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Exploring the biocontrol potential of rocket (*Eruca sativa*) extracts and associated microorganisms against *Verticillium* wilt

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Abstract

Aims: This study aimed to assess the impact of rocket (*Eruca sativa*) extract on *Verticillium* wilt in eggplants, explore rhizospheric microorganisms for disease biocontrol, and evaluate selected strains' induced systemic resistance (ISR) potential while characterizing their genomic and biosynthetic profiles.

Methods and results: Rocket extract application led to a significant reduction in *Verticillium* wilt symptoms in eggplants compared to controls. Isolated microorganisms from treated soil, including *Paraburkholderia oxyphila* EP1, *Pseudomonas citronellolis* EP2, *Paraburkholderia eburnea* EP3, and *P. oxyphila* EP4 and EP5, displayed efficacy against *Verticillium dahliae*, decreasing disease severity and incidence *in planta*. Notably, strains EP3 and EP4 triggered ISR in eggplants against *V. dahliae*. Genomic analysis unveiled shared biosynthetic gene clusters, such as ranthipeptide and non-ribosomal peptide synthetase-metallophore types, among the isolated strains. Additionally, metabolomic profiling of EP2 revealed the production of metabolites associated with amino acid metabolism, putative antibiotics, and phytohormones.

Conclusions: The application of rocket extract resulted in a significant reduction in *Verticillium* wilt symptoms in eggplants, while the isolated microorganisms displayed efficacy against *V. dahliae*, inducing systemic resistance and revealing shared biosynthetic gene clusters, with metabolomic profiling highlighting potential disease-suppressing metabolites.

Impact Statement

This research advances eco-friendly disease management in agriculture, showcasing the potential of plant extracts and rhizospheric microorganisms like *Paraburkholderia* sp. and *Pseudomonas citronellolis*.

Keywords: biological control; induced systemic resistance; *Paraburkholderia* sp.; *Pseudomonas citronellolis*; *Verticillium dahliae*

Introduction

The plant microbiome plays a crucial role in determining plant health and productivity, garnering significant attention in recent years. Manipulating the plant microbiome holds promise for reducing plant disease incidence, boosting agricultural production, cutting down chemical inputs, and curbing greenhouse gas emissions. This shift toward more sustainable agricultural practices has been highlighted (Arnault et al. 2023).

One method to manipulate the rhizosphere microbiome is by incorporating green manure into the soil (Jin et al. 2019). Brassica plants have long been acknowledged for their capacity to influence soil microbiota, a characteristic that has been leveraged for effective disease control, as initially observed by Papavizas (1966) and subsequently by several other researchers (Lu et al. 2010, Mazzola et al. 2015, DeWolf et al. 2023). Studies have revealed that brassica plants produce sulfur compounds known as glucosinolates, which break down into isothiocyanates. These compounds are toxic to many soil organisms, a process termed biofumigation (Sarwar and Kirkeagard 1998). They have proven effective in reducing soilborne fungal pathogens (Smolinska and Horbowicz 1999) and enhancing soil quality and crop yields (McGuire 2003). Nonetheless, some studies suggest that mechanisms beyond isothiocyanate production may also play a crucial role in reducing soilborne diseases caused by Brassica crops (Mazzola et al. 2001). For instance, Cohen et al. (2005) demonstrated that the suppression of *Rhizoctonia solani* by *Brassica napus* seedmeal was associated with specific changes in soil microbial communities and was unrelated to glucosinolate levels. Similarly, broccoli, as a soil amendment, exhibited plant-protective activity against *Verticillium dahliae* through a shift in soil prokaryote communities, potentially harboring numerous antagonists of fungal plant pathogens that contribute to biological disease suppression (Inderbitzin et al. 2018).

In our present study, we investigated the plant-protective activity of *Eruca sativa* (*Brassicaceae*), commonly known as rocket, water extracts against *V. dahliae* in eggplants. Rocket, an annual herbaceous plant found in the Mediterranean region, has demonstrated antifungal activity *in vitro* in previous studies (Rizwana et al. 2016), but its effectiveness in protecting plants against plant pathogens had not been explored until now. Notably, it has been observed that endogenous rocket

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microbiota, including *Pseudomonas*, thrives in rocket salad juice (Fagerlund et al. 2021). Therefore, rocket extracts hold the potential to foster a beneficial plant microbiome when applied to plants.

Conversely, *V. dahliae* represents one of the most pernicious soilborne pathogens affecting a variety of crops in temperate zones. This pathogen can persist in fields for extended periods, and the lack of a reliable, environmentally friendly soil fumigation strategy, coupled with the scarcity of plant resistance genes, makes it a significant global threat to agricultural production. Considerable attention has turned to biological control, particularly the selection of microorganisms with antibiotic activity against *V. dahliae*. Numerous microorganisms, primarily belonging to the *Bacillus* and *Pseudomonas* genera, have been tested against *V. dahliae* both *in vitro* and *in planta* (Cabanas et al. 2018, Ziazia et al. 2021). In several instances, these biological control agents (BCAs) were isolated from the rhizosphere of plants grown in soil substrates amended with *Verticillium* suppressive composts and extracts from various sources (Malandraki et al. 2008, Ziazia et al. 2021).

While antibiosis represents one of the most extensively studied modes of action of BCAs, another desirable mode of action is the induction of induced systemic resistance (ISR). ISR can protect plants against a broad spectrum of pathogens, has a lasting effect, and does not require direct contact between the BCA and the pathogen (Pieterse et al. 2014). Numerous microorganisms have been evaluated for their potential to trigger ISR against *V. dahliae* in various plants, such as *Arabidopsis thaliana*, eggplants, and olive trees (Gómez-Lama et al. 2014, Gkizi et al. 2016). The bacterial determinants responsible for activating ISR range from lipopolysaccharides to volatile compounds (Poulaki and Tjamos 2023) and interact with plant components like the flagellin receptor FLS2 (Gkizi et al. 2016).

The principal aim of our study was to ascertain the plant-protective potential of rocket extracts against *V. dahliae* in eggplants. Following the observation of the plant-protective effects of these extracts, we delved into the microbial aspect of their activity. We isolated rhizosphere microorganisms from plants treated with rocket extracts and investigated the biocontrol potential of these microorganisms against *V. dahliae*, along with their ability to induce ISR. Additionally, we identified the chemical compounds produced by the most efficacious isolate.

