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EFFICACY OF RHIZOBACTERIA AS BIOCONTROL AGENTS AGAINST VERTICILLIUM WILT IN EGGPLANT

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ABSTRACT

The study aimed to explore the influence of *Bacillus subtilis* (Bs1) and *Pseudomonas putida* (Psp1) on *Verticillium dahliae*, the causal agent of *Verticillium* wilt in eggplant, under *in vitro* and greenhouse conditions. Seventeen purified bacteria exhibiting broad-spectrum antifungal activity were isolated from soil in the North Sinai Governorate, Egypt. The isolates *Bacillus subtilis* (Bs1) and *Pseudomonas putida* (Psp1) were identified through 16S rRNA gene analysis. Assays of antibacterial activity showed that Bs1 and Psp1 produced the highest amounts of hydrogen cyanide (HCN), siderophores, and indole-3-acetic acid (IAA). Bs1 and Psp1 isolates recorded the highest levels of soluble phosphate in Pikovskaya's broth medium (6.39 and 6.46 ppm, respectively). Additionally, the highest values of soluble potassium in liquid medium were recorded by Bs1 and Psp1 isolates at 15.3 and 14.4 ppm, respectively. The maximum number of phenolic compounds was observed in Bs1 and Psp1, yielding 142.1 and 136.6 mg GA ml^{-1}. Under *in vitro* conditions, Bs1 and Psp1 demonstrated a strong ability to inhibit the mycelial growth of *V. dahliae*. A greenhouse experiment was conducted to evaluate the effects of Bs1 and Psp1 rhizobacteria, either individually or in combination, against *Verticillium* wilt using the long black hybrid cultivar. The fermentation broth of Bs1 and Psp1 reduced disease development (20%), decreased the disease index (13%), and achieved the highest control efficiency (86.4%). The combined treatment of Bs1 and Psp1 significantly increased both bud length (32.2 mm) and germination rate (97.5%) in eggplant. It also enhanced chlorophyll content and enzyme activity, with peroxidase (POD) and polyphenol oxidase (PPO) levels recorded at 6.2 U min^{-1} g^{-1} and 2.3 U min^{-1} g^{-1}, respectively, in eggplant treated with Bs1 + Psp1 compared to the control. Additionally, the combined treatment significantly reduced disease severity and demonstrated potential as a plant growth-promoting agent.

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INTRODUCTION

Eggplant (*Solanum melongena L.*) holds significant agricultural importance globally, serving as a vital vegetable crop in numerous regions around the world (Lan *et al*., 2017; Bilginturan & Hatat Karaca, 2021). Diseases pose a common challenge in eggplant

cultivation, and fungal diseases affecting eggplants encompass a range of conditions such as root rot, powdery mildew, white rot, gray mold, and wilt (Sulu *et al*., 2022; Kaniyasserya, 2023). Vascular wilt disease is a very destructive plant pathogens and could economically destroy the crop (Bilginturan &Hatat Karaca, 2021). *Verticillium dahliae* Kleb*.* is a pervasive soil-borne pathogen with a wide distribution, known for causing wilt disease. Its presence in the soil poses a significant threat to eggplant and various other crops, leading to substantial reductions in both yield and quality. (Meszka, 2013; Lan *et al*., 2017; Wang *et al*., 2021).

V. dahliae is a soil-dwelling fungus with a remarkable survival strategy, capable of persisting in the soil for over a decade in the form of microsclerotia. These resilient structures germinate when conditions are favourable, allowing the fungus to penetrate the elongated regions of plant roots and invade the xylem vessels. Once inside the root, *V. dahliae* hyphae proliferate rapidly within the elongation zone, gradually colonizing and obstructing the plant's vascular system. This invasion leads to the manifestation of wilt symptoms in affected plants, including stunted growth, wilting, discoloration, and eventual defoliation. Furthermore, *V. dahliae* produces microsclerotia, which can persist within plant residues and debris in the soil, facilitating its spread and survival between crop cycles (Reusche *et al*., 2014). Microsclerotia, disseminated through contaminated seeds, plant residues, and soil, pose a formidable challenge in agricultural settings due to their persistence and widespread distribution. Their capacity to accumulate within soil makes their eradication particularly daunting (Klosterman *et al*., 2009). Due to the absence of resistant eggplant cultivars to this pathogen, traditional methods such as crop rotation and soil fumigation have historically been employed to combat *Verticillium* wilt. However, both approaches are now discouraged. Rotating with non-host plants of *V. dahliae* is challenging due to the prolonged viability of its microsclerotia. Chemical treatments, while effective, are environmentally unfriendly, and major soil fumigants, including those utilized for sustained fumigation, have been banned under the Montreal Protocol (Panth *et al*., 2020). In response, biological control using beneficial microorganisms has emerged as a promising alternative for managing soil-borne diseases (Tomah *et al*., 2023). These microorganisms offer the potential for safe and environmentally friendly disease control, garnering increased attention as a viable solution (Tjamos *et al*., 2004; Lan *et al*., 2017). Recently, great attention has been given for the use of endophytic bacteria in plant protection and plant growth promotion opening newer windows for microbial exploitation (Peia *et al*., 2023). Endophytic bacteria could survive inside plants with little microbial competition. That leads to reliable suppression of vascular disease (Misaghi & Donndelinger, 1990). Gram-negative bacteria, such as *Pseudomonas* strains, have received significant attention in biological control research due to their ability to produce antimicrobial metabolites (Ni *et al*., 2022). Conversely, Gram-positive bacteria, including *Bacillus* spp., have been investigated primarily for their role in enhancing plant growth and managing plant diseases (Abd Alamer *et al*., 2020 and Wang *et al*., 2022).

This study aimed to explore the efficacy of novel biocontrol agents against *Verticillium* wilt in eggplant. Plant growth-promoting bacteria were isolated, subjected to in vitro screening for antagonistic activity, and subsequently assessed in plants for their ability to control *Verticillium* wilt while promoting plant growth. Antagonistic activity was evaluated using the dual culture technique on agar plates. Further characterization of biocontrol traits involved assessing protection against *V. dahliae*-induced *Verticillium* wilt in eggplant as addressed, both as individual and in combination treatments.

