



Original article

Pseudomonas spp. Mediate defense response in sugarcane through differential exudation of root phenolics

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ABSTRACT

Pseudomonas spp., a ubiquitous biocontrol agent, protects the plants from phytopathogens by suppressing them directly by reinforcing the plant's intrinsic defense mechanism. Root exudated phenolics play an important role in establishing the rhizobacteria population and cross the host boundaries in beneficial plant-microbe interaction. In this study, *Pseudomonas* spp. HU-8 & HU-9 antagonized the sugarcane red rot pathogen (*C. falcatum*) and showed a positive chemotactic response against different concentrations (10–30 μM) of synthetic phenolic acids like *p*-coumaric, vanillic, and 3,4 di-hydroxybenzoic acid. In a pot experiment, they effectively colonized the sugarcane rhizosphere and mediated defense response in sugarcane plants challenged with red rot pathogen *C. falcatum* by regulating the exudation of root phenolics under hydroponic conditions. They significantly induced the activity of the antioxidant enzymes CAT (1.24–1.64 fold), PO (0.78–1.61 fold), PAL (0.77–0.97 fold), and PPO (3.67–3.73 fold) over untreated plants in sugarcane. They also induced the total phenolic contents (TPC) in sugarcane in the presence (6.56–10.29 mg/g GAE) and absence (2.89–4.16 mg/g GAE) of the pathogen quantified through the Folin-Ciocalteu (FC) method. However, their effect was lower than that of the pathogen (4.34–8 mg/g GAE). The *Pseudomonas* spp. significantly colonized the sugarcane rhizosphere by maintaining a cell population of (1.0E + 07–1.3E + 08 CFU/mL). A significant positive Pearson's correlation was observed between the root exudated total phenolic contents, antioxidant enzymatic activities, and rhizospheric population of inoculated bacteria. The 16S rRNA and *rpoD* gene analysis showed sequence conservation (C: 0.707), average number of nucleotide differences (k: 199.816), nucleotide diversity, (Pi): 0.09819, average number of informative nucleotide sites per site (Psi: 0.01275), GC content (0.57), and polymorphic sites (n = 656). These diverse *Pseudomonas* spp. could be an ideal bio-inoculants for a broad range of hosts especially graminaceous crops.

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1. Introduction

Sugarcane (*Saccharum officinarum* L.), a major cash crop, is grown worldwide. Pakistan ranks 4th among sugarcane-producing countries in the world (GOP, 2019). The annual yield

of sugar cane is diminished severely due to fungal diseases. Red rot is the most devastating fungal disease that reduces cane yield by 5–50% worldwide (Viswanathan, 2021). Various disease control through general strategies in combination with; use of resistant varieties, agronomic/ cultural practices, fungicides, and biological control is employed to control the disease (Hossain et al., 2020).

Biological control using antagonistic rhizobacteria is an eco-friendly and sustainable practice to control red rot disease (Sharma et al., 2018). Plant Growth Promoting Rhizobacteria (PGPR) antagonizes microbes through direct suppression of pathogen (mucolytic enzymes; volatile organic compounds (VOCs); hydrolytic enzymes i.e., chitinase, β- glucanase; antibiotics, etc.) and/ or reinforcement of the plant's defense to combat the pathogen (Jayakumar et al., 2021; Rawat et al., 2021). The plant defense system consists of some proteins/ metabolites such as antioxidant

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enzymes. The induction of the plant defense system is termed induced systemic resistance (ISR) (Bano et al., 2017). Rhizobacteria have been widely reported as an elicitor of ISR (Singh et al., 2021). Although, numerous rhizobacteria have been characterized as bio-inoculants yet their commercial application is limited (Ali et al., 2020). The sub-optimal population of bio-inoculants in the plant's rhizosphere especially non-host leads to their inconsistent performance in the field. This inconsistency in field performance is the major limitation in the commercialization of bio-inoculants.

Rhizobacteria (the ingredient of bio-inoculant) should maintain their population in the rhizosphere and/ or endosphere of the host to perform consistently. This root colonization process is dependent upon the host-bacteria interaction in the highly complex soil environment (Haskett et al., 2021; Pane et al., 2020). Plant recruits rhizobacteria by exuding certain compounds termed root exudates, which include organic acids, amino acids, phytoalexins, vitamins, inorganic ions, purines, nucleosides, flavonoids, and sugars (Zhang et al., 2013; Bez et al., 2021). These root exudates act as signaling molecules/ chemoattractants for the colonization of beneficial rhizobacteria (Xiong et al., 2020). Root exudated organic acids and DIMBOA [2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one] improved the colonization of *Pseudomonas fluorescens* WCS365 and *Pseudomonas putida* KT2440 on tomato and maize crops (Sarma et al., 2015). *Medicago truncatula* (barrel medic/ medick) and *Medicago sativa* (alfalfa) released flavonoids and flavones (7,4'-dihydroxyflavone) to attract the plant growth-promoting rhizobacteria (Kowalska et al., 2007).

The root exudate-mediated plant-microbe interaction is highly specific to plant and microbial communities (Lombardi et al., 2018, Yasmin et al., 2020). As reported by Liu et al. (2017), the inoculation of *F. oxysporum* causing cucumber root rot, reduced the exudation of raffinose while increased that of tryptophan to promote the colonization of plant beneficial bacterium *Bacillus amyloliquefaciens*. Similarly, *P. fluorescens* WCS365 and *P. aeruginosa* P4 enhanced the production of organic acids, amino acids, and phenolic acids in tomatoes and peanuts (Gupta et al., 2020). Apart from root colonization, certain root exudates like phenolic compounds also play an important role in mediating plant defense (Rawat et al., 2021). They suppress the pathogens and induce systemic resistance in plants. The phenolics exuded by *Pisum sativum* L. (common/ garden pea), *Glycine max* L. Merr. (soybean), and *Vicia faba* L. (broad bean) suppressed various pathogens in the rhizobiome (Jain et al., 2015). The phenolic compounds/ polymers (lignin, lignans, and flavonoids, etc.) activate the phenylalanine ammonia-lyase (PAL) pathway to reinforce cell wall mediated defense against pathogens and act as ROS scavengers (Jiang et al., 2019; Rawat et al., 2021).

The composition of root exudated phenolics is similar among the monocotyledons and gramineous plants like sugarcane, wheat, maize (Akladiou et al., 2019). Accumulation of phenolics during biotic stress through phenylpropanoid pathway performs the dual function of attraction and repletion in plants surrounding (Mavrodi et al., 2021). So, exploring the PGPR mediated dynamics of root exudated phenolics could help in mimicking/ crossing the obstacles/ boundaries in the host-specific performance of PGPR. Hence, in the present study, we hypothesized the inoculation of antagonistic *Pseudomonas* spp. differentially regulate the defense system of sugarcane through the exudation of root phenolics.

