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CONSORTIUM OF COMPOST DWELLING AND RHIZO-BACTERIA ARE CONFEDERATE FIGHTER AGAINST SOIL BORNE PATHOGEN IN MAIZE

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ABSTRACT

Biocontrol emerges as a promising alternative to chemical methods in combating plant diseases, caused by soil borne pathogen. To explore this, an experiment was conducted during 2022-23 in Biochemistry Section in collaboration with Institute of Soil and Environmental Sciences University of Agriculture Faisalabad. Twenty-five strains of bacteria (PGPR and CDB) were secluded from rhizosphere of maize and decomposed compost respectively. The strains were re-streaked, multiplied and purified. The strains were then underwent comprehensive characterization, assessing traits like biosynthesis of auxin (IAA), solubilization of phosphate, study with methyl red, oxidase production, production of siderophore etc. and biofilm formation. Following this assessment, metabolite of competent strains of PGPR and CDB were extracted and identified. Further analysis involved extracting metabolites from these strains using methanol and subsequent analysis via HPLC for phenol and LC/MS for phanazine detection. Notably caffeic acid, chlorogenic acid and smaller quantities of ferulic acid were found in all the four strain's metabolites. Quantitative analysis via LC/MS revealed varied quantities of phenazine in the metabolites. Notably, Mb4 and Cb4 exhibited higher production of 2-hydroxy phenazine, while lesser production was observed in Cb9 and Mb7. These results showed that strains carried higher phanazine and phenol levels enabled them to enhance disease suppression. Moreover, they exhibited superior root colonization in diseased soil, can fostering plant growth even under pathogenic stress conditions. In conclusion, meticulously screened bacterial strains demonstrated the ability to suppress pathogens which may support crop growth amidst disease challenges.

KEYWORDS: Antibiotic compounds; disease confrontation; ferulic acid; bio metabolites; seedling blight; Pakistan

INTRODUCTION

Plant growth faces challenges from various factors, both living (biotic) and non-living (abiotic) factors including climatic conditions, soil properties, salinity, and pathogenic attacks (Ali *et al.,* 2017). To ensure sufficient and high-quality food, feed, and fiber production, managing plant diseases is crucial. Different methods like crop breeding, agronomic practices, and transgenic approaches are employed to prevent or mitigate plant diseases. Additionally, farmers often resort to chemical fertilizers and pesticides to boost crop yield, especially in stressful environments (Aziz *et al.,* 2020). While these agricultural inputs have significantly enhanced crop productivity and quality over the past century, their too much and improper use has directed to environmental pollution. This has prompted shifts in public perception regarding pesticide use in agriculture (Mustafa *et al.,* 2019; Abbas *et al.,* 2020). Less explored is the utilization of microbes for disease and pest management in plants. Seedling blight caused by the soil-borne *Fusarium oxysporum* exhibits 2 modes of deed: bio-trophic and necro-trophic. As a bio-trophic, it infiltrates the plant xylum via root hair and obstructs the movement of water and nutrients, leading to plant death. Being necrotrophic, nourishes on the deceased plant and spreads to neighboring plants population (Agrios, 2005). When plants face pathogenic attacks, they develop resistance against additional invasion(Spoel and Dong, 2012). Supportive microbe-plant interactions aid in more efficiently fortifying plant defenses, especially when these microbes are secluded from problem environments (Huang *et al.,* 2016). Plants inherent response to pathogen attacks includes the development of defense mechanisms (Spoel and Dong 2012; Maqsood *et al.,* 2020). The communication between favorable microbes and florae facilitates

a more robust defense system, particularly when these microbes originate from frazzled environments (Maqsood *et al.,* 2020; Huang *et al.,* 2012). Various PGPR exhibit different control planes of pest by manufacturing resistant compounds in crops. This can involve changes in levels of tryptophan, amino acids and phenylalanine in the stems (Walker *et al.,* 2011; Weston *et al.,* 2012). Viable agriculture aims to preserve the elusive stability of soil ecosystems and unravel the bacterial mysteries within the root zone. Plant Growth-Promoting Rhizobacteria serve multiple functions in the root zone, promoting evolution in soils whether healthy or unhealthy (Umar *et al.,* 2020; Naveed *et al.,* 2020). They performs as biocontrol agents against numerous soil-living pathogens, combating fungal, viral and bacterial diseases. These practices are becoming common in various regions worldwide. PGPRs compete for cosmos through root settlement (Minorsky 2008) and in diseased environments produce antiseptic compounds (Herman *et al.,* 2008), exert cure of plant diseases by generating antibiotics, triggering systemic or local resistance and mitigating harmfulness via the deprivation of xenobiotics (Khan *et al.,* 2019; Khan 2020). Compost Dwelling Bacteria (CDB) and PGPR serve as protective intermediaries for soil-living pathogens(Lowe *et al.,* 2012; Ali *et al.,* 2017). These microbes hinder the action of soil-living pathogens, including nematodes, oomycetes, bacterium and fungus(Ali *et al.,* 2017), while also enhancing growth of plant by encouraging confrontation against many pests of plants (Maqsood *et al.,* 2020). They foster progress of herb by defeating disease causing enemy (Ryu *et al.,* 2006) and manufacturing enzymes and antibiotics to combat pathogens (Karthikeyan *et al.,* 2006; Mustafa *et al.,* 2019), and by generating compounds like siderophore to compete with pathogens(Arora *et al.,* 2005). However, there's a growing concern about the use of these agriculturally important microbes for managing plant diseases. Favorable interactions between plants and microbes trigger systemic resistance in plants (Pozo and Azcón-Aguilar 2007). This interaction empowers plants to defeat pathogens (Pineda *et al.,* 2010). Floras exhibiting Systemic Acquired Resistance and repel the present pathogen and develop resistance against various other pathogens in subsequent infections (Navarre and Mayo 2004). These interactions have induced systemic resistance to control anthracnose in strawberries (Tortora *et al.,* 2012)and controls spot disease of bacteria in peppers (Hahm *et al.,* 2012). The purpose of this trial was to select strains (PGPR and CDB) based on their tendency to help growth and suppress diseases, considering characteristics such as auxin biosynthesis, chitinase, phanazene, and phenol (caffeic acid and chlorogenic acid) production. This selection process aimed to control soil-borne fungal diseases in maize crop.

MATERIALS AND METHODS

***Collection of pathogen (fungi)*:** Institute of Plant Pathology, Faisalabad, Pakistan has provided the required fungus (*Fusarium oxysporum*). It was extracted using the method of tissue segment culture (Rangaswami, 1958) and recognized through morphology of colony and infinitesimal structures (Singh, 1991). The pathogenic effect of the strain was confirmed using live plants in the cut stem method.

Isolation of PGPR: The Soil Bacteriology Section at the AARI, Faisalabad facilitated the extraction of rhizobacteria. The rhizosphere samples were gathered and kept at 4°C in plastic bags. To isolate PGPR, a procedure involving the suspension of 10% rhizosphere soil suspension in sterile distilled water, was prepared. It was followed by agitation on a rotary shaker for 10 minutes, was conducted. Subsequently, a 1 mL of the suspension was conveyed to a falcon tube of volume10 mL and shaken for 2 minutes. Dilution from 10^{-1} to 10^{-7} was performed. An aliquot of volume 0.1 mL of this inoculum was inoculated onto LB agar medium plates. These plates were then placed at 29°C for 72 hours. Well-isolated solo colonies were re-inoculated onto fresh LB agar plates, and incubated similarly (Luria and Burrous, 1957). Finally, 25 pure selected strains were marked codes and placed at cool dry place for storage (Table 2.1).

