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Evaluation of the Antagonistic Effect of *Pseudomonas* Rhizobacteria on Fusarium Wilt of Chickpea

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Abstract: This study aimed to isolate rhizobacteria belonging to the genus *Pseudomonas* with plant-growth-promoting properties that can be used in the control of chickpea wilt disease caused by *Fusarium oxysporum* f. sp. *ciceris* (Foc). The production of antifungal compounds by the isolated rhizobacteria was assessed against two Foc isolates, coded Foc-S1 and Foc-S2. Strains E1FP13, E1FP4, and E1PP7 were the most effective against Foc-S1, with percentages of 52.77%, 48.37%, and 47.97%, respectively, while E1PP6, E1FP13, and E1PP15 were the most effective against Foc-S2 with percentages of 52.20%, 52.09%, and 45.38%, respectively. All five isolates were identified as *Pseudomonas* species using 16S rRNA sequencing. The microscopic examination of the impact of the *Pseudomonas* strains on Foc revealed that all five strains caused morphological changes in Foc, such as granulation and condensation of the cytoplasm, fragmentation, and deformation of the hyphae. The strains produced several plant-growth-promoting compounds, such as cellulase, hydrogen cyanide, indole acetic acid, ammonia, siderophores, lipase, protease, and solubilized phosphate. They were also able to significantly increase chickpea growth and reduce wilt disease, with E1FP13 resulting in the highest disease reductions of 55.77% (Foc-S1) and 53.33% (Foc-S2). The results revealed that our isolates can make promising biocontrol agents for controlling chickpea wilt disease.

Keywords: *Pseudomonas*; Foc; biocontrol; plant-growth-promoting properties

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1. Introduction

Chickpea is considered to be one of the most valuable pulse crops around the world [1]. It provides energy and nutrients, such as proteins, lipids, carbohydrates, and minerals, and it also helps to maintain good health through non-nutritive components [2]. In a diet without animal proteins, it can present a solid substitute to vegetarians [3]. It is used in the livestock industry [1] and also contributes to soil fertility through azote fixation when included in crop rotations [4].

Fusarium oxysporum f. sp. *ciceris* (Foc) (Padwick) Matuo and K. Sato is a fungus that causes Fusarium wilt [5], a soil- and seed-borne disease [6]. It affects almost all chickpea-producing regions in the world [7]. It is a challenging pathogen, as it may survive in the soil and on crop remainders as chlamydospores for up to six years without its host [8]. The fungus can cause losses of yield up to 100% when conditions are adequate [9].

Several management strategies have been employed to control wilt disease. Crop rotation effectiveness has significantly decreased due to the persisting nature of the pathogen in the soil [8,10], and the use of resistant cultivars can have serious limitations due to the variability of the pathogen [11]. Chemical pesticides are no longer appealing due to their serious side effects on the applicator, the consumer, and the environment overall [12,13]. The search for a safer and more effective alternative has been the aim of many researchers

in the past few years; thus, interest in biological control has been increasing [14,15]. Among the diverse bacteria that exist in soil, the genus *Pseudomonas* seems to grab the attention of researchers due to its outstanding capacity in the management of phytopathogens through a multitude of mechanisms, ranging from the production of antibiotics, siderophores, and lytic enzymes to the release of volatile antifungal compounds into the atmosphere [16]. Numerous studies assessing the effect of these bacteria on phytopathogens have been published. *P. luteola* and *P. fluorescens* were reported by Abed et al. [14] for their ability to inhibit the growth of Foc. *P. fluorescens*-5 was also mentioned for its ability to inhibit the growth of *F. oxysporum* f. sp. *cumini* [17]. *P. aeruginosa* isolates ISO1 and ISO2 showed significant levels of antagonism toward *Fusarium solani* [15]. *P. frederiksbergensis* CMAA 1323 was also shown to control the growth of *Botrytis cinerea* affecting strawberry pseudofruits [18].

The overall goal of the present work was to isolate rhizobacteria belonging to the genus *Pseudomonas* from the rhizosphere of chickpea that could be used in the control of chickpea wilt disease. Specifically, we assessed their antagonistic activities against two Foc isolates, as well as their plant-growth-promoting properties (PGPP). The effect of the antagonists on the morphology of Foc isolates was also microscopically examined. The impact of the *Pseudomonas* isolates on chickpea growth and the control of wilt disease were evaluated under greenhouse conditions.

2. Materials and Methods

2.1. Source of Foc Isolates

Foc-S1 and Foc-S2 were isolated from chickpea plants showing typical wilt disease symptoms. The plants were collected from an infected chickpea field at the agriculture department of Ferhat Abbas University of Setif, Algeria, during the spring season of 2017. The Foc isolates were identified microscopically following the key of Nelson et al. [19] and through PCR using *Fusarium oxysporum* [20] and Foc [21] specific primers. The PCRs were carried out in a 25 µL volume containing: 1 µL of each primer (0.2 µM), 2.5 µL DNA template (25 ng), 8 µL of ultra-pure H_2O, and 12.5 µL of CSL-JADNA PCR reaction mixture. DNA amplifications were performed in a Applied Biosystems SimpliAmp thermal cycler (CA, USA) according to the specific cycle of each set of primers, as described by Mishra et al. [20] for *F. oxysporum* and Jimenez-Gasco and Jimenez-Diaz [21] for Foc. For electrophoresis, 2% (*w*/*v*) agarose gels in 10× TBE with 3 µL ethidium bromide stain were used. GeneOn 100bp Plus ladder was used as a molecular weight marker. Gels were examined under UV light in Vilbert Lourmat™ Ebox, and developed samples bands were compared with those of the molecular weight marker. The pathogenicity of the isolates was confirmed by conducting a pathogenicity test following the protocol of Hassan et al. [22]. Seeds from the chickpea line ILC 482 (susceptible to Foc) were used in the test and were provided by the National Institute of Agronomic Research of Algeria.

