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# Enhancing *Botrytis* disease management in tomato plants: insights from a *Pseudomonas putida* strain with biocontrol activity

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## Abstract

**Aims:** This study explores the biocontrol potential of *Pseudomonas putida* Z13 against *Botrytis cinerea* in tomato plants, addressing challenges posed by the pathogen's fungicide resistance. The aims of the study were to investigate the *in vitro* and *in silico* biocontrol traits of Z13, identify its plant-colonizing efficacy, evaluate the efficacy of different application strategies against *B. cinerea in planta*, and assess the capacity of Z13 to trigger induced systemic resistance (ISR) in plants.

**Methods and results:** The *in vitro* experiments revealed that Z13 inhibits the growth of *B. cinerea*, produces siderophores, and exhibits swimming and swarming activity. Additionally, the Z13 genome harbors genes that encode compounds triggering ISR, such as pyoverdine and pyrroloquinoline quinone. The *in planta* experiments demonstrated Z13's efficacy in effectively colonizing the rhizosphere and leaves of tomato plants. Therefore, three application strategies of Z13 were evaluated against *B. cinerea*: root drenching, foliar spray, and the combination of root drenching and foliar spray. It was demonstrated that the most effective treatment of Z13 against *B. cinerea* was the combination of root drenching and foliar spray. Transcriptomic analysis showed that Z13 upregulates the expression of the plant defense-related genes *PR1* and *PIN2* upon *B. cinerea* inoculation.

**Conclusion:** The results of the study demonstrated that Z13 possesses significant biocontrol traits, such as the production of siderophores, resulting in significant plant protection against *B. cinerea* when applied as a single treatment to the rhizosphere or in combination with leaf spraying. Additionally, it was shown that Z13 root colonization primes plant defenses against the pathogen.

## Impact Statement

This study reveals the biocontrol potential of *P. putida* Z13 against *B. cinerea* in tomato plants, providing a promising alternative to fungicides. It was demonstrated that Z13 possesses a number of biocontrol traits that enable the strain to prime the defense mechanisms of tomato plants against *B. cinerea*.

**Keywords:** biological control; induced systemic resistance; *PR1*; *PIN2*; plant diseases

## Introduction

*Botrytis cinerea*, commonly known as gray mold, poses a significant threat to over 200 plant species (Williamson et al. 2007). The predominant approach to managing this pathogen involves the widespread use of chemical fungicides. Unfortunately, the efficacy of this method is hampered by the pathogen's notorious resistance to fungicides, a consequence of its high genetic variability. One prominent victim of *B. cinerea* is the tomato, susceptible to infection in both greenhouse and open field conditions. Under optimal disease environmental conditions (mild temperatures between 15°C and 20°C and relative humidity above 90%), tomato yield losses can soar to as much as 40% (Carisse and Van der Heyden 2015).

To address this challenge, extensive research efforts have been directed toward exploring biological control methods for managing *B. cinerea* in tomato plants. Various microorganisms have been identified as promising biocontrol agents (BCAs) against *B. cinerea*, including *Pseudomonas* (Wang et al. 2021a), *Bacillus* (Li et al. 2023), *Streptomyces* (Boukaew et al. 2017), *Trichoderma* (Wang et al. 2021b), and yeasts (Agarbati et al. 2022). Among the widely employed BCAs, *Bacillus* spp. and *Pseudomonas* spp. stand out for their broad-spectrum antifungal activity, featuring prominently in commercial biopesticides (Abbey et al. 2019, Bolivar-Anillo et al. 2020). Mechanisms such as induced systemic resistance (ISR) and competition for nutrients contribute to the disease-suppressing actions of BCAs through antibiosis (Elad 2003, Verhagen et al. 2010). Remarkably, the plant protective activity of these BCAs often rivals that of traditional fungicides (Janisiewicz and Jeffers 1997).

Despite the wealth of research on *B. cinerea* biocontrol, few studies have systematically compared the efficacy of root versus foliar versus combined root and foliar treatments with BCAs (Toral et al. 2020). Root treatments are considered more cost-effective under greenhouse conditions, avoiding unnecessary wetting of leaf surfaces and influencing the root microbiome positively (Cucu et al. 2020). Such treatments may enhance nutrient uptake, combat soil pathogens, and trigger ISR against leaf pathogens (Jaiswal et al. 2020). In a notable study evaluating both foliar and root applications of *Bacil-*

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*lus velezensis* XT1 against *B. cinerea* in tomato plants, both treatments effectively reduced disease severity by triggering ISR (Toral et al. 2020). The significance of ISR as a mode of action against *B. cinerea* has been emphasized for various BCAs, including several *Pseudomonas* species (Zhu et al. 2021, Nguyen et al. 2022), though the impact of BCA application site on disease suppressiveness and defense response triggering remains largely unexplored.

Considering the aforementioned aspects, this study aimed to explore the biocontrol efficacy of the *Pseudomonas putida* Z13 strain against *B. cinerea* in tomato plants. Originally isolated from the rhizosphere of eggplants in soil with *Verticillium* wilt suppressive properties, strain Z13 has previously demonstrated protective effects on plants against *Verticillium dahliae* (Ziazia et al. 2021). The specific objectives were to: (i) assess Z13's ability to inhibit *B. cinerea* growth *in vitro*, while also evaluating its production of siderophores and its swarming and swimming capabilities; (ii) identify potential biocontrol traits encoded in the genome of Z13; (iii) monitor the persistence of Z13 in tomato leaves and the rhizosphere, (iv) evaluate Z13's biocontrol effectiveness against *B. cinerea* in tomato plants through root drenching and/or foliar spray application, and (v) observe the expression levels of plant defense-related genes *PR1* and *PIN2*, recognized markers of salicylic acid (SA) and ethylene/jasmonic acid (ET/JA)-dependent defense mechanisms, respectively, following Z13 and *B. cinerea* inoculation.

Therefore, the combined *in vitro* and *in silico* analyses will uncover the biocontrol capabilities of Z13. Additionally, tracking the population dynamics of Z13 in both leaves and the rhizosphere will guide the formulation of an effective application strategy for *in planta* experiments. Furthermore, monitoring the expression levels of *PR1* and *PIN2* provides valuable insights into the plant protective activity of Z13 against *B. cinerea*.

