Article

Soil Bacteria as Potential Biological Control Agents of *Fusarium* Species Associated with Asparagus Decline Syndrome

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Featured Application: The *Bacillus* strains FC37 and Hvs2 could be used as potential biocontrol agents as a sustainable and environmentally friendly control strategy for asparagus fields affected by the decline syndrome.

Abstract: To avoid the use of agrochemicals in agriculture, alternative methods are emerging to control plant pathogens. Some plant growth-promoting rhizobacteria (PGPR) can be used as biocontrol agents since they can induce protection against pathogens. The aim of this study was to evaluate the protective effect of several PGPR strains against the main *Fusarium* species involved in asparagus decline syndrome (ADS): *F. proliferatum*, *F. oxysporum* f. sp. *asparagi* and *F. redolens*. *In vitro* antagonism assays showed that all the bacteria inhibited the mycelium growth of the three *Fusarium* species. The most effective strains (*Streptomyces fradiae* Hvs6, *Bacillus paralicheniformis* Hvs2 and *Bacillus velezensis* FC37) were tested to evaluate their protective effect on asparagus plants inoculated with pathogenic *Fusarium* isolates. Strains FC37 and Hvs2 were the most effective in controlling pathogenic *F. proliferatum* and *F. oxysporum* f. sp. *asparagi*, but neither could protect against *F. redolens* isolates. The production of hydrolytic enzymes such as β -glucosidase, amylase and protease by these bacterial strains could be involved in the structural degradation of the fungal cell wall. In addition, the production of toxic volatile compounds, such as hydrogen cyanide, may inhibit the fungal growth, and the production of phosphate solubilizers could be related to the plant growth promotion. These results suggest that strains FC37 and Hvs2 could be used as potential biocontrol agents as a sustainable and environmentally friendly control strategy for ADS-affected fields.

Keywords: *Fusarium proliferatum*; *F. oxysporum*; enzymatic activity; *Bacillus paralicheniformis*; *Bacillus velezensis*; biocontrol; PGPR; hydrogen cyanide; phosphate solubilization

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1. Introduction

Asparagus (*Asparagus officinalis* L.) is a highly important crop worldwide. The crop has a great increase due to the demand of consumers for healthy foods. Spears have a high nutritional value and functional properties, providing dietary fiber and high content of natural antioxidants and other bioactive components, such as phenolic acids, flavonoids and ascorbic acid [1,2].

Asparagus decline syndrome (ADS) is one of the most important diseases of asparagus in most producing areas [3]. Symptoms consist of root rot of seedlings and mature plants that led to a drastic decrease in feeder roots and loss of their cortical parenchyma. The crowns present discoloration that evolves to necrosis, which extends to the base of spears. The adult plant shows progressive chlorosis, a wilting of fronds that resembles drought symptoms, and finally dies. Fields crops with previous ADS symptoms also display replanting problems, which imply premature uprooting of plants and prevents affected fields from being planted with asparagus. Several factors have been suggested to be involved in ADS, including abiotic factors, such as the accumulation in the soil of allelopathic compounds produced by the asparagus plant [4,5], the accumulation of herbicide

residues, nutrient starving or the alteration of the soil structure. Among biotic factors, ADS has been closely associated with the presence of pathogenic species of *Fusarium* in the soil. *Fusarium proliferatum*, *F. oxysporum* f. sp. *asparagi* and *F. redolens* were the main *Fusarium* species associated with ADS in Spain [6,7].

