



Article

Integrated Biological and Metabolomic Characterization Reveals the Multifunctional Potential of *Pseudomonas putida* V01 for Disease Suppression and Plant Growth Promotion

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Abstract

The increasing demand for sustainable crop protection strategies has intensified interest in plant-beneficial bacteria as alternatives to synthetic agrochemicals. In this study, the soil-derived bacterium *Pseudomonas putida* V01 was isolated and characterized for its antifungal and plant growth-promoting potential through an integrated approach combining biological assays, untargeted metabolomics, and in vivo plant experiments. Cell-free culture filtrates exhibited strong antifungal activity against major phytopathogenic fungi, completely inhibiting the growth of *Sclerotium rolfsii* and significantly reducing mycelial development of *Alternaria alternata* and *Fusarium proliferatum* by 40% and 20%, respectively. Volatile organic compounds (VOCs) selectively inhibited *Botrytis cinerea* and *A. alternata* by 28% and 10%, respectively, and affected sporulation of *F. proliferatum*. Metabolomic profiling through LC-qTOF-MS and GC-MS analyses revealed a chemically diverse metabolome, including putatively annotated diketopiperazines, cyclic peptides, phenolic compounds, and fatty acids. VOC profiling indicated ketones and alcohols as the predominant volatile classes, with 2-undecanone and 2-undecanol among the most abundant compounds detected. In vivo assays on wheat seedlings showed significant increases in shoot growth, biomass accumulation, and chlorophyll content compared with untreated controls. These findings indicate that *P. putida* V01 combines complementary antifungal and plant growth-promoting activities associated with a diverse repertoire of diffusible and volatile metabolites. The integrated biological and metabolomic characterization highlights its potential as a multifunctional microbial inoculant for sustainable crop production and disease management.



Academic Editor: Nikolai Ravin

Received: 11 June 2026

Revised: 24 June 2026

Accepted: 25 June 2026

Published: 28 June 2026

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Keywords: *Pseudomonas putida*; biological control; plant growth-promoting bacteria; volatile organic compounds; metabolomic profiling; antifungal activity; sustainable agriculture

1. Introduction

The intensive use of synthetic pesticides in agriculture has contributed substantially to crop protection and productivity but has also raised increasing environmental and

public health concerns, including soil and water contamination, adverse effects on non-target organisms, and the emergence of resistant phytopathogen populations [1,2]. These limitations have stimulated the search for sustainable alternatives capable of reducing reliance on chemical inputs while maintaining effective disease control. Among these alternatives, microbial biological control agents (BCA) have emerged as valuable tools for sustainable crop management due to their ability to suppress plant pathogens and promote plant health through multiple mechanisms [3,4].

Among plant-associated bacteria, *Pseudomonas* spp. has attracted considerable attention because of their remarkable metabolic versatility, ecological adaptability, and capacity to colonize diverse environmental niches, including the rhizosphere [5,6]. Members of this genus are known to exert beneficial effects on plants through direct and indirect mechanisms, including competition for nutrients and ecological niches, production of antimicrobial metabolites, induction of systemic resistance, enhancement of nutrient acquisition, and modulation of plant growth [7,8]. The ability of *Pseudomonas* spp. to synthesize a wide range of bioactive compounds has contributed to their extensive study as plant-beneficial bacteria [6].

Particular interest has been devoted to *Pseudomonas putida*, a ubiquitous soil and rhizosphere inhabitant recognized for its environmental adaptability, metabolic diversity, and plant-associated lifestyle. Beyond its well-established role in biodegradation and environmental biotechnology, *P. putida* is increasingly regarded as a promising plant growth-promoting rhizobacterium (PGPR) capable of enhancing plant performance and resilience under both biotic and abiotic stresses. Several strains have been reported to improve plant nutrition, stimulate root development, facilitate nutrient uptake, and contribute to disease suppression through the production of antimicrobial metabolites and signaling molecules [8,9]. The remarkable genomic and metabolic plasticity of *P. putida* enables the production of a broad repertoire of secondary metabolites that may contribute to both antagonistic and plant growth-promoting activities [10].

Among these metabolites, cyclic peptides, polyketides, phenolic compounds, fatty acids, and diketopiperazines (DKPs) have been associated with antimicrobial activity against a wide range of phytopathogens [5,7,10,11]. In addition to diffusible metabolites, *Pseudomonas* spp. produce volatile organic compounds (VOCs), which act as key mediators of long-distance interactions within plant-associated microbial communities. These low-molecular-weight compounds can inhibit fungal growth and sporulation, alter pathogen development, and influence microbial community dynamics [12–16]. Furthermore, bacterial VOCs may directly affect plant physiology by stimulating growth, enhancing photosynthetic performance, and activating defense-related pathways [17–19]. Ketones, alcohols, and related volatile compounds, including 2-undecanone and 2-nonanone, have been reported as important contributors to pathogen suppression and induced systemic resistance [15,16]. Importantly, increasing evidence suggests that microbial bioactivity often arises from the combined action of multiple metabolites rather than from a single dominant compound, highlighting the importance of adopting holistic analytical approaches for the characterization of beneficial microorganisms [5].

Recent advances in mass spectrometry-based metabolomics have provided powerful tools for investigating the chemical basis of microbial interactions and identifying metabolites potentially involved in biological activity [20,21]. Nevertheless, many studies on plant-beneficial *Pseudomonas* strains focus separately on antifungal activity, metabolomic characterization, or plant growth-promotion assays. Consequently, the relationships between metabolite production, antagonistic activity, and plant growth promotion remain poorly understood for many newly isolated strains. Integrative studies combining biological validation with metabolomic profiling are still relatively limited but are essential for

elucidating the mechanisms underlying multifunctional microbial phenotypes. However, little is known about the metabolome-driven mechanisms underlying the multifunctional activity of newly isolated *Pseudomonas putida* strains.

In this context, the present study aimed to characterize a newly isolated soil-derived strain, *Pseudomonas putida* V01, for its antifungal and plant growth-promoting potential using an integrated experimental framework. The strain was evaluated for its antagonistic activity against economically important phytopathogenic fungi, the effects of its volatile metabolites on fungal development, and its capacity to promote wheat growth under controlled conditions. To gain insights into the molecules potentially involved in these biological activities, metabolomic profiling of combined LC-qTOF-MS and GC-MS analyses of culture extracts was combined with headspace analysis of volatile metabolites. By integrating biological and metabolomic approaches, this study reveals novel insights into the mechanisms underlying the multifunctional activity of *P. putida* V01 and supports its potential as a promising microbial inoculant for sustainable crop production and disease management.