## Materials and methods

### Microbial isolates

The experiments utilized a *B. cinerea* isolate known for its pathogenicity against tomato plants (Kalogiannis et al. 2006). Cryopreservation of the fungal strain involved freezing a conidial suspension in 25% aqueous glycerol at −80°C. Prior to use, the fungus was transferred to potato dextrose agar (PDA) (Merck, Darmstadt, Germany) and incubated at 24°C for 5 days.

A spontaneously generated Z13 rifampicin-resistant mutant was employed throughout the experiments. The Z13 strain was cryopreserved by freezing a suspension of $10^8$ colony-forming units (cfu) $ml^{-1}$ in 25% aqueous glycerol at −80°C. Before utilization, Z13 was transferred to nutrient broth agar plus glycerol (NAG) and incubated at 28°C for 2 days.

### *In vitro* tests for Z13 biocontrol traits

The assessment of Z13 *in vitro* antagonism against *B. cinerea* followed the method outlined by Reddy and Patrick (1992). Briefly, Z13 was streaked ∼2.5 cm from the edge of a Petri dish (9 cm in diameter) containing PDA and allowed to grow for 48 hours. A 5-mm diameter disc taken from the edge of an actively growing colony of *B. cinerea* on PDA was then placed on the Petri dish, 2.5 cm from the edge opposite to Z13. The Petri dishes were incubated in the dark for up to 7 days at 25°C, and the growth of *B. cinerea* was quantified using ImageJ 1.46r software (http://imagej.nih.gov/ij).

For swimming and swarming motility assays, Z13 was grown for 5 days in a Petri dish with 1.5% yeast extract mannitol (YEM) agar medium at 28°C. An individual Z13 colony was selected and inoculated in the middle of a new Petri dish with 20 ml of 0.3% water–agar plus 10% YEM for swimming assays or 0.5% water agar plus 10% YEM for swarming assays. The extent of motility diameter was observed after 5 days of inoculation (Vicario et al. 2015).

The siderophore production assay employed M9 minimal medium supplemented with Chrome Azurol S (CAS). To prepare the medium, M9 minimal medium was first autoclaved and then supplemented with filter-sterilized, prewarmed $MgSO_4$ (2 mM final), $CaCl_2$ (0.1 mM final), sucrose (0.2% final), and Casamino acids (0.9% final). The CAS solution, following the protocol by Schwyn and Neilands (1987), was autoclaved separately and later combined with the M9 minimal medium before conducting the assay. Following an incubation period of 5 days at 28°C, the appearance of an orange coloration around a Z13 colony indicated siderophore production.

All *in vitro* experiments were conducted with a completely randomized design, featuring three replications and repeated twice.

### Z13 genome mining

The genome sequence of Z13 used in this study had been assembled and deposited in GenBank under the accession number: JAINFM000000000 (Ziazia et al. 2021). The DNA libraries were prepared using Ion Torrent technology and Ion Chef workflows, followed by sequencing with the S5XLS system. Analysis of primary data was conducted with Ion Torrent Suite, and *de novo* genome assembly was performed with SPAdes software, obtaining a genome completeness of 99.4%. Phylogenetic analysis showed that Z13 belongs to the species *P. putida* (Ziazia et al. 2021).

Genome comparisons were conducted using the BRIG (BLAST Ring Image Generator) program version 0.95 (Alikhan et al. 2011), utilizing the blastn option. The genome sequence of *P. putida* KT2440 served as the reference sequence, while the genome sequences of Z13 and other *Pseudomonas* strains previously reported for biocontrol activity (Table 1) were used as query sequences.

Mining for orthologous genes in the genome of Z13 was performed using the Basic Local Alignment Search Tool through protein–protein comparison (Camacho et al. 2008). Genes were considered orthologs when the e-value was $< 10^{-5}$. The genome was also screened for known secondary metabolite biosynthetic gene clusters (BGCs) using AntiSMASH 2.0 (Blin et al. 2013), employing a relaxed detection parameter. The KnownClusterBlast feature was used to compare the clusters of Z13 against the manually curated set of known BGCs from the MIBiG database.

### Monitoring of Z13 persistence in rhizosphere and leaves

The rhizosphere and leaf populations of Z13 were recorded at 3 and 10 dpi. To estimate rhizosphere populations, 2 g of rhizosphere soil (soil particles in close contact with roots within a distance of ∼1–5 mm) were collected from five plants per sam-

**Table 1.** Strains of *Pseudomonas* species used for comparative genomic analysis.

| Strain name | Biocontrol activity/plant growth promoting rhizobacterium (PGPR) | Genome size (bp) | GenBank accession number | References |
|---|---|---|---|---|
| *P. putida* Z13 | Biocontrol of *Verticillium* wilt in eggplants | 6.133.624 | JAINFM01 | Ziazia et al. (2021) |
| *P. putida* KT2440 | Induction of systemic resistance | 6.181.873 | AE015451.2 | Nelson et al. (2002) |
| *P. fluorescens* SBW250 | PGPR | 6.722.539 | AM181176.4 | Silby et al. (2009) |
| *P. simiae* WCS417 | Biocontrol of *Fusarium* wilt of carnation | 6.169.071 | CP007637.1 | Van Peer et al. (1991) |
| *P. protegens* Pf-5 | Biocontrol of *B. cinerea* through antibiotics production | 7.074.893 | CP000076.1 | Paulsen et al. (2005) |
| *P. aeruginosa* M18 | PGPR and biocontrol activity through antibiotics production | 6.327.754 | CP002496.1 | Wu et al. (2011) |

pling day. The collected soil was shaken for 45 min in 50 mM phosphate buffer at pH 7.0 containing 0.02% Tween 20. The suspension was then plated onto NAG medium supplemented with cycloheximide (100 $\mu$g ml$^{-1}$) and rifampicin (100 $\mu$g ml$^{-1}$). After 48 hours of incubation at 28°C, the number of bacterial cfu g$^{-1}$ of rhizosphere soil was determined.

The Z13 leaf population was determined by collecting leaf disks (1 cm in diameter) from the top, middle, and bottom parts of leaves from five plants per sampling day (3 and 10 dpi). The leaf disks were homogenized in 50 mM phosphate buffer at pH 7.02, and appropriate 10-fold dilutions were plated on NAG medium containing 100 $\mu$g ml$^{-1}$rifampicin and 100 $\mu$g ml$^{-1}$ cycloheximide. The plates were incubated at 28°C for 48 hours, and the bacterial population size cfu cm$^{-2}$ of leaves was determined.