2.2. Isolation of Pseudomonas from the Rhizosphere of Chickpea

The isolation of *Pseudomonas* from the rhizosphere was conducted as follows: 1 g of the soil surrounding the roots was mixed with 10 mL of sterile distilled water; serial dilutions were then prepared by mixing 1 mL of the previous solution with 9 mL of sterile distilled water, and so on, until dilution 10^{-5}. An amount of 1 mL of each dilution was spread on King B (KB) agar and incubated at 30 °C for 48 h [23]. After incubation, individual colonies were streaked onto new Petri dishes for purification. Isolates showing typical macroscopic, microscopic, and biochemical characteristics of *Pseudomonas*, as described by Cowan and Steel [24], were kept and conserved in glycerol stock at −20 °C for upcoming tests.

2.3. In Vitro Evaluation of Antagonistic Activities of Isolated Pseudomonas

Pseudomonas strains were evaluated for the production of antifungal compounds using co-culture assay following the protocol of Erdogan and Benlioglu [25] with some modifications: a 5 mm disc taken from seven-day-old Foc culture was placed onto one side of a 9 cm Petri dish containing Potato Dextrose Agar (PDA), and on the other side, 10 µL of bacterial

suspension (1.5×10^8 CFU mL^{-1}) was streaked at a distance of 3 cm from the mycelium plug. The production of volatile antifungal compounds was tested using the divided plate method, following the protocol of Dilantha Fernando and Linderman [26] with some modifications: a 5 mm disc taken from the Foc culture was placed onto one half of the divided plate containing PDA; then, 10 µL of bacterial suspension (1.5×10^8 CFU mL^{-1}) was streaked onto the other half containing KB agar. The plates were incubated at 30 °C for seven days, including plates without bacteria, which served as controls. Fungal growth was measured after seven days of incubation. Three replicates were used for each strain in both assays. The inhibition percentage was calculated using the formula of Etebarian et al. [27]:

% inhibition = [(diameter of Foc in control plate − diameter of Foc in sample plate)/diameter of Foc in control plate] × 100.

2.4. Effect of Pseudomonas Strains on Hyphae Morphology of Foc Isolates

To examine the impact of the bacterial antagonists on Foc isolates, a microscopic examination of the hyphae morphology of the Foc isolates treated with *Pseudomonas* strains (antagonism plates) and from the control plates was performed for the *Pseudomonas* strains with the highest inhibition percentages of each Foc.

2.5. Evaluation of PGPP

The *Pseudomonas* strains with the highest inhibition percentages of each Foc were selected and checked for their PGPP. Three replicates were used for each of the following tests.

2.5.1. Protease Production

Protease production was evaluated by inoculating *Pseudomonas* strains with 10% skim milk agar. The appearance of a clear zone around the colonies after incubation for three days at 30 °C was considered a sign of proteolytic activity [28].

2.5.2. α-Amylase Production

α-amylase production was evaluated by streaking *Pseudomonas* isolates on starch agar. After incubation at 30 °C for 48 h, the plates were amended with Gram's iodine solution, and isolates exhibiting clear zones around the colonies were marked as α-amylase producers [29].

2.5.3. Lipase Production

Lipase production was evaluated by inoculating *Pseudomonas* strains on a medium composed of (g L^{-1}): peptone (10), NaCl (5), $CaCl_2$ H_2O (0.1), agar (18), and Tween 80 at a final concentration of 1%. The appearance of opaque halos around the colonies after incubation for six days at 30 °C was noted as an indication of lipase production [30].

2.5.4. Cellulase Production

Cellulase production was evaluated by streaking *Pseudomonas* isolates on Carboxymethylcellulose (CMC) agar. After incubation at 30 °C for seven days, Gram's iodine solution was applied. Isolates showing clear halos around the colonies were marked as cellulase positive [31].

2.5.5. Hydrogen Cyanide (HCN) Production

HCN production was assessed as follows: sterile filter papers were soaked in HCN detection solution and placed on the lids of Petri plates containing KB agar supplemented with 4.4 g L^{-1} glycine and streaked with *Pseudomonas* isolates. The plates were then incubated at 30 °C for six days. The change in the paper color from yellow to light brown, brown, or reddish-brown was considered a sign of HCN production [32].

2.5.6. Indole Acetic Acid (IAA) Production

IAA production was estimated by streaking *Pseudomonas* isolates on Luria–Bertani agar supplemented with 5 mM L-tryptophan; the agar was then covered with sterile filter paper. After incubation at 30 °C for three days, the paper was recovered and treated with Salkowski reagent for 30 min. The change in the filter paper color from white to a reddish color was noted as a sign of IAA production [33].

2.5.7. Phosphate Solubilization

Phosphate solubilization was first screened by inoculating *Pseudomonas* strains on NBRIP agar. Strains showing clear halos around the colonies after incubation at 30 °C for 14 days were marked as phosphate solubilizers [34]. Quantification of the solubilized phosphate was estimated in NBRIP broth. Concentrations of the formed soluble phosphate were extrapolated by a standard curve drawn using known concentrations of KH_2PO_4 [35].

2.5.8. Ammonia Production

Pseudomonas strains were screened for their ability to produce ammonia by inoculation into tubes containing peptone water. After incubation at 30 °C for four days, Nessler's reagent was added to the tubes. Ammonia production was indicated by the development of a yellow to a brown color [36].

2.5.9. Chitinase Production

Chitinase production was evaluated by streaking *Pseudomonas* isolates on chitinase medium. Isolates showing clear zones around the colonies after incubation at 30 °C for five days were reported as chitinase producers [37].

2.5.10. Siderophores Production

Siderophores production was evaluated as follows: Chrome Azurol S (CAS) blue dye solution was prepared as described by Louden et al. [38]. A modified protocol of Hu and Xu [39] was used for the preparation of plates as follows: blue CAS agar was prepared by adding sterile blue dye solution into sterile agar at 10% of the final volume. *Pseudomonas* strains were streaked onto nutrient agar and incubated at 30 °C for 18 h. After incubation, plates were overlaid with the previously prepared blue CAS agar. The change of color from blue to yellow-orange was considered as positive siderophores production.