2. Materials and methods

2.1. Microorganism and culture condition

The antagonistic *Pseudomonas* spp. HU-8 (MF580377), and *Pseudomonas* spp. HU-9 (MF580378) isolated in a previous study (Ullah

et al., 2020), were obtained from Applied Microbiology and Biotechnology (AMB) Laboratory, Department of biosciences, COMSATS University Islamabad (CUI), Islamabad. A virulent strain of *Colletotrichum falcatum* was kindly provided by the Plant Pathologist (Fatima Sugar Research & Development (FSRD) Centre), Fatima Sugar Mills Limited, Kot Addu, Pakistan. The bacterial and fungal strains were maintained at 28 ± 2 °C on their respective media i.e., Luria-Bertani (LB) and potato dextrose agar (PDA) respectively.

2.2. Antagonistic activity

In vitro, antagonistic activity of HU-8 and HU-9 against *C. falcatum* was evaluated on potato dextrose agar (PDA). Fungal mycelial disc (5 mm) of five days old culture was placed in the center of the PDA plate. A 10 μ L drop of freshly grown bacteria in LB broth (10^8 CFU/mL) was spotted at an equal distance from the fungal disc. Sterile LB broth was used as a control. The inoculated plates were placed at 28 ± 2 °C for seven days (Verma et al., 2018). A percentage inhibition test was applied to check the inhibition of the pathogen by antagonistic bacteria was computed by using the following equation (Yasmin et al., 2020).

$$\text{Inhibition \% over control} = [(C - T)/C] \times 100$$

2.3. Chemotaxis assay

The Chemotactic assay was determined as described by Liu et al. (2019). Briefly, a 2 μ L drop of freshly grown bacteria (10^8 CFU/mL) in LB broth was placed in the center of soft agar (0.3%) plate containing M9 medium amended with different concentrations (10 μ M, 20 μ M, 30 μ M, 40 μ M) of phenolic compounds (3,4 di-hydroxybenzoic acid, Vanillic acid, and *p*-coumaric acid) as sole carbon source. Glucose and Casamino acids 2% (w/v) were used as positive and negative control respectively (Cremer et al., 2019; Elbing and Brent, 2019).

2.4. Growth of sugarcane plantlets and experimental design

Micropropogated sugar cane plantlets were obtained from Shakarganj Sugar Research Institute (SSRI), Jhang, Pakistan. The plantlets were gently uprooted from Murashige and Skoog (MS) medium and shifted in 50 mL of falcon tubes filled with Hoagland nutrient solutions as describe by Jabeen et al. (2015). The hydroponic nutrient solution was replaced on alternative days. The plants were placed in Lab conditions of 16/8 h (h) light/ dark period at 30 °C during October-November 2019 (Franklin et al., 2006). The experiment was conducted in three replicates by following the completely randomized design. Each replicate consisted of five plants.

2.5. Microbial inoculation

Micropropogated sugar cane plantlets were first established in hydroponic nutrient solutions for a week. Roots of micro propagated sugar cane plantlets were sterilized with mercuric chloride (0.1%), dipped in bacterial suspension (10^8 CFU/mL) for 3 h, and transplanted in Hoagland nutrient media with an additional 5 mL of the bacterial suspension. The bacterial population was harvested by centrifugation and suspension in fresh media.

Red rot pathogen *Colletotrichum falcatum* was grown on a PDA plate at 28 ± 2 °C for seven days. Fungal spores were harvested in 0.5% gelatin solution and adjusted to a density of 10^5 /mL. After seventy-two hours of bacterial inoculation, fungal spores were injected into plants through leaves and stem by syringe method (Khan et al., 2017).

2.6. Antioxidant enzymes activity

Three plants were harvested from each replication and frozen in liquid nitrogen. The plant shoots were chopped and mixed to make a representative sample. One gram of plant shoot was crushed in pre-chilled mortar and pestle by using liquid nitrogen. The ground tissues were suspended in phosphate buffer (0.1 M; pH 7.0) and centrifuged at 10,000 rpm, 4 °C for 12 min. The supernatant was used as a crude enzyme extract (Rais et al., 2017).

2.6.1. Catalase (CAT)

The activity of the CAT enzyme was assayed by preparing a mixture of enzyme extract (100 µL), phosphate buffer (100 mM, 1.7 mL), hydrogen peroxide (25 mM, 1.2 mL) and observing the absorbance (OD = 420 nm) at spectrophotometer (Specord-50 Analytik Jena Germany). The mixture of phosphate buffer, hydrogen peroxide, and heat-killed crude enzyme extract was used as control. The enzyme activity was expressed as U/min/mg of fresh weight (FW) as described by Rajeswari (2014).

2.6.2. Peroxidase (POD)

Peroxidase activity was assayed as described by Sofy et al. (2020). The reaction mixture (3 mL) containing 100 mM potassium phosphate buffer (0.9 mL); 1% H₂O₂ (0.5 mL); 0.05 M pyrogallol (1.5 mL) and crude enzyme extract (0.1 mL). The absorbance was recorded at 425 nm. Heat killed crude enzyme was used in control. The enzyme activity was expressed as U/min/mg FW.

2.6.3. Polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) activity was assayed as described by Zhang and Shao (2016). A mixture (3 mL) containing 0.1 M sodium phosphate buffer (1.3 mL); 0.1 M catechol (1.5 mL); and enzyme extract (200 µL). The enzyme extract was added to start the reaction and absorbance was observed at 420 nm. Heat killed crude enzyme was used in control. The enzyme activity was expressed in units (U/min/mg FW).

2.6.4. Phenylalanine ammonia-lyase (PAL)

Phenylalanine ammonia-lyase was assayed as described by Aoki et al. (1971). A reaction mixture (3 mL) consisted of 0.01 M L-phenylalanine (0.75 mL); 0.05 M borate buffer (2.15 mL) and 0.1 mL of crude enzyme extract. Phenylalanine conversion into cinnamic acid estimated at 290 nm and expressed as U/min/mg FW. Heat killed crude enzyme was used in the reaction mixture of the control treatment.

2.7. Quantification of root exudates

The root exudates were collected on alternative days for three weeks. The hydroponic solution was collected at each time interval and centrifuged at 10000 rpm for 10 min to remove the cells and debris. The supernatant was filtered through double-layered filter paper (Whatman no. 1) and lyophilized in a freeze dryer (Martin Christ, Germany). The freeze-dried root exudates were dissolved in methanol (1 mg/mL). The phenolics present in root exudates were quantified spectrophotometrically as described by Everette et al. (2010). A reaction mixture (3 mL), containing water (1.58 mL); F-C reagent (0.1 mL), and root exudates (20 µL), was homogenized and incubated at room temperature for 5 min. A 20% aqueous sodium carbonate solution (0.3 mL) was added to the mixture and incubated at 45 °C for 30 min. The absorbance of the mixture was measured at 750 nm electrometrically (Specord-50 Analytik Jena Germany). The phenolic acids concentration was expressed in terms of gallic acid equivalents (GAE), which is defined as the slope of test compound/ slope of gallic acid in the standard curve (Siddiqui et al., 2017).