MATERIALS AND METHODS

Isolation of Bacterial Strains

Soil samples were first collected from the rhizosphere of cultivated eggplant in fields in North Sinai, Egypt of cultivated eggplant in North Sinai Governorate, and subsequently air-dried. Following this, one-gram portions of these samples were mixed with 9 mL of sterile water and agitated in a shaker at 28°C for 1 hour. The resulting soil suspensions underwent dilution with sterile water and were then spread onto Glucose Nutrient Agar (GNA) medium (beef extract 3.0 gL^{-1}, yeast extract 1.0 gL^{-1}, peptone 5.0 gL^{-1}, agar 20 gL^{-1}) plates for the isolation of bacteria. Incubation of these plates occurred at 28°C for 5 days. After incubation, individual colonies were purified and separated to obtain monospore strains. Subsequent streaking of the purified colonies onto new GNA plates facilitated further purification, following the method of Dowson (1957). Finally, the isolated colonies were maintained on GNA medium and stored at 4°C until required for further experimentation.

***IN VITRO* EXPERIMENTS**

Effect of Antagonists (PGPR) on Linear Growth of *V. dahlia*

The fungal isolate of *V. dahliae*, obtained from eggplant, originated from the Plant Pathology Unit, Plant Protection Department of the Desert Research Center, Cairo, Egypt. An antagonism assay was conducted against *V. dahliae* using the dual culture technique. *V. dahliae* isolate was cultured on PDA plates at 28^0C for 6 days, following the method described by Lin *et al*. (2009). Subsequently, a 5 mm diameter agar disk of *V. dahliae* was positioned at the center of a plate, and a loopful of seventeen bacterial isolates, previously cultured on GNA medium for 5 days at 28^0C according to method of (Dowson, 1957), was inoculated on both sides of each plate. The plates were then incubated at 28^0C for 6 days. Control treatments involved inoculation solely with *V. dahliae*. Inhibition rates were calculated as per the procedure outlined by Kucuk &Kivanc (2003). Inhibition rate (%) was calculated by using formula (Watts *et al*., 1988): R I= 100 x (R2 - R1) / R2 where R1 was the distance between the inoculum of the pathogen and the inoculum of tested bacterial isolate, R2 was the colony growth of pathogen. This test was replicated twice, with three plates for each treatment serving as replicates. The best bacterial strains, based on their antifungal activity (inhibition rate), were selected for further studies.

Antibacterial Assay

The bacterial isolates were evaluated for their antimicrobial and promotion plant growth activities according to the mode of action as follows:

Hydrogen cyanide (HCN) production

In accordance with Wei *et al*. (1991), Whatman No. 1 filter paper pads were positioned on the top of plugged tubes. Each pad was immersed in 2 ml of sterile picric acid solution (composed of 2.5 g/l Picric acid and 12.5g/l Na_2Co_3) under aseptic conditions. The tubes were incubated at 28±2ºC for one week. This test was replicated twice, with three tubes for each treatment serving as replicates. Subsequently, the color change of the filter pads was monitored, and the production of hydrogen cyanide (HCN) by the antagonists was assessed using the following criteria:

No colour change (-): no HCN production

Brown colouration (+): weak HCN production

Brownish to orange (++): moderate HCN production

Complete orange (++++): strong HCN production

Siderophore production

Siderophore production was evaluated using the Chrome Azurol S (CAS) method as outlined by Alexander & Zuberer (1991). One milliliter of modified CAS assay solution (Hexadecyltrimethylammonium bromide (HDTMA) 0.21 mg, $FeCl_3.6H_2O$ 1 mM (in 10 mMHC1), CAS 2 mM, 2- [N-morpholino] ethanesulfonic acid (MES) 0.097g, 5-sulfosalicylic acid 0.87 mg) was combined with 1 mL of culture filtrate from the isolates. In 2ml tube under investigation. After allowing the mixtures to equilibrate for 3-4 hours, the absorbance was measured at 630 nm using a Jasco V-630 Spectrophotometer (UV 120-20). Control treatment was one milliliter of modified CAS assay solution without bacterial inoculation. This test was replicated twice, with three tubes for each treatment serving as replicates.

Indole acetic acid production

King's agar medium for flourescent *Pseudomonas* (Proteose peptone 20.0 gL^{-1}, K_2HPO_4 1.5 gL^{-1}, $MgSO_4$ 7 H_2O 1.5 gL^{-1}, Glycerol 5 ml, Agar 20 gL^{-1}) and nutrient agar medium for *bacilli* amended with 1 Mm tryptophan, was overlaid with a nitrocellulose membranes disk (82 mm- diameters). Agar plates were inoculated with loopfull of each of the tested isolates, then incubated for 3 days at 28±2°C. The plates were covered with a Whatman no.2 filter paper soaked with Salkowski reagent (12 g/l $FeCl_3$, 7.9M H_2SO_4). This test was replicated twice, with three tubes for each treatment serving as replicates. Organisms that produce Indole-3-acetic acid (IAA) were distinguished from those producing other indoles, which typically result in the production of a yellow to yellow-brown pigment. This distinction was made based on the characteristic pink to red color observed within 0.5 to 3 hours. (Bric *et al*.,1991).

Phosphate solubilization

The amount of soluble phosphate of the most efficient isolates was determined in Pikovskya's agar medium (Pikovskaya, 1948). One hundred ml of Pikovskya's broth medium amended with 0.5% tricalcium phosphate, were inoculated with standard inoculum ($3*10^8$ cfu ml^{-1}) of each bacterium individually then incubated under shake condition (150 rpm, 28±2°C) for 14 days. The amount of soluble phosphate was determined in culture filtrate by colorimetric method at 530 nm (Jackson, 1958). This test was replicated twice, with three tubes for each treatment serving as replicates.

Soluble potassium

Bacterial isolates were assessed for their capacity to release potassium (K) in Alexandrov broth media, supplemented with 1% muscovite mica. One milliliter of overnight culture from each isolate was added to 25 mL of Alexandrov broth as described by Hu *et al*. (2006) and then incubated for 2 weeks at 28±2°C. Each treatment was replicated three times. The quantity of K released into the broth was determined in triplicate flasks, comparing them with a set of uninoculated controls. Following a centrifugation step (10,000 rpm for 10 minutes), the available K content in the supernatant was measured using flame photometry (PFP7, Jenway, UK), as outlined by Sugumaran &Janarthanam (2007).

Gibberellin production

A nutrient medium totaling 100 mL was dispensed into 250 mL conical flasks and subsequently inoculated with the tested bacteria. These flasks were then placed in an incubator set at 28±2°C for 48 hours. Following incubation, the cultures underwent centrifugation at 10,000 rpm for 15-20 minutes at 4°C. The pH of the resulting culture supernatants was adjusted to 2.5 using 3.75N HCl. Subsequently, the supernatants were subjected to extraction three times using a mixture of ethyl acetate and $NaHCO_3$. Each treatment was replicated three times. The quantity of gibberellic acid present in the ethyl acetate phase was determined using a UV spectrophotometer set at 254 nm against a control blank, employing the methodology described by Mitter *et al*. (2002).