Extraction of compost dwelling bacteria (CDB): The isolation of CDB followed the adopted dilution-plate method outlined previously(Pugliese *et al.,* 2008). To begin, 1 g of compost samples was combined with sterile distilled water of volume 99 ml in a flask. The mixture was strongly vortexed for 10 minutes and diluted serially to concentrations of 10^{-2}, 10^{-4}, and 10^{-6}. Subsequently, 0.5 mL aliquots from these inoculums were inoculated onto Luria-Bertani (LB) agar media. The plates, carried inoculum were placed at 28°C for 2 days and re-inoculated till the isolated wholesome cultures of required strains were obtained. Selected strains were marked (coded) and placed at -40°C (Table 2.1).

***Characterization of isolates*:** Pure single colonies of the 12 selected strains for both PGPR and CDB underwent characterization to evaluate disease clampdown and promotion potential of plant growth. These assessments were conducted following established methods outlined in Systematic Bacteriology (Bergey's Manual) (see Table 3.1 and 3.2).

***Auxin biosynthesis*:** Screening for auxin biosynthesis

potential in PGPR and CDB was carried out by inoculating the isolates in broth culture of LB media for 4 days. Salkowski's reagent was used to measure Auxin biosynthesis potential as equivalents of IAA. The absorbance was observed at 535 nm on a visible light spectrophotometer alongside standards, considering the technique defined by (Sarwar *et al.,* 1992).

Table 2.1 Coding of the isolated PGPR and CDB strains

Sr. No.	Isolates from maize rhizosphere	Isolates from compost
1	Mb1	Cb1
2	Mb2	Cb2
3	Mb3	Cb3
4	Mb4	Cb4
5	Mb5	Cb5
6	Mb6	Cb6
7	Mb7	Cb7
8	Mb8	Cb8
9	Mb9	Cb9
10	Mb10	Cb10
11	Mb11	Cb11
12	Mb12	Cb12
13	Mb13	Cb13
14	Mb14	Cb14
15	Mb15	Cb15
16	Mb16	Cb16
17	Mb17	Cb17
18	Mb18	Cb18
19	Mb19	Cb19
20	Mb20	Cb20
21	Mb21	Cb21
22	Mb22	Cb22
23	Mb23	Cb23
24	Mb24	Cb24
25	Mb25	Cb25

Oxidase test: The test was conceded out to find the existence of required enzyme in bacterial isolates (Steel 1961). It involved swabbing the test cultures on dry slide of oxidase and observing purple/blue hue after one minute which confirms the positive results. ***Siderophore production***: Evaluation of siderophore production in the isolates was performed using CAS (chrome azurole S agar) as detailed by Clark and Bavoil (1994). Test organisms were streaked on chrome azurole S agar media containing plates and incubated at 30ºC for 120 hours. The colony showed a yellow-orange halo around it, indicated that siderophore is present. ***Starch hydrolysis***: Assessment of the preserved strains' ability for starch dissolution through the production of the enzyme called amylase was carried out (McFaddin and Jeans, 2000). Microbial isolates were inoculated on agar medium of starch and placed in incubator for 48 hours. Subsequently, 1% iodine solution was spread on the plates. A blue color showing colorless zone around the growth and indicated utilization of starch by the microorganisms. ***Catalase activity***: Catalase activity tests were conducted to ascertain the isolated strains' capacity to degenerate hydrogen per oxide by producing peroxidase or catalase enzyme (Mac Faddin 1980). This involved placing 1 mL of H_2O_2 (3%) on a spotless glass slide and emulsifying it with the 48 hours old isolated culture of bacteria. Presence or absence of air bubbles was observed to determine catalase activity. ***Chitinase activity***: Chitinase activity assessments were conducted using orthodox procedures (Skerman 1969). Chitin was integrated into LB agar medium, and test bacterial strains were inoculated onto these plates. The clearing zones around the bacterial colonies alarmed that Chitinase activity was active. ***Antagonism of phytopathogenic fungi***: In this assay, the required pathogenic fungi was streaked at four equal distance on Potato Dextrose Agar (PDA) plates, while CDB and/or PGPR were streaked on the plate center or peripheral points, respectively. This setup was replicated thrice and incubated at 28°C. Every 12 hours, pathogen inhibition was detected and diameter of the pathogen growth reticence zones was premeditated alongside the two vertical lines drawn at the midpoint of all inoculum plates. Following formula is used for percentage inhibition calculation(Vincent, 1947).

Mycelium Inhibition I (%) = (C - T) / C × 100

Where I = percent mycelium inhibition

C = mycelium growth in control,

T = mycelium growth in treatment.

Biofilm formation: Biofilm formation assays were conducted following the method described by (Peeters *et al.,* 2008). An LB media (broth) inoculated with required bacterial strains, containing 10^8 cells mL^{-1} was inoculated on 24 wells of a microtiter dish and placed in incubator at 37°C for 72 hours. After incubation, wells of microtiter containers were sterilized with 100 µL sterile distilled water to eliminate slightly connected planktonic bacteria. For fixation of biofilm, 99% ethanol (100 µL) was put to each well. The ethanol was removed after

15 minutes, plates were dried in air and stained with 0.5% crystal violet (100 µL). After washing with tap water, crystal violet color was released by putting 33% acetic acid (150 µL). Reading for each well was noted at 590 and 600 nm using a microplate reader (ELISA). Based on the characterization results, four highly effective strains, two from each of rhizobacteria (Mb4, Mb7) and compost inhabiting bacteria (Cb4, Cb9), were chosen for further investigation. ***Phenols production*****:** The strains of CDB (Cb4, Cb9) and PGPR (Mb4, Mb7) were cultured in broth culture of LB medium and placed in incubator at approximately 25°C for a period of 72 hours. After this period, it centrifuged at 4°C and 9,000 rpm to get the clear supernatant. Extraction of metabolites were carried out using methanol (HPLC grade) in a 1:1 ratio through vigorous shaking with separating funnels. The extracted organic phase was then analyzed for phenols (Fig 1) using HPLC by method described by (Mattila and Kumpulainen, 2002). ***Phenazine production*****:** Bacterial metabolites were extracted using ethyl acetate for phenazine determination. Broth samples of bacteria were mixed with ethyl acetate in a 1:1 ratio, and 10 µL of extracted samples were used for the LC/MS analysis (Mazurier *et al.,* 2009). ***Statistical analysis:*** Mean of data taken for two years was statistically analyzed through standard procedures (Steel *et al.,* 1997) using Statistics.8.1. Least significant difference test (LSD) at $p < 5\%$, were applied to evaluate treatment means differences.