The control of ADS is challenging because the causal agents can survive for long time in the soil and plant debris and can be disseminated by nursery plant material and irrigation water [8,9]. In addition, the commercial varieties have demonstrated high susceptibility to *Fusarium* infection, and tolerant or resistant varieties are not currently available. Chemical soil disinfection prior to planting is one of the most used control strategies for ADS. However, the drastic decrease in the use of chemical fungicides applied to agriculture, in accordance to the European strategy “Farm to fork”, aimed to prevent fungicide resistance and to avoid damages to human health and the environment, which has prompted the search for more sustainable alternatives. Biological control has emerged as one of the most sustainable control strategies. Fungal-based biological control agents such as non-pathogenic strains of *F. oxysporum* [10,11], arbuscular mycorrhiza [12], *Trichoderma harzianum* [13], and endophytic [14] and rhizospheric [15] fungi have been used for achieving tolerance to *Fusarium* root rot of asparagus. Regarding bacteria, *Streptomyces griseus* could control *Fusarium* diseases of asparagus *in vitro*, via antibiotic production [16], and species of *Pseudomonas* and *Serratia* could suppress *Fusarium* crown and root rot of asparagus [17]. Another asparagus disease, such as *Phytophthora* rot of asparagus, could also be controlled by the use of rhizospheric bacteria [18].

In this sense, the use of bacterial strains isolated from the soil or the rhizosphere of healthy plants as biological control agents has allowed reducing the incidence and severity of multiple fungal plant diseases. The modes of action are variable and consist of the competition for nutrients, the production of antifungal compounds such as hydrolytic enzymes, or the release of toxic substances which provide a broad antagonistic activity against different soil-borne phytopathogenic fungi [19]. In addition, some rhizospheric bacteria are able to induce the plant growth by producing plant growth promoting compounds such as auxin phytohormone [20] or facilitating the uptake of certain nutrients from the environment, such as nitrogen, potassium or phosphorus [21], which may help the plant fight pathogens [22].

The objective of this work was to evaluate the ability of five rhizospheric bacterial strains of the species *Bacillus amyloliquefaciens*, *B. paralicheniformis*, *B. velezensis*, *Brevibacterium frigoritolerans* and *Streptomyces fradiae* in the control of the main *Fusarium* species associated with ADS (*F. proliferatum*, *F. oxysporum* f. sp. *asparagi* and *F. redolens*), through both *in vitro* and *in planta* assays, and to propose possible mechanisms of action of the bacterial strains by characterizing the antifungal metabolites and growth promoter compounds produced by the biocontrol strains.

2. Materials and Methods

2.1. Fungal Isolates

Fusarium proliferatum isolates Fp3 and Fp86, *F. oxysporum* isolates Fo14 and Fo125, and *F. redolens* isolates Fr7 and Fr104 were obtained from symptomatic asparagus plants collected from ADS-affected asparagus fields [6] and conserved at the IFAPA's fungal collection, Seville, Spain.

2.2. Bacterial Strains

Five bacterial strains were used to evaluate their potential as antagonists and biological control agents: *Bacillus amyloliquefaciens* Adb14, *B. paralicheniformis* Hvs2, *B. velezensis* FC37, *Brevibacterium frigoritolerans* Hvs8 and *Streptomyces fradiae* Hvs6. All of them belonged to the IFAPA's bacterial collection. Strains Adb14 and FC37 were, respectively, isolated from strawberry plants and the rhizosphere of strawberry plants collected in Moguer (Huelva). Strains Hvs2, Hvs6 and Hvs8 were originally isolated from the rhizosphere of *Arthrocaulon* plants growing at Lebrija marshes (Seville). The strains were identified by 16S rDNA

sequencing using universal primers 27F-5'-AGAGTTTGATCCTGGCTCAG-3' and 1492-R-5' GGTACCTTGTACGACTT-3', and subsequently analyzed using the BLAST tool and EzBioCloud database. Finally, accession numbers from OU487633 to OU487636 were assigned to the sequences for FC37, Hvs8, Hvs6 and AdB1, respectively, and OU570686 for Hvs2 (Supplementary Materials 1).