Materials and methods

Pathogen preparation

In the experiments, we utilized a *V. dahliae* strain isolated from an infected eggplant and stored at −80°C. The fungus was transferred to a Petri dish containing potato dextrose agar (PDA) (Merck, Darmstadt, Germany) and incubated at 24°C for 5 days. Subsequently, a suspension of 10^7 conidia per ml of distilled sterile water (DSW) was prepared from a culture grown for 5 days at 24°C in a sucrose-sodium nitrate liquid medium (Sinha and Wood 1968).

Evaluation of rocket extracts against *V. dahliae* under glasshouse conditions

Rocket water extracts (100 g of macerated rocket leaves from 60 day old plants in 200 ml of H_2O) were applied to eggplants at the third-leaf stage of the cv. 'Black Beauty,' known for its high susceptibility to *V. dahliae*. The eggplants were individually grown in plastic pots (9 × 9 × 10 cm) containing autoclaved soil substrate (pH 6.0, black peat; Potground P; Klasmann), which was autoclaved twice within a 24-h interval for 1 h at 121°C and 1.2 atm, or in pots with nonautoclaved soil. The application involved soil drenching each plant with 10 ml of rocket extract. Five days later, the eggplants were infested with a 10 ml solution containing 10^7 *V. dahliae* conidia ml^{-1}. Control plants were treated with DSW. The eggplants were maintained at 25°C ± 3°C with a 16-h light and 8-h dark cycle. The experiment followed a completely randomized design with seven replications (plants) per treatment and was repeated thrice.

Symptoms were recorded every 5 days for a period of 25 days following pathogen application. Disease severity at each observation was calculated as a percentage of leaves showing *Verticillium* symptoms relative to the total number of leaves on each plant. Subsequently, the area under the disease progress curve (AUDPC) was calculated using the trapezoidal integration method for each plant. Disease expression was presented as a percentage of the maximum possible area for the entire experimental period, referred to as the relative AUDPC.

Isolation of rhizospheric microorganisms from rocket treated plants

Eggplants, cv. 'Black Beauty,' grown in nonautoclaved soil and treated with rocket extracts or DSW (mocks), as in the pathogenicity experiment, were uprooted 15 days after treatment to determine the rhizospheric microbial population level and isolate microorganisms from the rocket extract treatment. Soil particles attached to the roots of 5 eggplants per treatment and replication (three replications, 15 eggplants) were pooled into one sample and macerated in a mortar with 50 mM phosphate buffer, pH 7.02, containing 0.02% Tween-20 (PBT). The suspension was transferred to a Mc Cartney bottle containing 10 ml of PBT and placed in an orbital incubator at 100 rpm and 25°C for 45 min. Subsequently, 10-fold dilutions were prepared, and 500 ml of each dilution were plated on PDA Petri dishes and incubated for 2–4 days at 25°C. Single colonies from the highest dilution rate in the rocket-extract-treated plants were then selected for further experimentation.

In vitro activity against *V. dahliae* of the isolated microorganisms and siderophore production

The *in vitro* test against *V. dahliae* of the isolated microorganisms from the rhizosphere of the rocket extract plants followed the method outlined by Reddy and Patrick (1992). Briefly, the microorganisms were streaked ∼2.5 cm from the edge of a Petri plate (9 cm in diameter) containing PDA and allowed to grow for 48 h. A 5 mm diameter disc taken from the edge of an actively growing colony of *V. dahliae* on PDA was then placed on the plate, 2.5 cm from the edge opposite to microorganisms. The plates were incubated in the dark for up to ten days at 25°C, and they were visually inspected for inhibition of *V. dahliae* growth.

The efficacy of siderophore production by the isolated microorganisms was examined using M9 minimal medium supplemented with Chrome Azurol S (CAS). The minimal medium was prepared, autoclaved, and mixed with filter-sterilized, pre-warmed $MgSO_4$ (2 mM final), $CaCl_2$ (0.1 mM final), sucrose (0.2% final), and Casamino acids (0.9% final)

(Loewen 1984). The CAS solution was prepared according to the protocol developed by Schwyn and Neilands (1987), autoclaved, and combined with the minimal medium before the assay. The area of color conversion from blue to orange around the microbial colonies was observed after incubating for 5 days at 28°C.

Identification and genome mining of microorganisms

For full DNA sequencing of the microorganisms that reduced *V. dahliae* growth *in vitro*, the DNA was extracted from pure cultures using the NucleoSpin Microbial DNA kit (MACHEREY-NAGEL, Duren, Germany). The DNA was sequenced on an Illumina platform (Illumina NovaSeq6000, PE150 mode) at Eurofins Genomics (Constance, Germany). The program Trim-Galore V. 0.6.4 was used for the quality control of the sequencing by trimming the automatically detected adapters and removing low-quality sequences with quality and length cutoffs set to 30. The clean sequences were then used for the *de novo* genome assembly of each isolate. The program used for the *de novo* assembly was the SPAdes genome assembler v3.13.2 with default parameters (Bankevich et al. 2012). The *de novo* drafted genome assemblies were investigated using the fIDBAC platform for preliminary taxonomic identification and typing (Liang et al. 2021) (date of access: 2023–07-31). The preliminary taxonomies derived from the fIDBAC platform were used as a guide for the phylogenetic classification in the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker 2019) (date of access: 22/January/2024). The genomes were also screened for known secondary metabolite biosynthetic clusters using AntiSMASH 2.0 (Blin et al. 2013) (date of access: 27/January/2024).

In planta evaluation of microorganisms against *V. dahliae*

The microorganisms that reduced *V. dahliae* growth *in vitro* were further evaluated *in planta*. For this purpose, the microorganisms were incubated in liquid cultures of Nutrient Broth (NB) in an orbital incubator at 180 rpm at 28°C for 48 h. The microbial cultures were centrifuged at 5 000 g, 12°C for 5 min and then resuspended in 50 mM phosphate buffer, pH 7.02, before treating the plants. Similar to previous experiments, eggplants (cv 'Black Beauty') were individually grown in plastic pots (9 × 9 × 10 cm) containing nonsterilized soil substrate (pH 6.0, black peat; Potground P; Klasmann). The microbial isolates were applied at the third-leaf stage by soil drenching each plant with 10 ml of 10^7 cfu ml^{-1}. Five days later, the eggplants were infested with 10 ml of 10^7 *V. dahliae* conidia ml^{-1}. Control plants were treated with DSW. Eggplants were maintained at 25 ± 3°C with a 16-h light and 8-h dark cycle. The experiment was conducted with a completely randomized design with ten replications (plants) per treatment and repeated thrice. Disease severity and relative AUDPC were recorded and analyzed as previously mentioned.