2. Materials and Methods

2.1. Fungal Isolates, Plant Material and Reagents

Alternaria alternata, *Fusarium proliferatum*, *Sclerotium rolfsii*, *Botrytis cinerea* and *Rhizoctonia solani* were obtained from the culture collection of Plant Pathology, Department of Agricultural Sciences, University of Naples Federico II. All the microorganisms were maintained as spore suspensions in a 40% (v/v) glycerol solution at -20°C . All the microorganisms were cultivated on PDA (Potato Dextrose Agar, HiMedia Laboratories GmbH, Modautal, Germany). *Triticum aestivum* L. cv. Risciola plants were used in this study. Seeds were commercially obtained from SIS—Società Italiana Sementi (San Lazzaro di Savena, Italy).

All the reagents used for metabolomic profiling were LC-MS or GC-MS grade and were purchased from Merck (Merck KGaA, Darmstadt, Germany), unless otherwise stated.

2.2. Molecular Identification of *Pseudomonas putida* V01

Pseudomonas putida V01 was isolated from the soil rhizosphere of eggplant, located in Portici, Naples. Serial dilutions of the soil sample were plated on PCA (Plate Count Agar, HiMedia Laboratories GmbH, Modautal, Germany) and incubated at room temperature. After 48 h, individual colonies were selected and sub-cultured onto NA (Nutrient Agar, HiMedia Laboratories GmbH, Modautal, Germany) until pure cultures were obtained. Then, colonies (~1 mm in diameter) were transferred to 200 μL of ultrapure water and subjected to cell lysis by heating at 100°C for 10 min, followed by rapid cooling at -20°C for 3 min. The lysate was centrifuged, and the supernatant was used as template DNA for PCR amplification of the 16S rRNA gene using primers 16SF (5'-CGTTAGTAATCGTGGATCAGAATG-3') and 16SR (5'-TGTGACGGGCGGTGTGTA-3'). DNA amplification was performed using DreamTaq (Thermo Fisher Scientific, Segrate, Italy) in a 50 μL PCR reaction containing 5 μL of Buffer, 4 μL of dNTPs, 1 μL of forward primers, 1 μL of reverse primers, 2 μL of DNA template, 36.75 μL of ultrapure water and 0.25 μL of Dream Taq. The reaction cycle conditions were standardized as initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 45 s and extension at 72°C for 2 min and a final extension of 72°C for 7 min. PCR products were separated on a 1% agarose gel stained with SYBR Safe and visualized under UV light. Successful amplicons were purified with PureLink Quick PCR Purification Kit (Invitrogen, Thermo Fisher Scientific, Segrate, Italy) following the manufacturer's instructions and subjected to Sanger sequencing using the forward primer (16SF) by Eurofins Genomics. Taxonomic assignment

was performed using the BLASTn algorithm (available at <https://blast.ncbi.nlm.nih.gov>) against the NCBI nucleotide database. The sequences were adjusted using UGENE v48.1, concatenated, and further aligned with MUSCLE v3.8.31. The alignment file was then used as input to build a maximum likelihood (ML) phylogenetic tree using RAXML-HP v8.2.12, and the best-fitting nucleotide substitution model for the data was determined using MrModelTest (version 2.3). NJ and ML analyses were run under the TN93 (TrN) and GTR+I model, 1000 bootstraps. The tree was visualized using FigTree v1.4.4 (available at <http://tree.bio.ed.ac.uk/software/figtree/>, accessed on 22 June 2026) and further edited with Inkscape v0.92.

2.3. Antifungal Activity of Diffusible Metabolites

Bacterial cultures were grown in NB (Nutrient Broth, HiMedia Laboratories GmbH, Modautal, Germany) for 5 days at 28 °C with orbital agitation (150 rpm). Cultures were centrifuged at 10,000 rpm for 30 min, and the resulting supernatant was filtered through 0.45 µm and 0.22 µm syringe filters to obtain a sterile cell-free culture filtrate. Antifungal activity was evaluated against *A. alternata*, *F. proliferatum*, and *S. rolsii*. Petri dishes were filled with 16 mL of PDA supplemented with 4 mL of the culture filtrate or 4 mL of sterilised NB. Then, a fungal plug from a five-day-old colony was placed at the center of the plate and incubated at 28 °C for 3 days. Each experiment consisted of three biological replicates and was independently repeated twice with comparable outcomes. The diameter growth of the fungal colony was measured daily, and growth inhibition percentage was calculated as: $(D_c - D_t)/D_c \times 100$, where D_c is the colony diameter in the control, and D_t is the colony diameter in the presence of *P. putida* V01 culture filtrate.

2.4. Antifungal Activity of Volatile Organic Compounds

Antifungal activity of VOCs was tested against: *B. cinerea*, *A. alternata*, *F. proliferatum*, *R. solani* and *S. rolsii*. The bacterium *P. putida* V01 was inoculated into a Petri plate containing NA, and a 5 mm plug of a fungal pathogen was inoculated into another plate containing PDA. Lids were removed from each culture plate, and the plate bottoms with the bacteria or pathogen were placed face-to-face, then sealed with parafilm. Each experiment consisted of three biological replicates and was independently repeated twice with comparable outcomes. Plates were incubated at 28 °C for 5 days, colony diameter was recorded daily, and inhibition percentage was calculated as previously reported.

2.5. Metabolomic Profiling of *Pseudomonas. putida* V01 Extracellular Metabolites

Extraction of extracellular metabolites was performed following Fisher et al., 2024 [22]. Briefly, the bacterium was cultured in NB for 3 days on a rotary shaker at 150 rpm and 28 °C. Then, cultures were centrifuged at 10,000 rpm for 30 min, and the supernatant was sterilized using a 0.45 and 0.22 µm syringe filter. Culture filtrates were extracted three times with ethyl acetate (1:1 v/v) (Carlo Erba, Milan, Italy), and the organic layer was concentrated using a rotary evaporator at 37 °C under vacuum. The crude extract was subjected to metabolite profiling (non-volatile and semi-volatile) through liquid chromatography and gas chromatography coupled to mass spectrometry (LC-MS and GC-MS) analyses. For LC-MS analysis, an HP 1260 Infinity Series liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to a high-resolution quadrupole time-of-flight mass spectrometer model 6545 (qTOF, Agilent Technologies) was used, following Vitale et al. (2025) with some modifications [23]. Samples were resuspended in methanol at a final concentration of 0.1 mg mL⁻¹, and 5 µL was injected into a Kinetex® C18 column (100 × 2.1 mm, 2.6 µm, Phenomenex, Torrance, CA, USA). The following elution gradient, composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), was used for the separation: 5% B as starting condition, 80% B in 3 min, 98% B in 7 min, then 5% B in 2 min.

