [CrossRef]
16. De la Lastra, E.; Camacho, M.; Capote, N. Soil bacteria as potential biological control agents of *Fusarium* species associated with asparagus decline syndrome. *Appl. Sci.* **2021**, *11*, 8356. [CrossRef]
17. Lane, D.J. 16S/23S RRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematic*; Stackebrandt, E., Goodfellow, M., Eds.; John Wiley and Sons: New York, NY, USA, 1991; pp. 115–175.
18. Yoon, S.-H.; Ha, S.-M.; Kwon, S.; Lim, J.; Kim, Y.; Seo, H.; Chun, J. Introducing EzBioCloud: A taxonomically united database of 16s RRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 1613–1617. [CrossRef] [PubMed]
19. Benizri, E.; Courtade, A.; Picard, C.; Guckert, A. Role of maize root exudates in the production of auxins by *Pseudomonas fluorescens* M.3.1. *Soil. Biol. Biochem.* **1998**, *30*, 1481–1484. [CrossRef]
20. Alexander, D.B.; Zuberer, D.A. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biol. Fertil. Soils* **1991**, *12*, 39–45. [CrossRef]
21. De Freitas, J.R.; Banerjee, M.R.; Germida, J.J. Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). *Biol. Fertil. Soils* **1997**, *24*, 358–364. [CrossRef]
22. Viejobueno, J.; Albornoz, P.L.; Camacho, M.; de los Santos, B.; Martínez-Zamora, M.G.; Salazar, S.M. Protection of strawberry plants against charcoal rot disease (*Macrophomina phaseolina*) induced by *Azospirillum brasilense*. *Agronomy* **2021**, *11*, 195. [CrossRef]
23. Reznikov, S.; Vellicce, G.R.; González, V.; de Lisi, V.; Castagnaro, A.P.; Ploper, L.D. Evaluation of chemical and biological seed treatments to control charcoal rot of soybean. *J. General. Plant Pathol.* **2016**, *82*, 273–280. [CrossRef]
24. Avilés, M.; Castillo, S.; Borrero, C.; Castillo, M.L.; Zea-Bonilla, T.; Pérez-Jiménez, R.M. Response of strawberry cultivars: "Camarosa", "Candonga" and "Ventana" to inoculation with isolates of *Macrophomina phaseolina*. *Acta Hortic.* **2009**, *842*, 291–294. [CrossRef]
25. Hussey, R.S.; Barker, K.R. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Dis. Rep.* **1973**, *57*, 1025–1028.
26. Cesarz, S.; Eva Schulz, A.; Beugnon, R.; Eisenhauer, N. Testing soil nematode extraction efficiency using different variations of the Baermann-Funnel method. *Soil Org.* **2019**, *91*, 61–72. [CrossRef] [PubMed]
27. Koike, S.T.; Arias, R.S.; Hogan, C.S.; Martin, F.N.; Gordon, T.R. Status of *Macrophomina phaseolina* on strawberry in California and preliminary characterization of the pathogen. *Int. J. Fruit Sci.* **2016**, *16*, 148–159. [CrossRef]
28. Fang, X.L.; Phillips, D.; Li, H.; Sivasithamparam, K.; Barbetti, M.J. Severity of crown and root diseases of strawberry and associated fungal and oomycete pathogens in Western Australia. *Australas. Plant Pathol.* **2011**, *40*, 109–119. [CrossRef]
29. Bridge, J.; Page, S.L.J. Estimation of root knot nematode infestation level on roots using a rating chart. *Trop. Pest Manag.* **1980**, *26*, 296–298. [CrossRef]
30. Coolen, W.A. Methods for the extraction of *Meloidogyne* spp. and other nematodes from roots and soil. In *Root-knot nematodes (Meloidogyne Species)—Systematics, Biology and Control*; Lamberti, F., Taylor, C.E., Eds.; Academic Press: London, UK, 1979; pp. 317–329.