The plant growth conditions, Z13 growth and application were consistent with the *in planta* biocontrol experiments. The experiment was repeated three times, and samples were collected from five plants per sampling day.

## *In planta* biocontrol experiments

For the *in planta* biocontrol experiments, the Z13 isolate was cultivated in a nutrient broth and glycerol (NG) liquid culture, agitated in an orbital incubator at 28°C for 48 hours. Suspensions were then centrifuged at 5600 *g* and 20°C for 10 min. The resulting pellets were resuspended by vortexing in a 50-mM phosphate buffer at pH 7.02 before being used to treat the plants. The BCA isolate, Z13, was applied to 20-day-old tomato plants (cv Moneymaker) individually grown in plastic pots (9 cm × 9 cm × 10 cm) containing soil substrate (pH 6.0, black peat; Potground P; Klasmann), which had been autoclaved twice within a 24-hour interval for 1 hour at 121°C and 1.2 atm. Application of the Z13 strain was carried out through three methods: (i) drenching each plant with 10 ml of a $10^8$ cfu ml$^{-1}$ cell suspension; (ii) leaf spraying until runoff ($10^8$ cfu ml$^{-1}$); and (iii) or applying both treatments (root drenching and foliar spraying). Five days later, ten microliters of *B. cinerea* spores $5 \times 10^4$ spores ml$^{-1}$ (spores were harvested in sterile distilled water from a 10–12 days old culture in PDA plates and diluted in a quarter-strength potato dextrose broth) were placed on two leaflets per plant (10 plants per treatment per replication). Plants were kept for 5 days under high humidity (>90%) at 20 ± 3°C with a 16-hour light and 8-hour dark cycle. At 5 days postinoculation (dpi), the lesion spots were photographed for quantifying the lesion development using ImageJ 1.46r software (http://imagej.nih.gov/ij). The experiment was replicated three times, with a total of 30 plants per treatment.

## RNA isolation and quantitative PCR (qPCR) determination of *PR1* and *PIN2* transcript levels

For RNA analysis, the leaflets of five tomato plants from each treatment (mock; root drenching Z13, RDZ13; leaf spraying Z13, LSZ13; RDZ13/LSZ13; *B. cinerea*, Bc; RDZ13 + Bc; LSZ13 + Bc; RDZ13/LSZ13 + Bc) and experimental replication (a total of three replications) were collected and pooled into one sample at 1 and 3 dpi (after *B. cinerea* inoculation). The collected leaflets were ground to a fine powder using an autoclaved mortar and pestle, in the presence of liquid nitrogen, and stored at −80°C. Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen, Paisley, Renfrewshire, UK), following the manufacturer's instructions.

The RNA samples were treated with DNase I (Invitrogen), and their concentration was measured using a Nanodrop ND-1000 spectrophotometer (Saveen Werner, Malmo, Sweden). The RNA samples were treated with DNase I (Invitrogen) to eliminate traces of contaminating genomic DNA. The RNA concentration was measured on a Nanodrop ND-1000 spectrophotometer (Saveen Werner, Malmö, Sweden). First-strand cDNA was synthesized using SuperScript II (Invitrogen), following the manufacturer's procedure. The expression levels of *PR1* and *PIN2* were detected using the following primer sequences: for *PR1* (M69247), forward 5′-GAGGGCAGCCGTGCAA-3′ and reverse 5′-CACATTTTTCCACCAACACATTG-3′ (Block et al. 2005) and for *PIN2* (JN091682), forward 5′-TGATGCCAAGGCTTGTACTAGAGA-3′ and reverse 5′-AGCGGACTTCCTTCTGAACGT-3′ (Herman et al. 2008). qPCRs were performed in an Applied Biosystems StepOne Plus thermocycler and, for the amplification reactions, FastGene IC Green qPCR universal mix (NIPPON Genetics EUROPE GmbH) was used. The results were analysed with StepOne v.2.3 qPCR software. PCR cycling started with an initial step of denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. PCR efficiency for each amplicon was calculated by employing the linear regression method on log (fluorescence) per cycle number data, using Lin-Reg PCR software (Ramakers et al. 2003). The quantitative PCRs (qPCRs) were performed in duplicate. The absence of nonspecific products and primer dimers was confirmed by analyzing the melting curves. The expression levels of the actin gene (XM004249818), detected using the primer pair forward 5′-TTGCCGCATGCCATTCT-3′ and reverse 5′-TCGGTGAGGATATTCATCAGGTT-3′ (Herman et al. 2008), were used as an internal standard to normalize small differences in cDNA template amounts. For data analysis, average threshold cycle (Ct) values were calculated for each gene of interest based on three independent biological samples.