Equilibration time was set at 3 min. Flow rate was set at 0.2 mL min⁻¹. The instrument operated in positive ionization mode; ion source parameters were set as follows: gas temp 200 °C, sheath gas temp 400 °C, VCap 3000 V and nozzle voltage 1000 V. Mass spectra were acquired in the 100–1000 *m/z* range, at a scan rate of 2 spectra s⁻¹. A reference solution composed of purine (C₅H₄N₄ at *m/z* 121.050873, 10 µmol L⁻¹) and hexakis (¹H,¹H,³H-tetrafluoropentoxy)-phosphazene (C₁₈H₁₈O₆N₃P₃F₂₄ at *m/z* 922.009798, 2 µmol L⁻¹) was used to perform real-time mass correction. All acquisition parameters were set by using Mass Hunter Acquisition software version 12 (Agilent Technologies).

For GC-MS analysis, an Agilent 5977B MSD mass spectrometer coupled to an Agilent 8890 GC (Agilent Technologies) was used, following the method described by Lasmer et al., 2026 [24]. Samples were resuspended in ethyl acetate at a concentration of 0.1 mg mL⁻¹ and derivatized with 100 µL of *N*, *O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The reaction was conducted in an ultrasonic bath for 30 min at room temperature.

For the LC-MS dataset, metabolites were putatively annotated by matching their monoisotopic masses against an in-house database of fungal secondary metabolites and information reported in the literature. Annotations were successful when the mass deviation did not exceed 5 ppm.

For the GC-MS dataset, metabolite identification was achieved through spectral matching of the deconvoluted mass spectra with reference spectra collected in the National Institute of Standards and Technology (NIST) library and by comparing the experimentally determined retention indices with Kovats retention index values reported in the literature and reference databases. Annotations were considered reliable when the spectral match score exceeded 800, and the retention indices were in agreement with reference values.

2.6. Headspace Analysis of Volatile Organic Compounds

Bacteria were inoculated in 500 mL NB overnight, then 100 mL of the pre-culture was inoculated into 500 mL NB in a 2 L flask, and a Radiello® cartridge was attached to the lid. Bacteria were incubated for 7 days at 28 °C and 100 rpm. Then, cartridges were desorbed by using 5 mL of dichloromethane and concentrated to a final volume of 200 µL under a gentle nitrogen flow. Volatile compounds were analyzed using GC-MS. Separation was performed on an HP-5MS UI capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium was used as carrier gas at a constant flow rate of 1.00 mL min⁻¹. Samples (1 µL) were injected in splitless mode with the injector temperature set at 250 °C. The oven temperature program was as follows: initial temperature of 40 °C held for 1 min, increased to 200 °C at 10 °C min⁻¹ and held for 1 min, then raised to 300 °C at 30 °C min⁻¹ and maintained for 1 min. Mass spectrometric detection was carried out using electron ionization (EI) at 70 eV. The ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively, while the transfer line temperature was maintained at 250 °C. Mass spectra were acquired in full-scan mode over an *m/z* range of 50–550, with a solvent delay of 6.6 min. Compound identification was achieved as previously reported (Section 2.5).

2.7. In Vivo Biostimulant Activity

To evaluate the plant growth promotion effect of the bacterium, an in vivo assay was conducted on *Triticum aestivum*. Wheat seeds were disinfected with 1% NaClO and rinsed three times with sterile distilled water. Bacterial cells obtained by growth on NB (100 mL, 10⁹ CFU mL⁻¹) were resuspended in 100 mL of 0.9% NaCl (*w/v*). and subsequently used for seed coating. The seeds were shaken in the bacterial suspension at 80 rpm for 90 min; control seeds were treated with only 0.9% NaCl (*w/v*). Then, seeds were sown at a depth of 1 cm (3 replicates of 8 seeds each) in sterilized vermiculite in plastic pots (height 14 cm × 16 cm diameter). Pots were arranged in a fully randomized complete

block design, under standard conditions, in a plant growth room (25 °C, 16 h light/8 h dark, 65% humidity). All pots were watered daily with 10 mL of water. After 15 days, plants were treated by watering 50 mL per pot of a 10^9 CFU mL⁻¹ cell suspension in water. Negative control was treated with only water. The temperature was reduced to 18 °C to avoid heat stress on seedlings. After 30 days post-sowing, plants were removed carefully from the soil, biometric plant parameters (root and shoot length, and root and shoot fresh weight), and chlorophyll content (expressed as SPAD index) were measured. A total of 12 plants per treatment (three biological replicates, four plants each) were considered for statistical analysis.

2.8. Statistical Analysis

Data obtained from antifungal assays and in vivo biostimulant activity experiments were analyzed using SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA). Prior to statistical analysis, data were tested for normality and homogeneity of variances using the Shapiro–Wilk and Levene’s tests, respectively. Differences between treatments and control group (CN) were evaluated using Student’s *t*-test, and statistical significance was established at $p \leq 0.05$. Antifungal assay data were also analyzed by two-way ANOVA, considering fungal species and treatment as fixed factors. When significant effects were detected, means were compared using Tukey’s test at $p < 0.05$. Graphical representations were generated using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

Chemical classification of the identified metabolites was performed according to the ChemOnt chemical taxonomy using the ClassyFire database. Metabolite annotations were queried through the ClassyFire application programming interface (API) using custom scripts developed in R (version 4.5.0). For each metabolite, the corresponding ChemOnt taxonomy was retrieved, including kingdom, superclass, class, subclass, direct parent, and molecular framework. The direct parent category was used as the primary chemical classification level for data interpretation and reporting.