2.3. Dual Plate Confrontation Experiments

Bacterial suspensions were obtained by growing bacterial strains in nutrient broth (NB) liquid medium at 28 °C with continuous shaking (160 rpm) for 24 h. Bacterial cultures were then centrifuged at $10,000 \times g$ for 5 min and the pellet was resuspended in 0.03 M magnesium sulfate buffer to get a suspension of 10^9 bacteria/mL. Four droplets of each bacterial suspension (10 µL) were loaded into the periphery of Petri dishes (6 cm) containing Potato dextrose agar (PDA) and incubated at 28 °C for 24 h. Plates without bacterial inoculum were used as negative controls. Then, a 5-day-old mycelial disc (5 mm diam.) from the edge of an actively growing colony of each fungal pathogen was placed at the center of the PDA plates and incubated at 25 °C. Three replicates per dual confrontation were used. Percentage of inhibition of the pathogen development respect to the control was assessed at 7 days after confrontation and it was calculated using the formula 1:

$$\% = [(R_c - R_i) / R_c] \times 100 \quad (1)$$

where R_c is the radial growth of the fungal pathogen in the control plates (mm), and R_i is the radial growth of the fungal pathogen in the test plates (mm).

2.4. Growth Chamber Experiments

Growth chamber experiments were performed to evaluate the control efficacy of the three bacterial strains that showed the highest percentage of fungal growth inhibition: *S. fradiae* Hvs6, *B. velezensis* FC37 and *B. paralicheniformis* Hvs2. Asparagus seeds cv. Grande F1 were surface disinfested by immersion with 10% sodium hypochlorite for 10 min, subsequently rinsed with tap water and air dried on sterile paper in a laminar flow cabinet. Seeds were then immersed in a suspension of 10^8 bacteria/mL (prepared as explained above) and incubated at room temperature for 1 h with gentle agitation. Inoculated seeds were germinated on 1% agar–water Petri dishes at 25 °C in the dark. Non-inoculated seeds were immersed in sterile NB liquid medium. Once the radicle had emerged by 1–1.5 cm, seedlings were transplanted to incubation trays with sterile vermiculite and incubated in a growth chamber set at 25/18 °C (light/dark) with a 14 h photoperiod for 2 weeks. When plants reached an appropriate size, they were first inoculated by adding 1 mL of freshly prepared bacterial suspension (10^8 bacteria/mL) around each plant, and 24 h later by immersing the roots in a fungal suspension of 10^6 conidia/mL for 30 min. For fungal inoculum preparation, fungal isolates were cultured in Czapek–Dox broth on a rotary shaker (150 rpm) at 25 °C with a 12 h photoperiod for 10 days. The spore suspension was filtered through four layers of cheese cloth and adjusted with sterile distilled water to a concentration of 10^6 conidia/mL. Non-inoculated control plants were root-dipped in sterile Czapek–Dox broth. Plants were then transplanted into pots with 1.6 L of sterile vermiculite and maintained in a growth chamber set at 25/18 °C (light/dark) with a 14 h photoperiod (14,000 lux) for 60 days. Plants only inoculated with bacteria, only inoculated with fungi and non-inoculated plants were used as controls. Plants were watered as required and fertilized once a week with Hoagland medium [23]. Twelve plants were tested for each fungal-bacterial combination, with three plants per pot arranged in a completely randomized design.

2.5. Disease Assessments

Plants were evaluated weekly for disease severity based on a 1–5 scale, where 1 = 1–20%, 2 = 21–40%, 3 = 41–60%, 4 = 61–80% and 5 = 81–100% of the fronds showing chlorosis, necrosis or wilt [6]. The progression of the disease as area under the disease

progress curve (AUDPC) was calculated weekly throughout 2 months for each species. At the end of each experiment, the percentage of dead plants was determined. Plants were removed from the pots, the roots rinsed in tap water and the severity of the symptoms on the root system was assessed on a 1–5 scale such as that described above for the aerial part. The fronds and root wet weights were measured. Data were expressed as mean $\pm$ standard error of 12 replicate plants inoculated with each isolate. To confirm that the symptomatic and dead asparagus plants were affected by the inoculated *Fusarium* species, all dead plants and several randomly symptomatic plants per isolate were analyzed for recovery of the inoculated fungal isolate. Plants were taken out of the pots and roots, stems and fronds were surface sterilized independently in 1% sodium hypochlorite for 2 min, rinsed twice with sterile water and dried in a laminar flow chamber. Four small pieces from each plant tissue were cut and placed on PDA plates supplemented with 0.2% streptomycin. Plates were incubated at 25 °C for seven days in a 12 h photoperiod. *Fusarium* species were identified by morphological characteristics [24,25].

