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Isolation and Characterization of *Pseudomonas* sp. HX1, *Streptomyces luteogriseus* HR40, and *Streptomyces flavofungini* HR77 as Promising Biocontrol Agents Against Verticillium Wilt in Hops Affected by *Verticillium nonalfalfae*

Seyedehtannaz Ghoreshizadeh [1], Carla Calvo-Peña [1], Marina Ruiz-Muñoz [1], Maja Dobrajc [2], Sebastjan Radišek [2], Juan José R. Coque [1,*] and Rebeca Cobos [1,*]

[1] Instituto de Investigación de la Viña y el Vino, Escuela de Ingeniería Agraria, Universidad de León, 24009 León, Spain; sghore00@estudiantes.unileon.es (S.G.); ccalp@unileon.es (C.C.-P.); mruim@unileon.es (M.R.-M.)
[2] Slovenian Institute of Hop Research and Brewing, 3310 Žalec, Slovenia; maja.dobrajc@ihps.si (M.D.); sebastjan.radisek@ihps.si (S.R.)
* Correspondence: jjrubc@unileon.es (J.J.R.C.); rebeca.cobos@unileon.es (R.C.)

Abstract: *Verticillium* spp. are soil-borne fungal pathogens capable of infecting over 400 plant species via their root systems, with each species demonstrating a distinct host range. The primary host of *Verticillium nonalfalfae* is the hop plant (*Humulus lupulus*), which becomes affected by Verticillium wilt and, in severe cases, dieback. A dual screening approach was employed to isolate Streptomycetes as potential biocontrol agents from the hop root systems alongside other bacterial species from hop xylem. Three *Pseudomonas* strains from the xylem and three *Streptomyces* strains from the roots were selected based on their in vitro antifungal activity against *Verticillium dahliae* and/or *V. nonalfalfae*. Their potential for controlling Verticillium wilt was further assessed through in planta assays conducted under greenhouse conditions. The results demonstrated that *Pseudomonas* sp. HX1, *Streptomyces luteogriseus* HR40, and *Streptomyces flavofungini* HR77 significantly reduced the disease severity index by 32.56% compared to artificially infected control plants. In contrast, the commercial biocontrol product Serenade® ASO, containing *Bacillus amyloliquefaciens* QST 713, achieved a reduction of 13.96%. These findings underscore the potential of the selected bacterial strains as promising candidates for the biological control of Verticillium wilt in hops affected by *V. nonalfalfae*.

Keywords: antifungal activity; biocontrol; hops; *Humulus lupulus*; rhizosphere; soil-borne pathogen; *Streptomyces*; *Verticillium nonalfalfae*; Verticillium wilt

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Academic Editors: Josinaldo Lopes Araújo Rocha, Márcia Michelle Queiroz Ambrosio and Railene Hérica Carlos Rocha Araújo

Received: 14 March 2025
Revised: 22 April 2025
Accepted: 22 April 2025
Published: 25 April 2025

Citation: Ghoreshizadeh, S.; Calvo-Peña, C.; Ruiz-Muñoz, M.; Dobrajc, M.; Radišek, S.; Coque, J.J.R.; Cobos, R. Isolation and Characterization of *Pseudomonas* sp. HX1, *Streptomyces luteogriseus* HR40, and *Streptomyces flavofungini* HR77 as Promising Biocontrol Agents Against Verticillium Wilt in Hops Affected by *Verticillium nonalfalfae*. *Horticulturae* **2025**, *11*, 459. https://doi.org/10.3390/horticulturae11050459

1. Introduction

The hop plant (*Humulus lupulus*), a dioecious climbing plant of the *Cannabaceae* family, is primarily cultivated for its female inflorescences (hop cones). These cones are rich in secondary metabolites, including bitter acids and essential oils, which are integral to the brewing industry [1]. Hops play a vital role in beer production by imparting bitterness, flavor, and aromatic qualities. Furthermore, they are recognized for their therapeutic applications, such as managing insomnia, anxiety, and digestive disorders, due to their sedative properties [2]. Beyond their use in brewing, hops are utilized in various industries, including the manufacture of natural dyes, cosmetics, and food preservatives [3]. Hops are predominantly cultivated in temperate regions worldwide, with major production hubs in Europe, the United States, and Asia [4].

Verticillium wilt in hops is a vascular disease caused by the soil-borne fungal pathogens *Verticillium nonalfalfae* (formerly *V. albo-atrum sensu lato*) and *V. dahliae*. Of these, *V. nonalfalfae* is the most commonly isolated from hops and accounts for the majority of disease outbreaks. In contrast, infections caused by *V. dahliae* are relatively infrequent and typically less severe [5]. *Verticillium* spp. infect plants through the roots and subsequently disseminate via the vascular (xylem) systems. This infection results in the blockage of vascular vessels, which manifests as symptoms such as chlorosis, leaf curling, wilting, necrosis of lower leaves, and bine swelling. Upon dissection, infected vascular tissues exhibit medium-to-dark-brown discoloration [6,7]. By the time these symptoms become visible, the fungal pathogen has colonized all plant tissues, rendering effective chemical treatments unavailable. Consequently, implementing preventive measures to restrict fungal invasion is essential for managing Verticillium wilt.

Biocontrol agents (BCAs) have emerged as a sustainable and environmentally alternative to chemical fungicides, offering innovative solutions for plant disease management. BCAs consist of beneficial microorganisms that mitigate plant pathogens through diverse mechanisms, including antibiosis, competition for nutrients and physical space, parasitism, and the induction of systemic resistance in host plants [8]. The rhizosphere—a narrow soil region influenced by root exudates and microbial activity—serves as a rich reservoir of potential BCAs due to its remarkable microbial diversity [9]. In recent years, numerous promising BCAs have been identified for the management of Verticillium wilt in various crops, particularly olive trees. These BCAs encompass both fungal [10–14] and bacterial strains [15–19], including different bacterial strains isolated from the olive root endosphere [15,18] or rhizosphere [16,17,19]. Additionally, certain endophytic strains, such as *Pseudomonas aurantiaca* and other *Pseudomonas* spp., have demonstrated efficacy in controlling Verticillium wilt in cotton [20]. However, to the best of our knowledge, there is currently no information available regarding the use of BCAs for the management of Verticillium wilt in hops. Among the BCAs studied, *Streptomyces* spp. stand out as promising candidates for the biocontrol of soil-borne fungal pathogens [21]. The genus *Streptomyces*, comprising 743 validly named species (https://lpsn.dsmz.de/genus/streptomyces, accessed on 24 April 2025), is one of the most taxonomically diverse bacterial genera. *Streptomyces* spp. are renowned for their prolific production of antibiotics [22] and their ability to suppress a wide range of plant diseases, including those caused by soil-borne fungi [23,24]. Furthermore, *Streptomyces* spp. are prevalent across various soil types, representing approximately 10% of the total soil microbiome [25]. Several *Streptomyces* strains have demonstrated antifungal (AF) activity against *Verticillium* wilt in various crops such as cotton, potato, strawberry, tomato, and olive [19,26]. However, no data are currently available regarding the biocontrol potential of *Streptomyces* spp. against *Verticillium nonalfalfae*. Another promising strategy for managing soil-borne fungal pathogens capable of colonizing the xylem of hop plants is the isolation and characterization of bacterial endophytes with AF activity. Endophytic bacteria, which reside within plant tissues, are efficient colonizers that can enhance plant immune responses and establish symbiotic relationships with their host plants without inducing disease symptoms [27,28].

This study, therefore, aimed to evaluate the AF activity of putative BCAs isolated from the root systems of hop plants, including both rhizosphere-associated and endophytic strains, against *Verticillium nonalfalfae*. Additionally, the ability of these strains to suppress pathogen growth was investigated through both soil and in planta analyses. These findings have the potential to contribute to the development of sustainable alternatives to chemical fungicides, thereby promoting the health and productivity of hop plants.

2. Materials and Methods

2.1. Isolation of Culturable Streptomycetes Strains from the Rhizosphere of Hop Plants

Bacterial strains were isolated from the rhizosphere of hop plants (Nugget cultivar) cultivated under conventional agronomic practices in a field located in León, Spain (5°35′26.45″ W, 42°35′2.524″ N). Rhizosphere soil samples were collected from five individual plants by carefully excavating the surrounding root systems. Soil in direct contact with the roots was extracted using a sterile spatula, transferred into sterile 50 mL Falcon tubes, and stored in an icebox prior to preservation at 4 °C until further processing. Culturable Streptomycetes were isolated on starch casein agar (SCA) [29] and International *Streptomyces* Project 2 (ISP2) agar medium [30] following established protocols [19]. Plates were incubated at 30 °C for 5–7 days. Isolates were selected based on distinct morphological and cultural characteristics, including colony morphology, presence or absence of aerial mycelia, spore mass coloration, reverse colony pigmentation, and the production of diffusible pigments. Selected isolates were routinely cultured and maintained on MEY (Maltose Yeast Extract, Condalab, Madrid, Spain) medium [31] at 4 °C. Spore-producing isolates were preserved as spore suspensions in 40% glycerol at −20 °C.