Determination of total phenols

The total phenolic content in cell-free cultures of selected isolates was quantified using the Folin-Ciocalteu procedure, following the protocol outlined by Kumar& Min (2011). To initiate the assay, 200 µl of culture filtrate from the tested bacterium was added to test tubes containing 1 ml of Folin-Ciocalteu's reagent, followed by thorough shaking for 3 minutes. Subsequently, 4 ml of saturated Na2Co3 was introduced and mixed into the solution. After incubating for one hour, the total phenolic content was determined by measuring the developed blue color at 765 nm using an Optizen 2120 UV/Vis spectrophotometer. The blank consisted of 0.5 ml of 80% ethanol and reagents only. Each treatment was replicated three times.

Identification of Rhizobacteria (Bs1 and Psp1)

The most potent antagonistic bacteria (Bs1 and Psp1) were identified morphology according to (Bergey & Holt, 2000) and confirmed by molecular technique**,** which identified by 16S rRNA sequence. Genomic DNA extraction was conducted following the method outlined by Ausubell *et al*. (1987), and subsequent amplification was performed targeting the 16S rDNA gene, as described by Lane (1991). The amplification utilized universal 16S primers (F1: 5' AGAGTTT(G/C)ATCCTGGCTCAG 3'; R1: 5' ACGG/C) TACCTTGTTACGACTT 3'), as specified. Sequences of type strains were contrasted using the GenBank database at the National Centre for Biotechnology Information (NCBI) to identify the two bacterial isolates (Bs1 and Psp1). The Molecular Evolutionary Genetics Analysis (MEGA) program's neighbor-joining method was used to create phylogenetic trees. Identification was conducted in Sigma Scientific Services.

GREENHOUSE EXPERIMENTS

Impact of Bs1 and Psp1 on Seed Germination and Bud Growth

In greenhouse experiments, the eggplant cultivar (hybrid long black) was employed. After sterilization for 5 minutes in a 1% NaClO solution, eggplant seeds were subjected to triple rinsing in sterile distilled water (SDW) and then air-dried under sterile conditions. Following this treatment, the seeds were arranged on filter paper placed inside an 18 cm diameter Petri dish. The Petri dish was pre-soaked for 1 minute in bacterial fermentation broth. (10^8cfu/mL^{-1}). Incubation occurred for 6 days at 28°C. Control seeds were soaked in liquid sterilized medium. Each treatment was replicated three times, with each Petri dish containing ten seeds. This experiment was repeated three times. (Ajisha *et al*., 2021).

Effect of PGPR on Wilt Disease Severity and Growth Parameters of Eggplant Plant

To determine *in vivo* effects of PGPR (Bs1, Psp1) either as individual or combined on *V. dahliae,* a modification of the method from Bilginturan & Hatat Karaca (2021) was used. Four treatments were carried out: Bs1, Psp1, Bs1+ Psp1 and control treatment.

Eggplant seedlings (hybrid long black cv.) with 3-4 true leaves were dipped for 15 minutes into suspensions of the strains Bs1 or Psp1 which were cultured in GNA with 2% inoculum size at 28°C and 160 rpm for five days and concentrated at (10^8 cfu/mL^{-1}). For combined applications, the root tips of seedlings underwent initial trimming with a sterile scissor, followed by immersion in each suspension for 15

minutes. Pathogen inoculations involved additional immersion of seedlings in a *V. dahliae* suspension at a concentration of 10^8 cfu/mL^{-1}, prepared from 7-day-old PDA cultures, also for 15 minutes. The control treatment solely received *V. dahliae* inoculation. Subsequently, seedlings were transplanted into plastic pots containing a sterilized sand soil-peat mixture (2:1 v:v). The experiment consisted of 5 replications, each containing 5 plants. Disease severity and symptoms were assessed 60 days after pathogen infestation using the scale proposed by Jabnoun-Khiareddine *et al*. (2007). Disease severity Index (DSI) was determined according to Fang *et al*. (2013) and control efficacy (CE) according to Bae *et al*. (2021). Plant growth parameters including plant height (cm), leaf area (mm^2), fresh weight (g), dry weight of aboveground parts (g), and dry weight of roots (g) were also measured.

Determination of Non-Enzymatic Compounds

Chlorophyll content

At 40 days post-sowing, eggplant leaves weighing 0.1 g were finely chopped in a mortar. Subsequently, they were mixed with 0.5 mL of acetone and 10 mL of 80% acetone before being ground into a homogenate. The resulting extract was then transferred to a 25 mL volumetric flask containing 80% acetone, with 80% acetone serving as a blank test. Optical density (OD) readings were obtained spectrophotometrically at wavelengths of 663 nm and 645 nm (Gu *et al*., 2016).

Antioxidant enzyme activity

Enzyme extracts were prepared according to Urbanek *et al*. (1991).

Polyphenol-oxidase activity (PPO) was performed according to the method described by Oktay *et al*. (1995). The reaction mixture contained 600 µl of 0.1M catechol and 100 µl enzyme extract was completed to 3 ml with 50 mM phosphate buffer, pH 7). Absorbance at 240 nm was recorded by T60 UV VIS spectrophotometer.

Peroxidase activity (POD): POD activity was measured spectrophotochemically following the method described by Hammerschmidt *et al*. (1982). The reaction mixture (2.9 mL) comprised 0.25% (v/v) guaiacol in 10 mM sodium phosphate buffer (pH 6) containing 10 mM H2O2. A volume of 100 µl of the crude enzyme extract was introduced to start the reaction, and the rate of reaction was monitored using a T60 UV-VIS spectrophotometer at 470 nm per minute.

Catalase activity (CAT) was determined by the method of Chance & Maehly (1955). The reaction mixture contained 200µl of H2O2 (20 mM) and 100 µl enzyme extract was completed to 3 ml with 50 mM phosphate buffer, pH 7). Absorbance at 240 nm was recorded by T60 UV VIS spectrophotometer for 1 min. with an interval of 10 sec. for each sample. The activity is calculated from the extension coefficient for H2O2. The unit is defined as the amount of enzyme that decomposes one micromole of hydrogen peroxide per minute.

Statistical Analysis

The data were analyzed using a completely randomized design (CRD). Mean comparisons for various parameters were performed using SPSS statistical analysis software version 16. Mean separation was determined using one-way ANOVA and Duncan's multiple range test. Statistical significance was considered at $P < 0.05$.