2.6. Enzymatic Activity of Bacterial Strains

All the isolates were tested for the following biocontrol activities: protease, chitinase, amylase and β -glucosidase, and for the presence of volatile compounds (hydrogen cyanide, HCN). Protease, chitinase and amylase activities were recorded according to Mesa et al. (2015) [26], while β -glucosidase production was recorded following the method described by Gong et al. (2012) [27]. Briefly, for protease, chitinase and β -glucosidase activities, strains were grown in casein agar, minimal medium supplemented with colloidal chitin and Luria Bertani (LB) medium containing 0.1% esculin and 0.05% ferric ammonium citrate, respectively, and plates were incubated at 28 °C for 5 days until clearing (for protease and chitinase) or black (for β -glucosidase) zones could be seen around the colonies. For amylase activity, strains were inoculated on starch agar, incubated, and revealed by flooding the plates with lugol. The presence of volatile compounds was carried out joining the protocol of Bano and Musarrat (2003) [28], in which they used King medium supplemented with 0.44% glycine for growing the strains, and the protocol of Pacheco-Hernández et al. (2015) [29], in which a sterile filter paper saturated with a solution of picric acid were placed on the lid of the plate. Finally, the valuation of HCN production was done on a 0–3 scale according to Reetha et al. (2014) [30], where the initial yellow color (0) changed to dark brown (3).

2.7. Production of Plant Growth-Promoting Compounds

Bacteria were tested for their ability for auxin and siderophore production following the protocols of Benizri et al. (1998) [31] and Alexander and Zuberer (1991) [32], respectively. Briefly, for auxin production, bacteria were grown in NB medium supplemented with L-Try (100 mg L⁻¹) and IAA production was colorimetrically measured after adding Salkowski reagent (2% 0.5 FeCl₃ in 35% HClO₄ solution), and siderophore production was detected by the presence of orange halos on chrome azurol S (CAS) plates. For phosphate solubilization, transparent halos were recorded after the bacteria were grown in NB plates containing 0.5% K₂HPO₄ and 1% CaCl₂, following the procedure described by de Freitas et al. (1997) [33].

2.8. Data Analysis

Analyses of variance were performed for the fungal growth inhibitions produced by the different bacteria strains in the dual plate confrontation assays. Arcsine square root transformation was applied to inhibition percentages. Means were compared by LSD's significant difference test ($p \leq 0.05$).

Individual analysis of variance was performed (fronds and root weights) for each *Fusarium* isolate in the growth chamber experiments with plants. Means were compared by Tukey's whole significant difference test ($p \leq 0.05$). Additional non-parametric Kruskal–Wallis tests were performed when the assumption of the normality of the distributions was not sustainable (disease severity on fronds and roots, and AUDPC). All statisti-

cal analyses were carried out using Statistix 9 package software (Analytical Software, Tallahassee, FL, USA).

3. Results

3.1. Antagonism In Vitro

The strain Hvs6 significantly showed the highest percentage of fungal growth inhibition (81–84%) for all tested isolates of *F. proliferatum*, *F. oxysporum* f. sp. *asparagi* and *F. redolens*. FC37 and Hvs2 inhibited up to 73 and 82% the growth of the fungal isolates assayed, respectively. The Adb14 strain reduced the mycelial growth from 41 to 50%. Hvs8 showed the lowest ability for mycelial growth inhibition (10–20%; Figures 1 and 2).