RESULTS AND DISCUSSION

Bacterial strains**:** Numerous strains of rhizobacteria were extracted from root zone of maize, and CDB were isolated from finally decomposed organic matter (compost). Out of the isolated strains, 25 purified strains of PGPR (Mb) and twenty five of CDB (Cb) were selected (Table 2.1). These strains were assessed for their contribution in promotion of growth, initially focusing on proficient germination rates. Twelve proficiently germinated strains were selected for characterization via several biochemical traits (Table 3.1 and 3.2). One of the primary causes of significant annual agricultural losses is attributed to phytopathogenic fungi, particularly soil-borne pathogens. Fungi of name *Fusarium oxysporum* causing seedling blight in maize. Traditionally, fungicides have been employed for control, but biological control using plant-associated microbes presents an efficient and eco-friendly approach. Both PGPR and CDB displayed antagonistic behavior against *Fusarium oxysporum*. Compost, recognized for its disease-suppressing activity is attributed to its rich microbial community. It might be because of improved production of hormones that could triggered the particular enzymes activity (Gholami *et al.,* 2009) due to which obtainability of starch is increased and so primary sprouting is encouraged. In the inoculated treatments efficient action of mitochondrial enzymes may increase the oxygen supply (Fatima *et al.,* 2009). Numerous previous studies showed that PGPR act as disease suppressive agent, thus enhance the seed germination, growth and crop produce in diseased soil (Herman *et al.,* 2008; Srivastava *et al.,* 2010). The strains of PGPR and CDB produced different range of IAA it might be due to stage of growth, condition of culture and substrate availability(Bharathi *et al.,* 2004). Meticulous mechanism of PGPR is not clearly established for plant growth promotion even then numerous indices point towards PGPR role by manufacturing of plant-hormone, beating the poisonous beings, obtainability and sucking of phosphorus and inorganic nutrition (Pandey and Maheshwari, 2007). Compost dwelling bacteria, especially pseudomonads and some *Bacillus spp*, have demonstrated effective control against root diseases in agricultural crops (Noble and Coventry, 2005). Research worldwide focuses on managing diseases caused by fungus due to hostile microbes (Abaidoo *et al.,* 2011). Rhizobacteria acts not only as growth supporters but also as biocontrol managers, inhibiting the activity of pathogens, residing in the soil. Different Bacillus bacteria are recognized to manufacture various types of antibiotics and their hostile action against various plant pathogens (*Fusarium moniliforme*, *Phytophthora capsici*, and *Fusarium oxysporum*) has been noted in stressed environment (Tank and Saraf 2010). Studies have shown that bacterial strains of pseudomonas fluorescence and *Bacillus sp.* contribute to the resist of seedling blight in wheat caused by *Fusarium graminearum*, *Fusarium culmorum*, and *Microdochium nivale* (Ahmadzadeh *et al.,* 2004). These strains have chitinase production capabilities contribute to disease control eliminating harmful microorganisms. Previous experiments have underlined the potential of *Fusarium* and *Pseudomonas spp*. as biocontrol managers in response to extensive array of plant pathogens. Additionally, the strains' capacity to form a dense biofilm and their ability to produce chitinase (Karthikeyan *et al.,* 2006) and siderophores (Arora *et al.,* 2005) make them effective defenders against soil-borne plant pathogens, enhancing plant growth and inducing resistance against pests. Studies have also shown that compost-inhabiting microbes play a role in inhibiting seedling blight in maize and cowpea (Muhammad *et al.,* 2001). Enriching compost with beneficial microorganisms could potentially improve disease control efficiency

Table 3.1: Characterization of PGPR isolates for biochemical activities

PGPR	IAA equalants (µg mL^{-1})	Oxidase	Siderophore	Starch	Catalase	Chitinase	Antagonism (%)	Biofilm at OD	
								600 nm	5 9 5 nm
Mb1	7.9	++	_	_	++	_	72	1.243	0.976
Mb2	26.7	++	++	_	++	++	74	1.198	0.278
Mb3	6.7	_	_	++	_	_	80	1.279	0.557
Mb4	34.3	_	++	++	++	++	84	4.095	2.971
Mb5	36.5	++	_	++	_	_	83	2.018	1.775
Mb6	32.5	++	++	++	++	++	62	2.508	1.556
Mb7	39.6	++	++	++	++	++	85	3.314	2.860
Mb8	11.3	++	_	++	_	_	77	1.223	0.321
Mb9	9.5	++	_	++	_	_	69	1.972	0.687
Mb10	10.2	++	_	++	_	_	75	1.336	0.885
Mb11	10.9	++	_	++	_	_	79	1.143	0.932
Mb12	7.6	++	++	++	++	++	67	1.923	0.532

Table 3.2: Characterization of CDB isolates for biochemical activities

CDB	IAA equalants (µg ml^{-1})	Oxidase	Siderophore	Starch	Catalase	Chitinase	Antagonism (%)	Biofilm at OD	
								600 nm	595 nm
Cb1	10.2	++	++	++	++	-	83	2.341	1.29
Cb 2	12.0	_	++	_	++	-	73	1.121	0.32
Cb 3	9.3	_	_	++	_	_	76	1.921	0.41
Cb 4	19.5	++	++	++	++	++	86	3.210	2.78
Cb 5	9.3	++	++	_	_	++	79	1.223	0.932
Cb 6	10.6	++	++	++	_	_	78	0.997	0.432
Cb 7	7.3	++	_	_	_	++	81	1.912	0.567
Cb 8	11.6	++	++	++	++	--	84	2.117	1.56
Cb 9	17.6	++	++	++	++	++	88	4.081	2.54
Cb 10	10.3	++	_	_	_	_	76	1.321	0.23
Cb 11	09.0	++	_	++	++	_	84	1.732	0.79
Cb 12	10.3	++	_	_	++	_	78	1.436	0.75

and consistency (Muhammad and Amusa 2003). Among the PGPR strains, efficient IAA producers were Mb4 (34.3 µg mL-1), Mb5 (36.5 µg mL-1), Mb6 (32.6 µg mL-1), and Mb7 (39.6 µg mL-1) with Mb4 and Mb7 displaying additional characteristics such as higher siderophore production, catalase activity, chitinase production and thicker biofilm formation. These strains (Mb4 and Mb7) exhibited higher antagonistic effects (84% and 85% respectively) against *Fusarium oxysporum* compared to others (Table 3.1). Within the CDB strains, Cb4 and Cb9 showed higher IAA production (19.5 µg mL^{-1} and 17.6 µg mL^{-1} respectively) and positive characteristics in oxidase, siderophore production, starch, catalase, chitinase production and thicker biofilm formation. These strains also displayed higher antagonistic effects (ranging from 86% to 88%) against the pathogen. Other strains, such as Cb1, Cb2, and Cb8, exhibited varying degrees of IAA production and antagonism but differed in their capabilities regarding chitinase and antagonistic effects (Table 3.2). Based on these identified characteristics, 2 strains from PGPR (Mb4, Mb7) and 2 from CDB (Cb4, Cb9) were selected for identification before further tests. In response of diseased environment, excess growth of microbes make biofilm to compete for root niches and nutrition in infected plants (Haas and Défago 2005). To control the plant pathogen biofilm formation is linked with efficiency of plant pathogens control. Microbe's interaction is necessary for biocontrol of soil dwelling pathogens(Timmusk *et al.,* 2005). *Bacillus subtilis* make a bio-barrier in the form of biofilm on the roots of infected plants for protection from pathogens (Morikawa *et al.,* 2006). ***Identification of bacterial strains*:** Strains of bacteria were recognized via a technique of genomic DNA extraction from broth cultures of microbes using the Microbial DNA Isolation Kit and identified by Macrogen, Seoul, South Korea for sequence analysis. Bacterial strains were recognized as *Serratia spp*. (Cb4) and *Pseudomonas spp*. While (Cb9) *Bacillus spp*. (Mb4) and *Pseudomonas aeruginosa* (Mb7). The strains were belong to the family Enterobacteriaceae (Cb4), Bacillaceae (Mb4) and Pseudomonadaceae (Mb7 and Cb9) (Table 3.3). ***Determination of phenolic:*** Metabolites of identified strains were examined for phenolics and phenazine synthesis. Bacterial metabolites of selected strains revealed the presence of various phenolic compounds across all four strains, which were recognized by equating them with standards of known values (Figure 3.1). Significantly higher value of Caffeic acid, chlorogenic acid and Ferullic acid were found in the metabolites of Mb4, following by sinapic acid and vanillic acid. Mb7 and Cb9 displayed higher levels of vitamin C along with Caffeic acid, chlorogenic acid and Ferullic acid while other phenolic compounds (quercetin, gallic acid, p-coumaric and m-coumaric acid, cinnamic acid, and vanillic acid) were detected in lesser quantities in all the strains.