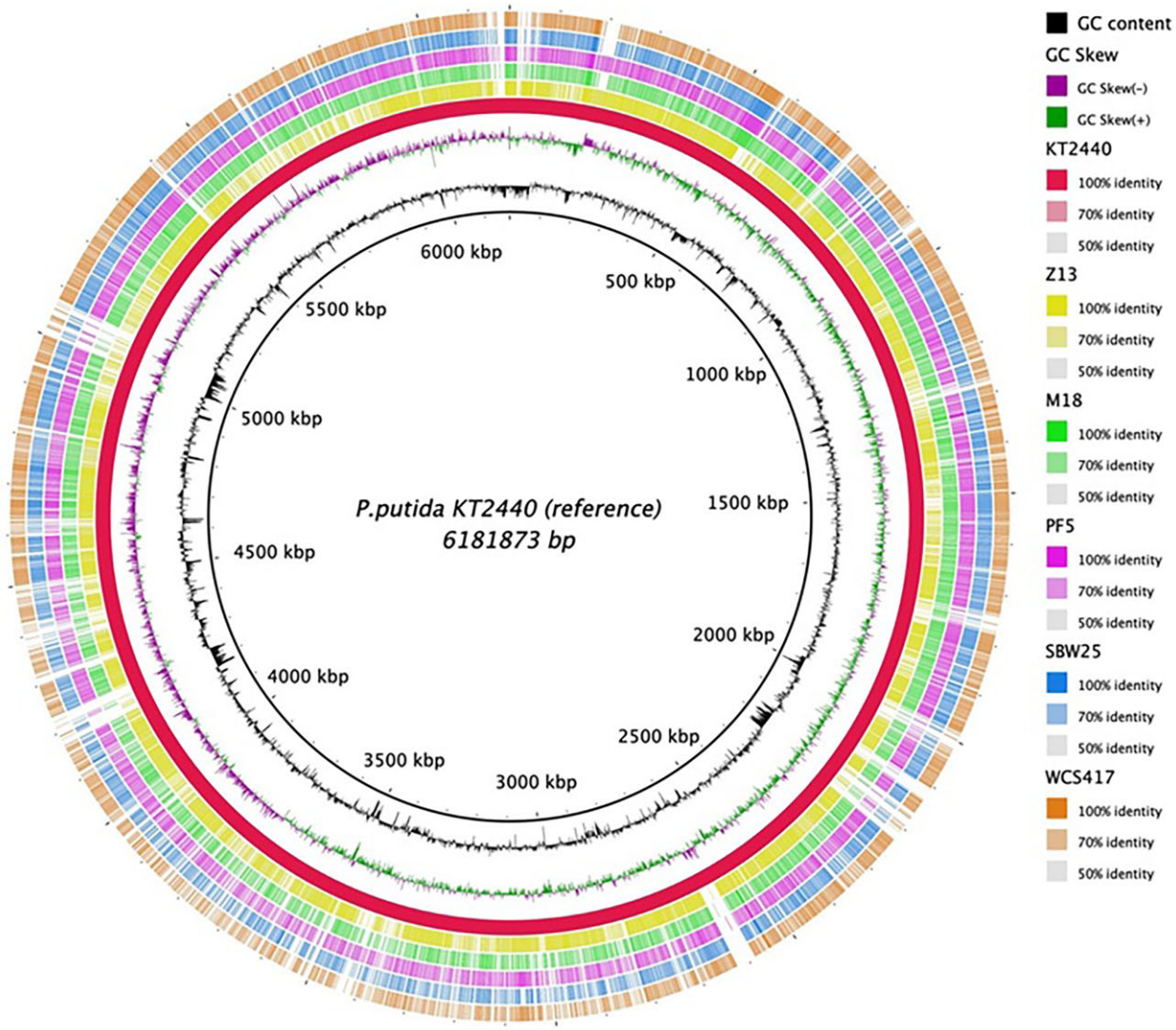


**Figure 1.** Circular representation of the whole-genome sequence of *P. putida* Z13 compared to *P. putida* KT2440, *P. fluorescens* SBW250, *P. simiae* WCS417, *P. protegens* Pf-5, and *P. aeruginosa* M18. The inner ring portrays the reference KT2440 genome with corresponding genetic coordinates. The colored rings (from inner to outer ring) portray: GC content, GC skew, and whole genome sequences of strains KT2440, Z13, M18, Pf-5, SBW250, and WCS417.

### Statistical analysis

Data on *B. cinerea* leaf lesions and gene expression were subjected to analysis of variance (ANOVA). When a significant ($P < .05$) F test was obtained for treatments, data were subjected to means separation by least significant difference (LSD) test.

## Results

### *In vitro* tests for Z13 biocontrol traits

The *in vitro* tests revealed that Z13 inhibits the growth of *B. cinerea* by *ca.* 40%, produces siderophores, and exhibits both swimming and swarming motility (Fig. S1). Therefore, Z13 possesses interesting traits for the biocontrol of *B. cinerea*. Siderophores may exert a direct and/or indirect action against the pathogen by inducing ISR in plants, while swimming and swarming demonstrate Z13's potential to colonize plant surfaces.

### Z13 genome mining and ISR triggering traits

BRIG analysis facilitated circular genome visualization and comparison between the whole genome sequences of Z13 and other *Pseudomonas* strains (Fig. 1). The analysis indicated a close genetic relatedness of the strains belonging to the *Ps. putida* group and demonstrated that most regions within their genomes were conserved. Subsequently, BlastP analysis revealed that the genome of Z13 has genes homologous to the gene cluster responsible for pyrroloquinoline quinone (PQQ) biosynthesis with 82%–96% identity (Figs 2 and 3; Tables S1 and S2). PQQ is an antioxidant and a plant growth-promoting substance. Additionally, several genes associated with lipopolysaccharide (LPS) biosynthesis were identified in the Z13 genome, as revealed by BlastP analysis against the NCBI database (Table 2). Besides the PQQ biosynthesis cluster, other genes that could potentially be involved in the synthesis of compounds with broad-spectrum antibiotic activity could not be identified in the genome of Z13. For instance, biosynthesis genes for the antimicrobial com-