Figure 1. Percentage of mycelial growth inhibition of *Fusarium proliferatum* (Fp), *F. oxysporum* f. sp. *asparagi* (Fo), and *F. redolens* (Fr) isolates by *Bacillus amyloliquefaciens* Adb14, *B. paralicheniformis* Hvs2, *B. velezensis* FC37, *Brevibacterium frigoritolerans* Hvs8, and *Streptomyces fradiae* Hvs6 bacterial strains. Results are the mean of three replicates and error bars correspond to the standard error calculated as the quotient of the standard deviation and the square root of the number of samples analyzed. Different letters indicate significant differences among bacterial strains based on the LSD test at $p \leq 0.05$.

Figure 2. Dual confrontation experiments showing the inhibition of the fungal mycelial growth of *Fusarium proliferatum* (isolates Fp3 and Fp86), *F. oxysporum* f. sp. *asparagi* (isolates Fo14 and Fo125) and *F. redolens* (isolates Fr7 and Fr104) by the bacterial strains Adb14, Hvs8, FC37, Hvs6 and Hvs2.

3.2. Biological Control of *Fusarium* Species in Growth Chamber Conditions

Non-inoculated plants and plants inoculated only with the bacterial strains did not show disease symptoms throughout the experiment. Control plants inoculated with the strain FC37 showed higher root weights than plants inoculated with strains Hvs2 and Hvs6 or non-inoculated (Table 1).

The strain FC37 showed the highest level of biocontrol. *Asparagus* plants coinoculated with *F. proliferatum* isolates Fp3 and Fp86, respectively, and strain FC37 reduced the plant mortality by 58 and 63% with respect to the control inoculated only with the fungal isolates. In addition, the weight of shoots and roots were significantly higher, and the severity of shoot and root symptoms, and the AUDPC were lower in plants coinoculated with *F. proliferatum* isolates and FC37 than in those inoculated only with the fungal isolates (Table 1).

A similar behavior was observed in plants inoculated with isolates of *F. oxysporum* f. sp. *asparagi*. The coinoculation of the fungal isolate Fo125 with the bacteria FC37 reduced the percentage of dead plants (24%) and produced significant higher root and shoot weights compared with plants inoculated only with the fungal isolate. The severity of roots was similar, although the shoot severity and AUDPC were lower in the coinoculated plants. Fo14 isolate did not produce dead plants, although the coinoculation with bacteria FC37 produced a significant increase in the root biomass and a reduction in root symptoms and AUDPC. However, no benefits of strain FC37 were observed in plants inoculated with any of the isolates of *F. redolens*, except for a slight increase in plant biomass observed in plants coinoculated with Fr104 and FC37, compared with those inoculated only with the fungus (data not shown).

The strain Hvs2 showed an intermediate ability to control *F. proliferatum* and *F. oxysporum* f. sp. *asparagi* isolates pathogenic to asparagus, reducing between 26 and 63% the percentage of dead plants, avoiding the loss of plant biomass, and reducing disease symptoms (except for isolate Fo125 of *F. oxysporum* f. sp. *asparagi*) in plants coinoculated with this bacteria and the respective Fusarium isolates (Table 1). No benefits of Hvs2 were observed in plants inoculated with any of the isolates of *F. redolens*.

The strain Hvs6 showed a lower ability to control *F. proliferatum* Fp3 and Fp86 isolates, reducing 14 and 51% the percentage of dead plants, respectively. In addition, the coinoculation of Hvs6 and Fp86 isolate avoided the loss of plant biomass and reduced the shoot and root disease symptoms, although to a lesser extent than FC37 and Hvs2 (Table 1). No beneficial effect of Hvs6 was observed on plants inoculated with *F. oxysporum* and *F. redolens* isolates.

Fungal isolates were reisolated from the roots of all inoculated plants although the percentage of fungal recovery was lower in plants coinoculated with any bacterial strain (data not shown).

Table 1. Percentage of dead plants, root and shoot fresh weight, symptoms severity and the area under the disease progress curve (AUDPC) for *in planta* assays of coinoculations of *Fusarium proliferatum* (isolates Fp3 and Fp86), and *F. oxysporum* f. sp. *asparagi* (isolates Fo14 and Fo125) and the bacterial strains FC37, Hvs2, and Hvs6. Data are mean $\pm$ standard deviation of 9–12 plants. For each fungal isolate, different letters indicate significant differences among treatments ($p \leq 0.05$).