3. Results

3.1. Molecular Identification of the Newly Isolated Strain *Pseudomonas putida* V01

The bacterial isolate formed circular, smooth colonies with regular margins on nutrient agar (NA) after 24 h of incubation at 28 °C. Molecular identification was performed through amplification of the 16S rRNA gene using universal bacterial primers. PCR amplification yielded a single amplicon of the expected size (approximately 1500 bp), indicating successful amplification of the target region. The resulting PCR product was sequenced, generating a high-quality partial 16S rRNA gene sequence of 426 bp, which was deposited in the GenBank database under accession number PZ558340. Comparative analysis using BLASTn (available at <https://blast.ncbi.nlm.nih.gov>) against sequences deposited in the NCBI database revealed a high level of similarity with members of the *Pseudomonas putida* group, including *P. putida*, *P. monteilii*, and *P. alloputida*. The highest similarity was observed with *P. putida* strain DWS_27 (99.06% identity; 99% query coverage). The phylogenetic tree, based on 16S rRNA genes from 20 isolates, revealed close relationships among members of the *Pseudomonas* genus (Figure S1). Isolate V01 clustered into a single group, showing a close relationship with five other strains of *P. putida* and *P. monteilii*. Accordingly, the isolate was assigned to *Pseudomonas putida* and designated *P. putida* V01.

3.2. Antifungal Activity of Non-Volatile Metabolites Produced by *Pseudomonas putida* V01

The antifungal activity of the cell-free culture filtrate was evaluated to assess the production of diffusible metabolites with inhibitory effects against phytopathogenic fungi. The cell-free culture filtrate of *Pseudomonas putida* V01 (Pse) significantly reduced the

mycelial growth of all tested pathogens compared with the untreated control (CN), although the magnitude of inhibition varied among fungal species (Figure 1 and Figure S2).

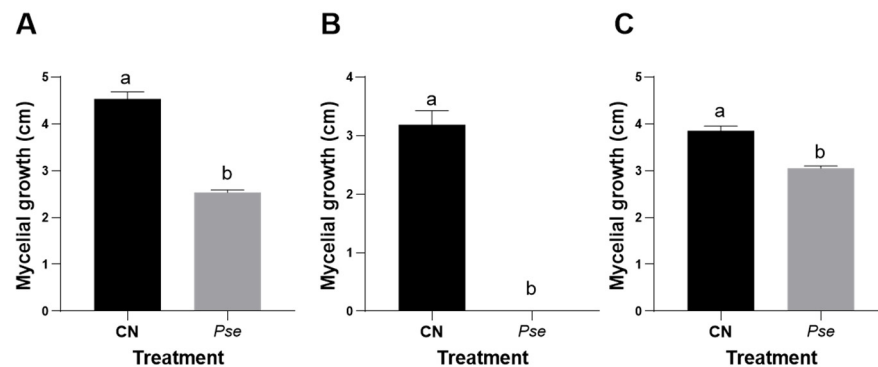


Figure 1. Antifungal activity of *Pseudomonas putida* V01 cell-free culture filtrate against different phytopathogenic fungi. Mycelial growth diameter of (A) *Alternaria alternata*, (B) *Sclerotium rolsii*, and (C) *Fusarium proliferatum* in the presence (Pse, grey bar) or absence (CN, black bar) of cell-free culture filtrate. Data are expressed as mean $\pm$ standard deviation of three biological replicates. Different letters indicate significant differences between treatments according to Student's *t*-test ($p \leq 0.05$).

The strongest inhibitory effect was observed against the soil-borne pathogen *Sclerotium rolsii*, whose growth was completely suppressed (100% inhibition, Figure 1B). In contrast, partial but significant growth reductions were recorded for *Alternaria alternata* and *Fusarium proliferatum*, showing approximately 40% and 20% inhibition, respectively, relative to the control treatment (Figure 1A,C). A two-way ANOVA revealed significant effects of pathogen species and treatment, as well as a significant pathogen $\times$ treatment interaction ($p < 0.001$), indicating that the inhibitory activity of *P. putida* V01 cell-free culture filtrate depended on the target fungal species.

3.3. Antifungal Activity of Volatile Organic Compounds VOC Produced by *Pseudomonas putida* V01

The antifungal activity of volatile organic compounds (VOCs) emitted by *Pseudomonas putida* V01 was evaluated using the overlapped plate assay. After three days of incubation, no significant differences in mycelial growth were observed for *Rhizoctonia solani*, *Sclerotium rolsii*, and *Fusarium proliferatum* exposed to bacterial VOCs compared with the untreated controls ($p > 0.05$; Figure S3), indicating the absence of detectable growth inhibition under the tested conditions. In contrast, exposure to VOCs significantly reduced the mycelial growth of *Botrytis cinerea* and *Alternaria alternata* (Figure 2). Growth inhibition reached approximately 28% for *B. cinerea* and 10% for *A. alternata*, demonstrating a selective antifungal activity of the volatilome produced by *P. putida* V01. A two-way ANOVA analysis revealed significant effects of pathogens, treatment, and their interaction ($p < 0.0001$), confirming that the response to VOC exposure was pathogen-dependent.

Although no significant reduction in colony diameter was observed for *F. proliferatum*, exposure to bacterial VOCs altered colony pigmentation and appearance compared with the untreated control (Figure 3). Colonies exposed to the bacterial volatilome exhibited a strong discoloration (Figure 3A) compared with the control treatment (Figure 3B).

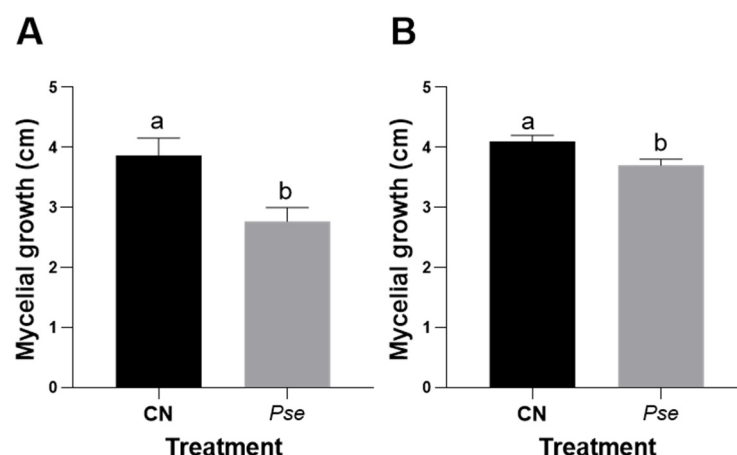


Figure 2. Antifungal activity of volatile organic compounds (VOCs) produced by *Pseudomonas putida* V01 against phytopathogenic fungi in the overlapped plate assay. Mycelial growth diameter of (A) *Botrytis cinerea* and (B) *Alternaria alternata* exposed to bacterial VOCs (Pse, grey bar) or grown in the absence of bacteria (CN, black bar). Data are expressed as mean $\pm$ standard deviation of three biological replicates. Different letters indicate significant differences between treatments according to Student's *t*-test ($p \leq 0.05$).