Split root bioassay

The ISR triggering ability of the five microorganisms, which exhibited *in vitro* antibiotic activity against *V. dahliae*, was examined in an eggplant split root system, as described by Liu et al. (1995). Following the protocol outlined by Liu et al. (1995), roots of cv 'Black Beauty' at the third leaf stage were uprooted and split into two halves. Each half of the root system was placed into separate plastic pots (9 × 9 × 10 cm) containing non-sterilized soil substrate (pH 6.0, black peat; Potground P; Klasmann). One half was drenched with 10 ml of 10^7 cfu ml^{-1} of the isolated microorganisms (prepared as previously described). Five days later, the other half was drenched with 10 ml of 10^7 *V. dahliae* conidia ml^{-1}. The experimental plants were maintained at 25 ± 3°C with a 16-h light and 8-h dark cycle. The experiment was conducted with a completely randomized design, with ten replications (plants) per treatment and repeated thrice. Disease severity and relative AUDPC were recorded and analyzed as previously mentioned.

RNA isolation and qPCR determination of *PR1* and *PR4* transcript levels

The expression levels of the defense related genes *PR1* and *PR4* were examined in the most effective microbial treatments of the split root experiment (EP3 and EP4). The stems (5 cm long stem segment cut at soil level) of seven plants from each treatment (mock, EP3, EP4, *V. dahliae*, EP3/*V. dahliae*, EP4/*V. dahliae*) and experimental replication (a total of 3 replications) were collected and pooled into one sample at 3 and 7 days post inoculation (dpi) of the pathogen. The collected stems 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 100 mg of ground tissue for each sample using TRIzol (Invitrogen) according to the manufacturer's instructions. The RNA samples were treated with DNase I (Invitrogen) to eliminate traces of contaminating genomic DNA. RNA concentration was measured using a spectrophotometer (ND-1000; NanoDrop). First-strand cDNA was synthesized using SuperScript II (Invitrogen) following the manufacturer's procedure. The expression levels of the *PR1* and *PR4* eggplant genes were detected using the following primer sequences: for *PR1* (AB222697), 5′-GCCGTGAAGATGTGGGTCGA-3′ and 5′-GCACATCCAAGTACGTACCGAGTT-3′; and for *PR4* (AB222698), 5′-GGACCGCTTTCTGTGGCCCCG-3′ and 5′-ATAAGGTGGCCTTGCTGGTAGCC-3′ (Kiba et al. 2006). Quantitative real-time PCRs (qPCR) were performed in duplicate. The absence of nonspecific products and primer dimers was confirmed by the analysis of melting curves. The expression level of the eggplant actin gene, detected using the primer pair ACTIN-F 5′-TTCCGTTGCCCAGAGGTCCT-3′ and ACTIN-R 5′-TTCCGTTGCCCAGAGGTCCT-3′ (Chen et al. 2007), was used as an internal standard to normalize small differences in cDNA template amounts. Average threshold cycle (Ct) values were calculated for each gene of interest based on three independent biological samples.

Untargeted metabolomics for *Pseudomonas citronellolis* EP2

The EP2 strain was cultured as described for the *in planta* experiments in NB medium, which also served as control. After 48 h of incubation, the liquid culture of EP2 was centrifuged at 5 000 g (12°C for 5 min), and the supernatant was filtered at 20 μm and stored at −20°C until further processing at BGI Genomics facilities (Hong Kong, China). The extraction of the metabolites was performed according to the following procedure: 100 μl of each sample (including a quality control, QC) was mixed with 700 μl of extractant (methanol: acetonitrile: water, 4:2:1, V/V/V) containing the internal standard

(d3-Leucine) in an Eppendorf tube. The mixture was placed at −20°C for 2 h, then centrifuged at 25 000 g (4°C for 15 min) and dried. After that, 180 μl of methanol: pure water (1:1 v/v) was added to the dried pellet, vortexed until fully dissolved in the reconstituted solution, and centrifuged at 25 000 g (4°C for 15 min). The supernatant was transferred to a new Eppendorf tube, and 20 μl of each sample was mixed into QC samples before LC-MS/MS analysis.

Chromatographic separation was performed on a Waters ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters, USA), and the column temperature was maintained at 45°C. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in the positive mode, and 10 mM ammonium formate (A) and acetonitrile (B) in the negative mode. The gradient conditions were as follows: 0–1 min, 2% B; 1–9 min, 2%-98% B; 9–12 min, 98% B; 12–12.1 min, 98% B to 2% B; and 12.1–15 min, 2% B. The flow rate was 0.35 ml min^{-1}, and the injection volume was 5 μl. Mass spectrometry conditions: Q Exactive (Thermo Fisher Scientific, USA) was used to perform primary and secondary mass spectrometry data acquisition. The full scan range was 70−1050 m/z with a resolution of 70 000, and the automatic gain control (AGC) target for MS acquisitions was set to 3×10^6 with a maximum ion injection time of 100 ms. The top three precursors were selected for subsequent MSMS fragmentation with a maximum ion injection time of 50 ms and a resolution of 17 500, with the AGC set to 10^5. The stepped normalized collision energy was set to 20, 40, and 60 eV. ESI parameters were set as follows: sheath gas flow rate was 40, auxiliary gas flow rate was 10, positive-ion mode spray voltage (|KV|) was 3.80, negative-ion mode spray voltage (|KV|) was 3.20, capillary temperature was 320°C, and auxiliary gas heater temperature was 350°C. The obtained mass spectrometry data were analyzed with Compound Discoverer 3.3 (Thermo Fisher Scientific, USA) software in combination with the BGI metabolome database, mzCloud database, ChemSpider online database, and MetaX software (Wen et al. 2017).

Results

Impact of rocket extract application on *Verticillium* wilt symptoms in eggplants

The application of rocket extracts on eggplants grown in non-autoclaved soil substrate resulted in reduced *Verticillium* wilt symptoms compared to controls and the treatment where rocket extracts were applied in eggplants grown in autoclaved soil. At 10 dpi, 22%–28% of the leaves in the noneffective treatments (controls and rocket extracts applied in autoclaved soil) exhibited wilt symptoms, whereas the respective percentage in the rocket extract-treated plants was 7% (Fig. 1). The development of the disease severity index progressed rapidly in the noneffective treatments over the following recording time points, reaching 53%–62% at 25 dpi. The main symptoms at the last recording were leaf necrosis and apoptosis in the control treatment, while milder symptoms like wilting were observed in the rocket extract-treated plants. The disease severity was 50% less in the rocket extract-treated plants grown in the non-autoclaved soil substrate compared to the non-effective treatments at 25 dpi. The difference in disease severity between treatments was also anticipated in the relative AUDPC analysis.