RESULTS

Isolation and Identification of the Rhizobacteria (Bs1 and Psp1)

Seventeen purified bacterial isolates have been isolated from the rhizosphere of healthy eggplant plants cultivated in North Sinai Governorate. Isolates have been coded from Bs1, Bs2, Bs3, Bs4, Bs5, Bs6, Bs7, Bs8, Bs9, Bs 10 to Psp11, Psp12, Psp13, Psp14, Psp15, Psp16, Psp17.

The most potent antagonistic bacteria (Bs1 and Psp1) were identified morphology and confirmed by using amplifying and sequencing the 16S rRNA techniques using sigma scientific services. The 16S rRNA sequences of the isolates were deposited in the National Center for Biotechnology Information NCBI /GenBank database system under accession numbers strain Bs1 OP984766 and strain Psp1 OP984769. By a variety of algorithms included in CLC free workbench, version 4.5.1, a phylogenetic tree was constructed using comparative analysis of the 16S rRNA genes (Figure 1 and 2). According to the findings, *Bacillus subtilis* strain Bs1 OP984766 and *Pseudomonas putida* strain Psp1 OP984769 share 99% identity with the 16S rRNA sequences of the Bs1 and Psp1 isolates, respectively.

In Vitro* Inhibitory Effects of Rhizobacterial Isolates against *V. dahliae

Through the result of dual culturing on PDA (Figure 3), Bs1 and Psp1 showed a strong ability (IR ≥ 85 &86%) to inhibit mycelium growth of *V. dahliae.*

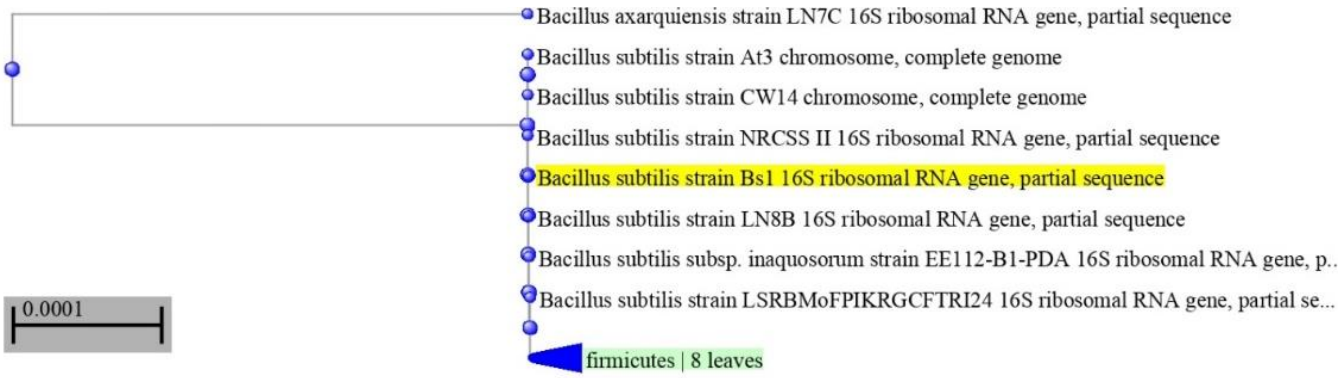

Figure 1. A neighbor-joining phylogenetic tree, constructed using 16S rRNA gene sequences, illustrates the relationships between isolate Bs1 and closely related taxa, showing high identity with *Bacillus subtilis* strain Bs1(OP984766). The phylogenetic tree displays 16S rRNA sequences from endophytic bacterium strain Bs1, compared with representative members of the *Bacillus* genus exhibiting over 98% identity.

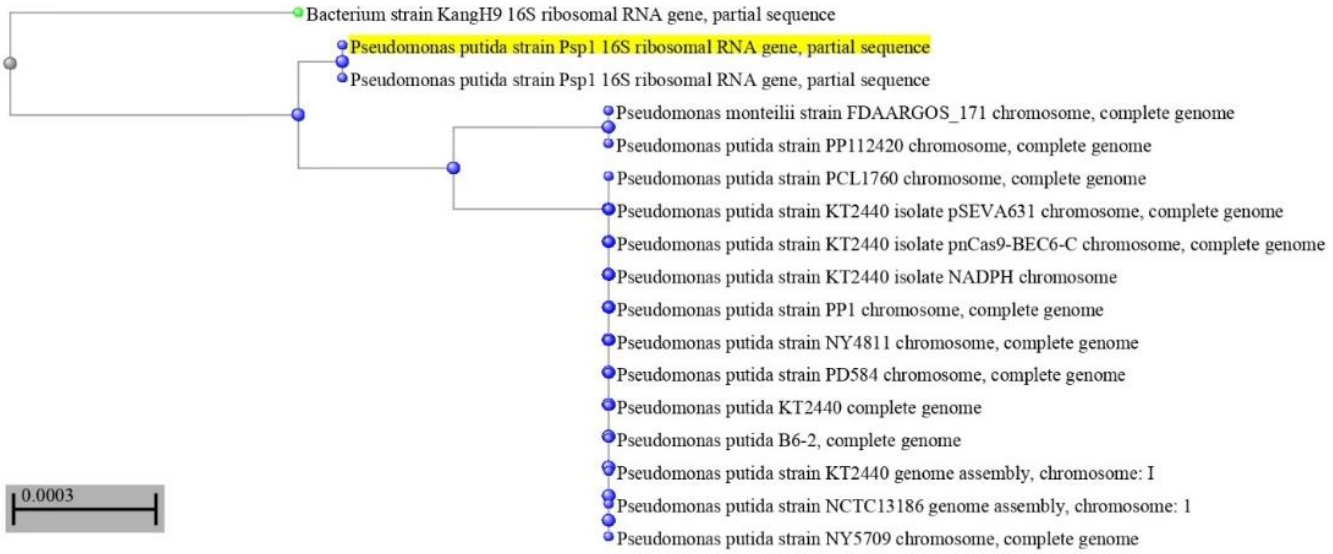

Figure 2. The phylogenetic tree, constructed based on 16S rRNA gene sequences, provides an overview of the relationships between isolate Psp1 and its related taxa, displaying high identity with *Pseudomonas putida* strain Psp1 (OP984769). The percentage of trees in which the associated taxa clustered in the bootstrap test (1000 replicates) is indicated next to the branches.

Antibacterial Assay and Plant Growth-Promoting Metabolites of Antagonists

Hydrogen cyanide (HCN) production

In this research, the *in vitro* production of hydrogen cyanide (HCN) by 17 antagonistic bacterial isolates was examined using the picric acid assay. Table 2 and Figure 4 revealed that bacterial isolates Bs1, Bs5, and Psp1 exhibited the highest HCN production, identified as strong HCN producers, resulting in the complete color change of the filter paper to orange.