2.2. Isolation of Culturable Streptomycetes from the Endosphere of Hop Roots

Endophytic *Streptomyces* strains were isolated from hop root tissues using the methodology described by Álvarez-Pérez et al. (2017) [32]. Sterilized root tissues were mechanically disrupted with a mortar and pestle, following by the addition of 20 mL of 0.9% (*w*/*v*) NaCl solution. The resulting samples were incubated at 28 °C with vigorous agitation (220 rpm) to facilitate the extraction of endophytic microorganisms. Serial tenfold dilutions were plated on SCA and ISP2 agar plates, which were incubated under the previously outlined conditions.

2.3. Isolation of Culturable Bacterial Strains from the Xylem of Hop Plants

Samples were collected from hop plants (variety Celeia) displaying visual symptoms of Verticillium wilt in a *V. nonalfalfae*-infested field located in the Savinja Valley, Slovenia (GPS coordinates: Y 50°61′65.424/X 12°39′75.556). Each sample consisted of 30 cm sections of bines taken from the basal part of symptomatic plants. Surface sterilization was performed using 2% sodium hypochlorite for 3 min to eliminate epiphytic fungi and bacteria, followed by thorough rinsing with sterile distilled water. The epidermis of the bines was removed with a sterile scalpel, and four 1 cm pieces of xylem tissue per bine were excised and placed onto potato dextrose agar (PDA) plates (Condalab). After 4 days of incubation at 25 °C in the dark, bacterial colonies that inhibited the growth of *V. nonalfalfae* from the xylem tissue were transferred to tryptic soy agar (TSA) plates (Condalab). All isolates were preserved in Luria-Bertani (LB) broth (Condalab) containing 20% glycerol and stored at −80 °C.

2.4. In Vitro Selection of Isolates Based on Antifungal Activity in Plate Assays

The AF activity of the isolated bacterial strains was assessed using in vitro plate assays, as described by Calvo-Peña et al. (2023) and Álvarez-Pérez et al. (2017) [19,32]. Briefly, bacterial isolates were inoculated onto MEY agar plates within a 1.0 cm^2 area, with four isolates per plate positioned 1 cm from the edge. An agar plug (0.5 cm in diameter) containing the *V. nonalfalfae* (hop pathotype PV1, isolate T2) [33] was placed at the center of each plate. The plates were incubated at 25 °C for up to 12 days, and growth inhibition zones were measured. To quantify the AF activity of the most effective isolates, individual bacterial strains were inoculated on MEY agar plates in a circular pattern, 1 cm from the plate's edge. A *V. nonalfalfae* T2 agar plug was placed at the center, and the plates were incubated at 25 °C for up to 10 days. The inhibition index (*I* index) was calculated using the formula *I* index (%) = $[(\mathrm{Rc} - \mathrm{R})/\mathrm{Rc}] \times 100$, where R represents the radius of the fungal colony in the presence of the bacterial isolate and Rc represents the maximum radius of the fungal colony in the control condition. Additionally, AF activity was evaluated against *V. dahliae* due to its significance as a pathogen affecting hop plants and other crops. All assays were performed in triplicate for each bacterial strain tested.

2.5. Preliminary Identification of Bacterial Strains by 16S rRNA and rpoD Gene Sequencing

Bacterial strains exhibiting the highest AF activity were identified through partial sequencing of the 16S rRNA gene. Genomic DNA was extracted following the protocol outlined by Hopwood et al. (1985) [34]. Amplification of the *16S rRNA* gene was performed using universal primers 27F and 1492R [35]. The resulting sequences were compared against reference sequences of type strains available in the EzTaxon-e database [36] (http://www.ezbiocloud.net/eztaxon/identify, accessed on 9 January 2025). Sequence alignments were conducted using MEGA11 software (http://www.megasoftware.net/, accessed on 10 January 2025). For species-level identification of *Pseudomonas* isolates, an internal fragment of the *rpoD* gene was sequenced in accordance with the methodology recommended by Girard et al. (2020) [37].

2.6. Identification of Streptomyces Isolates Through Multilocus Sequence Analysis (MLSA)

MLSA was employed to identify *Streptomyces* species based on five housekeeping genes: *atpD* (ATP synthase F1, β-subunit), *gyrB* (DNA gyrase B subunit), *recA* (recombinase A), *rpoB* (RNA polymerase, β-subunit), and *trpB* (tryptophan synthase, β-subunit) [38]. Gene amplification was performed using primers and conditions specified by Guo et al. (2008) [39] and Rong et al. (2009) [40]. A phylogenetic tree was constructed utilizing concatenated sequences from the five housekeeping genes. Sequences were manually trimmed to uniform lengths prior to alignment, which was carried out using MEGA11 software [41]. Reference sequences of type strains were retrieved from the *Streptomyces* Sequence Database maintained by the Buckley Lab at Cornell University (https://blogs.cornell.edu/buckley/streptomyces-sequence-database/, accessed on 19 January 2025). Phylogenetic relationships were inferred through the maximum likelihood method, applying the Kimura two-parameter (K2P) model to estimate nucleotide sequence divergence [42]. MLSA evolutionary distances were calculated using MEGA11 software, also employing the K2P model to estimate nucleotide sequence divergence [42]. As defined by Rong and Huang (2012) [38], strain pairs demonstrating an MLSA evolutionary distance of ≤ 0.007 were considered conspecific.

2.7. Genome Sequencing and Biosynthetic Gene Cluster Analysis

The genomes of strains HR31 and HR77 were sequenced using next-generation sequencing on the PacBio Revio platform provided by Macrogen Inc. (Seoul, Republic of Korea). De novo assembly was performed using Canu version 2.2 [43], and assembly quality assessed via QUAST-LG version 5.3.0 [44]. Genome annotation was conducted using the RAST-SEED webserver [45] (https://rast.nmpdr.org/, accessed on 12 February 2025). Species identification was further refined through digital DNA:DNA hybridization (dDDH) utilizing the Genome-To-Genome Distance Calculator (GGDC) version 2.1 [46], in conjunction with the Type (Strain) Genome Server (TYGS) v1.0 (https://tygs.dsmz.de/, accessed on 13 February 2025), a bioinformatics platform for whole-genome taxonomic analysis [47]. Biosynthetic gene cluster (BGC) identification in the genome sequences of HR31 and HR77 was performed using antiSMASH version 7.1.0 [48], with annotation results manually inspected for confirmation and refinement.

The genome sequences of strains HR31 and HR77 were deposited in the GenBank database under the accession numbers SAMN47195979 and SAMN47195978, respectively, with the associated BioProject accession number PRJNA1230872.

2.8. Plant Assays to Evaluate the Effectiveness of Selected Bacterial Isolates Against Verticillium Wilt Caused by V. nonalfalfae

The efficacy of selected bacterial isolates (HX1, HX2, HX3) and *Streptomyces* isolates (HR40, HR77, HR31) against Verticillium wilt caused by *V. nonalfalfae* was assessed through a pot experiment conducted under controlled conditions. One-year-old hop plants (variety Celeia, susceptible to Verticillium wilt) were used as test subjects. The soil was artificially contaminated with *V. nonalfalfae* (hop pathotype PV1, isolate T2). The experiment setup included the commercial bacterial fungicide Serenade® ASO (Bayer Crop Science, Leverkusen, Germany), containing *Bacillus amyloliquefaciens* (QST 713), as well as a negative control consisting of plants cultivated in infected soil without BCA treatment.

The *V. nonalfalfae*-contaminated soil was prepared by autoclaving field soil at 121 °C for 30 min, followed by inoculation with a mixture of conidial and mycelium suspension (1×10^7 conidia mL^{-1} and 1×10^2 mycelium fragments mL^{-1}) at a ratio of 100 mL per liter of soil. This resulted in a final concentration of 1×10^6 conidia g^{-1} of soil and 10 mycelium fragments g^{-1} of soil. To enhance contamination, pieces of *V. nonalfalfae*-infected hop bines (3–5 cm in length) were added at a dose of 100 mL per liter of soil.