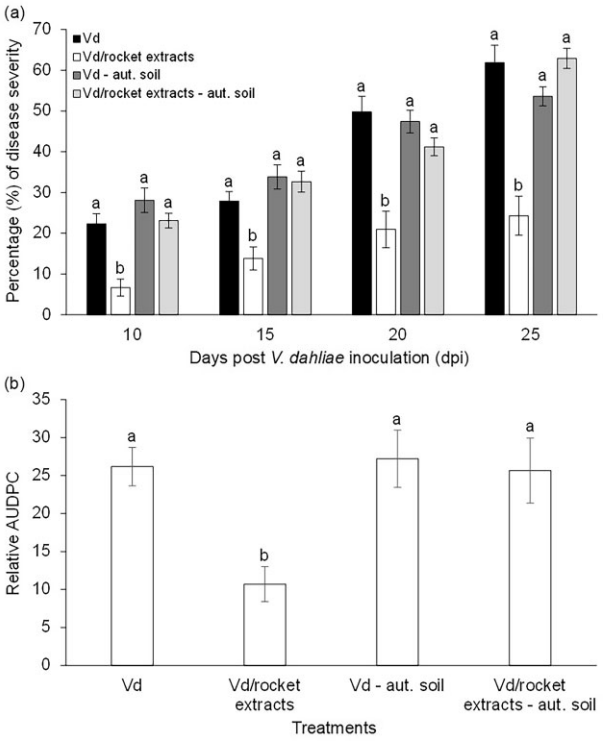

Figure 1. *Verticillium* (Vd) wilt disease severity in eggplants grown in pots containing autoclaved (aut), or not, soil substrate either amended or not with rocket water extracts (a). Disease ratings were plotted over time to generate disease progression curves. Subsequently the area under the disease progression curve (AUDPC) was calculated by the trapezoidal integration method and the disease was expressed as a percentage of the maximum possible area for the whole period of the experiment and it is referred as relative AUDPC (b). Columns represent represent the means of three biological repeats with 7 plants per treatment and repeated experiments ($n = 21$) and the vertical bars indicate the values of the SE. Columns with different letters, in (a) for each day, are statistically different according to LSD multiple range test (disease severity: 10 dpi $F_{3, 80} = 14.75$, $P < 0.001$; 15 dpi $F_{3, 80} = 11.87$, $P < 0.001$; 20 dpi $F_{3, 80} = 14.72$, $P < 0.001$; 25 dpi $F_{3, 80} = 24.77$, $P < 0.001$; Relative AUDPC: $F_{3, 80} = 21.53$, $P < 0.001$).

In vitro investigation of rhizospheric microorganisms from rocket extract-treated soil

The loss of the plant protective activity of the rocket extracts against *V. dahliae* in eggplants grown in autoclaved soil substrate led us to investigate the microbial origin of the plant protective activity of the rocket extracts. For this reason, we isolated rhizospheric microorganisms from eggplants grown in soil substrate treated with the rocket extracts and examined their *in vitro* activity against *V. dahliae*. The *in vitro* tests of the isolated microorganisms showed the efficacy of five strains (named EP1, EP2, EP3, EP4, and EP5) to reduce the growth of *V. dahliae* on PDA cultures (Fig. S1). It was also shown that these strains can produce siderophores under *in vitro* conditions (Fig. S2).

Genomic characterization and biosynthetic potential of isolated strains from the phylogenetic analysis

Draft genomes of all isolates were *de novo* assembled using the raw genomic data derived in the present study, that have become available at the Sequence Read Archive (SRA)

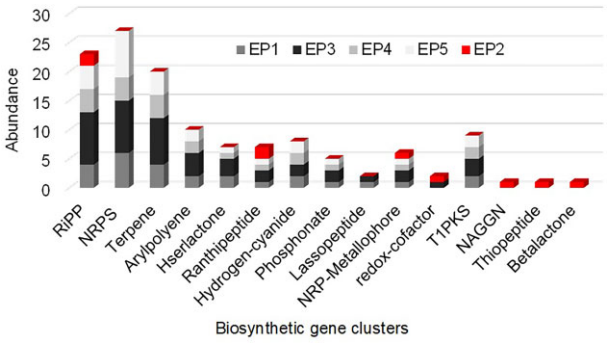

Figure 2. Biosynthetic gene clusters in the genome of the strains *P. oxyphila* EP1, *P. citronellolis* EP2, *P. eburnea* EP3, *P. oxyphila* EP4, and *P. oxyphila* EP5, as predicted by using the antiSMASH software.

with the code SUB13720666 and the BioProject code PRJNA1024950. The phylogenetic placement analysis was based on the whole drafted genome of the isolated strains (Table S1). The isolates, EP1, EP2, EP3, EP4, and EP5 were initially characterized by the fIDBAC platform as members of the *Paraburkholderia oxyphila*, *P.citronellolis*, *Paraburkholderia eburnea*, *P. oxyphila*, and *P. oxyphila* species, respectively. Subsequently, the phylogenetic placement analysis by the TYGS based on the whole draft genome placed the isolated strains closely to the fIDBAC suggested species, as shown in the phylogenetic trees presented in Figs. S3–S7.

Following the identification of the isolated strains, we examined the occurrence of biosynthetic gene clusters (BGCs) in their genome using the antiSMASH software. It was shown that the isolated strains had in common the ranthipeptide and the nonribosomal peptide synthetase (NRP)-metallophore BGC types (Fig. 2, Table S2–S6). The most similar known BGC suggested for ranthipeptide in the five strains was the siderophore pyoverdine, and the suggested NRP-metallophores were: rakicidin (EP1), pyoverdine (EP2), pacifibactin (EP3), variochelin (EP4), and plantaribactin (EP5). The antiSMASH analysis proposes the potential of the isolated strains to produce siderophores, as it was also confirmed *in vitro* (Fig. S2).

The comparison of the BGCs of the isolated *Paraburkholderia* sp. shows that they share in common the following BGC types: hydrogen cyanide, ribosomally synthesized and post-translationally modified peptides (RiPP), NRPs, terpene, phosphonate, arylpolyene, hserlactone, and type I polyketide synthase (T1PKS). Furthermore, the antiSMASH analysis predicted that the isolated *Paraburkholderia* sp. share in common in the hserlactone BGC type, the antifungal compound bacillomycin D; in terpenes, the antibiotic lagriene; in phosphonates, the antifungal yatakemycin; and in the NRPS-like, the siderophore crochelin A (Table S2, S4, S5, S6).