Siderophore production

To examine the ability of these isolates to produce siderophores, they were subjected to CAS assay. Table (2) reported that Bs1 and Psp1 isolates showed highly substantial amounts of siderophore production. Its corresponding values are 2.90 and 2.50 µMDFOM, respectively, followed by the Psp4 and Bs5 which showed 1.7 and 1.7 µMDFOM. The lowest significant production of siderophore was recorded by Bs8 and Bs3 isolates which gave 0.75, 0.63 µMDFOM, respectively.

IAA and gibberellins production

In this study, all the isolates were initially screened for producing indole acetic acid production. Table (2) and Figure 5 showed that 9 bacterial isolates were positive for IAA production. The production of gibberellins was in the range of 17.26 to 94.43 µg ml^{-1}.

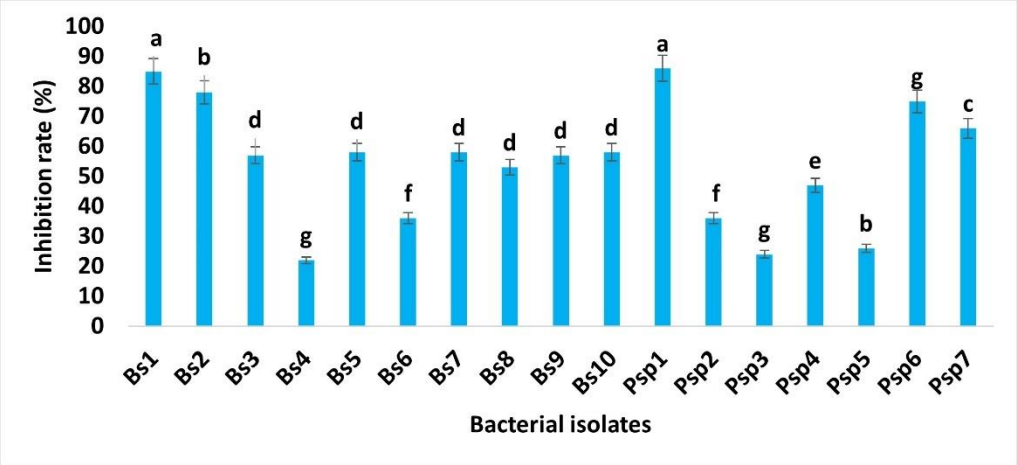

Figure 3. Effect of PGPR on inhibition rates of mycelial growth of *V. dahlia*.

The minimum potential was shown by Psp3 (17.26 µg ml^{-1}), whereas Bs1 and Psp1isolates recorded highest values where production ranged 94.43 – 86.66a µg ml^{-1}, which were significantly more than other isolates.

Nutrient solubilizing bacteria

Table (2) showed that all tested bacterial isolates were able to solubilize phosphate. Data showed significant variation between bacterial isolates for soluble phosphorus ranged from 1.45 to 6.46 ppm. The bacterial isolates Bs1and Psp1 recorded the highest amount of soluble phosphate on pikovskay's broth medium (6.39 and 6.46 ppm). On other hand, isolates Bs7, Psp2 and Psp3 gave the lowest significant values in phosphate solubilization.

The soluble potassium in liquid medium was observed with all bacterial isolates ranged from 2.7 to 15.3 ppm, the high values were recorded by Bs1and Psp1 isolates, ranged from 15.3 to 14.4 ppm Table (2).

Phenolic compounds production

The obtained results indicated that the maximum amount of phenolic compounds was yielded by Bs1and Psp1 bacterial isolates which ranged from 142.1 to 136.6 mg GA ml^{-1} (Table 2).

Table 2. Assessment of the plant growth-promoting activities of the bacterial isolates.

Bacterial isolate	HCN	Siderophore (µMDFOM)	IAA	Soluble Phosphate (ppm)	Soluble potassium (ppm)	Gibrillin (µg ml^{-1})s	Phenolic compound mg GA ml^{-1}
Bs1	++++	2.90a	+++	6.39 b	15.3 a	94.43 a	142.1 a
Bs2	-	1.93 e	-	4.75 c	4.5	37.85 f	123.8 c
Bs3	-	0.63 j	++	2.85 i	10.2 d	36.62 g	111.4 d
Bs4	+	1.54 h	++	4.72 d	-	14.87 n	-
Bs5	+++	1.72 g	+	-	12.1 c	64.66 c	95.3 e
Bs6	++	2.21 c	+	3.68 g	8.5 h	25.12 k	86.2 f
Bs7	++	-	-	1.47 m	7.3 j	-	-
Bs8	+	0.75 i	-	4.65 e	2.7 n	-	-
Bs9	+	-	-	3.98f j	6.8 k	46.22 d	95.4 e
Bs10	-	1.54 h	-	2.57 j	4.4 m	32.15 i	74.9 h
Psp1	++++	2.50 b	+++	6.46 a	14.4 b	86.66 b	136.6 b
Psp2	+	-		1.45 m	8.9 g	-	25.9 k
Psp3	++	2.10 d	++	1.54 l	-	17.26 m	58.9 j
Psp4	++	1.8 g	++	3.21 h	6.3 l	28.76 j	-
Psp5	++	-	+	2.28 k	9.5 e	43.89 e	85.9 g
Psp6	+	1.87 f	-	-	7.9 i	25.07 k	74.2 i
Psp7	-	-	-	-	9.2 f	35.62 h	-

Means with the same letter are not significantly different (P< 0.05).

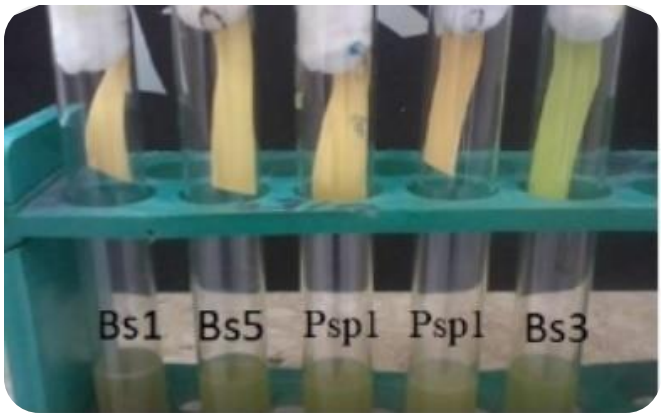

Figure 4. Assessment of hydrogen cyanid production of the most efficient antagonistic bacterial isolates *in vitro*.