Streptomyces isolates were cultivated in MEY or ISP2 liquid media in baffled flasks shaken at 120 rpm at 30 °C for 7 days. Similarly, HX1, HX2, and HX3 isolates were cultured in LB broth under identical conditions. Following incubation, the liquid cultures were centrifuged at 6000 rpm for 10 min in 50 mL tubes. The supernatant was discarded, and the resulting pellets were resuspended in an equal volume of sterile distilled water. For Serenade® ASO, a 1% solution was prepared using sterile distilled water. To estimate microbial concentrations, 1 mL of each microbial suspension was serially diluted tenfold in sterile water. Aliquots of 0.1 mL from each dilution were inoculated onto TSA (for HX1, HX2, HX3) and MEY agar (for *Streptomyces* strains) and incubated at 28 °C for 3 days. For inoculation, 10 plants per treatment were uprooted, and their roots cleaned with tap and distilled water. The roots were subsequently dipped into microbial suspensions for 15 min. Control plants were treated with sterile distilled water. The treated plants were transplanted into 2 L pots filled with *V. nonalfalfae*-contaminated soil, except for the negative control, which received sterile soil. To promote colonization by *Streptomyces* strains, five agar plugs containing sporulated mycelium were placed near the root systems in each pot.

Plants were maintained in a walk-in growth chamber (RK-13300, Kambič d.o.o., Semič, Slovenia) under controlled environmental conditions, with a 13 h light/11 h dark photoperiod provided by fluorescent light, temperatures set at 22 °C during the light period and 15 °C during the dark period, and 70% relative humidity. Upon the appearance of the first symptoms, plants were assessed weekly for the severity of foliar symptoms over a four-week period using a scale from 0 to 5 [49], where 0 = no leaf symptoms, 1 = 1–20% of leaf area wilted, 2 = 21–40%, 3 = 41–60%, 4 = 61–80%, and 5 = 81–100%. Following the final foliar assessment, the presence of the fungal pathogen was confirmed by its re-isolation from roots and stem tissues. Plants in which fungal infection was not confirmed were excluded from scoring. The disease severity index (DSI) for each treatment was determined as the mean wilt score of infected plants at each time point of assessment. The Area Under the Disease Progress Curve (AUDPC) was calculated based on the DSI values recorded during the four-week evaluation period using the following formula:

$$\text{AUDPC} = \sum_{i=1}^{n-1} \frac{(\text{DSI}_i + \text{DSI}_{i+1})}{2} \times (t_{i+1} - t_i)$$

where n represents the total number of plants evaluated and t corresponds to the time (in days post-inoculation) at the ith assessment. The AUDPC provides a cumulative measure of disease progression, taking into account both the severity of the symptoms and their duration over the evaluation period. This approach facilitates comparisons of disease progression across treatments and aids in assessing the effectiveness of the bacterial isolates in reducing disease severity [50].

2.9. Confrontation Assays

Potential negative interactions among the selected *Streptomyces* strains, as well as between *Streptomyces* and *Pseudomonas* strains, were evaluated using pairwise bioassays following the methodology described by Schrey et al. (2012) [51]. *Streptomyces* cultures were grown in ISP2 medium at 28 °C for three days, while *Pseudomonas* spp. were cultivated in Nutrient Broth (Condalab) at 30 °C for one day. For the bioassay, a 40 µL aliquot of the tester strain suspension was applied in a linear pattern along the lower section of a MEY agar plate. Once the tester strain had developed—marked by the onset of sporulation in *Streptomyces* strains—three parallel lines of the receiver strains were inoculated perpendicularly to the tester line. Each strain combination was evaluated in triplicate. The effect of the tester strain on the growth of the receiver strain, including substrate mycelium formation and sporulation (specifically for *Streptomyces* strains), was assessed at the onset of sporulation in the control cultures.

2.10. Analysis of AF Activity in Small-Scale Soil Tests

To assess AF activity in soil, small-scale experiments were conducted using 10 mL Falcon tubes, each containing 1 g of sterile soil, as previously described [19]. Spores of the tested strains were obtained by culturing on MEY agar plates at 28 °C for 6–8 days. The spores were collected in a 20% glycerol solution and subsequently quantified through tenfold serial dilutions followed by plating on MEY agar plates. The soil samples were inoculated with 100 µL of a bacterial suspension supplemented with 1% starch and 0.5% casein, resulting in a final concentration of 10^6 CFU g of soil^{-1}. Homogenization was achieved through vortex mixing and manual stirring with a spatula to ensure uniform inoculum distribution. The samples were incubated at 30 °C for 48 h. Subsequently, 2×10^6 spores g of soil^{-1} of *V. nonalfalfae* were added to each tube, followed by homogenization as described above. The study included a non-inoculated negative control and a positive control inoculated only with the fungal pathogen.

The tubes were incubated at 25 °C, and soil samples (100 mg) were collected 7 and 14 days after bacterial inoculation. Tenfold serial dilutions of the collected samples were prepared in sterile water, and 0.1 mL aliquots of each dilution were plated on PDA plates supplemented with 250 µg mL^{-1} chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) to quantify fungal growth. Additionally, SCA plates supplemented with 100 µg mL^{-1} pimaricin (VGP Pharmachem, Vic, Spain) and 50 µg mL^{-1} nalidixic acid (Sigma-Aldrich) were used to quantify the presence of the tested actinobacteria.

2.11. Soil Physicochemical Properties

Soil samples were collected from a depth of 20–30 cm, after the removal of the top 5 cm of superficial soil. Five samples were taken and combined for analysis. Soil texture and mechanical analyses were performed according to the standard sedimentation method, involving particle size distribution through a combination of sieving and sedimentation techniques (ISO 11277:2020) [52]. Organic carbon content was determined using the Walkley–Black wet oxidation method. The pH and electrical conductivity (EC) were measured in the supernatants of soil/water (1:2.5) suspensions after 25 min of shaking and 5 min of soil settlement, using a Crison micropH 2001 pH meter and a Crison 522 Conductivity Meter, respectively (Crison Instruments S.A., Alella, Spain). Soil nitrogen was determined by the Kjeldahl method [53], which quantifies all soil nitrogen except that in nitrates [54]. Available soil phosphorus was determined by ultraviolet–visible spectroscopy following treatment with the Olsen–Watanabe extractant [55]. Exchangeable potassium was extracted with successive aliquots of 1 M ammonium acetate and quantified by atomic absorption spectrometry (AAS) with a Unicam Model SOLAAR 969 (Unicam Ltd., Cambridge, UK). Micronutrients (Fe, Cu, Mn, and Zn) were determined using AAS in an air–acetylene flame [56]. Magnesium was analyzed using the $CaCl_2$ method [57] determined by AAS. Available boron was measured according to standard NF X31-122 [58], and humus content according to the ISO 14235:1999 [59]. Total carbonates and active carbonates were quantified using the Bernard calcimeter method, with active carbonates extracted using 0.2 N ammonium oxalate (Sigma-Aldrich).

2.12. Statystical Analysis

Data on Verticillium wilt severity in the in planta assays and the AF activity of the selected bacterial strains in the small-scale soil tests were statistically analyzed using R software version 4.4.3 [60]. The assumption of normality was assessed using the Shapiro–Wilk test (via the "stats" package), while homogeneity of variances was evaluated using Levene's test (via the "car" package) [61]. Based on the outcomes of these preliminary tests, the non-parametric Kruskal–Wallis test (from the "stats" package) was employed to evaluate significant differences between treatment and control groups. When significant effects were detected ($p < 0.05$), post hoc pairwise comparisons were conducted using Dunn's test with Bonferroni correction (via the "FSA" package version 0.9.5) [62] to control for Type I error. Statistical significance was defined as an adjusted p-value < 0.05. To aid in the visual interpretation of significant differences among treatments, grouping letters were assigned according to adjusted p-values using the "multcompView" package version 0.1–8 [63].

3. Results

3.1. Isolation of Culturable Streptomycetes from the Root System and Other Endophytic Bacterial Strains from the Xylem of Hop Plants and Their Antifungal Activity

A total of 100 putative Streptomycetes strains were isolated from the hop rhizosphere (designated HR1–HR100) based on their distinct morphological and cultural characteristics, including the color of substrate mycelium, aerial mycelium, exospores, and pigment production. An in vitro bioassay-based screening revealed that 37% of these isolates exhibited AF activity against *V. nonalfalfae*, while 35% demonstrated activity against *V. dahliae*.

Similarly, 85 endophytic strains (designated HE1–HE85) were isolated from the hop endosphere. Among these, 13 isolates (15.29%) showed AF activity against *V. dahliae*, whereas 16 isolates (18.8%) exhibited AF activity against *V. nonalfalfae*.

In the case of bacterial isolation from the xylem of hop plants infected with *V. nonalfalfae*, three isolates (HX1, HX2, and HX3, named after "Hop Xylem") were obtained. These strains clearly inhibited the growth of *V. nonalfalfae* developing on agar plates from xylem tissue fragments of hop infected bines. After selection, their AF activity on *V. nonalfalfae* was confirmed by in vitro bioassay tests.