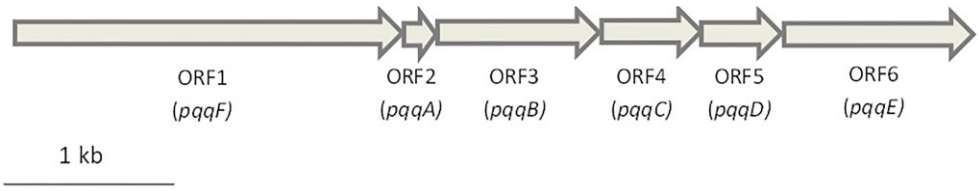


**Figure 2.** Genetic organization of the putative PQQ biosynthesis cluster on the genome of *P. putida* Z13.

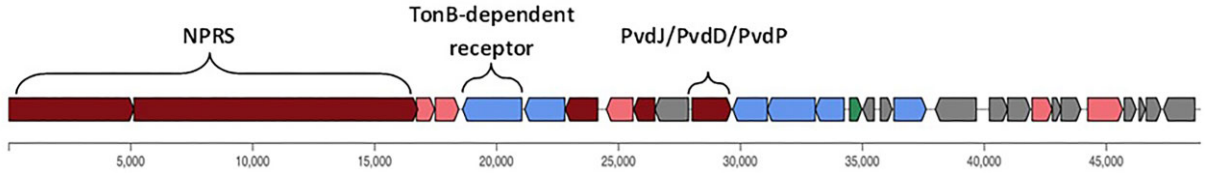


**Figure 3.** Pyoverdine BGC in the genome of *Ps. putida* Z13 as identified by AntiSMASH.

**Table 2.** Compilation of genes identified within the LPS biosynthetic cluster of strain *P. putida* Z13.

| Gene | Locus tag | Putative function | Structural domain |
|---|---|---|---|
| *lpxA* | K7A70_23045 | UDP-*N*-acetylglucosamine acyltransferase | Lipid A biosynthesis |
| *lpxC* | K7A70_01135 | UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase | |
| *lpxD* | K7A70_19830 | DP-3-O-(3-hydroxymyristoyl)glucosamine *N*-acyltransferase | |
| *lpxK* | K7A70_12705 | Tetraacyldisaccharide 4′-kinase | |
| *lpxB* | K7A70_19815 | Lipid-A-disaccharide synthase | |
| *lpxO* | K7A70_08370 | Lipid A hydroxylase | |
| *waaA* | K7A70_23045 | Lipid IV(A) 3-deoxy-D-manno-octulosonic acid transferase | Core oligosaccharide biosynthesis |
| *waaC* | K7A70_07530 | Lipopolysaccharide heptosyltransferase I | |
| *waaF* | K7A70_07535 | Lipopolysaccharide heptosyltransferase II | |
| *rfaq* | K7A70_07515 | Heptose kinase | |
| *inaA* | K7A70_07510 | Lipopolysaccharide kinase family protein | |
| *rfaP* | K7A70_07520 | Lipopolysaccharide core heptose(I) kinase | |
| *waaL* | K7A70_23090 | O-antigen ligase family protein | O-antigen chain biosynthesis |

pounds 2,4-diacetylphloroglucinol, phenazines and pyrrolnitrin, which are abundantly present in root-associated *Pseudomonas* strains (Loper et al. 2012), are not present in the genome of Z13. Furthermore, we utilized the antiSMASH algorithm to identify potential BGCs containing genes corresponding to secondary metabolites. In this analysis, antiSMASH predicted in the genome of Z13 the occurrence of BGCs with homology to the siderophores pyoverdine and crochelin A BGCs (Table S3), which is in agreement with the observed siderophore production under *in vitro* conditions (Fig. S1).

## *Pseudomonas putida* Z13 reduces *Botrytis* symptoms in tomato plants and colonizes effectively rhizosphere and leaves

The effectiveness of three application methods of *P. putida* Z13 against *B. cinerea* on tomato plants—root drenching, foliar spraying, and their combination—was assessed. The results indicated that the most effective treatment was the combination of root drenching and foliar spraying, followed by root drenching alone, whereas the application of Z13 as a foliar spray did not reduce disease severity compared to controls (Fig. 4). Consequently, it was inferred that Z13 does not exhibit a direct action against *B. cinerea* beyond its observed *in vitro* antibiotic activity against the pathogen (Fig. S1a). Nevertheless, foliar spray provided an additive effect on the plant protective activity of Z13 as a root-drenching bioinoculum, as evidenced by lower disease severity in the combination of foliar spraying and root drenching compared to the single treatment of root drenching.

In the same experiments, the population of Z13 in the rhizosphere and leaves was examined. It was revealed that Z13 persisted in the rhizosphere and leaves of the plants throughout the experimental period. The rhizosphere population was in the magnitude of $10^5$ cfu $g^{-1}$ of rhizosphere, and the leaf population was $10^4$ cfu $cm^{-2}$ at 3 and 10 dpi (Fig. 5). This result is consistent with the observed *in vitro* swimming and swarming motility of Z13 (Fig. S1). Therefore, it can be concluded that Z13 can effectively colonize the rhizosphere and leaves, and the lack of plant protective activity against *B. cinerea* when applied as a foliar inoculum, compared to root drenching, cannot be attributed to inconsistent colonization of Z13 on leaves.

## Expression of the defense related genes *PR1* and *PIN2*

The results of the pathogenicity experiment and the monitoring of the Z13 population in the rhizosphere and leaves suggest that the triggering of ISR is the main mode of action of Z13 against *B. cinerea* in tomato plants. Therefore, we examined the expression levels of *PR1* and *PIN2*, marker genes of SA and ET/JA dependent defenses, respectively, in Z13-treated tomato plants upon *B. cinerea* inoculation.

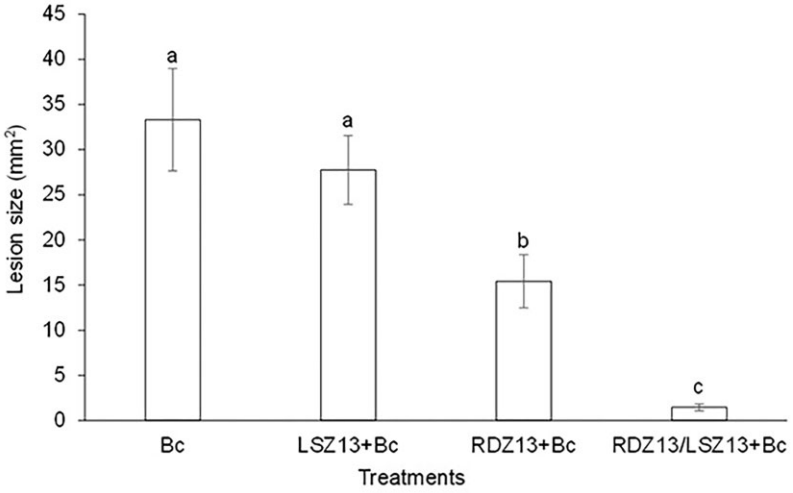


**Figure 4.** Lesion size ($mm^2$) of *B. cinerea*, Bc, on leaflets of tomato plants (cv Moneymaker) root drenched (RDZ13 + Bc) or/and leaf sprayed (LSZ13 + Bc; RDZ13/LSZ13 + Bc) with *P. putida* Z13, at 5 days after pathogen inoculation. The columns represent the means of three biological repeats with 20 leaflets (two leaflets per plant) per treatment and repeated experiments ($n = 60$). Columns with different letters are significantly different from each other, according to LSD multiple range tests ($F_{3, 236} = 92$, $P < .001$). The vertical bars indicate the values of the standard error.