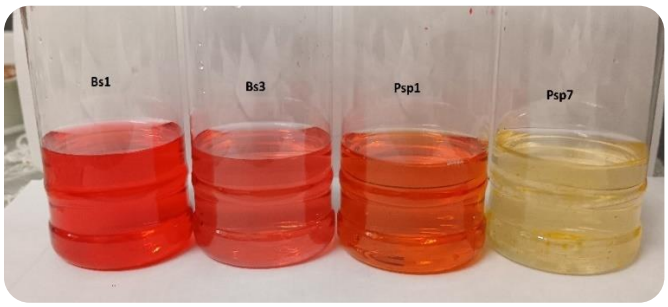

Figure 5. Assessment of indole acetic acid production of the most efficient antagonistic bacterial isolates *in vitro*.

Growth-Promoting Effects of Bs1, Psp1, and their Combination in Greenhouse Conditions

Broth bacterial fermentation of Bs1 and Psp1 positively influenced eggplant seed germination and bud growth, as indicated in Table 3. Eggplant buds inoculated with Bs1 exhibited enhanced bud length and germination rate, measuring 19.5 mm and 84.8%, respectively. Similarly, Psp1 treatment resulted in improved bud length (21.1 mm) and germination rate (76.5%), compared to the control treatment at 7.4 mm and 57.8%, respectively. Notably, the combined culture of Bs1 and Psp1 significantly increased both bud length (32.2 mm) and germination rate (97.5%) of eggplant.

Effect of PGPR on Growth Parameters of Eggplant Plant

Greenhouse experiments utilizing strains Bs1 and Psp1 fermentation broth on eggplant growth revealed a significant promotion in growth parameters, including plant height (cm), leaf area (mm^2), fresh weight (g), dry weight of aboveground parts (g), and dry weight of roots (g), compared to the control (Table 4). The utilization of strains Bs1 and Psp1 resulted in robust growth of eggplants with visibly greener leaves, as observed morphologically. It is hypothesized that the application of strains Bs1 and Psp1 stimulates the release of certain secretions that enhance plant growth, leading to the substantial increase observed in plant dry weight. Moreover, eggplants treated with the combination of Bs1 and Psp1 exhibited the highest dry weight of aboveground parts (6.3 g) and dry weight of roots (5.4 g), respectively.

Table 3. Effect of strains Bs1 and Psp1 on seed germination and bud growth of eggplant.

Treatment	Bud length (mm)	Germination rate (%)
Bs1	19.5 c	84.8 b
Psp1	21.1 b	76.5 c
Bs1+ Psp1	32.2 a	97.5 a
Control	7.4 d	57.8 d

Means with the same letter are not significantly different (P< 0.05).

Table 4. The effect of Bs1 and Psp1 fermentation broth on growth and development of eggplant.

Treatment	Height of plant/cm	Leaf area/mm^2	Fresh weight/g	Dry weigh of overground parts/g	Dry weigh of roots/g
Bs1	53.5 b	315.3 c	49.7 c	4.7 b	2.9 c
Psp1	49.7 c	346.6 b	57.4 b	4.2 b	3.1 b
Bs1+ Psp1	69.8 a	486.3 a	75.8 a	6.3 a	5.4 a
Control	36.2 d	194.9 d	20.5 d	1.7 c	1.6 d

Means with the same letter are not significantly different (P< 0.05).

Suppressive Effect of Bs1 and Psp1 on Disease Severity Index of *V. dahliae*

Data in (Table 5) indicated that Bs1 and Psp1 fermentation broth can suppress *Verticillium* wilt on eggplant. Combined Treatment of eggplant with Bs1 and Psp1 fermentation broth can dramatically decrease the rate of disease development in the plants (20%) and decrease the disease index (13%) and showed the highest control efficiency (86.4%).

Table 5. Control of eggplant *Verticillium* wilt in greenhouse by strains Bs1 and Psp1.

Treatment	Rate of diseased eggplant (%)	Disease index (%)	Control efficiency (%)
Bs1	58 a	36 b	62.5 c
Psp1	47 b	26 c	72.9 b
Bs1+ Psp1	20 c	13 d	86.4 a
Control	100 a	96 a	-

Means with the same letter are not significantly different (P< 0.05).

Determination of Non-Enzymatic Compounds

Determination of chlorophyll content

In comparison to the control group, treatments Bs1 and Psp1 exhibited higher total chlorophyll content in leaf activity, with values of 60% and 85%, respectively. The chlorophyll content was identified as a potential physiological factor contributing to the growth promotion effects of strains Bs1 and Psp1, as demonstrated in Table (6). Moreover, eggplants cultivated with a combination of bacterial cultures (Bs1 + Psp1) displayed the highest total chlorophyll content among the treatments. (9.7mg/100g fw).

Table 6. The effect of Bs1 and Psp1 fermentation broth on chlorophyll content (mg/100g fw).

Treatment	Chlorophyll a (mg/100g fw)	Chlorophyll b (mg/100g fw)	Total chlorophyll (mg/100g fw)
Bs1	3.5 c	2.9 c	6.4 c
Psp1	4.1 b	3.2 b	7.3 b
Bs1+ Psp1	5.4 a	4.3 a	9.7 a
Control	2.6 d	1.2 d	3.9 d

Means with the same letter are not significantly different (P< 0.05).

Effect of Bs1 and Psp1 on Enzyme Activity in Leaves

In the presence of *V. dahliae*, application of biocontrol agents resulted in plant growth promotion compared to the corresponding control regardless of the application. Data presented in Figure 6 showed the activities of polyphenol oxidase, peroxidase and catalase in fresh leaves of eggplant plant as influenced by bacterial inoculation in presence of *V. dahliae* after 40 days of planting. In this study, a correlation was found between bacterized treatments and increase of peroxidase (POD), polyphenol oxidase (PPO) and catalase (CAT) which possess direct antimicrobial activity. Peroxidase is a well-known enzyme involved in the oxidation of hydroxy-cinnamyl alcohol to produce lignin. This compound acts as a barrier to pathogen invasion and thus contributes to the host resistance mechanism. Peroxidase also produces free radicals and hydrogen peroxide (Sofy *et al*., 2021), which are toxic to several pathogens. The plants grown with bacterial culture (Bs1 and Psp1) with pathogenic fungi contained the highest activities of enzymes in significant values comparing to control treatment (un-inoculated plant). The corresponding values of POD were 6.2 U min^{-1} g^{-1} for eggplant treated with Bs1 + Psp1 compared to control treatment (2.1 U min^{-1} $g^{-1)}$. From this data, we recommend that the biocontrol agents were responsible for stimulation of resistance to *Verticillium* wilt disease in eggplant and can accelerate the defense response to stop fungal migration in the plant. Combined treatment of Bs1 + Psp1 recorded the maximum significant value of (PPO) at (2.3 U $min^{-1}g^{-1}$), whereas control treatment recorded the lowest value (0.7 U $min^{-1}g^{-1}$). Inoculated plants with Bs1 + Psp1 achieved catalase activity at (2.1 U $min^{-1}g^{-1}$) compared with the control treatment (Figure 6).