31. Villarreal-Delgado, M.F.; Villa-Rodríguez, E.D.; Cira-Chávez, L.A.; Estrada-Alvarado, M.I.; Parra-Cota, F.I.; de los Santos-Villalobos, S. The genus *Bacillus* as a biological control agent and its implications in the agricultural biosecurity. *Rev. Mex. Fitopatol.* **2017**, *36*, 95–130. [CrossRef]

32. Anayo, O.F.; Scholastica, E.C.; Peter, O.C.; Nneji, U.G.; Obinna, A.; Mistura, L.O. The beneficial roles of *Pseudomonas* in Medicine, Industries, and Environment: A Review. In *Pseudomonas Aeruginosa—An Armory within*; Sriramulu, D., Ed.; IntechOpen: London, UK, 2019. [CrossRef]
33. Ghazi, A.; Attia, E.A.; Rashed, N.M. Management of charcoal rot (*Macrophomina phaseolina*) infection in geranium (*Pelargonium graveolens* L.) using biocontrol agents and essential oils. *Environ. Biodivers. Soil Secur.* **2018**, *2*, 131–142. [CrossRef]
34. Pastrana, A.M.; Basallote-Ureba, M.J.; Aguado, A.; Akdi, K.; Capote, N. Biological control of strawberry soil-borne pathogens *Macrophomina phaseolina* and *Fusarium solani*, using *Trichoderma asperellum* and *Bacillus* spp. *Phytopathol. Mediterr.* **2016**, *55*, 109–120. [CrossRef]
35. Abd-El-Kareem, F.; Elshahawy, I.E.; Abd-Elgawad, M.M.M. Native bacteria for field biocontrol of black root rot in Egyptian strawberry. *Bull. Natl. Res. Cent.* **2022**, *46*, 82. [CrossRef]
36. Lovaisa, N.C.; Guerrero, M.F.; Delaporte, P.G.A.; Salazar, S.M. Response of strawberry plants inoculated with *Azospirillum* and *Burkholderia* at field conditions. *Rev. Agron. Noroeste Argent* **2015**, *35*, 33–36.
37. Guerrero-Molina, M.F.; Lovaisa, N.C.; Salazar, S.M.; Martínez-Zamora, M.G.; Díaz-Ricci, J.C.; Pedraza, R.O. Physiological, structural and molecular traits activated in strawberry plants after inoculation with the plant growth-promoting bacterium *Azospirillum brasilense* REC3. *Plant Biol.* **2014**, *17*, 766–773. [CrossRef] [PubMed]
38. Askary, T.H.; Pala-Martinelli, P.R.; Askary, T.H. *Biocontrol Agents of Phytonematodes*; CAB International: Wallingford, UK; Oxford, UK, 2015; ISBN 9788578110796.
39. Flor-Peregrín, E.; Azcón, R.; Salmerón, T.; Talavera, M. Biological protection conferred by *Glomus* spp. and *Bacillus megaterium* against *Meloidogyne incognita* in tomato and pepper. *IOBC/WPRS Bull.* **2012**, *83*, 215–218.
40. Turatto, M.F.; Dourado, F.d.S.; Zilli, J.E.; Botelho, G.R. Control potential of *Meloidogyne javanica* and *Ditylenchus* spp. using fluorescent *Pseudomonas* and *Bacillus* spp. *Braz. J. Microbiol.* **2018**, *49*, 54–58. [CrossRef]
41. Singh, N.; Kumar, S.; Bajpai, V.K.; Dubey, R.C.; Maheshwari, D.K.; Kang, S.C. Biological control of *Macrophomina phaseolina* by chemotactic fluorescent *Pseudomonas aeruginosa* PN1 and its plant growth promotory activity in Chir-pine. *Crop. Prot.* **2010**, *29*, 1142–1147. [CrossRef]
42. Yin, N.; Zhao, J.-L.; Liu, R.; Li, Y.; Ling, J.; Yang, Y.-H.; Xie, B.-Y.; Mao, Z.-C. Biocontrol Efficacy of *Bacillus cereus* Strain Bc-cm103 Against *Meloidogyne incognita*. *Plant Dis.* **2021**, *105*, 2061–2070. [CrossRef]
43. Egan, A.; Kakouli-Duarte, T. Observations on the interaction between plant growth-promoting bacteria and the root-knot nematode *Meloidogyne hapla*. *MicrobiologyOpen* **2022**, *11*, e1319. [CrossRef]