The collaborative impact of microbial interaction has positively influenced plant growth through various mechanisms, notably enhancing tolerance to all kind of stress (Barea *et al.,* 2005). Analysis via HPLC revealed bacterial metabolites. The occurrence of these phenolic compounds within metabolites underscores their capability in controlling fungal pathogens, as previously indicated by (Ge *et al.,* 2006). Cinnamic acid, for instance, is known as an allelochemical and has been observed to suppress *Fusarium spp* (Ye *et al.,* 2006). Resistant infected plants showed confrontation against insects and herbivores (Leiss *et al.,* 2009;Naseem *et al.,*, 2021) due to the presence of chlorogenic acid and its inhibition of growth and germination of *Fusarium spp* (Ling *et al.,* 2013) and its conidia. It mirrors its similarity in behavior to caffeic acid, which has also exhibited a trend of suppressing fungal pathogens (Beekrum *et al.,* 2003; Samapundo *et al.,* 2007). Our selected strains notably synthesized substantial amounts of caffeic acid and chlorogenic acid. Both acids are recognized for their ant oxidative, chemo-preventive, and antibiotic properties (Sato *et al.,* 2011). They exert unique antifungal effects, and their synergistic action contributes to a more robust antimicrobial impact. Released by both plants and microbes during stressful

Table 3.3: Identification of taxonomic characteristics of selected bacterial strains

Strain code	Closest match on the basis of 16S rRNA [accession number]	Bp length	Max score	Identity (%)	Tentative phylogenetic group
Mb4	*Bacillus megaterium* (NC 014103.1)	1131	1648	97	*Bacillaceae*
Mb7	*Pseudomonas aeruginosa* (NC002516.2)	1092	1984	99	*Pseudomonadaceae*
Cb4	*Serratia spp.* (NC015566.1)	1116	1622	97	*Enterobacteriaceae*
Cb9	*Pseudomonas fluorescens* (NC007492.2)	906	158	99	*Pseudomonadaceae*

conditions, these indole acetic acids and their derivatives participate in the plant's defense mechanisms against pathogenic attacks (Bharathi *et al.,* 2004). Secondary metabolites like caffeic acid and its derivatives play a crucial role in plants' defense mechanisms against biotic stress. They act as chemical barriers, swiftly recovering and inhibiting infections, eliminating pathogens, and minimizing damage to plants by being toxic to pests. (Martinez *et al.,* 2000) noted that plants accumulate caffeic acid in higher amounts during fungal attacks, thereby preventing brown rot, a fungal disease. Co-inoculation involving compatible bacterial strains yields superior outcomes compared to individual strains. Although combined inoculation of PGPR has been comprehensively explored for their beneficial impact on crop escalation, limited information exists regarding combined inoculation involving microbes, extracted from distinct environments like compost and rhizosphere. However, in our case, microbes from both habitats exhibited a synergistic effect against fungal pathogens. The microbes from these habitats release compounds like caffeic acid, chlorogenic acid, gallic acid, ferulic acid, ascorbic acid, and cinnamic acid, contributing to the synergy of co-inoculants. *Fusarium spp*. is supposed to be controlled by Cinnamic acid (Ye *et al.*,, 2006). Herbivores and insects are controlled by chlorogenic acid, causing resistance in host plant (Leiss *et al.,* 2009) conidial germination and growth of *Fusarium spp* is also inhibited by cholorogenic acid (Ling *et al.,* 2013). Caffeic acid plays a pivotal role in the plant's antioxidant mechanism, regulating defense responses against both biotic and abiotic stress(Xia *et al.,* 2011), thus facilitating normal physiological processes in plants. ***Determination of phenazine:*** The assessment of metabolites aimed to determine the presence of phenazine in the selected strains (Fig 3.2-3.8). The analysis indicated that all the four strains produced phenazine. Among these strains, Mb7 and Cb9, produced 2-Hydroxy phenazine (21 and 29% respectively), 2, 8-dihydroxy phenazine (5 and 27% respectively) and Phenazine-1- Carboxylic acid (17 and 15% respectively). Conversely, higher concentration of 2-hydroxy phenazine was produced by Mb4 (95%) and Cb4 (90%) compared to Mb7 and Cb 9. (Table 3.4). Phenazine production by all the four strains is also illustrated graphically instigated by LC/MS in Figures 3.2 to 3.8.

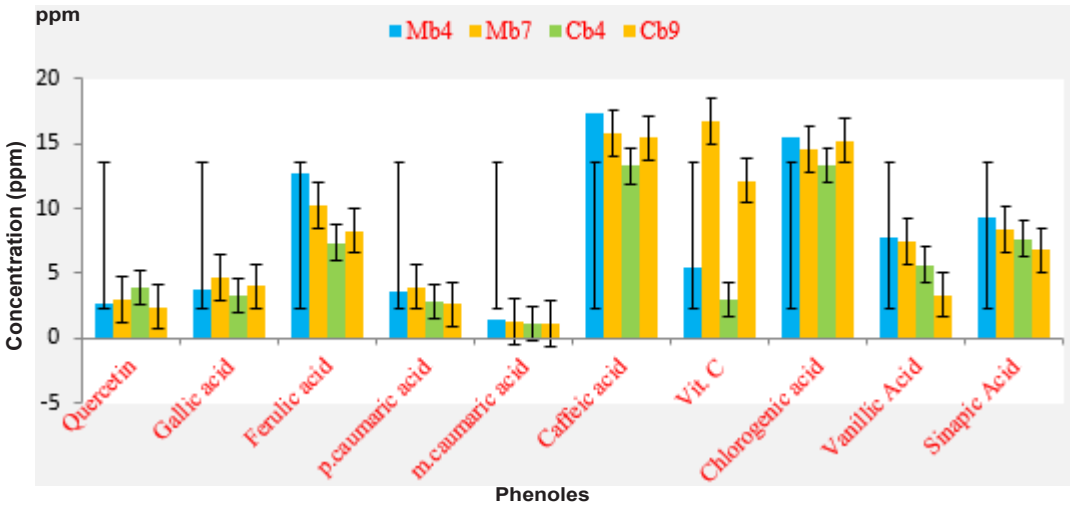

Fig. 3.1. Concentration of phenols in bacterial metabolite

Table 3.4: Phenazine production analysis of bacterial metabolite

Bacterial strains	2-Hydroxy phenazine (%)	2, 8-dihydroxy phenazine (%)	Phenazine-1-Carboxylic acid (%)
Mb4 (*Bacillus spp)*	95.0	-	-
Mb7(*Pseudomonas aeruginosa*)	21.0	5.0	17.0
Cb4 (*Serratia spp*)	90.0	-	-
Cb9 (*Pseudomonas spp*)	29.0	27.0	15.0