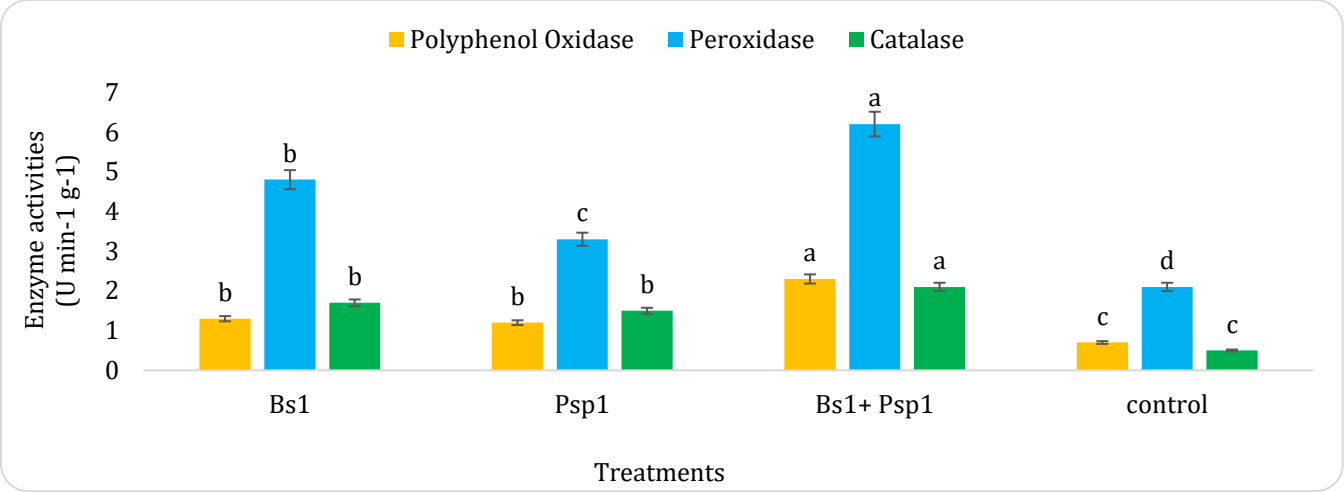

Figure 6. The effect of Bs1 and Psp1 fermentation broth on some enzyme's activity of eggplants.
Bars with different letters are significantly different (P< 0.05).

DISCUSSION

In an *in vitro* experiment, all tested PGPR isolates demonstrated a reduction in the mycelial growth of *V. dahliae* compared to the control. Notably, Psp1 and Bs1 exhibited the most significant ability to inhibit the mycelium growth of *V. dahliae* (IR ≥ 86% & 85%). These findings are consistent with Lin *et al*. (2009), who observed that the *B. subtilis* strain Jaas ed1 displayed potent antagonistic effects against *V. dahliae*, manifesting as a distinct inhibition zone of 9.2 mm. Additionally, the impact of secondary metabolites produced by *Pseudomonas* isolates on *Verticillium* species triggers a multifaceted transcriptional response. This response entails reduced growth, accompanied by measures for self-protection, along with the initiation of a shift in fungal growth direction (Harting *et al*., 2021). Furthermore, Wang *et al*. (2022) demonstrated that *B. velezensis* SBB strain exhibited a notable inhibitory effect on *V. dahliae* growth, particularly evident after 10 days of incubation at 28°C.

From assaying the antibacterial metabolites of 17 antagonistic bacterial isolates, data indicated that the Bs1, Bs5 and Psp1produced the highest amount of hydrogen cyanide. These results could be discussed in light in the findings of Kumar *et al*. (2015) who investigated that the rhizobacteria are capable of producing HCN. Also, these results are consistent with those obtained by Saber *et al*. (2015), who investigated the capabilities of *Alcaligenes*, *Aeromonas, Bacillus, Pseudomonas*, and *Rhizobium* in producing (HCN). HCN serves as a potent inhibitor of several metal enzymes, notably copper-containing cytochrome C oxidases. This compound is synthesized from glycine under the catalytic action of the HCN synthetase enzyme, which is typically associated with the plasma membrane of specific rhizobacteria (Rijavec &Lapanje, 2016). Among antimicrobial agents, hydrogen cyanide has been identified by numerous studies as a significant biocontrol trait, with *Bacillus* and *Pseudomonas* species notably demonstrating its production (Reetha *et al*., 2014; Anand *et al*., 2020).

In this study, Bs1 and Psp1 isolates exhibited notably high levels of siderophore production. Similarly, *B. velezensis* SBB was found to produce siderophores, suggesting its potential to compete with *V. dahliae* for iron availability via siderophore-mediated mechanisms, thereby inhibiting the growth of *V. dahliae* (Wang *et al*., 2022). Moreover, Mishra *et al*. (2017) highlighted the siderophore theory of biological control by rhizobacteria, with much evidence supporting this theory stemming from studies involving pyoverdine, a class of siderophores that constitute the fluorescent pigment of *Fluorescent Pseudomonads*. Plant-associated PGPR employ various strategies to acquire iron from the soil, one of which involves the synthesis and secretion of siderophores as selective ferric ion chelators (Dellagi *et al*., 2009; Wensing *et al*., 2010).

Nine bacterial isolates were positive for IAA production. The production of GA3 was ranged from 17.26 to 94.43 µg ml^{-1}. These obtained results could be supported in light of the findings of (Kumar *et al*., 2015; Jha & Saraf, 2015**)** who confirmed that many PGPB produce

cytokinins and gibberellins and some strains of PGPR can promote relatively large amounts of gibberellins, leading to enhanced plant shoot growth**.** Many PGPB can produce auxins to exert particularly strong effects on root growth **(**Jha &Saraf, 2015**).** Indole-3-acetic acid (IAA) is the most widely studied auxin produced by PGPB. It is involved in plant-microbe interactions (Ahemad &Kibret, 2014).