2.8. Root colonization

The colonizing ability of *Pseudomonas* spp. in the sugarcane rhizosphere was assessed on culture plates at respective stages of root exudate collection. The cell pellet was diluted serially (1–8), plated on selective medium (cloud S1), incubated, and counted. The inoculated strains were identified based on their reported morphological and functional traits (Ullah et al., 2020).

2.9. Nucleotide analysis and phylogenetic lineage

The nucleotide analysis and phylogenetic lineage of *Pseudomonas* spp. were conducted based on the 16S rRNA (MF347453, MF347454) and *rpoD* genes (MF580377, MF580378) of HU-8, HU-9 respectively which were sequenced in our previous study (Ullah et al., 2020). The nucleotide sequences of respective genes were accessed from “National Center for Biotechnology Information” (NCBI) <https://www.ncbi.nlm.nih.gov/>. The strains and their accession numbers are given in Table 1. The concatenated approach was applied to make a “super-gene” by head-to-tail alignment (Gadagkar et al., 2005) of both genes (16S rRNA and *rpoD*) through an online station http://www.bioinformatics.org/sms2/combine_fasta.html. This single sequence of different strains was aligned using the Muscle algorithm and trimmed for homozygosity in length on Molecular Evolutionary Genetics Analysis X (MEGA X). The homozygous sequences were analyzed at DNA sequence polymorphism (DNAsp) for various parameters like number of variable sites, nucleotide diversity (per site), number of polymorphic sites, the total number of mutations, Average number of nucleotide differences (k), Average number of nucleotide differences between populations, and nucleotide diversity, Pi(t). A phylogenetic tree was constructed on concatenated genes sequence through MEGA X based on a neighbor-joining algorithm with 1000 bootstrap values (Kumar et al., 2018).

2.10. Statistical analysis

The Percentage values were changed in arcsine values for statistical analysis and transformed back before the presentation. The numeric values of different treatments were subjected to the analysis of variance (ANOVA) and separated at least significant difference (LSD) $p \leq 0.05$.

3. Results

3.1. Antagonistic activity of *Pseudomonas* spp.

Pseudomonas spp. significantly inhibited the red rot pathogen *C. falcatum*. *Pseudomonas* spp. HU-8 showed maximum mycelial growth inhibition (64.5%) followed by that of *Pseudomonas* spp. HU-9 (48.05%).

3.2. Chemotactic response of bacterial strain towards different phenolic compounds

The swarming motility of HU-8 was better than that of the HU-9 strain under different concentrations of the synthetic phenolic compounds as compared to 2% glucose used as the sole carbon source. The highest swarming motility was observed (9–9.33 m m/24 h) in Vanillic acid and 3,4 di-hydroxybenzoic acid respectively followed by *p*-coumaric acid (7.33 mm/24 h). The chemotaxis response was highly dependent upon the concentration of compounds used as carbon sources. Bacteria followed a bell curve for chemotactic rings in response to different concentrations with the maximum at 20 µM and 30 µM during 48 to 72 h; except under

Table 1
Pseudomonas spp., their country of origin, length, and accession numbers of analyzed genes.

Strain Name	Country	Nucleotide length			Accession No
		16S rRNA	rpoD	Concatenated gene	
<i>Pseudomonas</i> sp. HU-1	Pakistan	1403	1752	3156	*MF347446 ** MF580376
<i>Pseudomonas</i> sp. HU-8	Pakistan	1412	1749	3154	*MF347453 **MF580377
<i>Pseudomonas</i> sp. strain HU-9	Pakistan	1140	960	2095	*MF347454 **MF580378
<i>P. poae</i> RE* 1-1-14	Austria	1539	1851	3279	***CP004045
<i>P. azotoformans</i> P45A	Canada	1538	1851	3279	***CP041236
<i>P. brassicacearum</i> DF41	Canada	1537	1848	3277	***CP007410
<i>P. chlororaphis</i> subsp. <i>aureofaciens</i> DSM 6698	Canada	1538	1848	3276	***CP027720
<i>P. azotoformans</i> F77	China	1537	1851	3279	***CP019856
<i>P. azotoformans</i> S4	China	1537	1851	3279	***CP014546
<i>P. mosselii</i> BS011	China	1544	1851	3279	***CP023299
<i>P. parafulva</i> CRS01 1	China	1537	1851	3280	***CP009747
<i>P. oleovorans</i> POT9AD	France	1537	1848	3277	***LR130779
<i>P. protegens</i> CHA0	Germany	1539	1848	3278	***LS999205
<i>P. lurida</i> MYb11	Germany	1537	1851	3279	***CP023272
<i>P. agarici</i> NCPPB 2472	Great Britain	1490	1848	3267	***CP014135
<i>P. fluorescens</i> SBW25	Great Britain	1537	1851	3279	***NC012660
<i>P. granadensis</i> CT364	Great Britain	1537	1848	3276	***CP069352
<i>P. granadensis</i> LMG 27,940	Great Britain	1537	1848	3276	***LT629778
<i>P. lurida</i> L228	Ireland	1538	1851	3279	***CP015639
<i>P. aeruginosa</i> DSM 50,071	Japan	1536	1854	3281	***CP012001
<i>P. putida</i> NBRC 14,164	Japan	1537	1851	3280	***NC021505
<i>P. simiae</i> WCS417	Netherlands	1537	1851	3279	***CP007637
<i>P. syringae</i> pv. <i>actinidiae</i> ICMP 20,586	New Zealand	1538	1851	3280	***CP017007
<i>P. syringae</i> pv. <i>actinidiae</i> str. Shaanxi-M228	New Zealand	1538	1851	3280	***CP032631
<i>P. graminis</i> PgKB30	South Korea	1538	1845	3273	***CP053746
<i>P. parafulva</i> JBCS1880	South Korea	1538	1851	3280	***CP031641
<i>P. psychrotolerans</i> CS51	South Korea	1544	1854	3282	***CP021645
<i>P. rhizosphaerae</i> DSM 16,299	South Korea	1537	1845	3273	***CP009533
<i>P. soli</i> SJ10	South Korea	1539	1851	3279	***CP009365
<i>P. poae</i> PMA22	Spain	1537	1851	3279	***CP063073
<i>P. monteilii</i> TCJ-CK1	Taiwan	1537	1851	3279	***CP040324
<i>P. alcaligenes</i> NEB 585	USA	1537	1839	3267	***CP014784
<i>P. azotoformans</i> LMG 21,611	USA	1537	1851	3279	***LT629702
<i>P. brassicacearum</i> LMG 21,623	USA	1537	1848	3276	***LT629713
<i>P. fulva</i> 12-X	USA	1524	1848	3276	***CP002727
<i>P. lini</i> DSM 16,768	USA	1537	1848	3276	***LT629746
<i>P. mediterranea</i> DSM 16,733	USA	1537	1848	3276	***LT629790
<i>P. oryzae</i> USDA-ARS-USMARC-56511	USA	1537	1854	3282	***CP013987
<i>P. poae</i> CAP 2018	USA	1538	1850	3278	***CP034537
<i>P. poae</i> LMG 21,465	USA	1537	1851	3279	***LT629706
<i>P. syringae</i> CC1557	USA	1539	1848	3279	***CP007014
<i>P. trivialis</i> LMG 21,464	USA	1537	1851	3279	***LT629760