Fungus-Bacteria Coinoculations	Dead Plants (%)	Root Fresh Weight (g)	Shoot Fresh Weight (g)	Root Severity	Shoot Severity	AUDPC
FC37	0	0.92 $\pm$ 0.43	0.20 $\pm$ 0.06	0.1 $\pm$ 0.3	0.7 $\pm$ 0.5	0.3 $\pm$ 0.4
Hvs2	0	0.79 $\pm$ 0.23	0.20 $\pm$ 0.05	0.1 $\pm$ 0.3	0.8 $\pm$ 0.8	0.2 $\pm$ 0.3
Hvs6	0	0.77 $\pm$ 0.22	0.20 $\pm$ 0.04	0.0 $\pm$ 0.0	0.8 $\pm$ 1.0	0.3 $\pm$ 0.5
Non-inoculated	0	0.73 $\pm$ 0.42	0.21 $\pm$ 0.08	0.1 $\pm$ 0.3	0.2 $\pm$ 0.4	0.0 $\pm$ 0.0
Fp3-FC37	33	0.11 $\pm$ 0.09 a	0.07 $\pm$ 0.04 a	2.6 $\pm$ 1.1	3.3 $\pm$ 1.5	2.3 $\pm$ 1.3
Fp3-Hvs2	58	0.08 $\pm$ 0.05 ab	0.05 $\pm$ 0.03 ab	2.6 $\pm$ 0.9	4.3 $\pm$ 1.1	2.9 $\pm$ 1.1
Fp3-Hvs6	67	0.04 $\pm$ 0.05 b	0.02 $\pm$ 0.02 b	3.2 $\pm$ 1.0	3.9 $\pm$ 1.9	2.7 $\pm$ 1.4
Fp3	78	0.04 $\pm$ 0.04 b	0.05 $\pm$ 0.04 ab	2.9 $\pm$ 0.6	4.1 $\pm$ 1.8	3.3 $\pm$ 1.4
Fp86-FC37	33	0.20 $\pm$ 0.19 a	0.08 $\pm$ 0.05 a	2.6 $\pm$ 0.9	3.3 $\pm$ 1.7	1.7 $\pm$ 1.0
Fp86-Hvs2	33	0.14 $\pm$ 0.11 ab	0.08 $\pm$ 0.05 a	2.6 $\pm$ 0.8	3.4 $\pm$ 1.4	2.0 $\pm$ 1.1
Fp86 Hvs6	44	0.12 $\pm$ 0.14 ab	0.07 $\pm$ 0.06 a	3.0 $\pm$ 1.2	3.3 $\pm$ 1.9	2.3 $\pm$ 1.8
Fp86	89	0.03 $\pm$ 0.01 b	0.02 $\pm$ 0.01 b	3.4 $\pm$ 0.5	4.7 $\pm$ 1.1	3.2 $\pm$ 0.8
Fo125-FC37	25	0.26 $\pm$ 0.10 a	0.11 $\pm$ 0.05 a	2.2 $\pm$ 1.0	2.7 $\pm$ 1.8	1.5 $\pm$ 1.2
Fo125-Hvs2	42	0.10 $\pm$ 0.04 b	0.07 $\pm$ 0.04 ab	3.0 $\pm$ 0.6	3.3 $\pm$ 1.7	2.3 $\pm$ 1.3
Fo125-Hvs6	67	0.06 $\pm$ 0.08 b	0.05 $\pm$ 0.06 b	3.3 $\pm$ 1.1	4.2 $\pm$ 1.4	2.7 $\pm$ 1.0
Fo125	33	0.12 $\pm$ 0.08 b	0.06 $\pm$ 0.03 b	2.2 $\pm$ 1.2	2.8 $\pm$ 1.9	1.9 $\pm$ 1.6
Fo14-FC37	0	0.79 $\pm$ 0.21 a	0.19 $\pm$ 0.05 a	0.5 $\pm$ 0.5	0.9 $\pm$ 0.5	0.2 $\pm$ 0.2
Fo14-Hvs2	0	0.67 $\pm$ 0.18 ab	0.16 $\pm$ 0.04 a	0.7 $\pm$ 0.5	0.5 $\pm$ 0.5	0.3 $\pm$ 0.4
Fo14-Hvs6	0	0.69 $\pm$ 0.16 ab	0.18 $\pm$ 0.04 a	0.8 $\pm$ 0.4	0.9 $\pm$ 1.1	0.4 $\pm$ 0.7
Fo14	0	0.53 $\pm$ 0.12 b	0.15 $\pm$ 0.05 a	0.7 $\pm$ 0.5	0.7 $\pm$ 1.0	0.3 $\pm$ 0.4