Investigation of rhizospheric microorganisms from rocket extract-treated soil for biocontrol of *Verticillium* wilt in eggplants

The *in planta* evaluation of the biocontrol activity of the isolated microorganisms EP1, EP2, EP3, EP4, and EP5 revealed their efficacy in reducing *Verticillium* wilt in eggplants (Fig. 3 and Fig. S8). It is notable that, in addition to reducing disease severity, they also significantly reduced disease incidence (data not shown). The disease incidence in the EP-treated plants ranged from 14% to 50% at 25 dpi, whereas the disease incidence in the control plants was 100%. In the most efficacious treatment, EP2, the plants remained asymptomatic until 25 dpi, when only 14% of the plants showed wilting leaves and the disease severity was 5%. Subsequently, the relative AUDPC values of the EP-treated plants were statistically lower than those of the control. Among the EP treatments, the EP2-treated plants had the lowest relative AUDPC values.

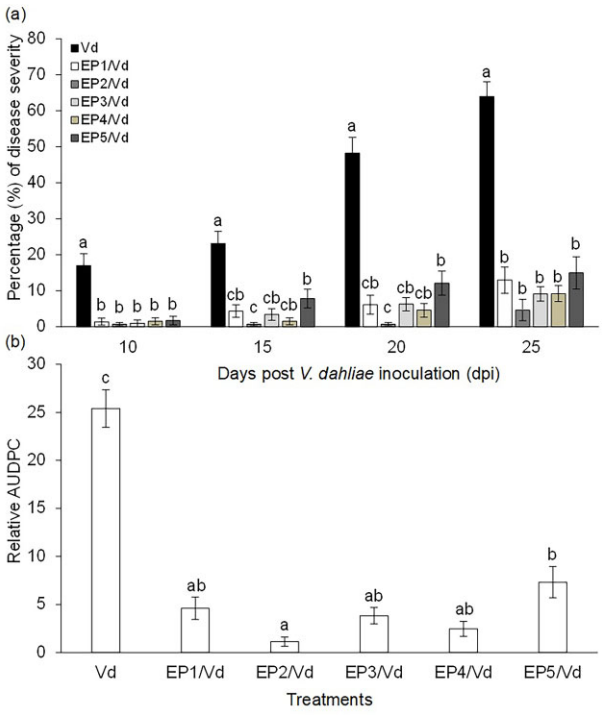

Figure 3. *Verticillium* (Vd) wilt disease severity in eggplants treated with the microbial strains isolated from the rhizosphere of eggplants treated with rocket water extracts (EP1: *P. oxyphila*; EP2: *P.itronellolis*; EP3: *P. eburnea;* EP4: *P. oxyphila*; EP5: *P. oxyphila*) (a). Disease ratings were plotted over time to generate disease progression curves. Subsequently the AUDPC was calculated by the trapezoidal integration method and the disease was expressed as a percentage of the maximum possible area for the whole period of the experiment and it is referred as relative AUDPC (b). Columns represent represent the means of three biological repeats with 7 plants per treatment and repeated experiments ($n = 21$) and the vertical bars indicate the values of the SE. Columns with different letters, in (a) for each day, are statistically different according to LSD multiple range test (disease severity: 10 dpi $F_{5, 120} = 17.19$, $P < 0.001$; 15 dpi $F_{5, 120}$=, 17.44 $P < 0.001$; 20 dpi $F_{5, 120} = 42.43$, $P < 0.001$; 25 dpi $F_{5, 120} = 43.72$, $P < 0.001$; Relative AUDPC: $F_{5, 120} = 52.55$, $P < 0.001$).

Assessing ISR potential of EP strains using a split root system

In order to investigate the ISR triggering potential of the EP strains, we employed the split root system devised by Liu et al. (1995). It was observed that strains EP1, EP2, and EP5 lost their plant protective activity in the split root system, as they exhibited disease severity statistically similar to the control throughout the experimental period. On the other hand, strains EP3 and EP4 maintained their plant protective activity against *V. dahliae*, as they reduced disease severity compared to controls (Fig. 4). It is noteworthy that the EP3 and EP4 treated plants showed ~2 and 8 times less symptoms, respectively, than controls at 10 and 15 dpi, respectively. The relative AUDPC analysis confirmed the plant protective activity of

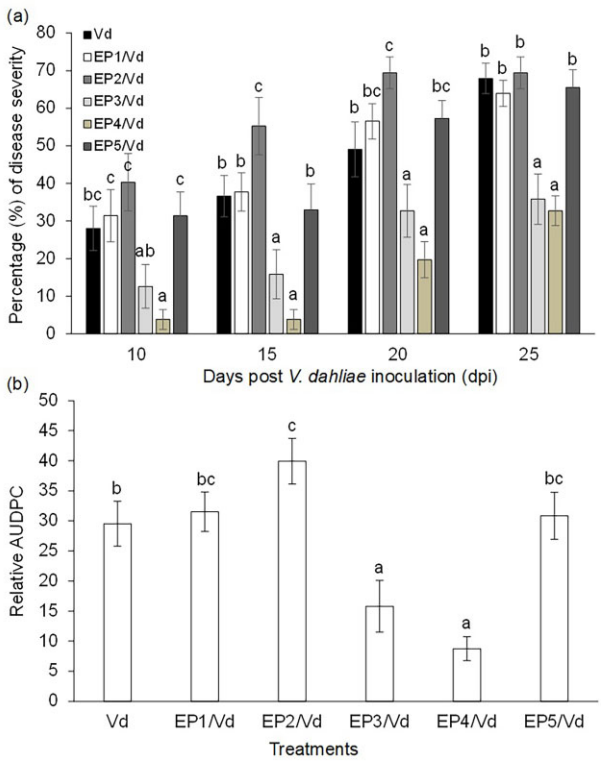

Figure 4. *Verticillium* (Vd) wilt disease severity in eggplants treated with the microbial strains isolated from the rhizosphere of eggplants treated with rocket water extracts in the split root experiment (EP1: *P. oxyphila*; EP2: *P. citronellolis*; EP3: *P. eburnea;* EP4: *P. oxyphila*; EP5: *P. oxyphila*) (a). Disease ratings were plotted over time to generate disease progression curves. Subsequently the AUDPC was calculated by the trapezoidal integration method and the disease was expressed as a percentage of the maximum possible area for the whole period of the experiment and it is referred as relative AUDPC (b). Columns represent represent the means of three biological repeats with 7 plants per treatment and repeated experiments ($n = 21$) and the vertical bars indicate the values of the standard error. Columns with different letters, in (a) for each day, are statistically different according to LSD multiple range test (disease severity: 10 dpi $F_{5, 120} = 17.19$, $P < 0.001$; 15 dpi $F_{5, 120}=$, 17.44 $P < 0.001$; 20 dpi $F_{5, 120} = 42.43$, $P < 0.001$; 25 dpi $F_{5, 120} = 43.72$, $P < 0.001$; Relative AUDPC: $F_{5, 120} = 52.55$, $P < 0.001$).