* 16S rRNA gene sequence accession number.

** *rpoD* gene sequence accession number.

*** 16S rRNA and *rpoD* genes sequence having same accession number as of whole genome.

vanillic acid initially, bacteria show higher swarming motility at 10 μ M concentration which slows down with the passage of time and higher concentration (20 μ M, 30 μ M, and 40 μ M) (Fig. 1). While relative chemotactic ring formation is shown in the right panel of Fig. 1 (b, d, f) with respective phenolic compounds at different concentrations.

Comparing of chemotactic rings diameter of on different concentration of respective media indicate that under vanillic acid bacterial swarming motility become less as we move higher concentration while there were random swarming motility diameters were observed under *p*-coumaric and 3, 4 di-hydroxybenzoic acids. Comparatively, strains showed higher outer ring formation upon ΔC_1 (20 μ M – 10 μ M) than the higher concentrations ΔC_2 (30 μ M – 20 μ M) and ΔC_3 (40 μ M – 30 μ M) (Fig. 2).

3.3. In planta experiment

Antagonistic bacteria improved antioxidant activity and root exudated total phenolic contents in challenged (*C. falcatum*) or inoculated sugarcane plant. It ultimately improved antagonistic

bacterial colonization and may help to cope with fungus pathogen under hydroponic conditions.

3.3.1. Antioxidant defense enzymes activity in sugarcane

PGPR significantly induced the activity of antioxidant enzymes i.e., Catalase (CAT), Peroxidase (POD), Polyphenol oxidase (PPO) & Phenylalanine Ammonia-Lyase (PAL) in sugarcane upon their inoculation as individual and/ or with the pathogen as compared to that of un-inoculated plants. However, the trend of each strain was variable dependent upon the enzyme type, mode of inoculation, and cultivar as discussed below.

3.3.2. Catalase (CAT) and Peroxidase (PO) activity

The highest CAT and PO activity was observed in the sugarcane plants inoculated with the PGPR and/ or challenged with *C. falcatum*. The *Pseudomonas* spp. enhanced CAT & PO activity (70.75–104.31 U/min/mg FW & 0.10–0.13 U/min/mg FW) in the plants challenged with *C. falcatum* as compared to the plants not inoculated with rhizobacteria but challenged with *C. falcatum* (57.43–90.91 U/min/mg FW & 0.09–0.12 U/min/mg FW). The *Pseudomonas* spp. also induced the CAT and PO activity (20.52–