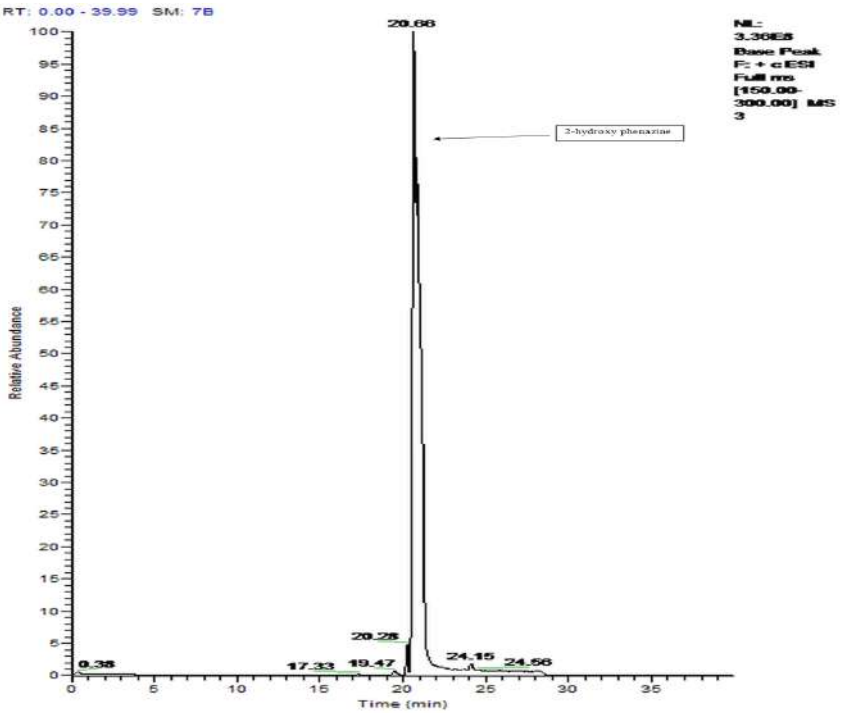

Fig. 3.2. Bacterial strain Mb4 (Bacillus spp.) production of Phenazine

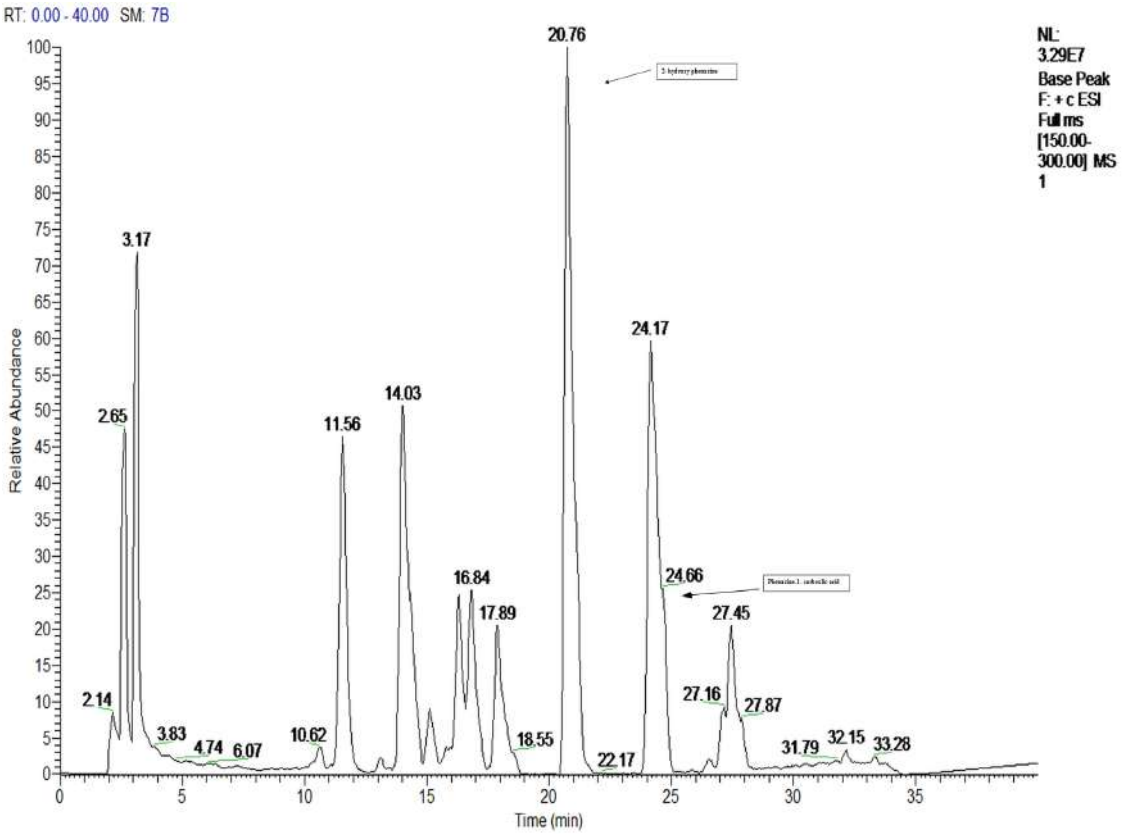

Fig. 3.3. Bacterial strain Mb7 (*Pseudomonas aeruginosa*) production of Phenazine