3.3. Biocontrol Enzymatic Activity of Bacterial Strains

The biocontrol-related enzymatic activities measured in the bacterial strains are shown in Figure 3. Strain Hvs2 showed the highest diversity of enzymatic activities. All strains produced protease and amylase, although FC37 presented the highest level of both. On the other hand, chitinase activity was the least common activity, being shown only by strain Hvs2. Production of toxic volatile HCN was observed in Hsv2 and Hvs8 strains, being more remarkable in the former.

Figure 3. Percentage of biocontrol enzymatic activities displayed by strains Adb14, FC37, Hvs2, Hvs6, and Hvs8.

3.4. Production of Plant Growth-Promoting Compounds

Results obtained from plant growth promoting test are shown in Table 2. All the tested strains showed only one PGP property, exception made of strain Hvs2 which showed no properties at all. It is noticeable the high siderophores production showed by strain Hvs6.

Table 2. Plant growth promoting compounds produced by bacterial strains Adb14, FC37, Hvs2, Hvs6 and Hvs8.

Bacterial Strain	Siderophore	Phosphate Solubilization	Auxin
Adb14	+	-	-
FC37	-	+	-
Hvs2	-	-	-
Hvs6	+++	-	-
Hvs8	-	-	+

4. Discussion

Soil-borne fungal diseases cause worldwide economic losses in agriculture. Alternative control methods are desirable to avoid using chemical fungicides, which can potentially be harmful to the environment and human health. Numerous studies have been published about the ability of PGPR to control soil-borne fungal diseases in different crops [34–36], however there are few studies based on PGPR to control *Fusarium* species involved in ADS, as far as we know. In this work, we have evaluated the potential of five PGP bacterial strains as antagonists and biological control agents of ADS-involved *Fusarium* species. Bacteria belonging to genera *Bacillus* (Adb14, Hvs2 and FC37), *Brevibacterium* (Hvs8) and *Streptomyces* (Hvs6) were selected because of their demonstrated biological control activity against different crop diseases tested earlier [37–40].

In vitro tests, through dual confrontation experiments, clearly showed that *S. fradiae* Hvs6, *B. velezensis* FC37 and *B. paralicheniformis* Hvs2 were the most effective strains to inhibit the fungal radial growth of the pathogenic *Fusarium* isolates, reaching from 73 to 84% inhibition. Interestingly, Hvs2 and FC37 were the bacterial strains that produced

the greatest diversity of cell wall degrading enzymes among the five strains tested. The production of cell wall degrading enzymes has been described as a mechanism used by biocontrol agents to control soil-borne pathogens [21,41]. Enzymes such as β -glucosidase, chitinase, amylase and protease secreted by biocontrol strains of PGPR provoke a direct inhibitory effect on the mycelium growth of fungal pathogens by degrading their cell wall [42].