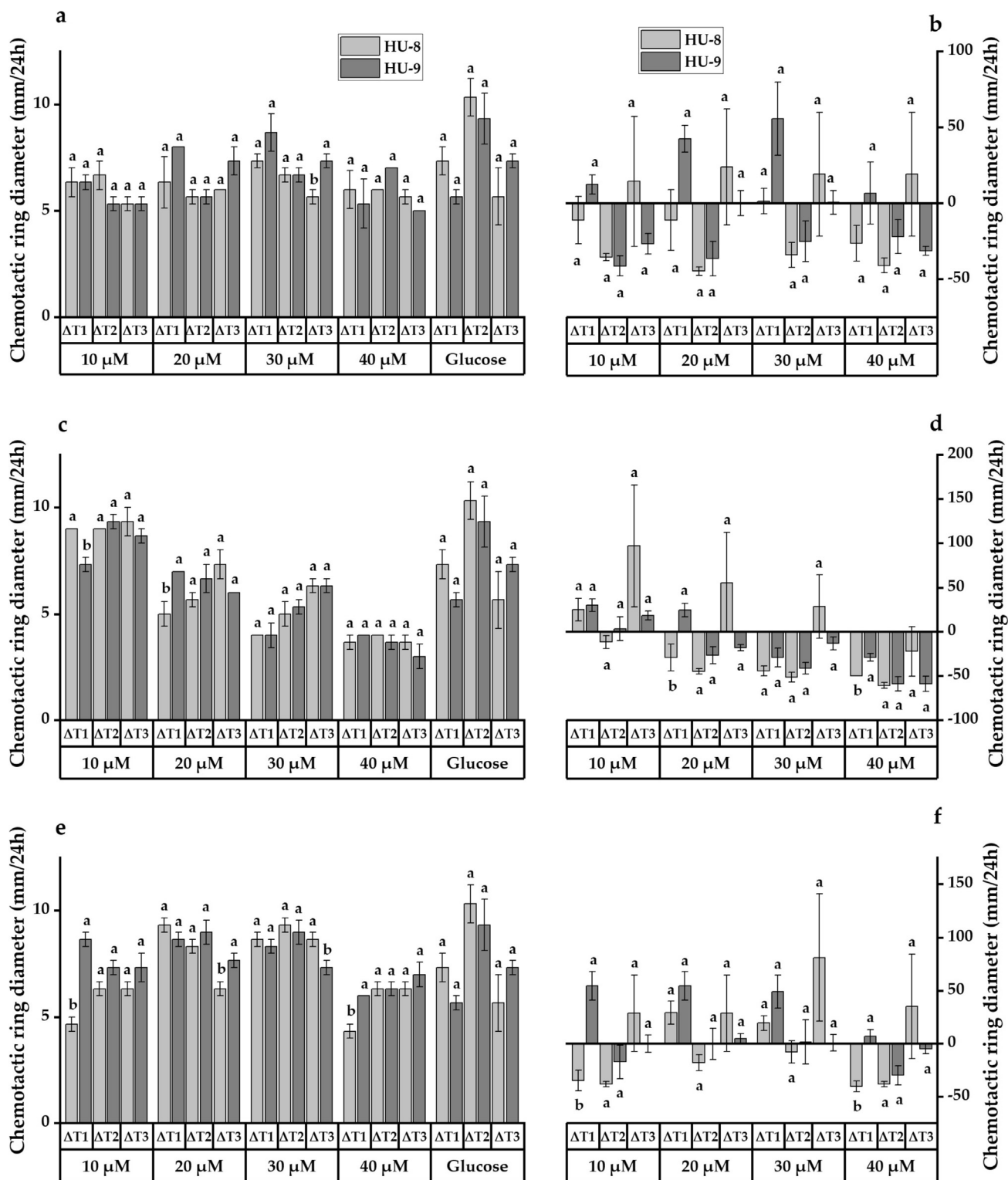


Fig. 1. Swarming motility of *Pseudomonas* spp. on different concentrations of phenolic compounds: An actual change in diameter of swimming motility of *Pseudomonas* spp. (Left panel); Relative swimming motility of *Pseudomonas* spp. against glucose (Right panel), *p*-coumaric acid (a, b), 3,4 di-hydroxybenzoic acid (c, d), and Vanillic acid (e, f). Values are the mean of three replicates; Columns with the variable letter are significantly different by least significant difference test (LSD, $p \leq 0.05$). The values were transformed to arcsine before applying ANOVA.

35.7 U/min/mg FW & 0.035–0.046 U/min/mg FW) over uninoculated plants (neither PGPR nor *C. falcatum*) (Fig. 3: a-d). There was a significant effect of clone on the CAT and PO activity (Table 2).

3.3.3. Polyphenol oxidase (PPO) and Phenylalanine Ammonia-Lyase (PAL) activity

The highest PPO and PAL activity was observed in the sugarcane plants inoculated with the PGPR and/ or challenged with *C. falca-*

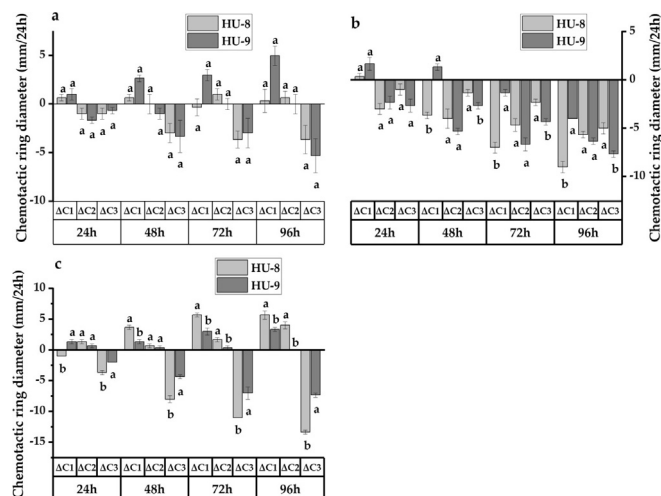


Fig. 2. Chemotaxis ability of *Pseudomonas* spp. on different concentration gradients of phenolic compounds. *p*-coumaric acid (a), Vanillic acid (b), and 3,4 di-hydroxybenzoic acid (c). Values are the mean of three replicates; Columns with the variable letter are significantly different by least significant difference test (LSD, $p \leq 0.05$). The values were transformed to arcsine before applying ANOVA.

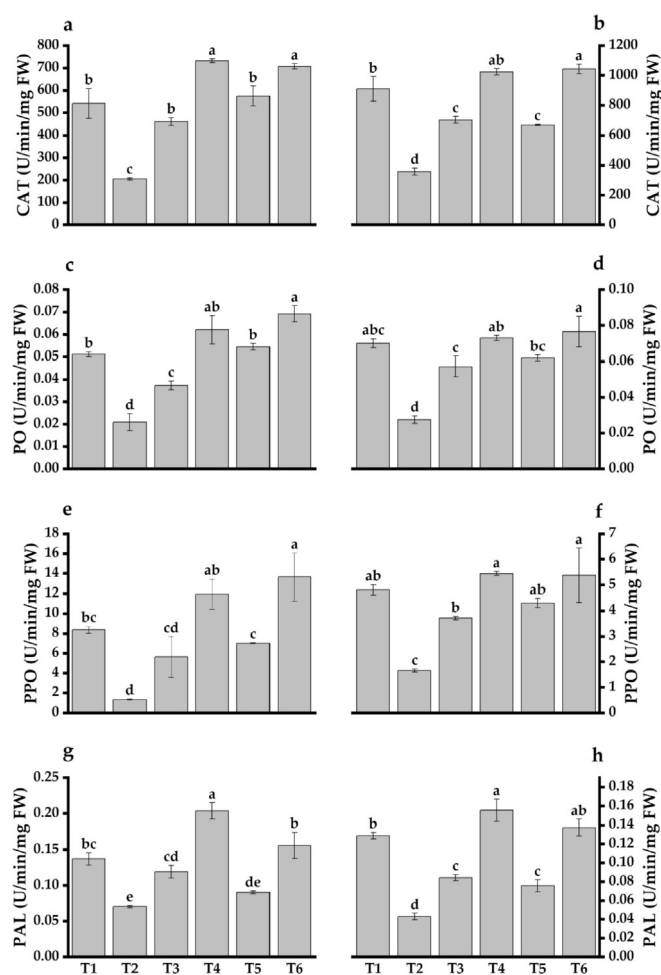


Fig. 3. Effect of *Pseudomonas* spp. on antioxidant defense enzyme in sugarcane. US-718 (Left pane); US-778 (Right pane); CAT (a, b); PO (c, d); PPO (e, f); PAL (g, h). T1 = *Colletotrichum falcatum*; T2 = Control; T3 = HU-8; T4 = HU-8 and *C. falcatum*; T5 = HU-9; T6 = HU-9 and *C. falcatum*. Values are the mean of three replicates; Columns with variable letters are significantly different by the least significant difference test (LSD, $p \leq 0.05$).

tum. The *Pseudomonas* spp. enhanced PPO & PAL activity (1.43–2.86 U/min/mg FW & 0.14–0.20 U/min/mg FW) in the plants challenged with *C. falcatum* as compared to the plants not inoculated with rhizobacteria but challenged with *C. falcatum* (1.10–2.01 U/min/mg FW & 0.13–0.14 U/min/mg FW). The *Pseudomonas* spp. also induced the PPO and PAL activity (0.96–1.76 U/min/mg FW & 0.08–0.12 U/min/mg FW) over un-inoculated plants (neither PGPR nor *C. falcatum*) (Fig. 3: e-h). There was a significant effect of clone on the PPO and PAL activity (Table 2).