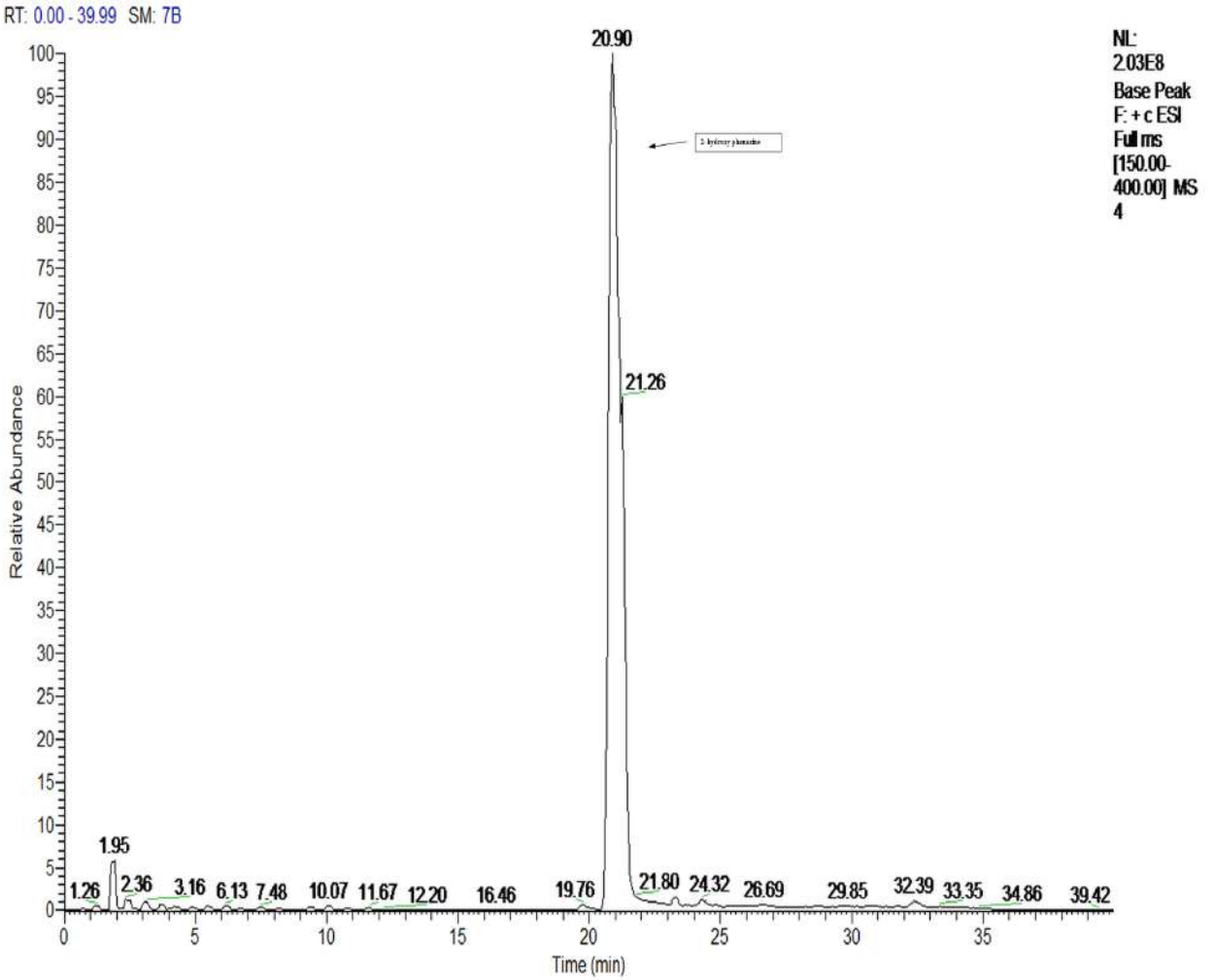

Fig. 3.4. Bacterial strain Cb4 (*Serratia spp.*) production of Phenazine

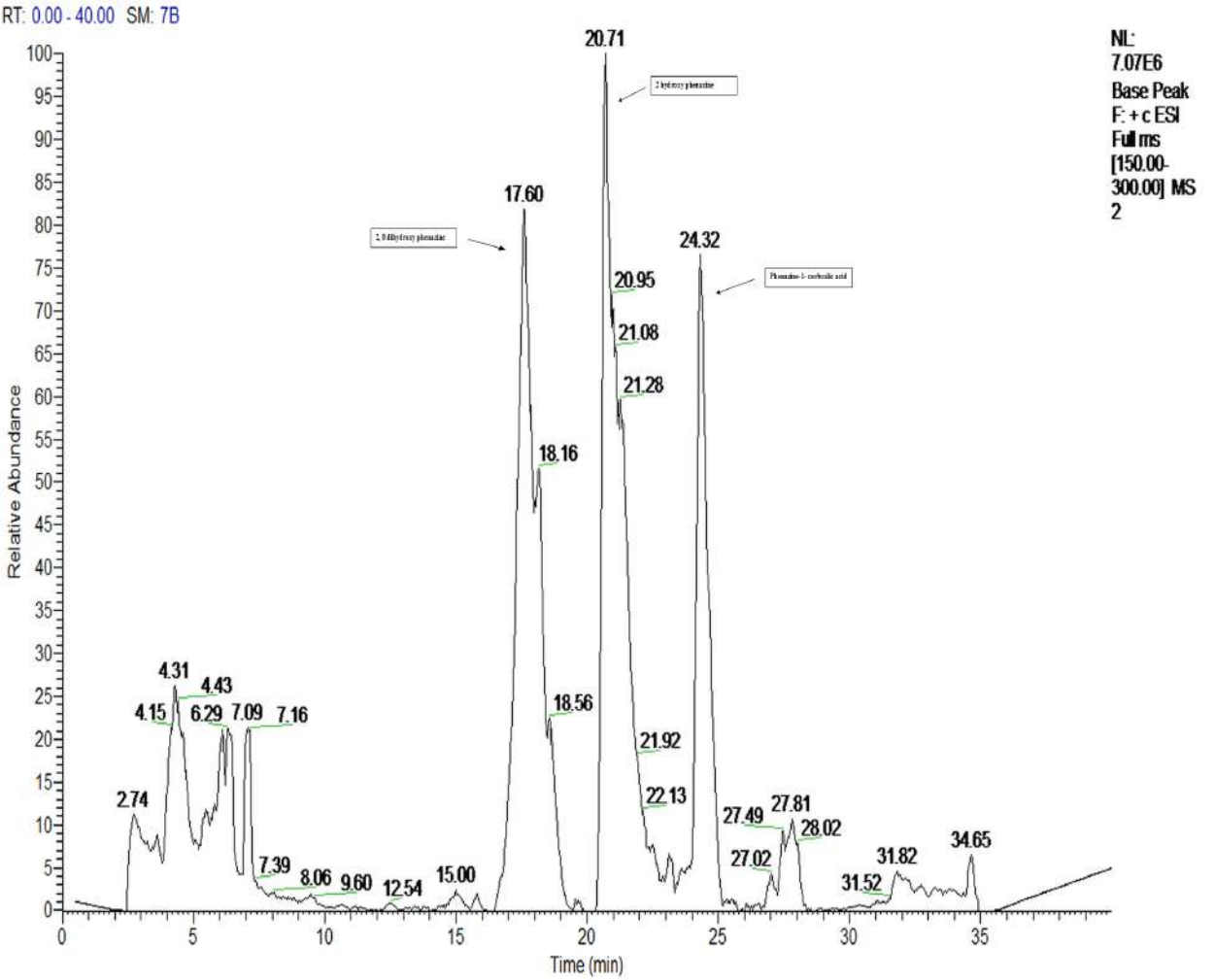

Fig 3.5. Bacterial strain Cb9 (*Pseudomonas spp.*) production of Phenazine

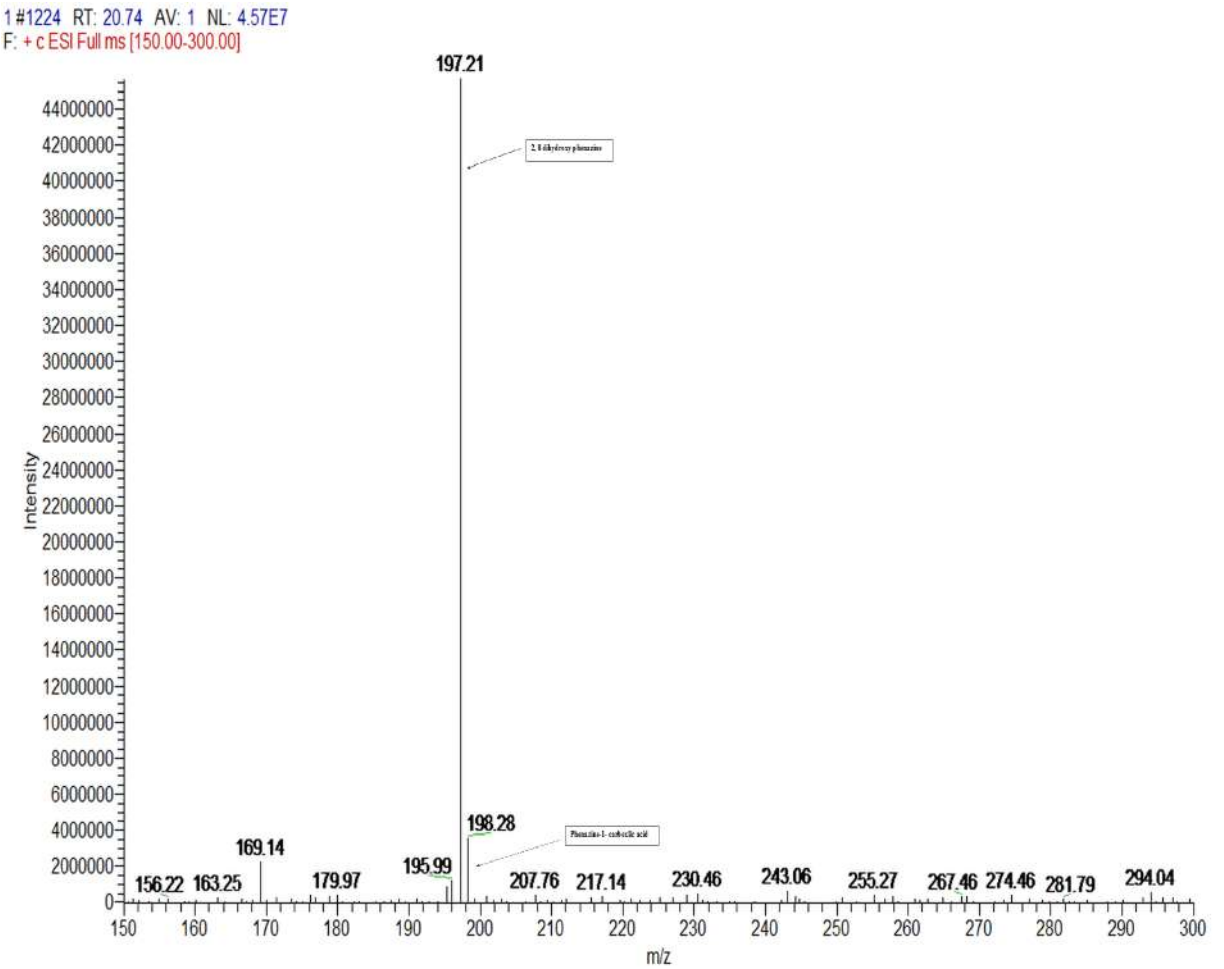

Fig. 3.6. Mass spectra of 2-hydroxy phenazine

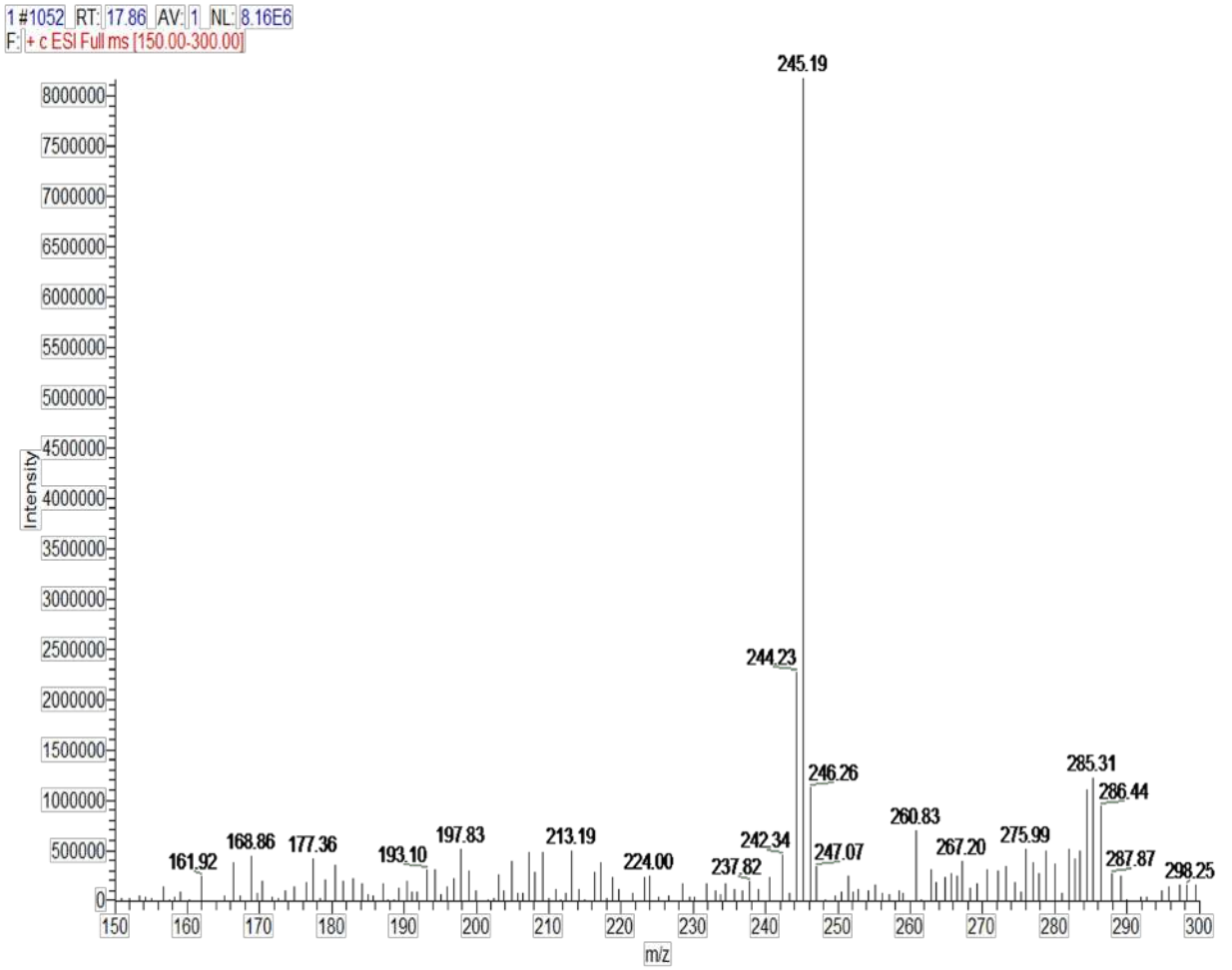

Fig. 3.7. Mass spectra of 2, 8-dihydroxyphenazine

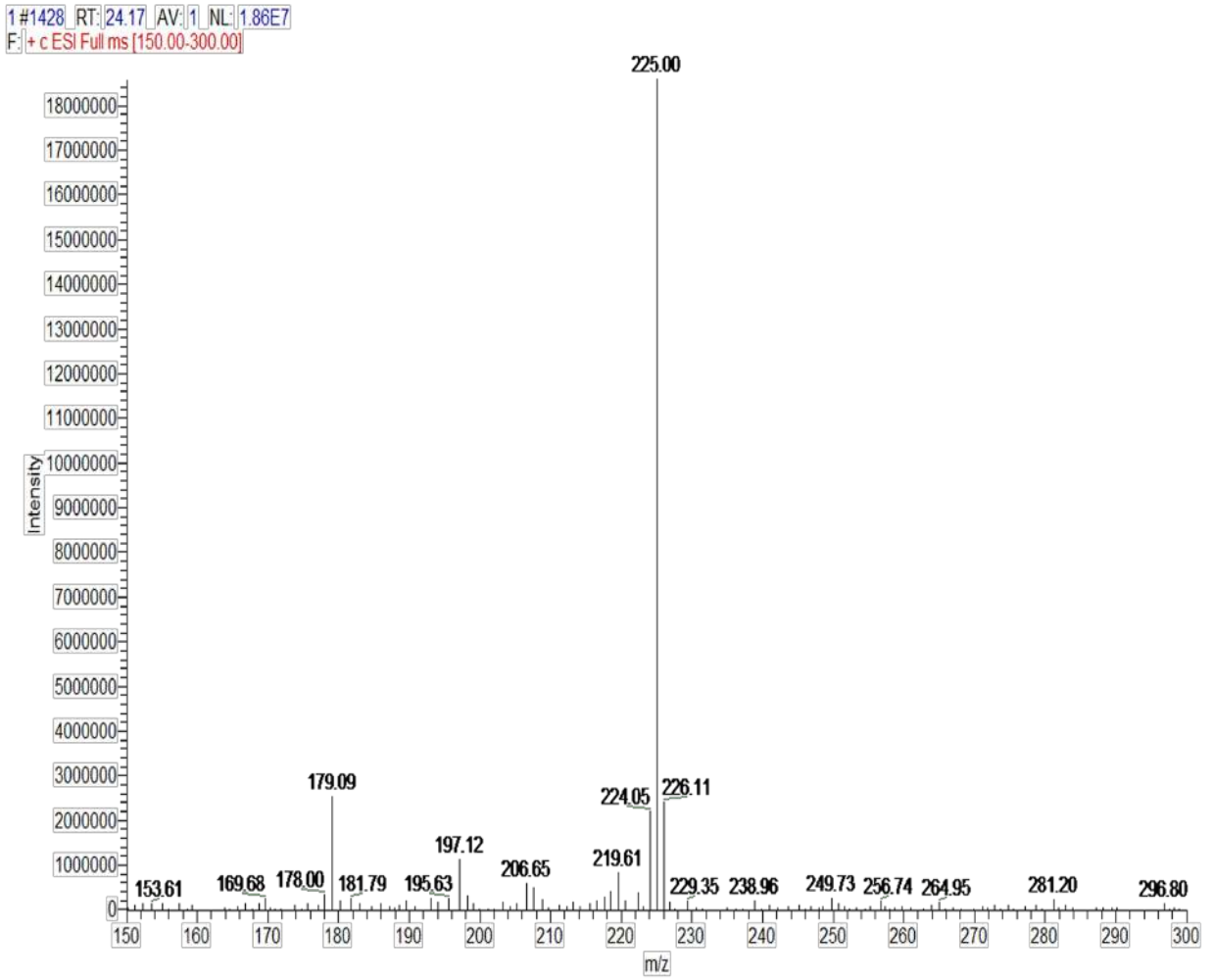

Fig. 3.8. Mass spectra of Phenazine-1-carboxylic acid

It has been previously established that the Pseudomonas spp control potatoes disease due to having phenazine carboxylic acid in their metabolite (Streptomyces scabies) (St-Onge *et al.,* 2011). Our findings demonstrate that each metabolites of bacteria, synthesized a substantial quantity of phenazine, as sensed via LC/MS. Due to diseased conditions, our selected strains generated phenazine to strive the disease causing fungus. This is supported by prior research indicating that Pseudomonas aeruginosa's secondary metabolites (Phanazine) inhibit fungal pathogens. The phenazine production across all the 4 strains demonstrated their potential in suppressing the pathogens. Numerous Pseudomonas spp. have been recognized as biocontrol agents for their production of antimicrobial phenazine (Raaijmakers *et al.,* 2009). Phenazine compounds are known to be primarily bacterial in origin (Pierson and Pierson 2010). In our experiment, Pseudomonas spp. synthesized 2-hydroxy phenazine, phenazine-1-carboxylic acid, and 2, 8 dihydroxy