2.6. Effect of Selected Pseudomonas Strains on the Shoot and Root Growth of Chickpea

This test was performed for the *Pseudomonas* strains with the highest inhibition percentages of each Foc. Seed bacterization was carried out by soaking disinfected chickpea seeds (ILC 482) overnight in a bacterial suspension (1.5×10^8 CFU mL^{-1}), to which CMC was added at a 1% concentration to assure the adherence of *Pseudomonas* strains onto the seeds [40]. Three replicates were used for each bacterial treatment, as well as for the control. Each replicate consisted of a pot (D: 15.5 cm and H: 13.5 cm) containing a sterilized mixture of soil, sand, and compost. Three seeds were used for each pot. Inoculated seeds were used for the treatments, and seeds soaked only in CMC water were used as control. Pots were irrigated every five days with 200 mL of water. After 30 days, shoot and root length, as well as shoot and root dry weight (after drying at 70 °C for a day), were measured.

2.7. Control of Wilt Disease of Chickpea by Selected Pseudomonas Strains

A pot experiment under greenhouse conditions (day/night cycle of 12/12 h, a temperature of 20 ± 2 °C, and 60 ± 2% relative humidity) was performed to evaluate the effect of selected *Pseudomonas* strains on chickpea wilt disease. The previously described sterilized soil mixture was infected with Foc isolates following the protocol of Hassan et al. [22]: five PDA discs of four-day-old Foc were transferred into flasks containing an autoclaved mixture of 100 g of barley and 100 mL of distilled water. After incubation at 30 °C for 14 days with shaking, the mixture was added to plastic pots containing the sterilized soil

mixture at a 2% concentration. Seed bacterization of chickpea line ILC 482 was performed as previously described. Three replicates were used for each bacterial treatment, as well as for the control. Each replicate consisted of a pot containing the infected soil mixture, and three seeds were used for each pot. Inoculated seeds were used for the treatments, and non-inoculated seeds served as control.

A scale was assigned to each plant based on the level of symptoms, (0: no symptoms; 1: yellowing or wilting of 1/3 of the plant; 2: yellowing or wilting of 2/3 of the plant; 3: yellowing or wilting of the whole plant; 4: plant dead) [41]. Disease severity (DS) was calculated as follows:

$$DS\ (\%) = [(\Sigma\ v \times n)/(N \times V)] \times 100.$$

The letter n is the number of plants in each scale value; v is the scale value; V is the maximum scale value, and N is the total number of plants observed [42]. The disease reduction (DR) percentage was calculated as follows:

$$DR = [(A - B)/A] \times 100.$$

The letter A is the percentage of disease in the control, and B is the percentage of disease with the application of bacteria [43].

2.8. Molecular Identification of Selected Pseudomonas

16S rRNA sequencing was performed to identify the selected *Pseudomonas* isolates as follows: DNA extraction was carried out using the commercial kit NucleoMag DNA Bacteria for DNA purification from bacteria and yeast (Macherey-Nagel Germany) by following the manufacturer's instructions. PCR was performed using primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′- TACGGYTACCTTGTTACGACTT -3′) in a reaction mixture containing: 2 µL genomic DNA (25 ng μL^{-1}), 0.2 µL Taq polymerase Promega (1U), 1 µL of each primer (0.5 µM), 0.2 µL dNTP (0.2 mM), 1.5 µL $MgCl_2$ (1.5 mM), 5 µL Taq Promega buffer, and ultra-pure water to a final volume of 25 µL. Conditions for PCR were as follows: initial denaturation at 95 °C/5 min followed by 35 cycles of denaturation at 95 °C/30 s; annealing at 53 °C/30 s and extension at 72 °C/45 s; and a final extension at 72 °C/7 min. The amplification products were separated by electrophoresis on a 1.5% agarose gel after staining with ethidium bromide (0.5 µg mL^{-1}). Fragments were visualized and photographed under UV using the Gel doc system from Biorad. PCR products were purified using NucleoSpin® Gel and PCR Clean-up kit following the manufacturer's protocol. Sequencing was conducted using Applied Biosystems' BigDye v3.1 kit and by following the method of Sanger et al. [44].

2.9. Data Analysis

To evaluate the significance between treatments, the results were statistically processed through the Student's t-test, analysis of variance (ANOVA), and Tukey's B test at $p < 0.05$ significance. Principal component analysis (PCA) was also performed to assess the variability of traits.

3. Results

3.1. In Vitro Evaluation of Antagonistic Activities of Isolated Pseudomonas

Seventeen *Pseudomonas* strains were isolated from the rhizosphere of chickpeas and evaluated for their antagonistic activities against the Foc isolates in vitro.

The results revealed a difference between the percentages of inhibition registered with each antagonism assay. The growth of Foc-S1 was significantly more reduced in the divided plate assay ($p < 0.0001$), with an average inhibition rate equal to 44.31% against 20.40% in the co-culture assay. Concerning Foc-S2, there was no significant difference between the two assays ($p = 0.085$), with an average inhibition equal to 26.75% in the co-culture assay against 19.91% in the divided plate assay (Table 1).

Table 1. Percentages of inhibition of Foc radial growth by antagonistic *Pseudomonas* isolates in each assay.