3.4. Root exudated total phenolic content in sugarcane

The *Pseudomonas* spp. significantly induced the total phenolic contents (TPC) in sugarcane as compared to that of control (uninoculated). Maximum TPC was observed in plants inoculated with *Pseudomonas* spp. HU-8 and pathogen (6.56–10.29 mg/g GAE) followed by that of *Pseudomonas* spp. HU-9 and pathogen (6.27–9.05 mg/g GAE). The *Pseudomonas* spp. also induced the TPC over an inoculated control (0.64–1.12 mg/g GAE). However, their effect was lower (2.89–4.16 mg/g GAE) than that of the pathogen (4.34–8.01 mg/g GAE) (Fig. 4 a, b). There was a significant effect of time and cultivar on the root exudated phenolics (Table 3).

3.5. Root colonization of PGPR

Pseudomonas spp. HU-8 and HU-9 significantly colonized the sugarcane rhizoplane in the presence as well as the absence of the pathogen stress. Inoculated strains maintained cell density of $1.0E + 07$ – $1.3E + 08$ CFU/mL of root homogenate on both clones US-778 and US-718 until the 3rd week. However, a decrease in cell population was observed from 1st week (4.5 – $4.4 E + 07$ CFU/mL) to 3rd week of inoculation (1.6 – $2.7 E + 07$ CFU/mL) as shown in Fig. 5. The effect of treatment, clone, time and their interactions are shown in Table 3.

3.6. Nucleotide analysis and phylogenetic lineage

The concatenated genes (16S rRNA & *rpoD*) showed the sequence conservation (C: 0.707) with six regions of different lengths from nucleotide no. 55 to (2896). There were 191 sites and an average number of informative nucleotide sites per site (Psi: 0.01275). DNA divergence between population analysis showed the different characteristics of the number of variable sites (S: 656), Number of mutations (1016), average number of nucleotide differences between populations (203.304); the average number of nucleotide differences (k: 199.82), nucleotide diversity (Pi (t): 0.09819); the average number of nucleotide substitutions per site between populations (Dxy: 0.099); the number of net nucleotide substitutions per site between populations (Da: 0.00351). Estimation of codon usage of concatenated gene population showed that all sequences contain protein-coding region from nucleotide site 1 to 3302; with an average number of analyzed codons: 1074.76 and average effective number of codons (ENC: 56.395, Table 4). The phylogenetic tree indicating the lineage of *Pseudomonas* spp. is shown in Fig. 6.

4. Discussion

Rhizobacteria induced systemic resistance mechanism for disease suppression and also involves in a cascade of changes in plant physiology such as suberization, lignification of the cell wall by phenol oxidation, and scavenging of reactive oxygen species through enhanced activity of antioxidant enzymes (Oliveira et al., 2016). In the present study, the rhizobacteria antagonized the red rot pathogen *C. falcatum*, enhanced the activity of antioxidant

Table 2
Analysis of variance for effect of *Pseudomonas* spp. on antioxidant defense enzyme in sugarcane.

Source	DF ^a	CAT (U/ min/ mg FW)			PO (U/ min/ mg FW)		PPO (U/ min/ mg FW)		PAL (U/ min/ mg FW)	
		F ^b	p ^c	F ^b	p ^c	F ^b	p ^c	F ^b	p ^c	
Treat	5	74.53	<0.001	37.31	<0.001	127.3	<0.001	52.88	<0.001	
Clone	1	134.66	<0.001	25.85	<0.001	312.8	<0.001	25.48	<0.001	
Treat*Clone	5	3.02	0.0297	1.11	0.3803	20.33	<0.001	1.4	0.26	
Error	24									
Total	35									

^a . DF: Degree of freedom.
^b . F: F value.
^c . P: Probability.

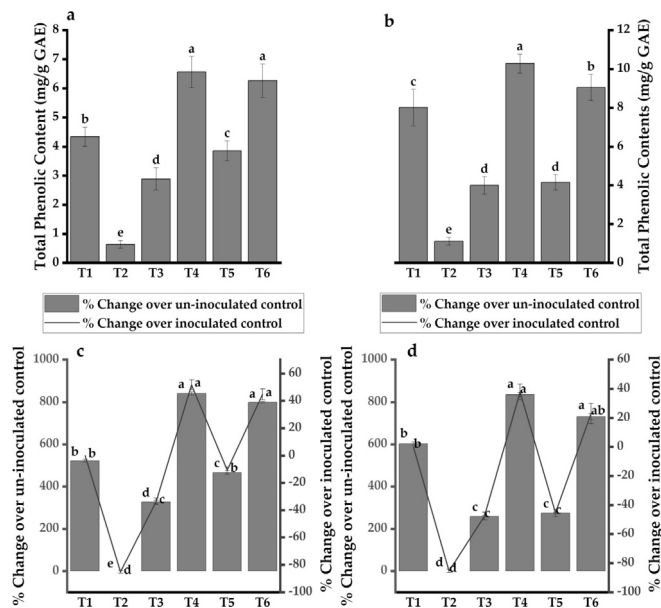


Fig. 4. Effect of *Pseudomonas* spp. on root exudated phenolic contents in sugarcane: (a) Total phenolic contents from sugarcane US-718 (Left pane); (b) US-778 (Right pane). Percent change in total phenolic contents over inoculated control (*C. falcatum*) and un-inoculated control (untreated) in US-718 (c); US-778 (d). Values are the mean of three replicates; the Column with the variable letters is significantly different by the least significant difference test (LSD, $p \leq 0.05$). T1 = *Colletotrichum falcatum*; T2 = Control; T3 = HU-8; T4 = HU-8 and *C. falcatum*; T5 = HU-9; T6 = HU-9 and *C. falcatum*.

enzymes in the sugarcane cultivars, colonized the sugarcane rhizosphere, and regulated the exudation of root phenolics. Suppression of *C. falcatum* by rhizobacteria has been reported in earlier studies (Hassan et al., 2010; Zia et al., 2019) but in this study, rhizobacteria

Table 3
Analysis of variance for colonization of *Pseudomonas* spp. and root exudated total phenolic contents (TPC) of sugarcane.

Source	DF ^a	Colony Forming Unit			*Total Phenolic Content (TPC)	
		F ^b	p ^c	F ^b	p ^c	
Treat	5	36.27	<0.001	452.24	<0.001	
Clone	1	0.04	0.844	86.32	<0.001	
Week	2	6.08	0.0036	231.64	<0.001	
Treat*Clone	5	1.34	0.2557	2.26	0.0229	
Treat*Week	10	2.33	0.0193	14.37	<0.001	
Clone*Week	2	0.5	0.6075	5.41	0.0065	
Treat*Clone*Week	10	0.24	0.9909	3.16	0.0021	
Error	72					
Total	107					

*: The values were transformed to sign arch before applying ANOVA.
^a . DF: Degree of freedom.
^b . F: F value.
^c . P: Probability.

were non-indigenous i.e. isolated from the wheat. These non-indigenous rhizobacteria (*Pseudomonas* spp.) suppressed the *C. falcatum*, a pathogen of sugarcane red rot, and induced the defense response in sugarcane.