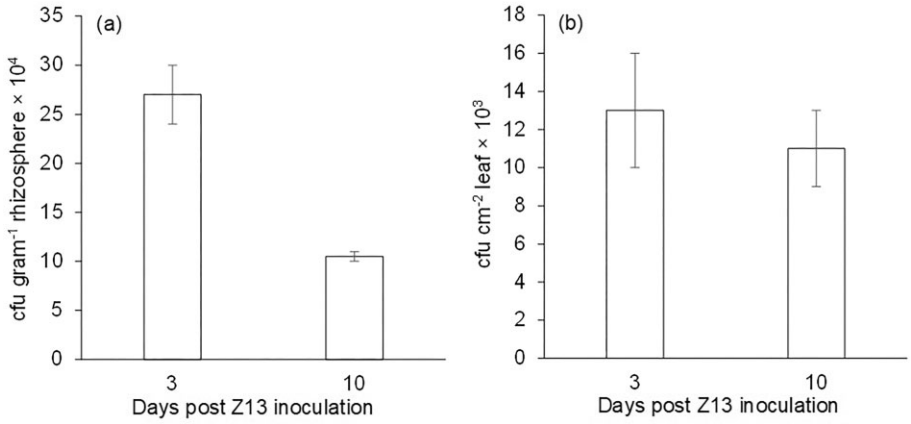


**Figure 5.** Rhizosphere (a) and leaf (b) population of *P. putida* Z13 at 3 and 10 days after application by root drenching (a) and leaf spraying (b) at tomato plants cv Moneymaker. The columns represent the means of three biological repeats ($n = 3$). The vertical bars indicate the values of the standard error.

The transcriptomic analysis revealed that both genes, *PR1* and *PIN2*, were downregulated in the absence of the pathogen, except in the case of *PR1* in plants where Z13 was applied as a foliar spray and root-drenching inoculum (Fig. 6). Upon pathogen inoculation, *PIN2* was upregulated in the tomato plants that were root-drenched with Z13 as a single treatment or in combination with foliar spray at 1 dpi (Fig. 6a). However, the expression of *PIN2* was downregulated in all treatments at 3 dpi (Fig. 6b).

The expression of *PR1* was upregulated in the pathogen-inoculated tomato plants that were foliar sprayed with Z13 as a single treatment or in combination with root drenching at 1 dpi (Fig. 6c). Similar to the case of *PIN2*, the expression of *PR1* was downregulated in all treatments at 3 dpi, except in the case of the control pathogen-inoculated plants (Fig. 6d). Therefore, the expression of *PR1* and *PIN2* exhibited an opposite trend between the Z13 treatments upon *B. cinerea* inoculation at 1 dpi. It can be suggested that the expression of *PR1* and *PIN2* is influenced by the plant site of Z13 inoculation; where foliar application of Z13 results in the upregulation of *PR1*, and root application of Z13 results in the upregulation of *PIN2*.

## Discussion

*Botrytis cinerea* is one of the most significant plant pathogens undermining tomato production worldwide. Despite the availability of various chemical disease management strategies, the ease with which *B. cinerea* develops resistance to fungicides poses a major obstacle in disease management. Therefore, the use of BCAs becomes a significant alternative to fungicides for managing *Botrytis* disease.

In our study, we explored the biocontrol activity of the BCA, *P. putida* Z13, against *B. cinerea* in tomatoes. For this purpose, we evaluated the efficacy of Z13 in reducing *Botrytis* infection through root drenching and/or foliar spray applications. It was observed that the combination of foliar spray and root drenching was the most efficacious treatment. Interestingly, the application of Z13 as a root drenching treatment reduced *Botrytis* symptoms, whereas the foliar treatment was ineffective. Monitoring the Z13 leaf population revealed its efficacy in colonizing leaf surfaces; therefore, it can be ruled out that Z13 foliar application was ineffective against *B. cinerea* due to its inefficacy to colonize leaves. It is also evident that the *in vitro* observed antibiotic activity of Z13 against *B. cinerea*