Isolates	**Foc-S1**		**Foc-S2**	
Assays	**Co-Culture**	**Divided Plate**	**Co-Culture**	**Divided Plate**
E2PP3	4.66 ± 2.48 ^{a}	44.70 ± 4.09 ^{b}	17.13 ± 4.32 ^{abc}	23.62 ± 3.25 ^{bcd}
E1PP14	11.81 ± 5.59 ^{abc}	44.51 ± 2.14 ^{b}	14.96 ± 4.38 ^{ab}	12.57 ± 7.62 ^{ab}
E1FP14	9.36 ± 3.64 ^{ab}	24.89 ± 4.09 ^{a}	22.98 ± 2.78 ^{abcd}	32.69 ± 5.85 ^{cde}
E2PP4	17.43 ± 1.06 ^{bcd}	43.95 ± 8.90 ^{b}	17.81 ± 0.24 ^{abc}	22.69 ± 5.80 ^{abcd}
E1PP15	21.85 ± 3.27 ^{cdef}	47.43 ± 8.58 ^{b}	14.69 ± 1.23 ^{ab}	**45.38 ± 4.68 ^{e}**
E2PP7	24.10 ± 3.90 ^{def}	44.22 ± 2.29 ^{b}	13.24 ± 6.67 ^{a}	19.37 ± 6.75 ^{abc}
E2PP5	20.49 ± 1.29 ^{bcde}	44.61 ± 0.09 ^{b}	17.18 ± 8.82 ^{abc}	19.17 ± 7.68 ^{abc}
E1FP8	22.16 ± 2.02 ^{cdef}	45.01 ± 0.41 ^{b}	22.75 ± 5.54 ^{abcd}	14.25 ± 4.86 ^{ab}
E2PP8	17.08 ± 3.38 ^{bcd}	43.51 ± 0.37 ^{b}	28.72 ± 5.28 ^{cdef}	18.56 ± 8.86 ^{abc}
E1FP9	24.59 ± 2.97 ^{def}	44.89 ± 0.51 ^{b}	23.97 ± 1.99 ^{abcde}	13.09 ± 2.64 ^{ab}
E1PP2	22.95 ± 3.31 ^{cdef}	43.77 ± 2.47 ^{b}	27.36 ± 0.19 ^{bcde}	20.49 ± 6.92 ^{abc}
E2PP6	30.10 ± 8.48 ^{ef}	45.00 ± 1.34 ^{b}	21.12 ± 5.74 ^{abcd}	12.26 ± 4.34 ^{ab}
E1PP7	25.61 ± 2.81 ^{def}	**47.97 ± 5.04 ^{b}**	32.33 ± 4.67 ^{def}	35.55 ± 3.37 ^{de}
E1FP4	23.24 ± 1.41 ^{cdef}	**48.37 ± 6.31 ^{b}**	40.52 ± 4.43 ^{f}	6.88 ± 0.24 ^{a}
E2PP2	33.03 ± 8.54^{f}	44.19 ± 2.26 ^{b}	35.70 ± 3.68 ^{ef}	19.88 ± 4.57 ^{abc}
E1PP6	19.06 ± 0.73 ^{bcde}	43.44 ± 6.67 ^{b}	**52.20 ± 4.51 ^{g}**	7.02 ± 2.18 ^{a}
E1FP13	19.24 ± 1.00 ^{bcde}	**52.77 ± 2.32 ^{b}**	**52.09 ± 2.65 ^{g}**	15.06 ± 6.13 ^{ab}
Mean	20.40	44.31	26.75	19.91

The values given are means (n = 3) with standard deviations. Means in the same column followed by the same letters are not significantly different at $p < 0.05$.

Isolates E1FP13, E1FP4, and E1PP7 gave the highest inhibition percentages against Foc-S1, with values equal to 52.77%, 48.37%, and 47.97%, respectively. All three values were registered with the divided plate assay (action of volatile compounds). In the case of Foc-S2, isolates E1PP6, E1FP13, and E1PP15 registered the highest inhibition rates of 52.20%, 52.09%, and 45.38%, respectively, with co-culture assay (mainly through the action of diffusible compounds) for E1PP6 and E1FP13 and with divided plate assay (action of volatile compounds) for E1PP15 (Table 1) (Supplementary Materials, Figure S1).

The ANOVA analysis showed that the results in both assays (co-culture assay and divided plate assay) for both pathogens (Foc-S1 and Foc-S2) were highly significant ($p < 0.0001$). All traits were subjected to PCA to estimate the contribution of each trait and to assess the total level of variability. Two components (PC1-PC2) gave eigenvalues >1.0 and contributed to the explanation of 75.19% of the information. PC1 explained 50.19% of the total variability; the two pathogens were linked to it, as well as the two assays. PC2 explained 25.00% of the total contribution to variability; all strains of *Pseudomonas* were linked to it.

3.2. Effect of Pseudomonas Strains on Hyphae Morphology of Foc Isolates

The comparison between the microscopic observations of the Foc isolates in the antagonism plates and the control plates revealed a clear difference between the two. In the case of Foc-S1, the hyphae treated with E1FP13 appeared discontinued (fragmented), empty, and without septa (Figure 1D), while for E1PP7 and E1FP4, the hyphae appeared empty of their content with the presence of granulations (vesicles); the septa were also absent (Figure 1B,C). In the case of Foc-S2, the hyphae treated with E1PP15 were devoid of septa with condensed cytoplasm (Figure 2D). With regard to E1PP6 and E1FP13, the hyphae appeared empty, devoid of septa for the most part, with the presence of granulations; some areas were also deformed (swollen) (Figure 2B,C). Both controls (Figures 1A and 2A) displayed the characteristic Foc appearance without any changes in the hyphae morphology.

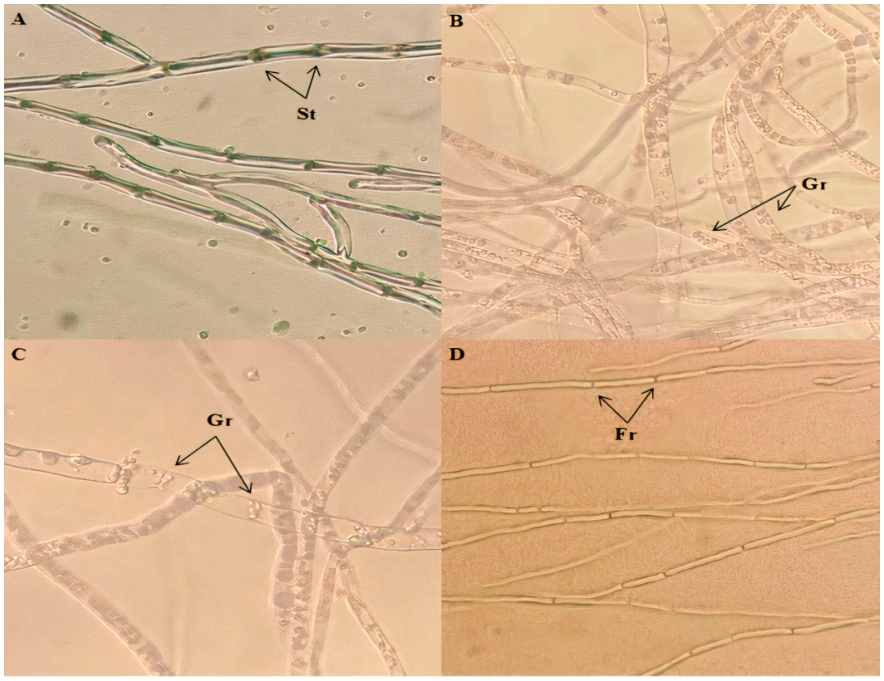

Figure 1. Light microscopy observations of the impact of selected *Pseudomonas* strains on Foc-S1 hyphae morphology (40×). (**A**) Control; (**B**) Effect of E1PP7; (**C**) Effect of E1FP4; (**D**) Effect of E1FP13. (St) Septa; (Gr) Granulations; (Fr) Fragmentations.