The six Streptomycete strains demonstrating the highest AF activity in in vitro assays, along with the three strains isolated from the xylem, were individually tested to calculate their inhibition index (I index) against both *V. dahliae* and *V. nonalfalfae* (Figure 1). The I index values for *V. dahliae* ranged from 12.5% (strain HX1) to 100% (strain HX3). In the case of *V. nonalfalfae*, the I index values varied between 15% (strain HX1) and 100% (strains HR40, HR77, and HR31) (Figure 1).

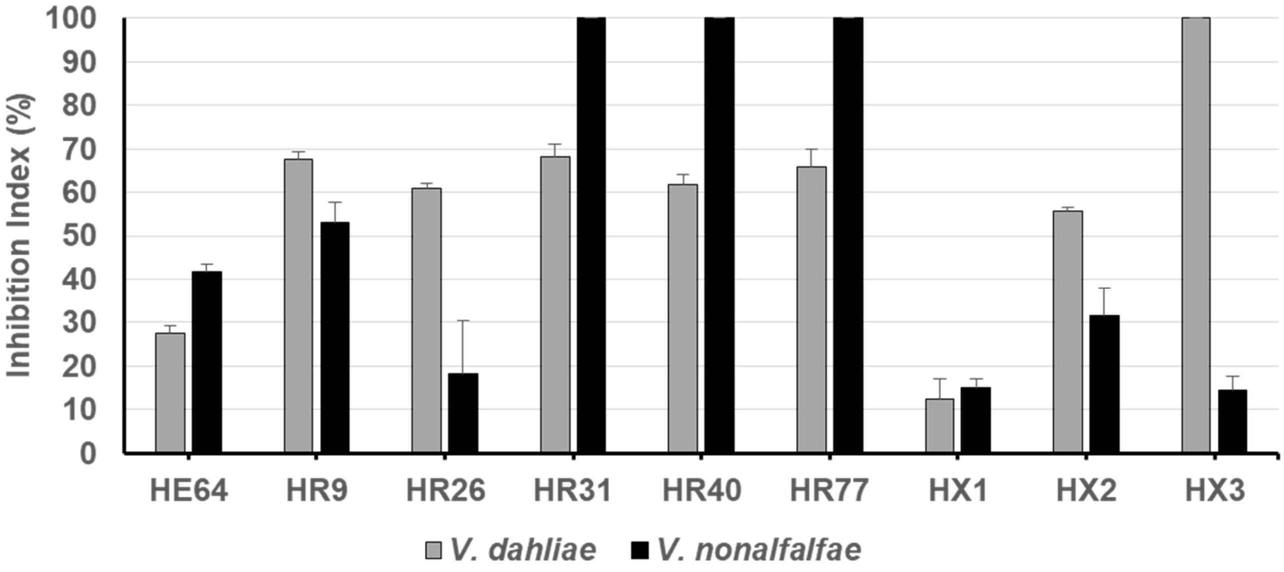

Figure 1. Antifungal activity of promising strains isolated from root endosphere (HE), hop rhizosphere (HR), and xylem of hop plants (HX) against *V. dahliae* and *V. nonalfalfae*. Values presented are means of two independent experiments, each conducted in triplicate. Error bars indicate standard deviation (SD) of mean.

3.2. Molecular Identification of the Selected Isolates by Partial Sequencing of 16S rRNA and rpoD Genes and Multilocus Sequence Analysis (MLSA)

Partial sequencing of the 16S rRNA gene of the six selected Streptomycete strains isolated from hop roots identified them as members of the *Streptomyces* genus. Given the high genetic complexity of the *Streptomyces* genus and the limited resolution of 16S rRNA sequencing for species differentiation, an MLSA was conducted using five housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*). The GenBank accession numbers of the DNA sequences corresponding to these housekeeping genes are provided in Table 1.

Table 1. GenBank accession numbers of DNA sequences corresponding to partially sequenced genes used in MLSA analysis of different *Streptomyces* sp. strains characterized.

Strain	**GenBank Accession Numbers**					
	16S rRNA	***atpD***	***gyrB***	***recA***	***rpoB***	***trpB***
HE64	PQ638391	PQ654892	PQ654898	PQ654886	PQ654904	PQ654910
HR9	PQ638389	PQ654890	PQ654896	PQ654884	PQ654902	PQ654908
HR26	PQ638386	PQ654887	PQ654893	PQ654881	PQ654899	PQ654905
HR31	PQ638390	PQ654891	PQ654897	PQ654885	PQ654903	PQ654909
HR40	PQ638387	PQ654888	PQ654894	PQ654882	PQ654900	PQ654906
HR77	PQ638388	PQ654889	PQ654895	PQ654883	PQ654901	PQ654907

Once the partial sequences of the housekeeping genes were obtained, a phylogenetic tree was generated (Supplementary Material, Figure S1) and the corresponding MLSA evolutionary distances were calculated (Table 2).

Table 2. Identification of selected isolates by MLSA analysis and evolutionary distances from phylogenetically nearest strains.

Strain	MLSA (Kimura 2-Parameter) Distance *					
	HE64	**HR9**	**HR26**	**HR31**	**HR40**	**HR77**
Streptomyces sp. HE64						
Streptomyces sp. HR9	0.1032					
Streptomyces sp. HR26	0.1033	0.1184				
Streptomyces sp. HR31	0.1105	0.1050	0.1129			
Streptomyces sp. HR40	0.0663	0.1152	0.1053	0.1123		
Streptomyces sp. HR77	0.0869	0.1170	0.0829	0.1144	0.0910	
Streptomyces flavofungini NRRL B12307	0.1092	0.0958	0.0919	0.1134	0.1144	0.0160
Streptomyces luteogriseus NRRL B12422	0.0672	0.1157	0.1048	0.1133	0.0040	0.0901
Streptomyces rectiviolaceus NRRL B16374	0.1171	0.1214	0.0646	0.1152	0.1125	0.0968
Streptomyces robefuscus NRRL B5743	0.1034	0.0020	0.1205	0.1164	0.1147	0.1231
Streptomyces rubiginosohelvolus CGMCC 4.0127	0.0329	0.1069	0.1047	0.1086	0.0725	0.0860
Streptomyces sirii CCTCC AA2024094	0.1259	0.1156	0.1340	0.0020	0.1276	0.1348

* Strain pairs having ≤0.007 MLSA evolutionary distance (highlighted by shading) were considered conspecific based on guideline empirically determined by Rong and Huang in 2012 [38].

The analysis revealed that the endophytic isolate HE64 is a putative new species closely related to *Streptomyces rubiginosohelvolus* (Figure 2A) with an MLSA evolutionary distance of 0.0329. HR9 was identified as *Streptomyces robefuscus* (Figure 2B) with an MLSA evolutionary distance of 0.002 (less than 0.007). Isolate HR26 is a putative new species in the *Streptomyces* genus closely related to *Streptomyces rectiviolaceus* (Figure 2C), with an MLSA evolutionary distance of 0.0646, whereas HR31 was identified as *Streptomyces sirii* (Figure 2D), exhibiting an MLSA evolutionary distance of 0.002. The HR77 strain is closely related to *Streptomyces flavofungini* (Figure 2E) with an MLSA evolutionary distance of 0.016, suggesting it could represent a new species within the genus. Finally, HR40 was assigned to *Streptomyces luteogriseus* (Figure 2F), with an MLSA evolutionary distance of 0.004.

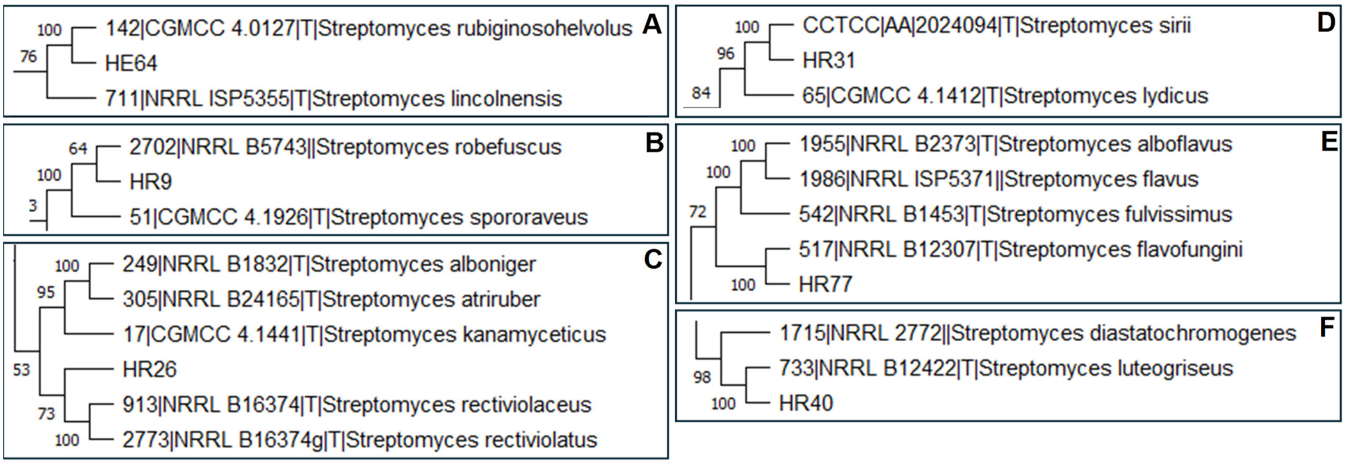

Figure 2. Partial details of the Neighbor-Joining tree generated from the MLSA analysis. The figure illustrates the positions of the selected strains: *Streptomyces* sp. HE64 (**A**), *Streptomyces* sp. HR9 (**B**), *Streptomyces* sp. HR26 (**C**), *Streptomyces sirii* HR31 (**D**), *Streptomyces flavofungini* HR77 (**E**), and *Streptomyces luteogriseus* HR40 (**F**). For the complete tree, refer to Supplementary Materials, Figure S1.