Phenolics (a benzene ring with hydroxyl group) play important role in plant physiology to overcome stresses (abiotic/ biotic) lignin and pigment biosynthesis (Bhattacharya et al., 2010). These compounds repel or kill microorganisms to protect plants but can also be subverted by microbes and used for their advantage. In the present study *Pseudomonas* spp. showed chemotactic response against the different concentrations of phenolics which may help them in colonization on different host act as a chemoattractant (Bhattacharya et al., 2010) and microbial community changer (Zhou and Wu, 2018). Microbial populations induced/ selected depend upon the concentration of phenolics and the ability of bacteria to utilize these compounds as carbon sources (Blum et al., 2000). Colonization of *Pseudomonas* spp. on sugarcane plant induce defense enzymes production against the pathogen.

The antagonistic bacteria improved antioxidants activities in sugarcane which are an important determinant of ISR and scavenge the reactive oxygen species (Zia et al., 2019). These findings are similar to the earlier reports where antagonistic bacteria stimulate various pathways in plants like phytohormones and proline biosynthesis to improve the membrane permeability and defense system including antioxidant enzymes under biotic stress (Amna et al., 2020; Singh et al., 2021).

Differential induction of antioxidant enzymes like CAT, POD, PPO, and PAL was observed upon inoculation of rhizobacteria to the sugarcane plants either with or without pathogen. This variation may be due to the mechanisms underlying the induction of system resistance such as microbial-based activation through cell surface pattern recognition receptors (PRRs) (Backer et al., 2018; Khanna et al., 2019) and/ or their metabolites-based activation like volatile compounds, salicylic acid, and methyl jasmonate (MeJA)

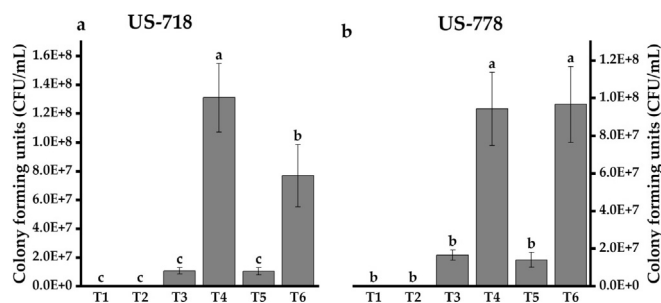


Fig. 5. *Pseudomonas* spp. colonization in sugarcane: Bacterial population (a) US-718; (b) US-778. Values are the mean of three replicates; Columns with the variable letters are significantly different by least significant difference test (LSD, $p \leq 0.05$). T1 = *Colletotrichum falcatum*; T2 = Control; T3 = HU-8; T4 = HU-8 and *C. falcatum*; T5 = HU-9; T6 = HU-9 and *C. falcatum*.

Table 4
Diversity of *Pseudomonas* spp. based on 16S rRNA and *rpoD* genes.

Genetic parameters	<i>rpoD</i>	16S rRNA	*Concatenated
Average number of analyzed codons:	603.619	470.191	1074.762
Effective number of codons, (ENC)	49.824	60.373	56.395
G + C content (selected region), (G + C)	0.595	0.539	0.57
Average number of sites:	1823.81	1419.69	3243.52
Sequence conservation (C)	0.578	0.876	0.707
Average number of nucleotide differences, (k)	163.02	38.908	199.816
Average number of nucleotide differences between populations:	168.952	39.007	203.304
Average number of nuc. subs. per site between populations, (Dxy):	0.18186	0.0352	0.0999
Number of net nuc. subs. per site between populations, (Da)	0.01309	0.00018	0.00351
Nucleotide diversity, (Pi)	0.17548	0.03512	0.09819
Average number of informative nucleotide sites per site (Psi)	0.03316	0.00125	0.01275
Number of sites with information	194	18	191
Number of variable sites (S)	505	153	656
Number of mutations	840	184	1016

* Concatenated genes are constructed through head-to-tail alignment of 16S rRNA and *rpoD* gene.

(Poveda, 2020). Rhizobacteria-mediated induction of the antioxidant enzymes in sugarcane has been reported in numerous studies (Amna et al., 2020; Singh et al., 2021).

In this study, the unit activity of CAT, PO, PPO, and PAL was found lower in the sugarcane plants. However, it was highly induced by the rhizobacteria over control. This might be due to the difference in sugarcane cultivar, microbial strain, and growing conditions (Amna et al., 2020). Highly controlled conditions of the hydroponic experiment could allow strains to exhibit their maximum potential as compared to the complex soil environment.

CAT improves plant defense but also a role in the aging and senescence of plant cells (Yang and Poovaiah, 2002). PO activity leads to the lignification process or produces antimicrobial radicals to inhibit the pathogen (Choudhary et al., 2016). Polyphenol oxidase (PPO) mainly oxidase phenols to improve defense against pathogens and also normalize ROS impact in plants under biotic stress (Sharf et al., 2021). PAL activity plays a role in plant defense through lignin production (Song, 2019; Sofy et al., 2020). In this study, PGPR also induced antioxidants activity in absence of a pathogen but it was lower than that of the pathogen. This could either be due to the greater stress imposed by the pathogenic fungi or higher production of ISR elicitors in rhizobacteria, a consequence of quorum sensing. The pathogen causes toxicity in cells through the generation of reactive oxygen species (ROS) (Sharma

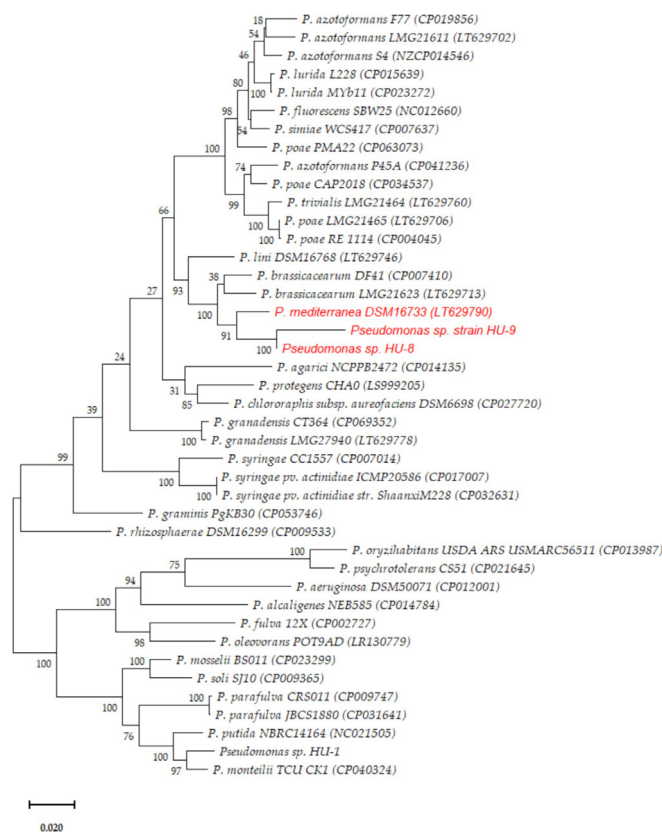


Fig. 6. Phylogenetic tree based on the concatenated sequences of 16S rRNA and *rpoD* genes. Redline letters for indication of *Pseudomonas* spp. analyzed in this study showed consensus with *P. mediterranea* DSM16733 (LT629790). Concatenated gene sequence (16S rRNA + *rpoD*) based evolutionary history was inferred by 42 nucleotide sequences with a total of 3302 positions in the final data set.