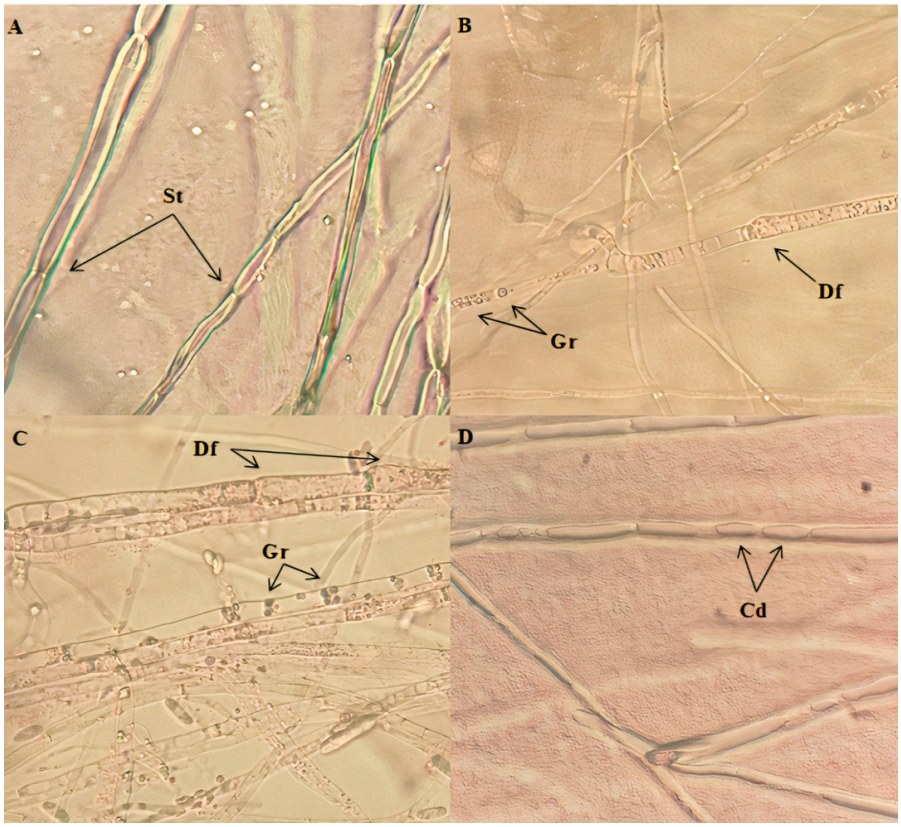

Figure 2. Light microscopy observations of the impact of selected *Pseudomonas* strains on Foc-S2 hyphae morphology (40×). (**A**) Control; (**B**) Effect of E1FP13; (**C**) Effect of E1PP6; (**D**) Effect of E1PP15. (St) Septa; (Gr) Granulations; (Df) Deformations; (Cd) Condensations.

3.3. Evaluation of PGPP

All selected *Pseudomonas* strains were positive for phosphate solubilization; the quantitative estimation showed highly significant variability between the strains ($p < 0.0001$) with values ranging from 57.66 to 240.69 µg P mL^{-1}. The highest solubilization values were recorded with E1FP4 and E1PP7, which released 240.69 µg P mL^{-1} and 223.09 µg P mL^{-1}, respectively. All isolates were found positive for the production of siderophores, IAA, and ammonia. Three isolates, E1PP7, E1PP15, and E1PP6, were found positive for the production of HCN. The screening for the different enzymes revealed that all isolates were negative for α-amylase and chitinase production but were positive for cellulase production. The results of the production of lipase and protease were very variable, with E1PP7 and E1PP15 exhibiting the highest activity (Table 2).

Table 2. Evaluation of plant-growth-promoting and antifungal properties of selected *Pseudomonas*.

Tests	E1PP7	E1FP4	E1PP15	E1PP6	E1FP13
α-amylase production	-	-	-	-	-
Lipase production	++++	+	+++	-	+
Protease production	+	-	++	-	-
Cellulase production	+	+	+	++	+
Chitinase production	-	-	-	-	-
HCN production	++	-	+	++	-
IAA production	+	+	++	+	++
Ammonia production	+++	+	++	+	+
Siderophores production	++	+++	++	+++	+++
Qualitative phosphate solubilization	+	+	+	++	++
Quantitative phosphate solubilization (µg mL^{-1})	223.09 ± 18.99 ^{a}	240.69 ± 0.06 ^{a}	173.80 ± 1.47 ^{b}	62.82 ± 6.01 ^{c}	57.66 ± 11.40 ^{c}

(-) no activity, (+) low, (++) moderate, (+++) high, (++++) very high. The values given are means ($n = 3$) with standard deviations. Means followed by the same letter are not significantly different at $p < 0.05$.

3.4. Effect of Selected Pseudomonas Strains on the Shoot and Root Growth of Chickpea

The inoculation of seeds with the selected *Pseudomonas* revealed that, aside from E1PP15, all isolates were able to significantly increase shoot length, as well as shoot dry weight in comparison to the control ($p < 0.0001$). Isolate E1PP7 gave the best results with a shoot length of 35.77 cm (increased by 42.68%) and a shoot dry weight of 0.32 g (increased by 88.24%). Aside from E1PP6, all isolates were able to significantly increase root length in comparison to the control ($p < 0.0001$), with E1PP7 giving the highest length of 20.53 cm (increased by 83.80%) followed by E1FP13 with a length of 17.97 cm (increased by 60.88%). All isolates were able to significantly increase root dry weight in comparison to the control ($p = 0.003$). There was no significant difference between root dry weights of seeds treated with isolates E1FP13, E1FP4, E1PP7, and E1PP15. However, seeds treated with isolate E1PP6 registered the lowest root dry weight. Nonetheless, it was still significantly higher than the root dry weight of the control (Table 3) (Supplementary Materials, Figure S4).