EP3 and EP4 against *V. dahliae* and also revealed that the EP2 treated plants had the highest disease score among treatments.

ISR by EP3 and EP4 strains in eggplants: expression analysis of defense-related genes *PR1* and *PR4*

The results of the split-root experiment revealed that EP3 and EP4 strains may trigger ISR in eggplants since they reduced disease severity compared to controls. Therefore, the expression of the defense-related genes *PR1* and *PR4* was investigated at 3 and 7 dpi. For *PR1*, it was shown that EP4 upregulated its expression in the absence of the pathogen, whereas EP3 did not have a significant effect on its expression (Fig. 5). Interestingly, the application of *V. dahliae* resulted in the upregulation of *PR1* both in the controls and the BCA-treated plants (EP3/*V. dahliae*, EP4/*V. dahliae*) at 3 dpi, while at 7 dpi, the expression of *PR1* remained upregulated only in the EP3/*V. dahliae* and EP4/*V. dahliae* treated plants. For *PR4*, it was observed that its expression was not upregulated by EP4 and EP3 in the absence of the pathogen, especially in the case of EP4 where its expression was downregulated. Upon pathogen inoculation, the expression of *PR4* was significantly upregulated in the BCA-pretreated plants at 3 dpi, being higher than controls, while at 7 dpi, the expression of PR4 in the EP3/*V. dahliae* was statistically equal to controls but still the highest among treatments in the case of the pathogen-challenged EP4 treated plants.

Metabolomic profiling of *P. citronellolis* EP2

A principal component analysis (PCA) revealed that EP2 samples were clustered separately from controls along the horizontal axis, describing 93% of the variation. Additionally, a volcano plot showed that 411 metabolites were significantly upregulated in the EP2 group versus control (Fig. 6). Among the upregulated metabolites, we selected those for further analysis where the ratio of EP2 versus control was over 2 and the match to known compounds was over 80% (Table S7). These metabolites are mainly classified into the metabolic pathway of amino acids. Further analysis showed the presence of putative antibiotics (2-Hydroxyphenylacetic acid, Pyrrole-2-carboxylic acid, Kynurenic acid, Phenyllactic acid, Isobutyric acid, Dethiobiotin, Isoquinoline, Palmitic acid, 2,5-Dimethylpyrazine, D-(+)-Camphor, *cis*-4-Decenoic acid), phytohormones (Indole-3-acetic acid, Jasmonic acid, Indole-3-methyl acetate), a plant nutrient assimilation (Gluconic acid), and a systemic acquired resistance inducer (Glycerol 3-phosphate) compound (Table S7).

Discussion

The management of *Verticillium* wilt caused by *V. dahliae* presents a widespread threat to dicotyledonous plants worldwide. Given the limitations of chemical control methods, researchers have been actively exploring alternative, sustainable strategies for disease management. Towards this direction, we explored the potential of rocket extracts to mitigate *Verticillium* wilt in eggplants, investigated the microbial nature of the observed plant protection, and isolated, identified and examined the biocontrol potential of microorganisms isolated from the root system of eggplants treated with the rocket extracts.

Our pathogenicity experiments revealed the effectiveness of rocket extracts in protecting plants against *V. dahliae*. This finding is consistent with shifts observed in the rhizosphere microbial community in previous studies, where soil amendment with brassica plant extracts induced beneficial changes in soil prokaryotes, thereby enhancing plant growth and protection against various pathogens. For instance, incorporating broccoli residues into *Verticillium* conducive soil has been shown to reduce disease severity in eggplants, increase plant height, and alter soil prokaryote communities (Inderbitzin et al. 2018, Ogundeji et al. 2022). Notably, among the enriched bacterial genera were *Burkholderia* and *Pseudomonas*, known for their antifungal properties (Inderbitzin et al. 2018). Similarly, our study identified *Paraburkholderia* and *Pseudomonas*, both recognized for containing biocontrol strains effective against plant pathogens.

Furthermore, genome analysis revealed that EP1, EP4, and EP5 belong to the species *P. oxyphila*, while EP3 is classified as *P. eburnea*, and EP2 is identified as *P. citronellolis*. Members of the *P. oxyphila* and *P. eburnea* species are gram-negative, aerobic, nonspore-forming bacteria from the family *Burkholderiaceae* (Otsuka et al. 2011). This family is recognized for comprising species with beneficial effects on plants, attributed to