Partial sequencing of the 16S rRNA gene confirmed that the three bacterial strains isolated from hop xylem belonged to the *Pseudomonas* genus. However, only the HX3 strain could be identified at the species level. Specifically, isolate HX3 was identified as *P. putida*, as the *rpoD* partial sequence showed a 99.83% homology to the *P. putida* JCM 20005 strain

(GenBank accession number LC752193.1). This value exceeds the 98.0% cutoff defined for differentiating *Pseudomonas* spp. strains at the species level using this molecular marker [37]. HX1 and HX2 are closely related to *P. fluorescens*, with the *rpoD* partial sequence showing 97.06% and 97.03% homology to *P. fluorescens* YK-310 strain (GenBank accession number CP071797.1), respectively.

Since the analysis of the AF activity of the isolates HR40, HR77, and HR31 confirmed its very high effectiveness in inhibiting the in vitro growth of *V. nonalfalfae* (I index values of 100 were obtained for all three strains, as previously shown in Figure 1), we decided to analyze their genomes to elucidate the potential production of AF compounds.

3.3. Genome Analysis of *Streptomyces Flavofungini* HR77 Strain

The MLSA analysis previously established that strain HR77 shared close similarity with *Streptomyces flavofungini*, exhibiting a genetic distance of 0.016. Since the species delimitation threshold within the *Streptomyces* genus based on MLSA is defined as 0.007 [38], this initial finding suggested that strain HR77 could potentially represent a novel species. Furthermore, due to the limited genomic information available for *S. flavofungini*, whole-genome sequencing was conducted to explore potential AF compounds biosynthesized by this strain.

For strain HR77, de novo genome assembly yielded a single contig comprising 9,363,350 bp with a GC content of 72.17%, differing marginally by 0.09% from the type strain *S. flavofungini* JCM 4753. Species identification was further corroborated through TYGS analysis, which revealed a d4DDH value of 95.3%, surpassing the 70% threshold for species delineation [64]. These results underscore the significance of genome-wide methodologies in accurate taxonomic classification. Additionally, quality assessment using QUAST indicated that 95.66% of the HR77 genome aligned to the type strain reference genome, with an average sequencing depth of 71×, indicating high-quality genome coverage.

The antiSMASH analysis identified a total of 29 putative biosynthetic gene clusters (BGCs) in *S. flavofungini* HR77 (Supplementary Material, Table S1). These included six polyketide synthase (PKS) clusters (PKS I and PKS II), four nonribosomal peptide synthetase (NRPS) clusters, five terpene clusters, two siderophore clusters, two ribosomally synthesized and post-translationally modified peptide (RiPP-like) clusters, and ten hybrid clusters. Among these, ten exhibited a high degree of similarity ($\geq$80%) to known clusters, with four clusters—responsible for the biosynthesis of albaflavenone, ectoine, geosmin, and isorenieratene—demonstrating 100% similarity. The siderophores griseobactin and coelichelin showed similarities of 92% and 81%, respectively. Additionally, one BGC exhibited 86% similarity to the cluster involved in the biosynthesis of undecylprodigiosin, an antimicrobial compound known to disrupt bacterial membranes [65,66].

In relation to the putative AF compounds produced by HR77, the genome was found to harbor a PKS cluster exhibiting 90% similarity to spectinabilin, a polyketide noted for its diverse derivatives with nematicidal and AF properties [67]. Moreover, several BGCs displayed lower similarity to entries in current databases yet were associated with reported AF activity. Notably, an NRPS-like cluster exhibited 60% similarity to desertomycin, a compound with established known AF properties [68]. Another BGC showed 25% similarity to the cluster responsible for producing 67-121C, a macrolide polyene complex with documented AF activity [69]. Additionally, two clusters displayed low similarity—24% and 13%—to kutzneride 2, a compound also recognized for its AF activity [70].

3.4. Genome Analysis of *Streptomyces luteogriseus* DSM 40483 Type Strain

The MLSA analysis confirmed the classification of strain HR40 within the *Streptomyces luteogriseus* species. A search of available genomic data revealed the existence of 23 fully sequenced genomes of this species in the NCBI database (accessed 28 February 2025). Consequently, sequencing the genome of strain HR40 was deemed unnecessary, as the existing datasets were considered sufficient to capture the genetic diversity of the species. Instead, an antiSMASH analysis was performed on the genome of the *S. luteogriseus* DSM 40483 type strain. This analysis identified 30 putative BGCs, including eight terpene clusters, alongside a diverse array of PKS, NRPS, RiPP-like, and siderophore-related pathways (Supplementary Material, Table S2). Of these, ten BGCs displayed high similarity to known clusters. These included those responsible for synthesizing albaflavenone, citrulassin D, coelichelin, desferrioxamine B/E, ectoine, flaviolin, geosmin, and informatipeptin, all showing 100% similarity. Additionally, clusters responsible for the biosynthesis of hopene and a spore pigment demonstrated 92% and 83% similarity, respectively. Notably, one BGC exhibited 88% similarity to oligomycin, a macrolide antibiotic with proven AF activity [71–73].

3.5. Genome Analysis of *Streptomyces sirii* HR31 Strain

As previously noted, initial MLSA analysis suggested that strain HR31 could represent a new species, leading to the decision to sequence its genome. However, during the preparation of this manuscript, a new species, *S. sirii*, was described [74].

Whole-genome sequencing of strain HR31 was performed, resulting in a de novo assembly comprising a single contig of 8,600,360 bp with a GC content of 71.46%. Digital DNA-DNA hybridization analysis revealed a d4DDH value of 94.2% and a G + C content difference of 0.02% in comparison to the *S. sirii* BP-8 type strain. Alignment with the reference genome indicated genome coverage of 95.80%, with an average sequencing depth of 38×. These findings unequivocally identified strain HR31 as *S. sirii*.

AntiSMASH analysis of the genome of *S. sirii* HR31 identified 37 putative BGCs (Supplementary Material, Table S3). These included seven PKS clusters (encompassing both PKS I and PKS II), five NRPS clusters, six terpene clusters, three siderophore clusters, three RiPP-like clusters, and thirteen hybrid clusters. Among these, eight BGCs demonstrated high similarity ($\geq$80%) to known clusters, including ectoine (100%), isomigrastatin (100%), SapB (100%), isorenieratene (85%), a spore pigment cluster (83%), lagmysin (80%), and two siderophores—legonoxamine A-B/desferrioxamine B and ethylenediaminesuccinic acid hydroxyarginine (EDHA)—both with 100% similarity. Despite these findings, none of these metabolites have been reported to exhibit direct AF activity, although *S. sirii* HR31 demonstrated strong AF activity against *Verticillium* spp.

3.6. In Planta Efficacy of Selected Bacterial Isolates Against Verticillium Wilt Caused by *V. nonalfalfae*

A pot trial was conducted under controlled growing chamber conditions to assess the efficacy of three bacterial strains isolated from hop xylem (HX1, HX2, HX3) and three *Streptomyces* sp. strains isolated from the root system (HR40, HR77, HR31) against the Verticillium wilt pathogen *V. nonalfalfae*. The commercial bacterial fungicide Serenade® ASO (1% dilution) served as the standard treatment, while sterile water was used as the negative control. Quantification of the prepared microbial inocula revealed varying concentrations (Supplementary Material, Table S4), which were attributed to differences in biomass production processes and organism types. The results indicated a 100% disease incidence across all microbial treatments and the negative control (plants infected but untreated with BCAs), with typical symptoms of Verticillium wilt observed in all treatments. However, at the end of the experiment (42 days post-pathogen inoculation), significantly reduced disease severity was observed in plants treated with *Pseudomonas* sp. isolate HX1 (DSI 2.9) and the *Streptomyces* isolates HR40 (DSI 2.9) and HR77 (DSI 2.9) compared to the negative control (DSI 4.3) (Figure 3A,B). Additionally, plants treated with the commercial fungicide

Serenade® ASO displayed a lower DSI value (3.7) in comparison to the control plants. However, no statistically significant differences were detected between the Serenade® ASO treatment and the negative control (Figure 3B).