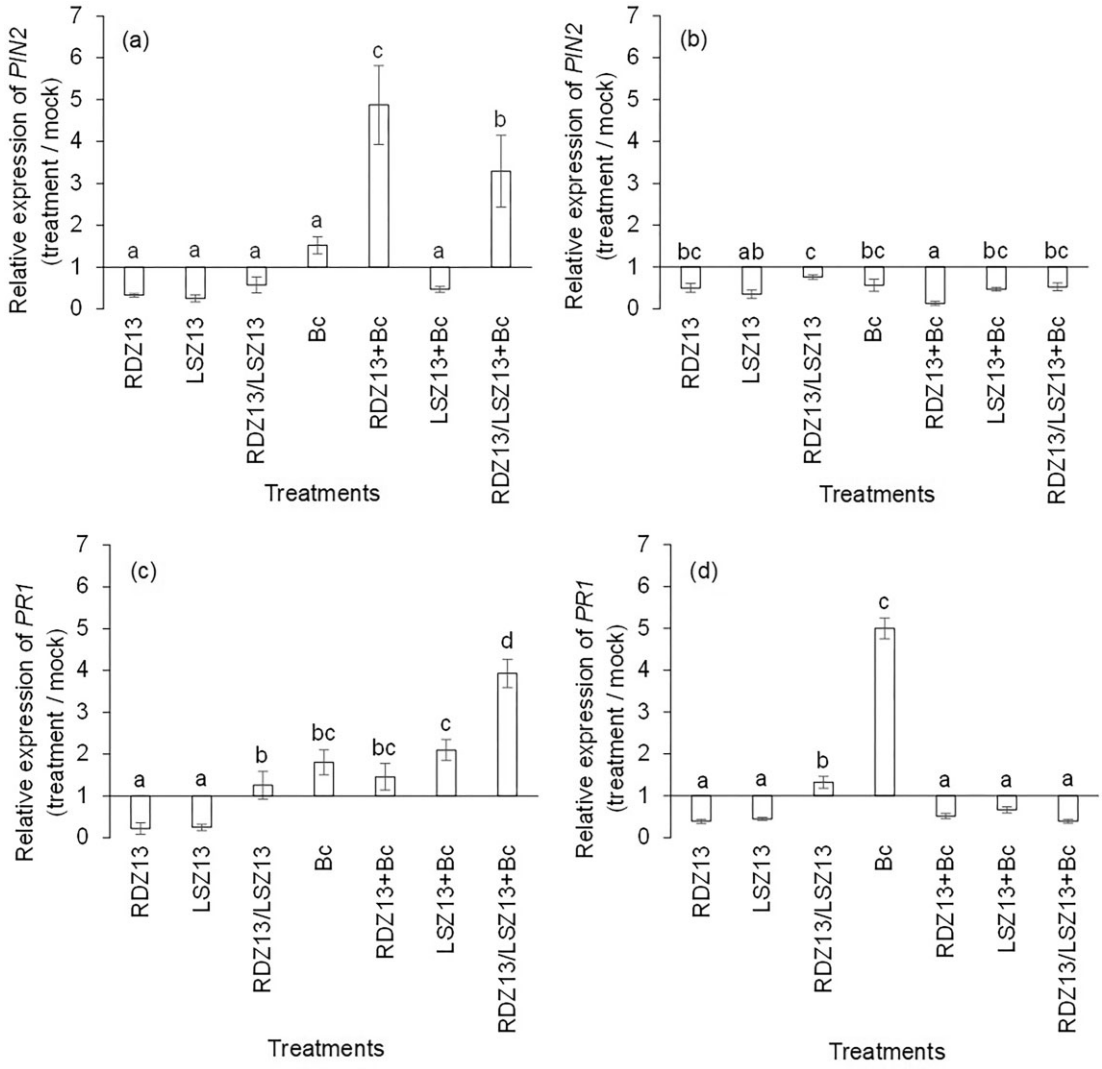


**Figure 6.** Fold changes in relative transcript abundance of the *PIN2* (a) and (b) and *PR1* (c) and (d) of tomato plants (cv Moneymaker) root drenched (RDZ13) or/and leaf sprayed (LSZ13; RDZ13/LSZ13) with *P. putida* Z13 and challenged inoculated with *B. cinerea* (Bc), at 1 (a) and (c) and 3 (b) and (d) days after pathogen inoculation. Transcript levels of the examined genes were normalized to the expression of *ACTIN* (XM004249818) gene measured in the same samples and expressed relative to the normalized transcript levels in mock-inoculated plants. Each column represents average data with SE from three independent biological samples (leaf tissues from five plants per biological sample). At each sampling day, columns with different letters are statistically different according to LSD multiple range (*PIN2* $F_{6,\,14} = 13.18$, $P < .001$ at 1 dpi, $F_{6,\,14} = 3.11$, $P < .05$ at 3 dpi; *PR1* $F_{6,\,14} = 22.06$, $P < .001$ at 1 dpi, $F_{6,\,14} = 200.59$, $P < .001$ at 3 dpi).

was not reflected *in planta*. This is not surprising since microbial antibiotic production depends on various external factors such as pH, oxygen, and water activity (Raaijmakers et al. 2002). Overall, the efficacy of Z13 to significantly reduce *Botrytis* symptoms when applied as a root drenching treatment suggests that Z13 triggers ISR. Likewise, the plant protective activity of the *P. putida* strains BTP1 and WCS358 against *B. cinerea* has been attributed to the triggering of ISR. Bacterial determinants of WCS358 involved in inducing systemic resistance in *Arabidopsis* are the fluorescent siderophore pseudobactin and the O-antigenic side chain of the LPS, since LPS and pseudobactin mutants had lost their ability to induce resistance against *B. cinerea* in tomato plants; also, LPS and pseudobactin isolated from WCS358 triggered ISR (Meziane et al. 2005).

The bioinformatics analysis of the Z13 genome revealed the existence of genes involved in ISR activation. These genes are associated with the biosynthesis of LPS, the siderophore pyoverdine and PQQ (Fig. 7). The characterization and description of the LPS biosynthetic cluster in novel BCAs like Z13 is important due to the significant variability observed in LPS clusters among different *Pseudomonas* strains (Jayaraman et al. 2020). This variability plays a role in the differential effectiveness of ISR inducibility. For example, in radish, purified LPS of the *Pseudomonas* model strains WCS417 and WCS374 triggered ISR, whereas that of WCS358 did not, indicating strain-specific differences in LPS (Leeman et al. 1995). The ISR triggering activity of LPS is also influenced by element availability in the BCA vicinity, since the O-antigenic side chains of the LPS of both *P. fluorescens* WCS417 and *P. fluorescens* WCS374 were major determinants of ISR against *Fusarium* wilt in radish under iron-replete conditions, but not under iron-limiting conditions (Meziane et al. 2005). Unknown iron-regulated factors in WCS374 and WCS417 caused ISR un-

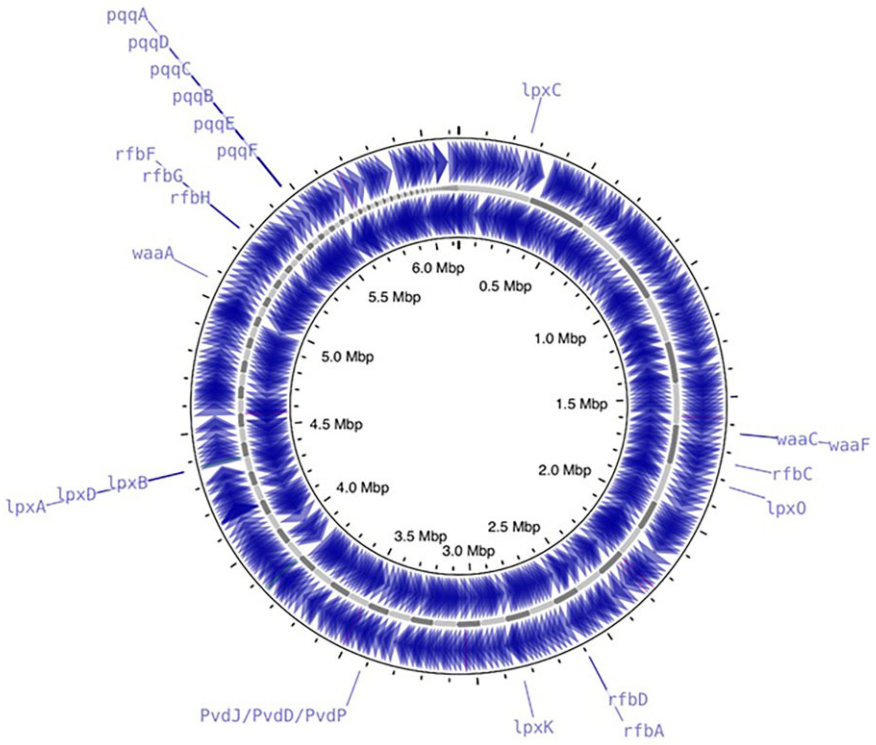


**Figure 7.** Genetic potential of strain *P. putida* Z13, showed in genes involved in the biosynthetic clusters of PQQ (*pqqA, pqqB, pqqC, pqqD, pqqE*, and *pqqF*), pyoverdine (*PvdD, PvdJ*, and *PvdP*) and LPSs (*rfbA, rfbC, rfbD, rfbF, rfbG, rfbH, waaA, waaC, waaF, lpxA, lpxB, lpxC, lpxD, lpxK*, and *lpxO*). Circular genome visualization was created with Proxee (Grant et al. 2023).