Table 3. Effect of selected *Pseudomonas* strains on the shoot and root growth of chickpea.

Selected *Pseudomonas*	Shoot Length (cm)	Root Length (cm)	Shoot Dry Weight (g)	Root Dry Weight (g)
E1PP6	28.80 ± 0.69 ^{b}	12.13 ± 1.33 ^{a}	0.23 ± 0.02 ^{b}	0.14 ± 0.04 ^{ab}
E1FP13	30.17 ± 1.46 ^{b}	17.97 ± 0.96 ^{bc}	0.26 ± 0.01 ^{b}	0.20 ± 0.01 ^{b}
E1FP4	30.23 ± 1.66 ^{b}	14.83 ± 1.56 ^{ab}	0.27 ± 0.02 ^{b}	0.18 ± 0.02 ^{b}
E1PP7	35.77 ± 1.10 ^{c}	20.53 ± 0.38 ^{c}	0.32 ± 0.04 ^{c}	0.20 ± 0.04 ^{b}
E1PP15	23.57 ± 0.57 ^{a}	14.53 ± 1.19 ^{ab}	0.18 ± 0.02 ^{a}	0.17 ± 0.01 ^{b}
Control	25.07 ± 1.87 ^{a}	11.17 ± 2.40 ^{a}	0.17 ± 0.02 ^{a}	0.11 ± 0.01 ^{a}

The values given are means ($n = 3$) with standard deviations. Means in the same column followed by the same letters are not significantly different at $p < 0.05$.

3.5. Control of Wilt Disease of Chickpea by Selected Pseudomonas Strains

The pot experiment revealed that the DS was significantly lower ($p = 0.002$) in plants inoculated with the selected *Pseudomonas* strains (E1FP13, E1FP4, and E1PP7) in comparison to the uninoculated control infected with Foc-S1. There was no significant difference ($p = 0.489$) in the DR between E1FP13, E1FP4, and E1PP7, with values equal to 55.77%, 48.08%, and 46.16%, respectively (Table 4) (Supplementary Materials, Figure S2).

Table 4. In vivo effect of selected *Pseudomonas* strains on Foc isolates.

Strains	Foc-S1		Strains	Foc-S2	
	DS%	DR%		DS%	DR%
E1FP13	31.94 ± 6.36 ^{a}	55.77 ± 8.81 ^{a}	E1FP13	38.89 ± 4.82 ^{a}	53.33 ± 5.78 ^{b}
E1FP4	37.50 ± 4.17 ^{a}	48.08 ± 5.78 ^{a}	E1PP6	44.44 ± 9.62 ^{ab}	46.67 ± 11.55 ^{ab}
E1PP7	38.89 ± 9.62 ^{a}	46.16 ± 13.33 ^{a}	E1PP15	58.33 ± 0.00 ^{b}	30.00 ± 0.00 ^{a}
Control	72.22 ± 12.73 ^{b}	00.00	Control	83.33 ± 8.34 ^{c}	00.00

The values given are means ($n = 3$) with standard deviations. Means in the same column followed by the same letters are not significantly different at $p < 0.05$.

The DS was also significantly lower ($p = 0.0002$) in plants inoculated with the selected *Pseudomonas* strains (E1FP13, E1PP6, and E1PP15) in comparison to the uninoculated control infected with Foc-S2. There was a significant difference ($p = 0.021$) among the DR results, with isolate E1FP13 giving the highest percentage of 53.33%, followed by E1PP6 (46.67%) and E1PP15 (30%) (Table 4) (Supplementary Materials, Figure S3).

3.6. Molecular Identification of Selected Pseudomonas

The sequences of selected *Pseudomonas* strains were compared with other sequences using BLAST. The search was limited to sequences from type material, and the identification was based on homology percentage with the reference sequences. Results are presented in Table 5, with accession numbers of each isolate received from NCBI after sequences submission.

Table 5. BLAST results of selected *Pseudomonas*.

Isolates	Accession	Best Match	PI *	E Value
E1PP6	MT774541	*Pseudomonas frederiksbergensis*	99.55%	0.0
E1FP13	MT774542	*Pseudomonas frederiksbergensis* strain LMR 708	99.56%	0.0
E1PP7	MT774544	*Pseudomonas granadensis* strain F-278,770T	100%	0.0
E1PP15	MT774545	*Pseudomonas granadensis* strain F-278,770T	99.77%	0.0
E1FP4	MT774548	*Pseudomonas frederiksbergensis* strain LMR708	99.89%	0.0

* PI: Percentage identification.

Phylogenetic trees were also generated by the neighbor-joining method using the MEGA-X program with Bootstrap values based on 1000 replications. Sequences from the BLAST search with the highest similarity percentages were selected and used for the construction of the trees. *Rhizobacter gummiphilus* was used as an out-group bacterium. Results of the analysis revealed that the closest relative to isolates E1PP6, E1FP13, and E1FP4 was *Pseudomonas frederiksbergensis* with which they had the highest similarity percentages (>99.50%), while isolates E1PP7 and E1PP15 were closely related to *Pseudomonas granadensis* (100% and 99.77% identity, respectively) and *Pseudomonas soyae* (99.72% and 99.65% identity, respectively). Results can be found in Supplementary Materials (Figures S5–S9).

4. Discussion

In this study, *Pseudomonas* isolates were used as potential biocontrol agents against Foc. Several studies have demonstrated the efficacy of biocontrol agents in controlling this damaging phytopathogen [45–47]. The biocontrol activity of our *Pseudomonas* strains against Foc was confirmed through multiple assays.