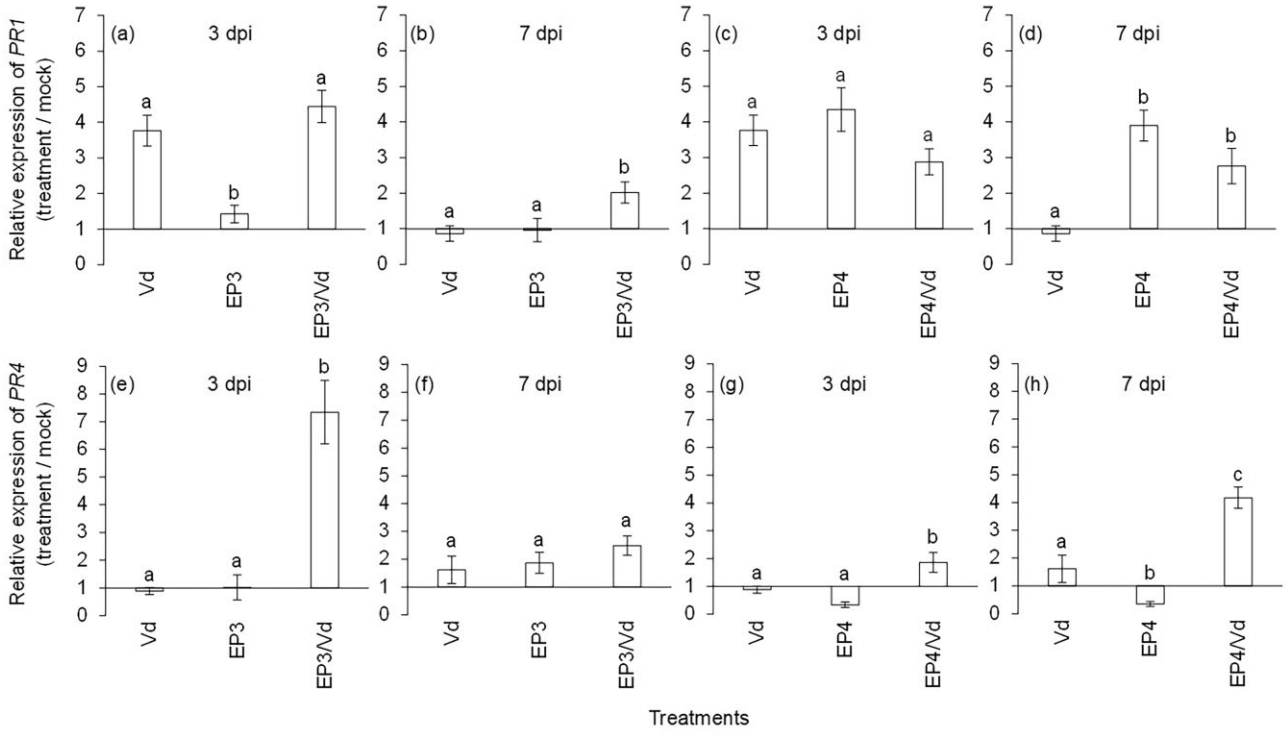

Figure 5. Fold changes in relative transcript abundance of *PR1* (a, b, c, d) and *PR4* (e, f, g, h) of eggplants (cv Black beauty) root drenched with *P. eburnea* (EP3) or *P. oxyphila* (EP4) and challenged inoculated with *V. dahliae* (Vd), at 3 (a, c, e, g) and 7 (b, d, f, h) days post pathogen inoculation (dpi). Transcript levels of the examined genes were normalized to the expression of ACTIN (Chen et al. 2007) 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 (stem tissues from seven plants per biological sample). Columns with different letters are statistically different according to LSD multiple range (a: $F_{2,6} = 16.4$, $P < 0.01$; b: $F_{2,6} = 5.2$, $P < 0.05$; c: $F_{2,6} = 2.38$, p = 0.17; d: $F_{2,6} = 14.79$, $P < 0.01$; e: $F_{2,6} = 8.36$, $P < 0.05$; f: $F_{2,6} = 1.2$, p = 0.36; g: $F_{2,6} = 11.87$, $P < 0.01$; h: $F_{2,6} = 22.44$, $P < 0.01$).

the production of secondary metabolites and volatile compounds, as well as their ability to enhance plant tolerance against pathogens (Riera et al. 2018). While extensively studied as biocontrol agents against soilborne pathogens such as *Fusarium oxysporum* (Ahmad et al. 2022), they have not been previously investigated against *V. dahliae in planta*. Thus, this study represents the first report on the biocontrol efficacy of *Paraburkholderia* species against *V. dahliae*.

Genome mining of the *Paraburkholderia* species suggested that the four strains, along with *P. citronellolis* EP2, may share a common ability to produce the siderophore pyoverdine. Indeed, *in vitro* tests demonstrated the efficacy of the isolated strains in siderophore production. Siderophores, besides their direct action against pathogens, may also induce systemic resistance trigger ISR in plants (Maurhofer et al. 1994). Previous studies have shown the *in vitro* antagonistic activity of purified pyoverdine against *F. oxysporum* (Lemanceau et al. 1992). Additionally, pyoverdine appears to play a significant role in ISR, as evidenced by its induction of resistance in tomato mediated by *P. putida* WCS358, whereas the pvd—mutant did not induce resistance (Meziane et al. 2005). Besides the potential production of pyoverdine, *Paraburkholderia* species may also produce a different siderophore, as indicated by the prediction of crochelin A formation through bioinformatics analysis (Baars et al. 2018).

Moreover, the antiSMASH analysis of the isolated *Paraburkholderia* species suggests the potential production of antibiotic compounds, including bacillomycin D, lagriene, and yatakemycin. Bacillomycin D, a cyclic lipopeptide primarily synthesized by *Bacillus subtilis* strains, has been documented to inhibit several plant pathogens such as *Fusarium graminearum* (Gu et al. 2017). Lagriene, known to be produced by *Paraburkholderia* sp. and *P. gladioli* Lv-StA, exhibits antibiotic activity against *Bacillus thuringiensis* and *Mycobacterium vaccae* (Florez et al. 2017). Yatakemycin, a potent DNA alkylating agent, demonstrates antimicrobial and antitumor properties, inhibiting the growth of plant pathogenic fungi such as *Aspergillus flavus* (Mullins et al. 2017). Consequently, the isolated *Paraburkholderia* species hold the potential to produce antibiotic compounds that may account for the reduced symptoms of *Verticillium* wilt observed in plants treated with these strains.

Furthermore, the split-root experiment revealed the ability of the *P. eburnea* EP3 and *P. oxyphila* EP4 strains to trigger ISR in plants. This finding was corroborated by transcriptomic analysis, which demonstrated the upregulation of defense-related genes *PR1* and *PR4* in EP3- and EP4-treated plants following *V. dahliae* inoculation. *PR1* and *PR4* serve as marker genes for salicylic acid (SA) and ethylene/jasmonic acid (ET/JA)-dependent plant defenses, respectively, and ISR development is associated with the coordinated expression of these two defense pathways (Thomma et al. 1998). Our experiments revealed that both bacterial strains, EP3 and EP4, elicited the expression of *PR4* and *PR1* upon pathogen inoculation at 3 and 7 dpi, respectively. *PR4* encodes chitinase, which cleaves fungal cell wall chitin, generating fragments that may act as signaling molecules to further induce plant defenses (Van Loon et al. 2006). Conversely, the properties