der low-iron conditions. Likewise, in *Ps. putida* BTP1, an unknown iron-regulated metabolite Cx appeared to be responsible for ISR in beans against *B. cinerea* (Ongena et al. 2002). Under iron-limiting conditions, it can be suggested that siderophores like pyoverdine trigger ISR. Pyoverdine is a major class of siderophores synthesized by fluorescent strains of *P. putida*, *P. syringae*, and *Pseudomonas aeruginosa* (Bouizgarne 2013) and has a high affinity for Fe(III) (Fe-pyoverdine, K = 1032) (Meyer et al. 1996). Moreover, pyoveridin has been highly related to ISR since it has been proven that repression of its synthesis increases plant susceptibility to *B. cinerea* (Trapet et al. 2016). In addition, purified pyoverdines from *P. putida* WCS358 were efficient in inducing ISR against *B. cinerea* in beans and tomatoes (Meziane et al. 2005). Accordingly, De Vleesschauwer et al. (2008) demonstrated that pyoverdine was the determinant responsible for *P. fluorescens* WCS374-induced ISR in rice against the leaf blast pathogen *Magnaporthe oryzae*. Investigations in tobacco cell suspensions indicated that the pyoverdine siderophores from *P. putida* WCS358 and *P. fluorescens* WCS417 strains are perceived by cells and mediate defense-related early signaling events (Van Loon et al. 2008). Additionally, the analysis of Z13 genome revealed the presence of the antioxidant compound PQQ, which is also known as an inducer of ISR in tobacco and rice against *Rhizoctonia solani* (Peng and Zhang 2022). PQQ may act in combination with other compounds like pyoverdine, as it has been reported in the case of pyocyanin which, in combination with SA or the SA-containing siderophore pyochelin, produced by *P. aeruginosa* 7NSK2, acts as determinants for induced resistance against *B. cinerea* (Audenaert et al. 2002).

The transcriptomic analysis of *PIN2* and *PR1*, marker genes of the ET/JA and SA pathway, respectively, revealed their upregulation in the Z13-treated plants only after *B. cinerea* application at 1 dpi. This suggests a priming effect where various induced resistance phenomena are associated with an enhanced capacity for the rapid and effective activation of cellular defense responses, which are induced only after contact with a challenging pathogen (Conrath et al. 2002). The plant defense responses are mainly comprised of two main distinct pathways regulated by SA and ET/JA. These pathways act antagonistically to each other, where the SA pathway is linked to defense responses against biotrophs and the ET/JA pathway is linked to defense against necrotrophs (Spoel et al. 2007); however, it has been reported that SA and JA signaling both provide resistance to *B. cinerea* and are not in antagonism (Zhang et al. 2017). Indeed, in our experiments, the expression of *PR1* and *PIN2* was simultaneously upregulated in the most efficacious treatment (application of Z13 in roots and leaves) against *B. cinerea*. Nevertheless, the pathogenicity and transcriptomic results suggest the importance of *PIN2* and consequently of the ET/JA-dependent defenses since *PIN2* upregulation was observed in the plant-protective treatments of Z13; whereas the expression of *PIN2* was not upregulated in the nonprotective Z13 leaf treatment. This result is in accordance with the documented role of the ET/JA-dependent defenses against necrotrophs, like *B. cinerea* (El Oirdi et al. 2011). In similar studies, it has been shown the upregulation of *PIN2*, as a marker gene of the ET/JA-dependent defenses, upon *B. cinerea* inoculation of plants primed by chemical compounds or BCAs (Yu et al. 2022). In most of these studies, *PIN2*, among other examined defense-related genes, reached its maximum expression level at 24 hours after *B. cinerea* inoculation, similarly to our study (Chun-Hao et al. 2018).

Interestingly, the analysis of *PR1* and *PIN2* expression in the Z13/*B. cinerea*-treated plants suggests that it depends on the site of Z13 application on the plant. The application of Z13 in roots upregulated the expression of *PIN2*, while the spraying of Z13 on leaves upregulated the expression of *PR1*. It can be suggested that different plant sites react differently to Z13 and/or Z13 produces different ISR signaling compounds in roots compared to leaves due to different microenvironmental conditions. This is not surprising since it is known that minerals and carbon sources affect microbial metabolite production and consequently the plant protective activity of BCAs (Duffy and Defago 1999).

In conclusion, our study on the biocontrol activity of *P. putida* Z13 against *B. cinerea* in tomatoes reveals promising insights for sustainable disease management. Given *B. cinerea*'s threat to global tomato production and the limitations of chemical fungicides due to resistance, our research underscores the potential of BCAs as a viable alternative. The combined application of Z13 through root drenching and foliar spray emerges as the most effective treatment, reducing *Botrytis* symptoms. Notably, the foliar treatment proves ineffective, emphasizing the importance of understanding tissue specificity in plant responses to BCAs. The analysis of Z13's genome unravels genes associated with ISR, particularly in LPS biosynthesis, pyoverdine, and PQQ. This study sheds light on *P. putida* molecular mechanisms and emphasizes the need to consider microenvironmental conditions for optimizing biocontrol strategies in sustainable agriculture.

## Ethical approval statement

The study does not require local ethics committee approval.

## Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

*Conflict of interest*: None declared.

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## Author contributions

Litsa Ampntelnour (Investigation, Formal Analysis), Eirini G. Poulaki (Conceptualization, Investigation, Formal Analysis), Vasilis Dimitrakas (Investigation, Formal Analysis), Maria Mavrommati (Investigation), Grigorios G. Amourgis (Investigation) and Sotiris E. Tjamos (Supervision, Writing—review and editing).

## Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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