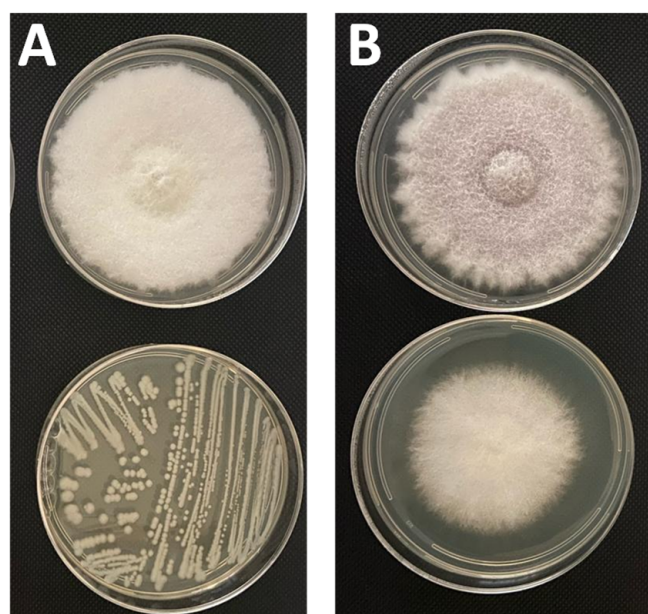


Figure 3. Effect of volatile organic compounds (VOCs) produced by *Pseudomonas putida* V01 on pigmentation of *Fusarium proliferatum* in the overlapped plate assay. (A) *F. proliferatum* grown on PDA in the presence of an NA plate inoculated with *P. putida* V01. (B) *F. proliferatum* grown on PDA in the presence of an uninoculated NA plate (control). Exposure to bacterial VOCs resulted in a marked reduction in fungal pigmentation without affecting colony growth.

A comparative overview of the responses of all tested pathogens to diffusible and volatile metabolites produced by *P. putida* V01 is presented in Table 1. Diffusible metabolites generally exhibited stronger antifungal activity, completely suppressing the growth of *S. rolfii* and substantially reducing the growth of *A. alternata* and *F. proliferatum*. In contrast, VOC-mediated inhibition was more selective, affecting mainly *B. cinerea* and, to a lesser extent, *A. alternata*, while no growth inhibition was detected against *S. rolfii* and *R. solani*.

Table 1. Comparative susceptibility of the tested phytopathogenic fungi to diffusible metabolites (cell-free culture filtrate) and volatile organic compounds (VOCs) produced by *Pseudomonas putida* V01. Values are expressed as percentage growth inhibition relative to the corresponding untreated controls. nd = not determined.

Pathogen	Diffusible Metabolites (%)	VOCs (%)
<i>Alternaria alternata</i>	44.10	9.73
<i>Botrytis cinerea</i>	nd	28.33
<i>Fusarium proliferatum</i>	20.73	2.65
<i>Rhizoctonia solani</i>	nd	0
<i>Sclerotium rolfsii</i>	100	0

3.4. Metabolomic Profiling of Extracellular Metabolites and VOCs Produced by *Pseudomonas putida* V01

To characterize the metabolic profile of *P. putida* crude extract, complementary LC-qTOF-MS and GC-MS analyses were performed, enabling the detection of non-volatile, semi-volatile, and moderately polar metabolites. LC-qTOF-MS profiling of the crude extract revealed a total of 685 molecular features. Following feature filtering and annotation procedures, several compounds were putatively identified. Among these, metabolites previously reported to possess antimicrobial or antifungal activity were selected for further interpretation and are presented in Table 2. The identified compounds belong to different chemical classes, including amino acid derivatives, terpenoids, indole alkaloids, diketopiperazines, and phenazine alkaloids, several of which have been previously reported to possess antimicrobial properties. According to ChemOnt classification, the identified metabolites mainly belonged to organoheterocyclic compounds and benzenoids, and included phenazines and derivatives, indoles, diketopiperazines, aniline derivatives, and cyclic ketones.

Table 2. Putatively identified metabolites detected in the crude extract of *Pseudomonas putida* V01, 72 h after inoculation, by LC-qTOF-MS metabolomic profiling. Annotations were assigned through accurate-mass database matching and filtered according to signal quality, mass accuracy (<5 ppm), and annotation score (≥ 80). Chemical classification was assigned according to the ChemOnt taxonomy using the ClassyFire database.

Compound Name	Chemical Classification	RT (min)	Molecular Formula	<i>m/z</i> (Da)	Calculated Neutral Mass (Da)	Mass Error (ppm)
2-Aminophenol	Aniline and substituted anilines	1.075	C ₆ H ₇ NO	110.0601	109.0528	0.28
Methylenomycin B	Cyclic ketones	1.106	C ₈ H ₁₀ O	105.0698	122.0732	0.27
Indole-3-carboxaldehyde	Indoles	1.599	C ₉ H ₇ NO	146.0600	145.0528	0.42
Cyclo (Pro-Tyr)	Diketopiperazines	4.934	C ₁₄ H ₁₆ N ₂ O ₃	261.1235	260.1163	0.66
Cyclo (Leu-Pro)	Diketopiperazines	5.027	C ₁₁ H ₁₈ N ₂ O ₂	211.1443	210.1370	0.66
Cyclo (Phe-Pro)	Diketopiperazines	5.198	C ₁₄ H ₁₆ N ₂ O ₂	245.1286	244.1214	0.81
Cyclo(Pro-Val)	Diketopiperazines	5.441	C ₁₀ H ₁₆ N ₂ O ₂	197.1284	196.1212	0.05
Pyocyanin	Phenazines and derivatives	6.745	C ₁₃ H ₁₀ N ₂ O	211.0866	210.0794	0.31

Moreover, a total of 240 molecular features were detected by GC-MS metabolomic profiling of the crude extract. Compound annotation was achieved through comparison of deconvoluted mass spectra with the NIST spectral library, combined with retention index verification using Kovats indices. Fourteen metabolites were putatively identified (Table 3). According to ChemOnt classification, the identified compounds mainly belonged to fatty acids and conjugates, diketopiperazines, hydroxypyrimidines, piperidinones, ureas, and

triterpenoids. Diketopiperazines represented the most abundant group, accounting for five of the fourteen identified metabolites, including cyclo(Ala-Leu), cyclo(Ile-Val), cyclo(Gly-Pro), cyclo(Leu-Pro), and cyclo(Phe-Pro). In addition, several medium- and long-chain fatty acids, together with squalene and other bioactive metabolites, were detected.