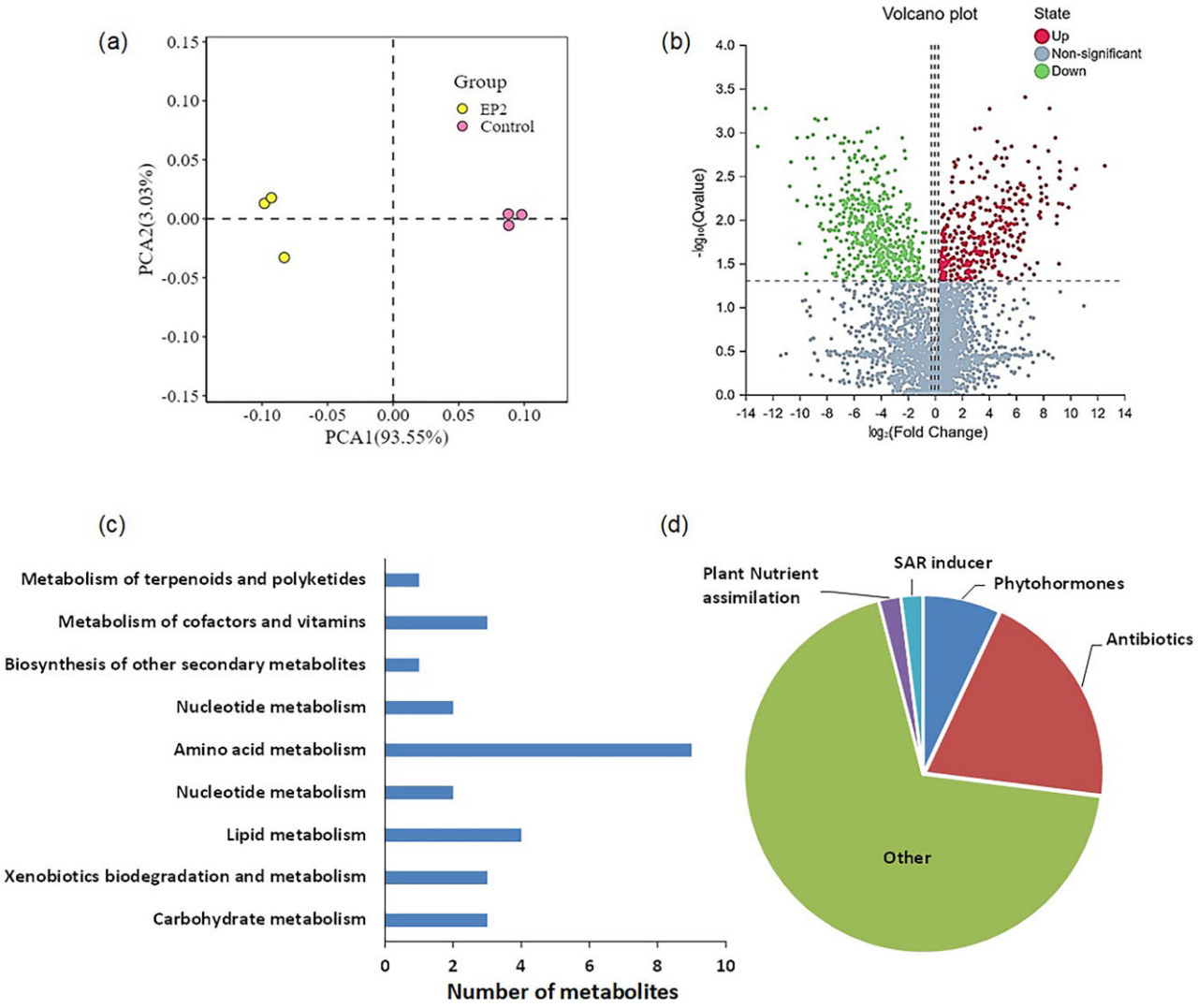

Figure 6. PCA of the metabolites of the *P. citronellolis* EP2 and control (noninoculated growth medium, nutrient broth) samples (3 samples per treatment) (a) and volcano plot (b). The EP2 metabolites, where the ratio of the quantity of the EP2 metabolites versus control was over 2 and the match to known compounds was over 80%, were categorized in metabolomic pathways (c) and biocontrol traits (d). In the volcano plot, the abscissa is the fold change converted by $\log_2$, and the ordinate is the q-value (*P*-value) converted by $\log_{10}$; with i) green are the down regulated significant difference metabolites, ii) red are the up regulated significant difference metabolites, and iii) gray are the non significant metabolites.

of *PR1* remain largely unclear, though its importance in protecting against biotrophic pathogens has been noted in studies discussing ISR triggered by BCAs and host—*V. dahliae* interactions (Derksen et al. 2013, Gkizi et al. 2016).

Similar to the isolated *Paraburkholderia* species, *P. citronellolis* has not previously been investigated as a biocontrol agent against *V. dahliae*. Historically, *P. citronellolis* has garnered recognition for its proficiency in hydrocarbon degradation, including isoprenoid compounds like citronellol, from which it derives its name, and remediation of complex oily sludge contaminations (Bhattacharya et al. 2003), suggesting its potential application in bioremediation efforts to clean up oil-contaminated sites. Our study unveils the plant protective potential of *P. citronellolis* against plant diseases. One possible mode of action of this strain against *Verticillium* wilt may involve the secretion of antibiotic compounds, as evidenced by its ineffectiveness in protecting plants in the split-root experiment, in addition to the previously discussed possibility of producing the siderophore pyoverdine. Untargeted metabolomic analysis of EP2 metabolites revealed several compounds with antifungal activity, including isoquinoline and palmitic acid, which have demonstrated efficacy against soilborne plant pathogens such as *R. solani*, *F. oxysporum* f. sp. *vasinfectum*, *Sclerotinia sclerotiorum*, and *F. oxysporum* f.sp. *niveum* (Zhao et al. 2019, Ma et al. 2021).

In conclusion, this study investigated the potential of rocket extracts to mitigate *Verticillium* wilt in eggplants and examined the associated microbial dynamics. The findings revealed the plant protective activity of rocket extracts, which correlated with shifts in the rhizosphere microbial community, particularly enriched with biocontrol strains such as *Paraburkholderia* and *Pseudomonas*. Notably, this study marks the first report of the biocontrol efficacy of *Paraburkholderia* species against *V. dahliae*, underscoring their potential as sustainable disease management agents. Genome analysis unveiled the production potential of siderophores and antibiotic compounds by the isolated strains, suggesting mechanisms for disease suppression.

Additionally, the study demonstrated the ability of certain strains to induce systemic resistance in plants, offering further insights into the complex plant-microbe interactions underlying disease suppression. Furthermore, the potential of *P. citronellolis* as a biocontrol agent against *V. dahliae* was revealed, highlighting its ability to produce antibiotic compounds with antifungal activity. Overall, these findings contribute valuable knowledge to the development of sustainable strategies for combating *Verticillium* wilt and other plant diseases.

Acknowledgment

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

Eirini G. Poulaki (Conceptualization, Formal analysis, Investigation), Ioanna Karamichali (Formal analysis, Investigation, Writing – review & editing), Orestis Lianos (Investigation), Vasilis Alexopoulos (Investigation), Vasilis Dimitrakas (Investigation), Grigorios G. Amourgis (Investigation), and Sotirios E. Tjamos (Supervision, Writing – review & editing)

Data availability

The raw genomic data derived in the present study are available at the Sequence Read Archive (SRA) with the code SUB13720666 and the BioProject code PRJNA1024950. The data of the pathogenicity and gene expression experiments will be shared on reasonable request to the corresponding author.

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