phenazine, while Bacillus and Serratia spp. produced only 2-hydroxy phenazine. Phenazines, produced by pseudomonads, are heterocyclic nitrogen-containing secondary metabolites and exhibit significant potential as antifungals against various fungi (Dietrich *et al.,* 2013; Naseem *et al.,* 2021). Pseudomonas spp. demonstrates growth promotion and antibiosis through the synthesis of phenazine, pyrrolnitrin-type antibiotics, and indole derivatives (Grassmann *et al.,* 2002). Phenazine serve as cell signals regulating gene expression patterns, electron shuttles to alternate terminal acceptors, modify cellular redox states, contribute to biofilm formation, and enhance bacterial survival (Mavrodi *et al.,* 2006). Phenazine-1-carboxylic acid, produced by several Pseudomonads, improves survival in soil environments and has proven essential for the biological control activity of specific strains (Dietrich *et al.,* 2013). The population of bacteria, specifically concerning growth promotion and plant protection, holds particular significance in the context of sustainable agriculture. Pseudomonas spp. genetically modified to yield phenazine showed enhanced capabilities in suppressing plant diseases in field-grown wheat (Glandorf *et al.,* 2001). Most microbes synthesized and release one or more compounds having antibiotic action, which have shown effectiveness in suppressing plant pathogens and related diseases. These antibiotics must be synthesized in adequate amounts in proximity to the pathogen causing biocontrol.

CONCLUSION