Table 3. Putative metabolites detected in *Pseudomonas putida* V01 crude extract, 72 h after inoculation, by GC-MS metabolomic profiling. Compound annotation was performed by matching deconvoluted mass spectra against the NIST spectral library and verifying retention indices using Kovats index values. Only compounds showing high-quality spectral matches and consistent retention index assignments were retained. Chemical classification was assigned according to the ChemOnt taxonomy using the ClassyFire database. TMS = trimethylsilyl derivative.

Compound	Chemical Classification	RT (min)	RI
Lactic Acid, 2TMS	Alpha hydroxy acids and derivatives	5.684	1092
2-Piperidinone, TMS	Piperidinones	6.766	1168
Urea, 2TMS	Ureas	8.032	1259
Nonanoic acid, TMS	Medium-chain fatty acids	9.632	1377
Thymine, 2TMS	Hydroxypyrimidines	10.282	1427
Cyclo(Ala-Leu), 2TMS	Diketopiperazines	11.438	1523
Cyclo(Ile-Val), 2TMS	Diketopiperazines	11.657	1547
Cyclo(Gly-Pro), TMS	Diketopiperazines	13.186	1688
Myristic acid, TMS	Long-chain fatty acids	13.716	1765
Cyclo(Leu-Pro), TMS	Diketopiperazines	13.838	1791
Palmitic Acid, TMS	Long-chain fatty acids	14.510	1959
Stearic acid, TMS	Long-chain fatty acids	15.207	2153
Cyclo(Phe-Pro), TMS	Diketopiperazines	15.290	2176
Squalene	Triterpenoids	18.568	2773

Headspace VOCs produced by the bacterial strain were characterized by GC-MS using adsorbent cartridges, which allowed the identification of compounds actively released into the surrounding atmosphere during bacterial growth. A total of nine volatile compounds were identified (Table 4). According to the ChemOnt classification, the volatile profile was mainly composed of ketones, fatty alcohols, caprolactams, and alkanes, including both linear and branched hydrocarbons. The VOC profile was strongly dominated by the methyl ketone 2-undecanone, which showed the highest relative peak area, followed by 2-undecanol and tetradecane. Additional methyl ketones, including 2-nonanone and 2-decanone, were detected at lower relative abundances. Several medium- to long-chain hydrocarbons, such as tetradecane, hexadecane, and methyl-branched alkane derivatives, were also identified.

3.5. In Vivo Plant Growth-Promoting Activity of *Pseudomonas putida* V01

The biostimulant potential of *Pseudomonas putida* V01 was evaluated in wheat seedlings under greenhouse conditions. Thirty days after sowing, no significant differences were observed in all root parameters measured (i.e., root length, fresh and dry weight) between treated and untreated plants ($p > 0.05$; Figure S4). In contrast, bacterial treatment significantly enhanced shoot growth and physiological parameters (Figure 4). Compared with untreated controls, shoot length (SL) increased by approximately 25% (Figure 4A), whereas shoot fresh weight (SFW) and shoot dry weight (SDW) increased by 145% and 125%, respectively (Figure 4B,C). Furthermore, chlorophyll content, measured as SPAD values, increased by 59% following bacterial inoculation. The stronger effect observed on biomass accumulation and chlorophyll content than on shoot elongation suggests that *P. putida* V01 primarily promoted plant vigor and biomass production under the tested conditions.

Table 4. Volatile organic compounds (VOCs) identified in the headspace of *Pseudomonas putida* V01 cultures by GC-MS analysis. Compound annotation was performed by matching mass spectra against the NIST spectral library and verifying retention indices using Kovats index values. Chemical classification was assigned according to the ChemOnt taxonomy using the ClassyFire database. Relative peak area (%) was calculated by normalizing each compound's peak area to that of the most abundant compound (2-undecanone), which was assigned a value of 100. RT = retention time; RI = retention index.

Compound	Chemical Classification	Relative Peak Area (%)	RT (min)	RI
2-Nonanone	Methyl ketones	1.01	8.725	1308
2-Decanone	Methyl ketones	1.32	10.249	1424
Caprolactam	Caprolactams	1.74	11.105	1493
2-Undecanone	Methyl ketones	100	11.734	1556
2-Undecanol	Fatty alcohols	15.81	11.793	1562
Tetradecane	Alkanes	9.81	13.102	1681
3-methyl-pentadecane	Branched alkanes	1.94	15.217	2156
Hexadecane	Alkanes	2.92	15.557	2244
3-methyl-eptadecane	Branched alkanes	1.04	17.429	2607