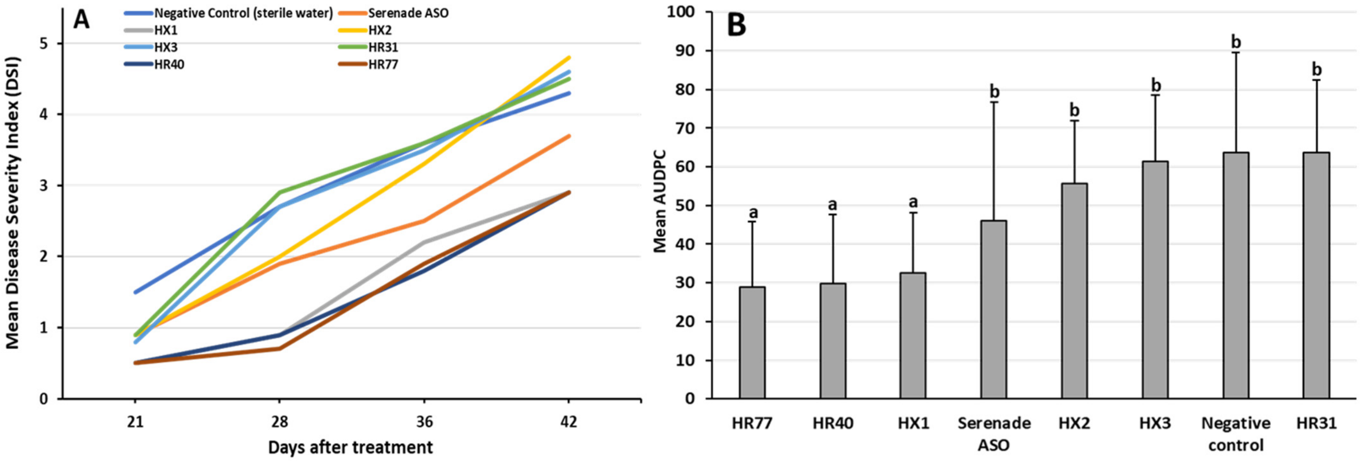

Figure 3. (**A**) The progression of the disease severity index (DSI) over time in *Humulus lupulus* cv. Celeia plants treated with selected *Pseudomonas* sp. and *Streptomyces* sp. isolates. (**B**) The Area Under the Disease Progress Curve (AUDPC) corresponding to each treatment. Error bars represent the standard deviation (SD) of the mean. Different letters above the bars denote statistically significant differences between treatments (adjusted *p*-value < 0.05). The results are presented as the means of two independent experiments.

These findings demonstrate that treatments incorporating *Pseudomonas* sp. HX1, *Streptomyces* sp. HR40, and *Streptomyces* sp. HR77 yielded a substantial 32.56% reduction in DSI values. In contrast, the commercial treatment with Serenade® ASO achieved a non-significant reduction of 13.96% in DSI values (Figure 3A) when compared to the control plants.

3.7. Strain-Specific Patterns of Inhibition in Interaction Bioassays

Based on the findings from the in planta experiments, the strains *Pseudomonas* sp. HX1, *S. luteogriseus* HR40, and *Streptomyces* sp. HR77 demonstrated the highest efficacy in reducing the incidence of Verticillium wilt caused by *V. nonalfalfae* in hop plants. The potential for negative interactions (cross-inhibition) between these strains was assessed to enable future field trials on commercially cultivated plots using these three promising BCAs. Consequently, co-cultured bioassays were performed. As depicted in Figure 4, no evidence of cross-inhibition was observed between the HX1 strain and the HR40 and HR77 strains. However, partial growth inhibition was detected when HR40 served as the "receiver strain" and HR77 as the "tester strain", with an inhibition index of 24.4% (±0.17). This level of negative interaction is considered acceptable for their combined application in field conditions. Overall, these results exclude the presence of any quantitatively significant cross-inhibition between these three strains, thereby supporting their joint application in future field trials to validate their efficacy under real cultivation conditions.

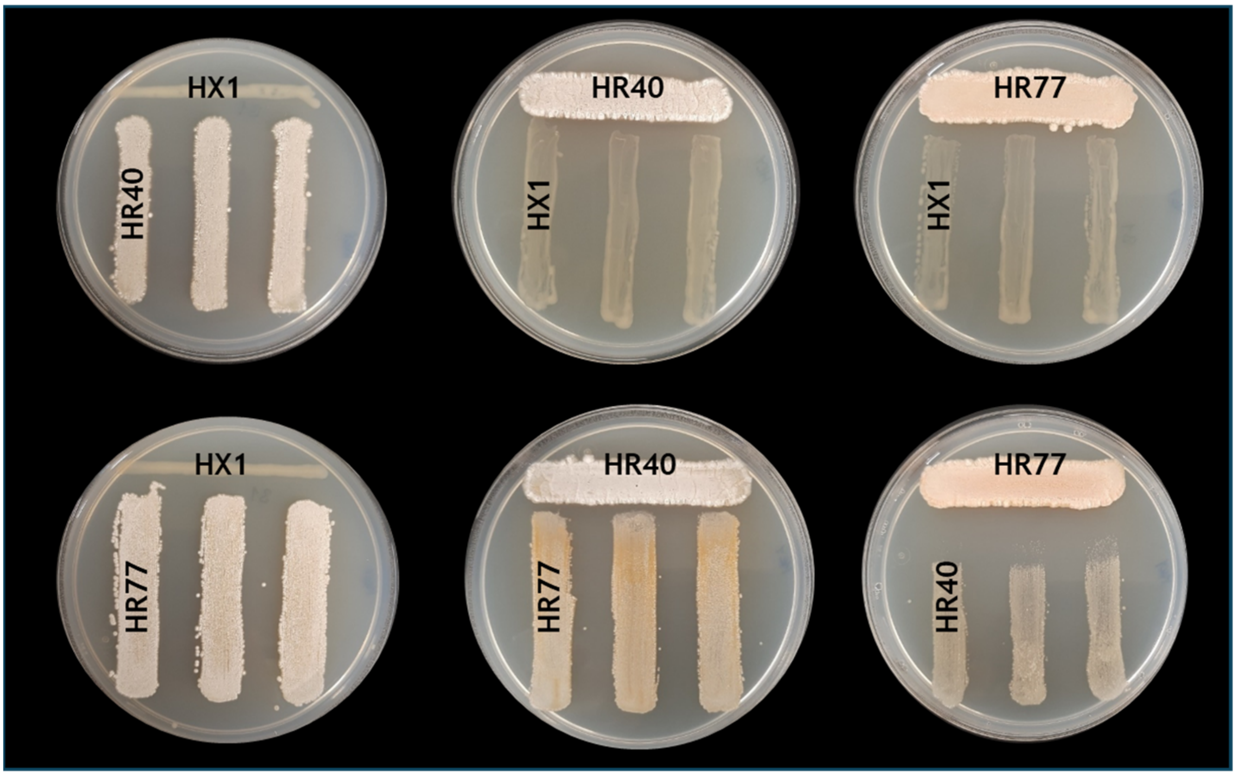

Figure 4. Analysis of cross-interactions between HX1, HR40, and HR77 strains. Co-culture bioassays were conducted on Petri dishes, with each interaction replicated three times ($n = 9$).

3.8. Analysis of the Antifungal Activity of the Selected Strains in Small-Scale Soil Tests

Building upon the results from the preceding co-culture bioassays and considering the low level of negative interaction (<25.0%) observed between strains HR40 and HR77, small-scale soil tests were conducted to evaluate their efficacy. These tests were conducted to evaluate the capacity of the strains, both individually and in combination, to inhibit the development of *V. nonalfalfae*, and to rule out any potential adverse effects on the soil that may occur. Sterile soil samples were inoculated with the HR40 and HR77 strains, either individually or in combination, followed by inoculation with *V. nonalfalfae*. The presence of the pathogen was quantified 7 and 14 days post-inoculation (Figure 5). The soil used in this study was classified as loamy according to the USDA standard classification, with a pH of 7.98, an organic matter content of 6.59%, and a total nitrogen content of 0.32%. These were identified as the most characteristic attributes of the soil, with no additional notable data. A complete description of the physical–chemical properties of the soil is provided in Supplementary Material, Table S5.