The underground plant parts not only provide water and nutrients but also protect the plant during biotic stress. In the rhizosphere, the root serves as an intermediary signaling agent between plants and microbial populations. Root-microbes interaction figure out the functions and community of microbial population in the root zone. Interactions between plants and pathogens regulate rhizo-secretion, impacting the biological effects on plants directly and indirectly and shape the overall microbial habitats. Plants engage in dynamic interactions with greatly diverse microbial populations. Plant roots react differently for pathogens than of beneficial microbes, highlighting the need for rapid pathogen elimination and stimulation of beneficial microbe growth for plant survival. Selected strains produced antifungal agents including phenazine, chitinase, and siderophore for promoting growth of plant and control disease under stress circumstances.

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Sr. No.	Author's name	Contribution	Signature
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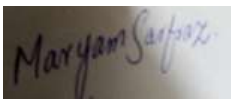
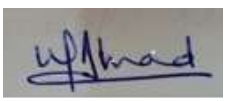
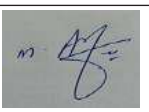
3.	Muhammad Sarfraz	Critically reviewed the manuscript	
4.	Maryam Sarfraz	Analyzed the data statistically	
5.	Muhammad Arfan-ul-Haq	Assisted the research work	
6.	Waqar Ahmad	Analyzed the data statistically	
7.	Muhammad Abubakar Siddique	Assisted in research	
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