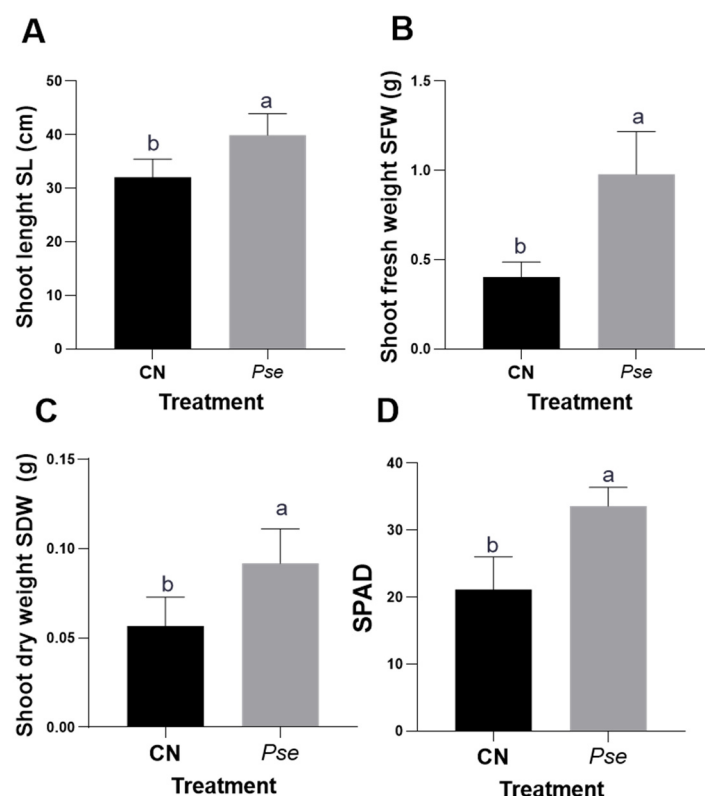


Figure 4. (A): Shoot length (SL), (B): Shoot fresh weight (SFW), (C): shoot dry weight (SDW) and (D): chlorophyll content expressed as SPAD values in wheat seedling treated with *Pseudomonas putida* V01 (Pse, grey bar) or untreated (CN, black bar), 30 days after sowing. Results are expressed as mean $\pm$ standard deviation of three replicates, four plants each. Different letters indicate significant differences between treatments according to Student's *t*-test ($p \leq 0.05$).

4. Discussion

Microbial inoculants are increasingly recognized as sustainable alternatives to synthetic agrochemicals because they can simultaneously suppress plant pathogens and enhance crop performance. In this study, a bacterial isolate obtained from the eggplant rhizosphere was identified as *Pseudomonas putida* V01 and exhibited both antifungal and

plant growth-promoting activities, supporting its potential application in sustainable crop management. The rhizosphere represents a highly dynamic and nutrient-rich ecological niche that hosts microbial communities, many of which possess beneficial properties. Among these, members of the genus *Pseudomonas* are widely recognized as effective plant growth-promoting rhizobacteria (PGPR) due to their ability to colonize plant roots, compete with phytopathogens, and produce several bioactive metabolites involved in plant nutrition, stress tolerance, disease suppression, and plant–microbe communication [6,7,9].

The cell-free culture filtrate of *P. putida* V01 displayed strong but differential antifungal activity against the tested pathogens, completely inhibiting *Sclerotium rolsii* while significantly reducing the growth of *Alternaria alternata* and *Fusarium proliferatum*. The observed broad-spectrum antifungal activity is consistent with previous studies describing the ability of *Pseudomonas* spp. to suppress phytopathogenic fungi through the production of antibiotics, lytic enzymes, siderophores, and volatile organic compounds. However, the magnitude of inhibition often varies among target pathogens, reflecting differences in fungal physiology, cell wall composition, and susceptibility to specific microbial metabolites [7,25,26]. The complete inhibition of *S. rolsii* observed in the present study is particularly noteworthy. This pathogen showed a high susceptibility to the diffusible metabolites produced by the strain, although the specific compounds responsible were not experimentally identified. Previous studies have shown that specific metabolites produced by *Pseudomonas* spp., including phenazines and lipopeptides, play a key role in suppressing hyphal growth and stem rot disease caused by *S. rolsii* [27]. Therefore, a similar involvement of such compound classes in our system is highly plausible, even if their specific roles remain to be functionally validated. Importantly, the comparison between volatile and diffusible fractions revealed a clear differential response of this fungus, as VOC exposure did not result in any detectable growth inhibition, indicating that the main antifungal activity against *S. rolsii* is associated with diffusible compounds.

In contrast, VOCs exerted selective action, significantly suppressing *Botrytis cinerea* and *A. alternata* while showing no efficacy against *S. rolsii*, *R. solani*, or *F. proliferatum*. This targeted activity is consistent with the recognized role of volatile metabolites produced by *Pseudomonas putida* in pathogen suppression [28]. However, since these data reflect the biological activity of whole volatiles, further studies will be required to attribute these effects to individual volatile compounds.

Interestingly, VOC exposure altered colony pigmentation and morphology in *F. proliferatum* despite having no significant effect on radial growth. This observation indicates that volatile metabolites may interfere with fungal developmental processes rather than directly inhibiting vegetative growth. Similar VOC-mediated modulation of fungal differentiation has been reported in several bacterial–fungal interaction systems [12,13,16]. Overall, these findings suggest that the antagonistic activity of *P. putida* V01 is pathogen-specific and relies on multiple mechanisms mediated by both diffusible and volatile metabolites.

Metabolomic profiling revealed a chemically diverse extracellular metabolome comprising diketopiperazines (DKPs), amino acid derivatives, phenazine-related compounds, fatty acids, terpenoid-like metabolites, and other bioactive molecules. These compound classes are commonly associated with microbial competition, signaling, and antimicrobial activity in rhizosphere environments. In particular, DKPs are widely recognized as multifunctional microbial metabolites involved in antimicrobial activity, interspecies communication, and quorum-sensing regulation [29–31]. Several DKPs were tentatively identified, including cyclo(Pro-Tyr), cyclo(Leu-Pro), cyclo(Phe-Pro), cyclo(Pro-Val), cyclo(Ala-Leu), cyclo(Ile-Val), and cyclo(Gly-Pro). Members of this compound family have been frequently associated with antimicrobial and antifungal activities and may collectively contribute to the antagonistic effects observed in vitro [32–36]. A metabolite tentatively annotated as py-

ocyanin was also detected in the crude extract. Although this annotation was not confirmed using authentic standards, phenazine compounds, a group of redox-active metabolites, are widely recognized for their role in microbial antagonism and may have contributed to the antifungal activity observed in this study [5,6]. Phenazine-producing *Pseudomonas* strains have previously been shown to suppress *S. rolfii* and other phytopathogenic fungi [27], which aligns with the chemical profile observed in the present study. However, its direct contribution to antifungal activity was not experimentally verified.

Headspace analysis showed that the volatilome was dominated by ketones and alcohols, particularly 2-undecanone and 2-undecanol. These compounds have been widely reported as important mediators of microbial antagonism and plant–microbe interactions, contributing to membrane disruption, growth inhibition, and signaling processes [12–15]. Additional VOCs, including 2-decanone, 2-nonanone, tetradecane, and hexadecane, have also been associated with antimicrobial activity, plant growth promotion, and the modulation of plant defense responses [13,17,19,37]. Although these compound-specific roles are supported by literature reports, the co-occurrence of multiple bioactive classes in our data suggests that the biological activity of *P. putida* V01 results from the combined action of several metabolites rather than from a single dominant molecule.