In the in vitro antagonism test, the *Pseudomonas* strains reduced the mycelial growth of the Foc isolates with varying inhibition percentages. The results of the antagonistic effect of isolated *Pseudomonas* strains revealed that the growth of Foc-S1 was significantly more reduced in the divided plate assay (effect of volatile compounds) than in the co-culture assay, whereas for Foc-S2, there was no significant difference between the two assays. Typically, a higher growth inhibition percentage would be expected with the co-culture assay rather than with the divided plate assay, considering that in the co-culture assay, Foc is exposed to both diffusible and volatile antifungal metabolites, whereas in the divided plate assay, Foc is only exposed to the volatile antifungal metabolites. However, our results stated above revealed otherwise. This can be explained by the diffusion of antibacterial metabolites by Foc into the medium during the co-culture assay, which negatively affected the ability of *Pseudomonas* strains to inhibit fungal growth, thus resulting in a lower inhibition percentage. Meanwhile, in the divided plate assay, the partition in Petri dishes protects the *Pseudomonas* strains from potential antibacterial compounds released by Foc. The most known metabolite is fusaric acid, a mycotoxin produced by several species in the genus *Fusarium* [48]. The production of fusaric acid by Foc was reported by Türkkan and Dolar [49]. This mycotoxin is capable of decreasing the production of 2, 4-diacetylphloroglucinol (2,4-DAPG), an antifungal metabolite synthesized by the antagonistic strain *P. protegens* Pf-5 [50]. Similar results to ours have been reported by Kumari and Khanna [51].

E1FP13, E1FP4, and E1PP7 were the *Pseudomonas* strains with the highest inhibition percentages against Foc-S1. They registered the highest inhibition rates with volatile compounds, which is consistent with their ability to produce volatile antifungal compounds. The assessment of their PGPP revealed that E1FP13 and E1FP4 produced ammonia, while E1PP7 produced ammonia and HCN. A recent study conducted by Vlassi and Nesler [52] confirmed the involvement of ammonia released by *Lysobacter capsici* AZ78 in the inhibition of *Rhizoctonia solani*. HCN emitted by *P. putida* R32 and *P. chlororaphis* R47 was shown to be responsible for inhibiting the mycelial growth of the phytopathogen *Phytophthora infestans* [53]. HCN targets the electron transport chain of the pathogens, which leads to a deficiency in their ATP production, thus limiting their growth and development [51].

Microscopic examination of the effect of E1FP13 on Foc-S1 revealed fragmented and empty hyphae without septa. Similarly, Attia et al. [54] have reported hyphal fragmentation of *Alternaria solani* after treatment with plant-growth-promoting rhizobacteria. These results can be interpreted by the lysis of the hyphae that led to the loss of their content, which explains the empty appearance. *P. monteilii* PsF84 was shown to cause hyphal lysis of *Fusarium oxysporum* through the action of 2, 4-Di-tert-butylphenol, a volatile antifungal compound [55]. In the case of E1PP7 and E1FP4, the hyphae appeared empty of their content with the presence of granulations (vesicles). The same results were observed by Barka et al. [56] when *Botrytis cinerea* was treated with *Pseudomonas* sp. strain PsJN. In another study, a volatile antifungal compound released by *P. brassicacearum* and identified as dl-Limonene was also shown to cause the formation of granulations inside *Sclerotinia sclerotiorum* hyphae [57]. The alterations observed with Foc-S1's hyphae were caused by the volatile antifungal compounds released by E1FP13, E1FP7, and E1FP4. These compounds can be ammonia and HCN, as they can be other volatile antifungal compounds produced by these strains [58–60].

Isolates E1PP6 and E1FP13 gave the highest inhibition rates against Foc-S2 with diffusible compounds, while E1PP15 acted through the action of volatile compounds. The results of PGPP revealed that E1PP15 was able to produce HCN and ammonia, which correlates positively with its volatile antagonistic activity. Isolates E1PP6 and E1FP13 were both able to produce siderophores, which are low molecular weight compounds that

chelate ferric iron [61]. Di Francesco and Baraldi [62] reported that competition for iron through the release of siderophores by antagonists reduced the mycelial growth of the pathogenic fungus *Monilinia laxa.* In another study, it was shown that rhizobacteria that had a strong antagonistic effect against *Pyricularia oryzae* were the ones that registered the best siderophores production [63]. Besides the production of siderophores, isolate E1FP13 was also positive for the production of lipase, an enzyme responsible for the degradation of the fungal cell wall [64]. Isolate E1PP6 did not produce any of the tested diffusible lytic enzymes, which suggests that other enzymes, such as β-glucanase, involved in the degradation of the fungal cell wall [65] or other mechanisms, such as the production of cyclo (Pro-Val), a cyclic dipeptide with antifungal activity produced by *P. frederiksbergensis* CMAA 1323 [18], and the production of antibiotics [66], may have been employed.

The microscopic examination of the effect of E1PP15 on Foc-S2 revealed that this isolate caused a condensation of the hyphae's cytoplasm; the same observation was reported by Giorgio et al. [57] when *Sclerotinia sclerotiorum* was treated with *Pseudomonas* spp. USB2104. E1PP6 and E1FP13 caused granulations, emptiness, and deformations (swelling) of the hyphae, which were also observed by Arora et al. [67] when *Rhizoctonia solani* was treated with *Pseudomonas* PGC2. Likewise, empty and swollen hyphae were reported by Chiranjeevi et al. [68] when *Rhizoctonia bataticola* was treated with the crude metabolite extract of *Bacillus subtillis*. The granulations could be cytoplasm remains formed due to cytoplasm leakage following hyphal damage caused by the hydrolytic enzyme, lipase, produced by E1FP13. In the case of E1PP6, which did not produce any of the tested lytic enzymes, other mechanisms, as mentioned above, may have been responsible for the observed morphological changes.

All five isolates were shown to synthesize IAA, a phytohormone that increases the plant's nutrients intake by enhancing the root surface [69]. All isolates were positive for the production of cellulase, involved in the increase in organic matter in the soil [70]. All five isolates were also able to solubilize phosphate; however, the qualitative assay results did not match those of the quantitative assay, as the highest solubilization values were observed with the strains that gave the weakest solubilization halo in the plate assay. The same results were observed by Nautiyal [34]. Phosphate plays a vital role in plant development. Despite its abundance in the soil, most of it remains unavailable to plants. Phosphate-solubilizing microorganisms can hydrolyze the insoluble phosphate forms into a soluble form that can be easily assimilated by plants [71].