As illustrated in Figure 5, at 7 days post-inoculation, the HR40 strain significantly reduced the viable cells of *V. nonalfalfae* in the soil by 99.81%, achieving a three-order-of-magnitude reduction in pathogen levels. The HR77 strain yielded a reduction of 99.58%. When the two strains were applied in combination, a significant difference in pathogen viability was observed, with the combined treatment achieving a reduction of 99.96%. At 14 days post-inoculation, analysis revealed that the HR40 strain reduced pathogen survival by 98.41%, while the reduction achieved by HR77 was quantified at 94.53%. Joint application of both strains resulted in a significant reduction in pathogen survival, estimated at 99.97%.

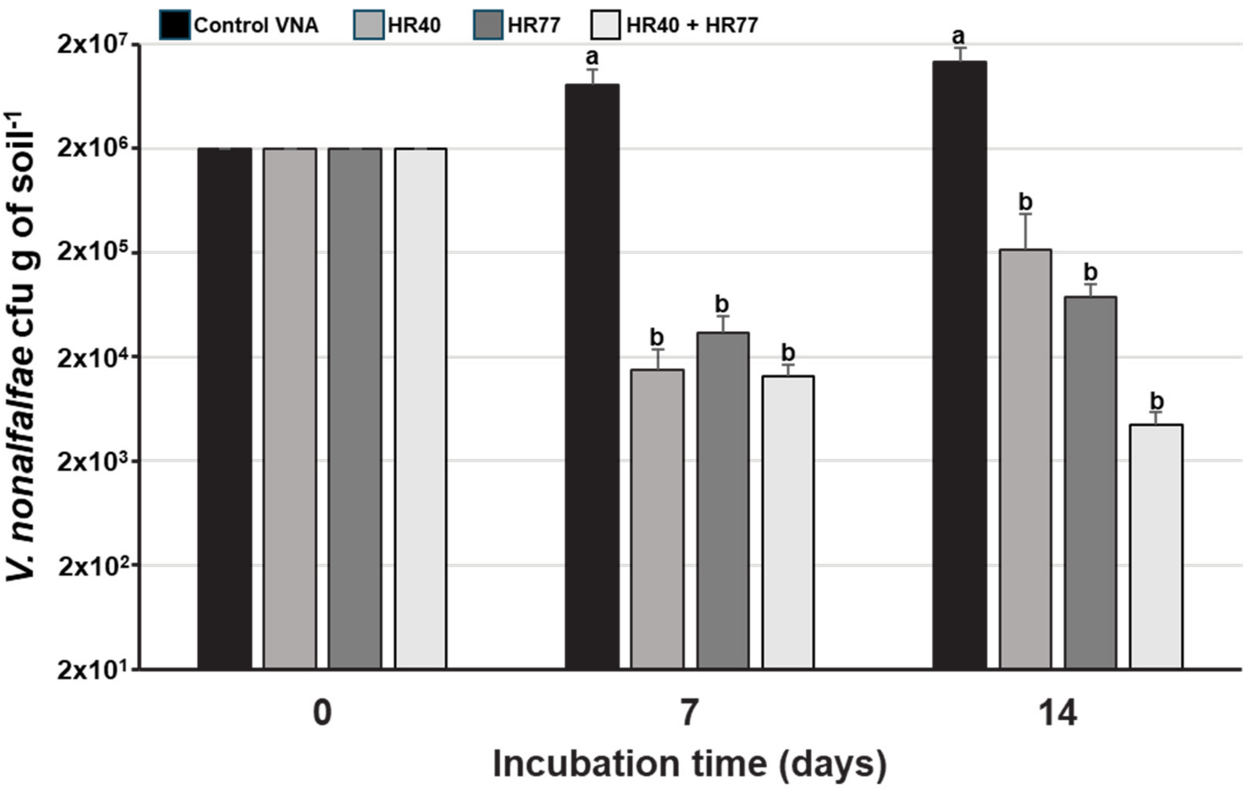

Figure 5. A small-scale in vitro soil assay to assess the AF activity of the selected strains HR40 and HR77 against *Verticillium nonalfalfae* (VNA). The values shown represent the means of two independent experiments conducted in triplicate. Error bars indicate the standard deviation (SD) of the mean. Different letters above the bars indicate statistically significant differences between treatments (adjusted p-value < 0.05).

4. Discussion

To the best of our knowledge, no BCAs have been identified as exhibiting AF activity against *Verticillium* wilt in hops caused by *V. nonalfalfae*. Consequently, this study aims to isolate and select BCAs to combat this destructive disease in hops. To identify potential BCAs, we employed two distinct methodologies. First, bacterial strains from the genus *Streptomyces* were isolated from the endosphere and rhizosphere of the hop plant's root system. *Streptomyces* spp. are widely recognized as promising candidates for the biocontrol of soil-borne fungal pathogens [21,75], with several *Streptomyces* strains previously demonstrating AF activity against *V. dahlia*, which affects a variety of crops [19,26].

Given that soil-borne fungal pathogens such as *Verticillium* spp. partially complete their life cycle in soil and infect plants via the root system, the isolation of BCAs from the root system represents a logical approach. The identification of *Streptomyces* strains in the rhizosphere of hop plants indicates their adaptability to proliferate within soil environments, from which they are subsequently recruited by the plant into the rhizosphere. These strains have the potential to function as a primary barrier, mitigating fungal infection within the plant. Notably, highly effective BCAs targeting Verticillium wilt in olive trees have been isolated from the rhizosphere [16,17,19] or the endosphere of roots [15,18]. Endophytic BCAs, capable of colonizing the interior of the root system, could offer a secondary line of defense if the pathogen breaches the root system. By exploring this dual approach, both rhizosphere-associated and endophytic *Streptomyces* strains exhibiting AF activity against *V. nonalfalfae*—and also *V. dahliae*—were successfully isolated from the hop plant's root system. These findings underscore the significance of both rhizosphere and endosphere environments for the selection of BCAs against various fungal phytopathogens, such as those affecting olive [19] and grapevine [32], among other crops.

A secondary protective strategy involves the isolation of bacterial BCAs from the xylem of plants infected with *V. nonalfalfae*. The capacity of *Verticillium* spp. to infiltrate the xylem of host plants it infects is well established, utilizing this pathway to disseminate towards the plant's upper tissues, including leaves and branches [76]. Endophytic BCAs capable of colonizing the xylem could serve as a tertiary defensive barrier.

Following the selection of the most promising BCAs, it is imperative to identify them at the genus level, or preferably at the species level, as this information could provide insights into the potential AF compounds they produce. MLSA serves as a robust tool for differentiating novel strains within established species of the genus *Streptomyces* and for identifying putative new species within this complex genus. In both this and previous studies, MLSA has proven effective in delineating species-level distinctions and detecting novel species among various rhizosphere and/or endophytic strains isolated from the root environments of olive [19] and grapevine plants [32]. The case of the isolate HR31 is particularly illustrative. Initial MLSA analysis positioned this strain within a subclade containing *Streptomyces lydicus* and *Streptomyces chattanoogensis*, with MLSA evolutionary distances of 0.0837 and 0.0841, respectively. These results indicated that HR31 represented a new *Streptomyces* species. However, in December 2024, a novel species, *Streptomyces sirii*, was identified and characterized from the rhizosphere of bamboo plants [74]. Subsequent MLSA analysis confirmed that the HR31 isolate belonged to this newly described species, as evidenced by an MLSA evolutionary distance of 0.002 (Table 2).

The fact that the three endophytes isolated from the xylem of hop plants belong to the genus *Pseudomonas* suggests a strong capacity of different species within this genus to penetrate the xylem. Indeed, species of *Pseudomonas* spp. have been widely reported as endophytes of different plants [77]. They can colonize plant tissues without eliciting disease symptoms in host plants [27]. The AF activity of different *Pseudomonas* spp. is well documented and is exerted through various mechanisms, such as the production of diffusible compounds [78], volatile organic compounds [79], or siderophores [80]. However, as of now we do not have any direct evidence of the AF mechanism exerted by the *Pseudomonas* sp. HX1 strain.

For the *Streptomyces* strains under investigation, an in silico analysis using antiSMASH enabled the identification of numerous BGCs within their genomes, some of which are implicated in the biosynthesis of metabolites with AF activity. Specifically, for the *Streptomyces flavofungini* HR77 strain, this analysis revealed a BGC with 90% similarity to the AF compound spectinabilin, positioning this compound (or a structurally similar one) as the primary candidate responsible for its AF activity. Moreover, the HR77 genome harbors BGCs with low similarity to known pathways associated with AF compounds, such as desertomycin, kutzneride 2, or the macrolide polyene 67-121C. This finding suggests that HR77 may synthesize novel bioactive compounds. Hybrid PKS-NRPS clusters are particularly noteworthy due to their structural diversity, which frequently gives rise to unique biological activities. The discovery of such clusters highlights the necessity for further functional characterization, as they may encode metabolites with AF or other antimicrobial properties yet to be explored. Additionally, while *S. flavofungini* is known to produce flavofungin [81], the corresponding biosynthetic cluster remains uncharacterized in publicly available databases. This raises the possibility that some of the unclassified BGCs in HR77 are involved in the biosynthesis of flavofungin or structurally related compounds. Investigating these pathways could yield valuable insights into the diversity of secondary metabolites produced by this species and their potential applications.