Variability among bacterial isolates in solubilizing nutrients, particularly phosphorus and potassium, was evident in the data. Mehnaz (2016) investigated the effectiveness of *B. megaterium*, a commercially available product marketed as Phosphorus (BioPower Lanka, Sri Lanka). This strain was found to reduce phosphate fertilizer requirements for plantation crops by up to 75%. Additionally, P-solubilizing bacteria such as *P. striata*, *B. polymyxa*, and *B. megaterium* have been commercialized as Phosphate Solubilizing Bacteria (PSB). These bacteria convert insoluble phosphate into a soluble form by producing organic acids, thereby enhancing its availability for plant uptake and nutrition. Furthermore, Hu *et al*. (2006) isolated two *Bacillus* sp. strains capable of solubilizing phosphate and potassium from soils. They employed a modified medium containing phosphorite and potassium minerals such as kaolinite and potassium feldspar. Archana (2007) also noted the potential of *Bacillus* sp. in solubilizing potassic minerals.

The maximum amount of phenolic compounds was yielded by Bs1and Psp1 bacterial isolates which ranged from 142.1 to 136.6 mg GA ml^{-1}**.** Saito *et al*. (2018) found that Coriobacteriaceae, Enterobacteriaceae, Fusobacteriaceae and *Clostridium* clusters can produce 100 µM of phenol. Also, Musilova *et al*. (2016) recorded that the smaller amounts of complex secondary metabolites (such as phenolic compounds) can attract specific microbes in the rhizosphere.

Bacterial isolates Bs1 and Psp1 were evaluated on eggplant seed germination and bud growth, demonstrating significant improvements in both processes. This study found that these strains not only promoted eggplant seed germination but also stimulated bud growth, resulting in enhanced seedling growth. Similarly, Lan *et al*. (2017) reported the growth-promoting effects of various concentrations of *Purpureocillium lilacinum* QLP12 fermentation on eggplant seed germination, bud growth, and seedling growth. Eggplant bud growth exhibited notable improvements in bud length and germination rate, with increases to 12.2 mm and 76.7%, respectively, compared to 6.8 mm and 60.2% for the control group.

The application of tested PGPRs, either individually or in combination, resulted in a notable increase in various plant growth parameters of eggplant plants. These findings align with those reported by Lan *et al*. (2017) who observed that *P. lilacinum* QLP12 contributed to vigorous growth and greener leaves in eggplants based on morphological observations. It is hypothesized that QLP12 induces the release of certain secretions that facilitate significant growth enhancement, as evidenced by the observed substantial increase in plant dry weight. Similarly, Abo-Koura *et al*. (2019) demonstrated the effectiveness of three strains (*P. polymyxa*, *B. nakamurai* and *B. pacificus*) either alone or in combination, in successfully colonizing wheat plants. These strains stimulated significant increases in various growth parameters and vigour index, indicating their potential as growth-promoting agents for wheat plants.

Under greenhouse conditions, the DSI of eggplant affected by *Verticillium* wilt was significantly influenced by treatments involving PGPRs. Lin *et al*. (2009) documented the potent antifungal activity of *B. subtilis* Strain Jaas ed1 and its cell-free filtrate against *V. dahliae*. The application of the strain's cell suspension effectively managed *Verticillium* wilt in eggplant, with its control efficiency surpassing that of the cell-free filtrate following *V. dahliae* inoculation. Furthermore, Deketelaere *et al*. (2017) identified a predominance of endophytic *Bacillus* and *Pseudomonas* isolates with potential efficacy against *Verticillium*. Madhi &Jumaah (2020) noted that the utilization of *B. subtilis* resulted in a reduction in the severity of root rot disease and an increase in the fresh and dry weight of both vegetative and root components of eggplant. Notably, *Bacillus* spp. has demonstrated efficacy in controlling *V. dahliae* in eggplant, resulting in a disease reduction of 65% and subsequent yield improvement (Li *et al*., 2008). Similarly, Pei *et al*. (2023) demonstrated that the application of *B. subtilis* strain P10 on tomato seedlings effectively suppressed the severity of *Verticillium* wilt.

Application of bioagents (Bs1 and Psp1), either individually or in combination, to eggplant plants led to a significant elevation in total chlorophyll levels compared to the control group. This increase in chlorophyll content serves as an indicator of the plant's health status and contributes to enhancing its resistance against diseases

(Formisano *et al*., 2021). Similarly, Lan *et al*. (2017) observed that eggplants treated with *P. lilacinum* QLP12 exhibited a 47.83% increase in total chlorophyll content in the leaves compared to the control group.

The inoculation of PGPRs, whether individually or in combination, resulted in increased activities of POD, CAT, and PPO enzymes. The interaction effect of PGPRs on this enzyme activity demonstrated maximum enhancement in plants treated with the combination of bacteria Bs1 + Psp1 compared to control plants without PGPR treatments. These results clearly indicate the positive role of PGPRs in upregulating POD, CAT, and PPO activities in eggplants under biotic stress. Similarly, Heidari & Golpayegani (2012) reported that the treatment with rhizobacteria led to the highest concentration of catalase activity. In another study, the treatment with *B. subtilis* SL-44 was found to increase the activity of defense-related enzymes such as SOD, POD, CAT, PAL, and PPO, thereby activating cellular defense responses in pepper plants (Wu *et al*. 2019). Additionally, Pei *et al*. (2023) observed higher activities of POD, SOD, and CAT enzymes in plants challenged with *V. dahliae* and treated with *B. subtilis* strain P10. Many investigators supported this idea since they stated that there are positive relationships between peroxidase enzyme and resistance developed in plants (Eid *et al*., 2024). In the same manner, *B. safensis* (BS-22) was also the best treatment for increasing chitinase, POD, PAL and β-1,3-glucanase activities which denote plant resistance against diseases as these enzymes are pathogenesis-related proteins in strawberry plants (El-Batal *et al*., 2024).

CONCLUSIONS

Based on laboratory and glasshouse experiments, the tested bioagents (*Bacillus subtilis* (Bs1) and *Pseudomonas putida* (Psp1)) proved to have great potential to control *Verticillium* wilt in eggplant. Inoculation of eggplant seedlings with PGPR enhanced the plant tolerance to *Verticillium* wilt via plant vigour, growth parameters, induced plant defense and antagonistic effect. Indeed, combined inoculation resulted in significant increase in the measured parameters and caused a significant decrease in disease severity that indicated the possibility of using seedling inoculation with microbial control agents as potential candidate to control *Verticillium* wilt in eggplant. In further research, it is needed to improve and applicable new bioformulations especially for field treatments.

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