The evaluation of the effect of selected *Pseudomonas* strains on the shoot and root growth of chickpea revealed that isolates E1PP6, E1FP13, E1FP4, and E1PP7 were able to significantly increase shoot length and shoot dry weight, which correlates positively with their ability to produce several PGP compounds, such as ammonia, cellulase, siderophores, and solubilize phosphate. Although E1PP15 was also shown to produce these compounds, it did not improve shoot growth like the rest of the isolates. This result may be explained by the fact that, unlike in the in vitro tests, E1PP15 was not able to synthesize these metabolites in vivo. Concerning the growth of the roots, isolates E1PP15, E1FP13, E1FP4, and E1PP7 were able to significantly increase root length and root dry weight, which concurs well with their ability to produce IAA. IAA plays an important role in the development of plant roots; in a recent study, *P. moraviensis* wild type was reported for its ability to increase wheat root area in comparison to its IAA-deficient mutants [72]. Regarding E1PP6, this isolate did not improve the root length; however, it significantly increased the roots' dry weight, and this can be interpreted by an increase in the growth of lateral roots through the production of IAA. The positive effect of IAA-producing bacteria on the number of plant's lateral roots has been demonstrated by Herlina et al. [73]. Our results are in line with other studies that have demonstrated the capacity of *Pseudomonas* strains to improve chickpea growth [74–76]. Our study confirms the ability of the *Pseudomonas* isolates to promote chickpea growth by increasing shoot and root length, as well as their dry weight.

The pot experiment revealed that all selected *Pseudomonas* strains were able to significantly decrease the disease severity caused by both Foc isolates. The in vitro antagonistic

effects (percentages of inhibition) of E1FP13, E1FP4, and E1PP7 against Foc-S1 were not significantly different from their in vivo effects (DR percentages). Similarly, there was no significant difference between the in vitro and in vivo antagonistic effects of E1PP6 and E1FP13 against Foc-S2. However, the antagonistic effect of isolate E1PP15 was significantly lower in the in vivo test, which can be attributed to lower production of antifungal compounds than that expressed in the in vitro assay. A recent study conducted by Besset-Manzoni et al. [77] showed that biocontrol agents may not always perform the same way in the in vivo antagonism assay as they do in the in vitro antagonism assay. In this study, the selected *Pseudomonas* strains were shown to control Fusarium wilt of chickpea caused by Foc; various studies have demonstrated the potential of *Pseudomonas* strains in the protection against phytopathogens [14,15,17,53]. Our results revealed that the *Pseudomonas* isolates produced various antifungal compounds and caused some damage to the Foc hyphae. Moreover, the isolates improved chickpea growth through the production of several metabolites that play an important role in the provision and acquisition of nutrients. The improvement of plant growth contributes to the biocontrol process, as it confers a better resistance to plants against pathogens, thus limiting their negative impact on the plants. The combination of all these mechanisms explains the significant reduction in disease severity by our isolates when applied as seed treatments.

The BLAST results and the phylogenetic analysis confirmed that the selected rhizobacteria belong to the genus *Pseudomonas*. Isolates E1PP6, E1FP13, and E1FP4 were closely related and best matched with *P. frederiksbergensis*, a bacterium that is usually associated with abiotic stress reduction, such as the degradation of pesticides [78] and the enhancement of cold stress and salt stress tolerance [79]. A few studies have been carried out on the antagonistic and PGPP of *P. frederiksbergensis*. A study performed by Ferchichi et al. [80] revealed that *P. frederiksbergensis* LB113 was able to produce HCN and inhibit mycelium growth of *Macrophomina phaseolina* and *Alternaria alternate* in PDA. *P. frederiksbergensis* PgBE39 and PgBE45 were reported for their antifungal activity against *Cylindrocarpon destructans* and *Botrytis cinerea* [81], while *P. frederiksbergensis* G62 was reported by Ben Zineb et al. [82] for its ability to produce siderophores, HCN, IAA, and solubilize phosphate. Isolates E1PP7 and E1PP15 were closely related to two *Pseudomonas* species, *P. soyae* and *P. granadensis*, with the latter having the highest similarity percentages with the two isolates. *P. granadensis* strain 100 was reported by Riera et al. [83] for its ability to produce siderophores, while *P. granadensis* PMK4 was shown to produce IAA, siderophores, and solubilize phosphate [84].

5. Conclusions

In brief, our results revealed that the *Pseudomonas* strains have the potential to be used as biocontrol agents in the management of wilt disease and as plant-growth-promoting bacteria in chickpea plants.

6. Future Perspective

In the future, it would be of interest to further study the ability of our *Pseudomonas* strains to control Foc under different stress conditions, such as cold, high salinity soils, and soils polluted with pesticides, and to test their effect alone and in consortium with other bacteria against Foc and other phytopathogens. Other experiments and field evaluations of the isolated *Pseudomonas* strains should be carried out to reveal in detail the underlying mechanisms of their biocontrol activity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12030429/s1, Figure S1: Inhibition of Foc growth by isolated *Pseudomonas*. (1) Antagonism plate, (2) Plate without bacteria (control); Figure S2: Biocontrol of Foc-S1 by isolated *Pseudomonas*. (1) Treatment with E1FP13, (2) Treatment with E1PP7, (3) Control, (4) Treatment with E1FP4; Figure S3: Biocontrol of Foc-S2 by isolated *Pseudomonas*. (1) Treatment with E1PP6, (2) Treatment with E1PP15, (3) Control, (4) Treatment with E1FP13; Figure S4: Promotion of chickpea growth by *Pseudomonas* isolates. (1) Control, (2) Treatment with E1FP13, (3) Treatment with E1PP7, (4) Treatment with E1PP6, (5) Treatment with E1FP4, (6) Treatment with E1PP15; Figure S5:

Phylogenetic tree of strain E1PP6 constructed by neighbor-joining method with Bootstrap values supporting the branches shown at the nodes; the bar indicates the number of substitutions per site; Figure S6: Phylogenetic tree of strain E1FP13 constructed by neighbor-joining method with Bootstrap values supporting the branches shown at the nodes; the bar indicates the number of substitutions per site; Figure S7: Phylogenetic tree of strain E1PP7 constructed by neighbor-joining method with Bootstrap values supporting the branches shown at the nodes; the bar indicates the number of substitutions per site; Figure S8: Phylogenetic tree of strain E1PP15 constructed by neighbor-joining method with Bootstrap values supporting the branches shown at the nodes; the bar indicates the number of substitutions per site; Figure S9: Phylogenetic tree of strain E1FP4 constructed by neighbor-joining method with Bootstrap values supporting the branches shown at the nodes; the bar indicates the number of substitutions per site.

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