et al., 2020). This ROS leads to the higher production of antioxidant enzymes which scavenge ROS and maintain balance to avoid cell injury (Khanna et al., 2019). Our findings are inconsistent with the earlier studies where PGPR induced POD activity even in absence of a pathogen is reported (Asthir et al., 2009; Minaeva et al., 2018). However, we found a lower effect of these rhizobacteria on the non-indigenous host than that of the indigenous host except for PPO activity (Zia et al., 2019; Ullah et al., 2020).

Microbial invasion in plants alters their metabolomics to establish symbiosis. Some of these secondary metabolites include organic acids, phenolics, and flavonoids which are exudates via roots. These root exudates shape the bacterial community in the rhizosphere to establish a plant-bacterial synergetic association (Khanna et al., 2019b; T. A. et al., 2020). In this study, the rhizobacteria stimulated the exudation of root phenolic in sugarcane. A higher concentration of phenolic acids was observed upon the combined inoculation of the antagonistic bacteria and *C. falcatum*. These findings again predict the production of elicitors through altered metabolomics of rhizobacteria under the regulation of quorum sensing (Rosier et al., 2018).

A less induction of phenolics was observed upon the individual inoculation of rhizobacteria as compared to that of the pathogen. This might be due to the hypersensitive infection caused by the fungal pathogen (Jayapala et al., 2019). A differential effect of time on root exudated phenolic was also observed in this study. The root exudated phenolics were higher in concentration initially and diminished laterally. Microbial-mediated induction of root exudates is well documented (Wallis and Galarnau, 2020). As reported by Jayapala et al. (2019), rhizobacteria induced a higher

Table 5

Pearson correlation among total phenolic content (TPC), the antioxidant enzyme of sugarcane clone US–718, and antagonistic bacterial colony forming units (CFU).

	TPC	CAT	PO	PPO	PAL	CFU
US–718						
TPC	1					
CAT	0.98**	1				
PO	0.96**	0.97**	1			
PPO	0.97**	0.97**	0.98**	1		
PAL	0.89*	0.83*	0.74	0.79	1	
CFU	0.8	0.74	0.67	0.78	0.87*	1
US–778						
TPC	1					
CAT	0.98**	1				
PO	0.90*	0.96**	1			
PPO	0.93**	0.97**	0.99**	1		
PAL	1.00**	0.98**	0.90*	0.92**	1	
CFU	0.76	0.74	0.63	0.68	0.74	1

* $p \leq 0.05$.** $p \leq 0.01$.

phenolic acid concentration in chili (*Capsicum annum*) on the 5th day of post-inoculation (dpi).

The root exudates also help the plant to recruit the rhizobacteria and maintain an optimal population density in the rhizosphere which is a prerequisite to benefit the plant against biotic/ abiotic stress. In this study, *Pseudomonas* spp. maintained their population 10^7 - 10^8 colony forming units (CFU), which is necessary to execute the biocontrol activity and other beneficial effects on plants (Rais et al., 2018; Zia et al., 2019). The cell population of these strains was higher than the critical/ threshold level of effective plant-microbe interaction. PGPR utilizes the phenolic compounds as the carbon source to maintain their population in the rhizosphere. Moreover, the phenolics have strong antimicrobial activity and their secretion is highly correlated with the disease susceptibility and resistant trait of varieties. In the current study, a strong correlation was observed between the induction of antioxidant enzymes, root exudated phenolics, and rhizobacteria population in the rhizosphere (Table 5).

Evolutionary relationship between pedigree and ancestor based on conserved and variable regions in sequences of genes. But accurate phylogenetic analysis depends upon highly-conserved and motif regions of molecular sequence (Grundy and Naylor, 1999). Higher variable regions in 16S rRNA lead to inconsistency and lesser discrimination between species of different bacteria. But still can be used to identify genera and bacterial taxonomy. While *rpoD* or σ^{70} (protein-encoding genes) evolves at a higher rate through gene duplication than rRNAs, with rare horizontal gene transfer (Lalucat et al., 2020).

Genetic parameter analysis provided variation in housekeeping genes of the population which helped in discrimination between the different strains and species. It also provided information on highly active genes and horizontal gene transfer between the species. The two genes sequence arrangement as “concatenated supergene” into a single sequence provides much accuracy and least variance for distance estimation (Gadagkar et al., 2005). HU-8 and HU-9 showed sequence homology with *Pseudomonas mediterranea* as shown in Fig. 6.

5. Conclusions

Plant growth-promoting rhizobacteria antagonize *C. falcatum* in dual culture assay. These antagonistic bacteria had better root colonization and maintain their population in non-host crop plants under lab conditions. This ultimately leads to improve plant defense through antioxidant enzyme and also differentially induce total phenolic compounds in presence or absence of the pathogen.

The ability of host shift adaption due to variations in *rpoD* gene which were estimated in *Pseudomonas* spp. of the sugarcane clones and Exploring the PGPR modulated root exudate secretion can act as plant immunization against fungal pathogens. The time and strain-dependent exudation of root compounds help in optimizing the dosage as well as the time of application of bio-inoculants to increase the crop yield and control diseases.

Declarations: I have not taken any material from any source except referred. The findings of this study are a part of the Ph.D. studies of Faluk Shair.

Ethics approval: Not Applicable.

Consent to participate: All authors consent to participate in this manuscript.

Consent for publication: All authors consent to publish this manuscript in the Saudi Journal of Biological Science.

Availability of data and material: Data will be available on request to the corresponding or first author.

Code availability: Not Applicable.

CRediT authorship contribution statement

Faluk Shair: Writing – original draft, Methodology, Data curation, Formal analysis, Conceptualization. **Humaira Yasmin:** Project administration, Formal analysis, Resources. **Muhammad Nadeem Hassan:** Conceptualization, Supervision, Project administration, Formal analysis, Resources. **Othman M. Alzahrani:** Revision, Statistical analysis, Funding. **Ahmed Nourelddeen:** Revision, Statistical analysis, Funding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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