In planta challenge assays suggested protection against pathogenic isolates of *F. proliferatum* and *F. oxysporum* f. sp. *asparagi* by the three bacterial strains. FC37 displayed the best protection effect, which could be related to the higher protease and amylase activities exhibited by this strain. In addition, plants inoculated with the FC37 presented higher root and shoot weights than non-inoculated plants or inoculated with the other two bacteria, suggesting that this strain could also stimulate the asparagus growth. *B. velezensis* FC37 is the only tested strain able to solubilize phosphorus, suggesting that FC37 could protect the plant by both, limiting the growth of fungi by producing cell wall degrading-enzymes, and enhancing plant growth by converting unavailable forms of phosphorus into forms available for subsequent uptake by the plant. Furthermore, it was found that in addition to some fungal cell wall degrading enzymes [43], *B. velezensis* strains can produce volatile compounds which can affect the growth of plant pathogenic fungi including *Fusarium* spp. [44]. It could be possible that strain FC37 produces a volatile compound that it has not been tested in this work. In the same way, the mechanism of action of strain Hvs2 could be related to the production of hydrogen cyanide (HCN), which is a toxic volatile compound that affects the respiratory system of pathogenic fungi resulting in their growth inhibition [45]. However, whether volatile compounds play a role in biocontrol *in planta* has not yet been confirmed. On the other hand, Hvs2 could effectively control the *F. proliferatum* isolates and the *F. oxysporum* Fo14 isolate but not the *F. oxysporum* Fo125 isolate. The ability of controlling pathogenic *Fusarium* depends not only on the fungal species (e.g., *F. redolens* cannot be controlled by any of the assayed bacteria), but within the same species, the factor isolate also influences in the interaction. Just as within a bacterial species some strains work better than others as biocontrol agents, fungal isolates within the same species can be differentially controlled. The genus *Fusarium*, and especially *F. oxysporum*, is not easy to control due in part to the high genetic variability of this species [6,46]. A combination of these two *Bacillus* strains should be assessed to determine their synergistic effect on asparagus plant protection.

Hvs6 strain only showed the ability to control *F. proliferatum* isolates and to a lesser extent than those exhibited by the other two bacteria. This fact contrasts with its *in vitro* behavior in which Hvs6 produced the greatest percentage of mycelium growth inhibition. It is probable that the environment of the asparagus rhizosphere is not appropriate for the establishment of this strain. The asparagus plants can produce allelopathic compounds [47] that could affect the composition of the rhizosphere and interfere in the establishment of some bacterial species. The high production of siderophore by this strain does not seem to affect the growth of asparagus plants. Siderophores are iron-chelating compounds that play a key role in DNA synthesis and respiratory systems [48]. Some *Bacillus* species can secrete siderophores under conditions of iron limitation [49]. The use of fertilizer solutions containing iron, such as Hoagland, used to water asparagus plants in growth chamber experiments, could have inhibited the production of siderophores by this strain, although this function could be active in cropping soils lacking this element.

Frequently, strains expressing the best activities *in vitro* are not always the strains showing the best results *in vivo* and vice versa [50]. The potential of the bacteria to produce such a compound *in vitro* does not necessarily correlate with their antagonism *in planta*. In this way, *F. redolens* growth could be controlled *in vitro* but none of the tested bacteria resulted in a good biological control agent for *F. redolens* isolates *in vivo*. Even though *F. redolens* is phylogenetically close to *F. oxysporum*, there must be essential differences between the species that determine their control by a specific bacterial strain or a type of mechanism. However, since *F. proliferatum* and *F. oxysporum* have been proved to be the

most pathogenic and common *Fusarium* species involved in asparagus decline syndrome, respectively [6,7], FC37 and Hvs2 could be used as potential biocontrol agents against the main *Fusarium* species involved in ADS.

5. Conclusions

The present work provides for two rhizospheric *Bacillus* strains (*B. velezensis* FC37 and *B. paralicheniformis* Hvs2) which produce cell wall degrading enzymes, antifungal metabolites and plant growth promoter compounds. Their effectiveness has been proved both *in vitro* and *in planta* challenge experiments, although their ability to control ADS needs to be further evaluated in field conditions. If these two *Bacillus* strains were effective in future field trials, they could be used as suitable biocontrol agents for controlling ADS caused by *F. proliferatum* and *F. oxysporum* f. sp. *asparagi*, avoiding or reducing the use of chemical fumigants.

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