Beyond pathogen suppression, *P. putida* V01 significantly promoted wheat growth under greenhouse conditions. Inoculated plants exhibited increased shoot length, biomass accumulation, and chlorophyll content, whereas root growth parameters remained unaffected. The stronger effects observed on shoot biomass and SPAD values suggest that the strain primarily enhanced plant physiological performance and photosynthetic efficiency rather than root system development. Similar growth responses have been reported for plant growth-promoting rhizobacteria (PGPR), including *Pseudomonas* spp., which can improve plant vigor through multiple mechanisms such as phytohormone production, improved nutrient acquisition, modulation of plant metabolism, and emission of bioactive volatile organic compounds [8,9,18,19]. Although the absence of measurable effects on root growth may appear unexpected considering the rhizospheric origin of the isolate, PGPR-mediated growth promotion is highly strain- and host-dependent. Several *Pseudomonas* strains have been reported to enhance shoot growth and chlorophyll accumulation without inducing significant changes in root architecture [7–9]. Therefore, the marked increase in chlorophyll content observed in the present study suggests that *P. putida* V01 may have primarily influenced plant physiological processes related to photosynthetic efficiency and biomass production rather than directly stimulating root elongation or branching.

Taken together, these results indicated that *P. putida* V01 exhibited a dual mode of action, combining antifungal activity mediated by both diffusible and volatile metabolites with positive effects on plant growth. These combined activities are particularly relevant for sustainable agriculture, where microbial inoculants are increasingly expected to provide multiple agronomic benefits while reducing dependence on synthetic inputs [38–40]. From an applied perspective, the ability of a single microbial strain to combine antifungal activity with biostimulant effects represents a valuable attribute for the development of next-generation bioinoculants. The presented results highlight the usefulness of integrating biological assays with metabolomic approaches to better understand the mechanisms underlying beneficial plant–microbe interactions.

Some limitations of this study should nevertheless be acknowledged. Metabolite annotation was based on database matching of accurate mass data, thus remaining putative and requiring further structural confirmation. Furthermore, because no direct functional validation or bioassays with pure compounds were performed, the proposed relationships between specific metabolite classes and the observed biological effects should be interpreted as hypotheses rather than verified causal mechanisms. Further studies involving

structural confirmation, metabolite purification, and bioactivity-guided assays will be necessary to establish direct links between individual compounds and the observed biological effects. Moreover, the efficacy of microbial biocontrol agents under field conditions is often influenced by environmental variability and interactions within the native soil microbiome. Therefore, future studies should focus on validating the performance of *P. putida* V01 under greenhouse and field conditions, as well as exploring suitable formulation strategies to enhance strain stability, shelf life, and rhizosphere colonization capacity.

5. Conclusions

Pseudomonas putida V01 exhibited a multifunctional phenotype, combining pathogen-dependent antifungal activity with plant growth-promoting effects in wheat. Both diffusible metabolites and volatile organic compounds were involved in fungal suppression, with VOCs showing selective effects on fungal growth and development. Metabolomic profiling revealed a diverse set of putative secondary metabolites, including diketopiperazines, phenazine-related compounds, fatty acids, and volatile ketones and alcohols, indicating a chemically complex extracellular metabolome potentially associated with the observed biological activities. In addition, *P. putida* V01 significantly improved wheat shoot growth and chlorophyll content under greenhouse conditions, indicating enhanced plant physiological performance. Overall, these results support the potential of *P. putida* V01 as a promising candidate for sustainable crop protection and growth promotion. Future studies should focus on validating its performance under field conditions and on experimentally verifying the role of individual metabolites in mediating its biological activities.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/applmicrobiol6070074/s1>, Figure S1: Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequences showing the relationship between strain V01 and closely related members of the genus *Pseudomonas*. The tree was reconstructed using RAXML from aligned 16S rRNA gene sequences retrieved from the NCBI GenBank database. Strain V01 (highlighted in red) clustered within the *Pseudomonas putida* group together with reference strains of *P. putida*, *P. monteilii*, and *P. alloputida*, supporting its assignment to *Pseudomonas putida*; Figure S2: Representative images of the antifungal assays performed with the cell-free culture filtrate of *Pseudomonas putida* V01. (A) *Alternaria alternata* and (B) *Sclerotium rolfsii*. For each pathogen, the upper plate corresponds to the untreated control (CN), whereas the lower plate corresponds to the treatment with the cell-free culture filtrate of *P. putida* V01 (*Pse*). Photographs were taken after 3 days of incubation; Figure S3: Antifungal activity of volatile organic compounds (VOCs) produced by *Pseudomonas putida* V01 against phytopathogenic fungi in the overlapped plate assay. Mycelial growth diameter of (A) *Fusarium proliferatum*, (B) *Rhizoctonia solani*, and (C) *Sclerotium rolfsii* exposed to bacterial VOCs (*Pse*, grey bar) or grown in the absence of bacteria (CN, black bar). Data are expressed as mean $\pm$ standard deviation of three biological replicates. Different letters indicate significant differences between treatments according to Student's *t*-test ($p \leq 0.05$); Figure S4: A: Root length (RL), B: Root fresh weight (RFW), C: Root dry weight (RDW) in wheat seedling treated with *Pseudomonas putida* V01 (*Pse*, grey bar) or untreated (CN, black bar), 30 days after sowing. Results are expressed as mean $\pm$ standard deviation of three replicates, four plants each. Different letters indicate significant differences between treatments according to Student's *t*-test ($p \leq 0.05$).

Author Contributions: Conceptualization, S.L.W., M.L. and F.V.; methodology, S.P. and A.P.; investigation, A.P. and A.S.; data curation, A.P., G.I. and A.S.; writing—original draft preparation, A.P., G.I. and F.V.; writing—review and editing, A.P., G.I., A.S. and F.V.; project administration, F.V., S.L.W. and S.P.; funding acquisition, S.L.W., M.L., F.V. and S.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by Versalis S.p.A. within the PhD. program in ‘Sustainable agricultural and forestry systems and food security’—University of Naples Federico II.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors thank doctors Anna Gaspari, Carmen Scamardella and Sonia Marcone for their assistance during in vitro and in vivo experiments and during GC-MS analysis of volatile metabolites.

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

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