The case of the *Streptomyces sirii* HR31 strain presents a greater degree of complexity, as none of the metabolites encoded by the BGCs identified in its genome appear to be associated with the biosynthesis of compounds with established AF activity. Nonetheless, *S. sirii* HR31 has been shown to exhibit significant AF activity against *Verticillium* spp., implicating the potential involvement of bioactive metabolites. This AF activity may result from synergistic interactions among various metabolites or from as-yet-unknown mechanisms. Furthermore, HR31 possesses BGCs with low similarity to known AF clusters, such as auroramycin (8%) [82] and blasticidin S (7%) [83]. Although these clusters exhibit only limited homology to established pathways, their presence in HR31 underscores its potential as a source of novel AF compounds.

Meanwhile the antiSMASH analysis of the genome of the type strain *Streptomyces luteogriseus* DSM 40483 suggests that oligomycin is the most likely candidate metabolite responsible for the AF activity exhibited by strain HR40 against *V. nonalfalfae*.

In planta analyses conducted to evaluate the effectiveness of the selected bacterial strains demonstrated that treatments utilizing *Pseudomonas* sp. HX1, *Streptomyces* sp. HR40, and *Streptomyces* sp. HR77 resulted in a significant reduction of 32.56% in DSI values. In comparison, the commercial treatment with Serenade® ASO achieved only a 13.96% reduction in DSI values. These findings indicate that the three bacterial isolates may alleviate the pathogen's detrimental effects on the plant, either by decreasing its infectivity in the soil or by limiting its virulence once the plant has been infected. Furthermore, the data suggest that isolates HR40 and HR77 could be more effective than the other treatments tested, as their application concentrations were two and three orders of magnitude lower than those of the commercial product Serenade® ASO and the *Pseudomonas* sp. HX1 strain, respectively (Supplementary Material, Table S4).

As the selected bacterial strains were applied by immersing the plant root system in a bacterial suspension, the positive effects observed in the aforementioned strains may indicate a notable ability of these microorganisms to effectively colonize, or at least establish themselves within, the rhizosphere environment of the treated plants. For *Pseudomonas* sp. HX1, this capability may extend to penetration into the plant xylem. However, the absence of specific molecular markers for these strains has hindered the verification of this assumption, presenting a research gap to be addressed in future studies. Conversely, the lack of a protective effect observed for strains HR31, HX2, and HX3 may be attributable to inadequate colonization of the plant root environment under the tested conditions.

Although many microorganisms identified as potential BCAs exhibit strong AF activity in in vitro assays, their efficacy under soil conditions remains uncertain. Small-scale soil tests are essential for evaluating the potential effectiveness of the selected BCAs when present in a soil sample. Results from such experiments demonstrated that strains HR40 and HR77 exhibit significant AF activity against *V. nonalfalfae* while persisting and proliferating in soil, at least in environments with comparable physicochemical properties. Nevertheless, certain soil characteristics, such as highly acidic or alkaline pH levels, could adversely affect the survival of these strains and thereby diminish their AF activity. Consequently, further studies are needed to assess their behavior across a broader range of soil types. Interestingly, the negative effect observed during in vitro tests, wherein strain HR77 appeared to impact the activity of strain HR40, was not evident in the small-scale soil tests. If this effect does occur in soil, it does not appear to be sufficient to counteract the potential synergistic effects of the AF compounds produced by strains HR40 and HR77 in such assays.

Finally, we would like to mention that the promising results obtained from the in planta analysis, which confirmed the efficacy of strains *Pseudomonas* sp. HX1, *Streptomyces luteogriseus* HR40, and *Streptomyces flavofungini* HR77 in controlling Verticillium wilt in hops affected by *V. nonalfalfae*, underscore the need for further characterization of these isolates. A particularly valuable research direction involves identifying the compounds responsible for their AF activity. This could be accomplished through the purification of these compounds via Vacuum Flash Chromatography, followed by their identification using liquid chromatography–mass spectrometry (LC-MS) analysis, nuclear magnetic resonance (NMR) spectroscopy, or other complementary analytical techniques.

We are currently conducting a long-term field assay in which the three selected isolates are being applied to commercial hop plots as a biofertilizer. The development of specific molecular markers for each strain will be critical in evaluating their capacity for survival and establishment in agricultural soils, as well as in quantifying their presence and potential for colonization of the rhizosphere environment or the interior of the plant. This is particularly significant for the endophytic strain *Pseudomonas* sp. HX1.

Additionally, long-term metataxonomic studies will be necessary to assess the impact of repeated application of these isolates over successive years on soil microbiota. The primary objective of this research is to determine whether continued application can foster the development of soil characteristics capable of suppressing this highly detrimental pathology in hops.

5. Conclusions

The endosphere and rhizosphere of hop plants have been identified as favorable microenvironments for isolating *Streptomyces* strains exhibiting AF activity against *V. dahliae* and *V. nonalfalfae*. An in vitro screening strategy employing bioassay techniques facilitated the selection of six potential BCAs to combat Verticillium wilt caused by *V. nonalfalfae*. MLSA, based on the partial sequencing of five housekeeping genes, has proven to be a reliable method of identifying species within the genus *Streptomyces* as well as detecting isolates that may represent new species. Additionally, various species within the genus *Pseudomonas* were found to colonize the xylem of hop plants, some of which demonstrated antagonistic activity against *V. nonalfalfae*. In planta analyses evaluating the efficacy of the selected BCAs for controlling Verticillium wilt identified *Pseudomonas* sp. HX1, *Streptomyces luteogriseus* HR40, and *Streptomyces flavofungini* HR77 as the most promising candidates. These isolates significantly reduced disease progression in hop plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11050459/s1, Figure S1: *Streptomyces* phylogenetic tree inferred from concatenated partial sequences of the housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*) of rhizosphere isolates HR9, HR26, HR31, HR40, and HR77, and endophytic strain HE64 with type strains obtained from the ARS Microbial Genomic Sequence Database server; Table S1: Predicted Biosynthetic Gene Clusters (BGCs) in the genome of *S. flavofungini* HR77 using antiSMASH; Table S2: Predicted biosynthetic gene clusters (BGCs) in the genome of *S. luteogriseus* DSM 40483 using antiSMASH; Table S3: Predicted Biosynthetic Gene Clusters (BGCs) in the genome of *S. sirii* HR31 using antiSMASH; Table S4: Concentrations of the different inocula used in the in planta tests to evaluate the efficacy of the different selected bacterial isolates (HX1, HX2, HX3, HR31, HR40 and HR77) to reduce the incidence of Verticillium wilt in hops caused by *V. nonalfalfae*; Table S5: Physicochemical properties of the soil used in the small-scale soil test.

Author Contributions: Conceptualization, S.R., R.C. and J.J.R.C.; methodology, S.G., M.D. and C.C.-P.; investigation, S.G., M.D. and C.C.-P.; bioinformatics analysis, M.R.-M.; writing—original draft preparation, S.R., R.C., M.R.-M. and J.J.R.C.; writing—review and editing, S.R., R.C., M.R.-M. and J.J.R.C.; funding acquisition, S.R. and J.J.R.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financed through a PRIMA grant (Section 2-2021) and is part of the project PCI2022-132966, funded by the Ministry of Science and Innovation (MCIN), the State Investigation Agency (AEI) (DOI/10.13039/501100011033), and project N4-0270, funded by the Slovenian Research and Innovation Agency (ARIS), and the European Union "NextGenerationEU"/Recovery Plant, Transformation, and Resilience (PRTR). Marina Ruiz-Muñoz was supported by a postdoctoral contract financed by the Ministry of Science and Innovation (MCIN), the State Investigation Agency (AEI) (DOI/10.13039/501100011033), and the European Union "NextGenerationEU"/Recovery Plant, Transformation, and Resilience (PRTR). Carla Calvo-Peña was first supported by a predoctoral contract from the Junta de Castilla y León and the European Social Fund (EDU/601/2020) and later by a postdoctoral contract financed by the Ministry of Science and Innovation (MCIN), the State Investigation Agency (AEI) (DOI/10.13039/501100011033), and the European Union "NextGenerationEU"/Recovery Plant, Transformation, and Resilience (PRTR).

Data Availability Statement: The data are contained within the article.

Acknowledgments: We thank Rebeca Otero-Suárez and Paola Callado Tejerina for their technical support.

Conflicts of Interest: The authors declare no conflicts of interest.

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