GMR
The Original

ASSESSMENT OF PLANT GROWTH-PROMOTING RHIZOBACTERIA: ANTAGONISTIC ACTIVITIES AND DETERMINATIVE FACTORS

Kahkashan Arzoo[1], Erayya*[1], Smita Puri[2], Mahesh Kumar[3], Md Shamim[3], Rekha Balodi[4], Devanshu Dev[1], Shoji Lal Bairwa*[5], J. Kumar[6]

[1]Department of Plant Pathology, Bihar Agricultural University, Sabour, Bhagalpur, Bihar
[2] Department of Plant Pathology, Jawaharlal Nehru Krishi Vishwavidyalaya Jabalpur, M. P
[3] Department of Molecular Biology and Genetic Engineering, Bihar Agricultural University, Sabour, Bhagalpur, Bihar
[4] ICAR National Research Centre for Integrated Pest Management, New Delhi, India
[5] Department of Agricultural Economics, Bihar Agricultural University, Sabour, Bhagalpur, Bihar
[6]G. B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand
***Corresponding Authors:** erayyapath@gmail.com, smabm.bhu@gmail.com

ABSTRACT

Seventy plant growth-promoting rhizobacteria that were isolated from the soils of rhizosphere and rhizoplane of various crops in Uttarakhand's plains were assessed for their antagonistic potential. Antagonistic activity was assessed against four fungal pathogens, namely Bipolaris sorokiniana, Colletotrichum gloeosporioides f. sp. mangiferae, Fusarium oxysporum f. sp. lycopersici, and Rhizoctonia solani, using the dual culture plate technique. Among the seventy isolates, thirty showed inhibition of all four pathogens' radial growth of mycelium in vitro. Considerable diversity in antagonistic behavior was observed among the isolates. Isolates Pfa-50 and Pfa-22 showed more than 50% inhibition against all test pathogens, except Fusarium oxysporum, where inhibition was limited to 39.1% and 46.9%, respectively. 16S rRNA gene amplification confirmed the identity of majority of Gram-negative isolates to be Pseudomonas spp. whereas all the Gram-positive isolates were Bacillus spp. The underlying mechanisms employed by the thirty selected isolates to control these four pathogens in vitro were investigated by evaluating their ability to synthesize hydrogen cyanide (HCN), chitinase and other antimicrobial secondary metabolites. Out of the thirty antagonistic isolates, twenty-two showed positive responses for both HCN production and chitinase activity. Isolates lacking both chitinase and HCN production were further examined for their ability to synthesize antimicrobial secondary metabolites. Thin-layer chromatography (TLC) analysis of extracted metabolites revealed that all fluorescent Pseudomonas isolates produced pyoluteorin (PLT), 2,4-diacetylphloroglucinol (DAPG), and phenazines. Isolates Pfa-50, Pfa-2, and Pfa-65 produced pyrrolnitrin (PRN), while isolates Pfa-50, Pfa-2, Pfa-11, and Pfa-24 also produced indole-3-acetic acid (IAA). Secondary metabolites produced by Bacillus spp. included macrolactins, amicoumacins, surfactins, phenazines, IAA, salicylic acid (SA), and other bioactive compounds, all of which possess well-established antimicrobial properties. The screened rhizobacterial isolates demonstrate significant potential as biological control agents and warrant further evaluation under field conditions. These isolates may be effectively incorporated into integrated pest management strategies and have promising applications in organic farming systems.

KEYWORDS: Biocontrol, PGPR, Chitinase, HCN, Secondary metabolites, Plant disease,

INTRODUCTION

Agricultural productivity and global food security are significantly undermined by pathogens and pests, with plant diseases alone accounting for an estimated 21–30% yield loss in major global crops (Savary et al., 2019). The historically indiscriminate application of synthetic chemicals, has led to emergence of long-term resistance in pathogens to these chemical control methods (Lucas, 2011). Because synthetic fungicides and pesticides have historically been applied indiscriminately, pathogens have developed long-term resistance to these chemical control methods. Due to this, the recurrence and prevalence of some threatening plant diseases have increased. The reliance on chemical pesticides has consequently become a major challenge to environmental sustainability and human health and they often pose a serious threat to microbial diversity, soil fertility and sustainable agricultural output. To address this environmental issue, it is crucial to adopt agricultural practices that not only manage diseases and boost productivity but also protect environmental quality by mitigating pesticide toxicity and restoring soil fertility.

To come for the rescue, microbial biological control agents (MBCAs) have emerged as an effective eco-friendly strategy for managing diseases in plants, improving plant growth, increasing yield and solving many ecological and sustainability issues associated with chemicals. Among the MBCAs, some plant growth-promoting rhizobacteria acting as MBCA stand out as a significant alternative (El-Saadony et al, 2022; Qiao et al., 2017; Alwahshi et al., 2022). PGPR colonizes the rhizosphere as well rhizoplane of the plants and stimulate their growth via many direct and indirect mechanisms (Antoun and Klbpper, 2001). The synthesis of plant hormones, the solubilization of phosphates and insoluble potassium and biological nitrogen fixation are examples of direct mechanisms of plant growth. They can also improve the growth indirectly by nutritional competition with pathogens, thus protecting the plants from various diseases. PGPR effectively outcompete deleterious microorganisms and pathogens through rapid root colonization and efficient nutrient sequestration.

By dominating the ecological niche and depleting available resources, these beneficial bacteria create an environment that suppresses the growth of competing organisms (Salomon et al., 2017; Abd El-Mageed et al., 2020). Depriving the pathogens of iron from their environment by secretion of siderophores which have iron chelating properties, PGPR control various plant diseases (Deb and Tatung, 2024; Srivastava et al,2022; Srivastava et al,2020; Radzki et al., 2013;). Other strategies of biological control adopted by PGPR include synthesis of antibiotics, antifungal metabolites (Wang et al,2024; Raaijmakers et al., 2002), bacteriocins(Nazari et al,2020; Srivastava et al 2020; Srivastava et al 2022; Subramanian and Smith, 2015; Abriouel et al., 2011), HCN(Shehrawat et al, 2022; Abd El-Rahman et al, 2019), volatile organic compounds (Wei et al, 2025), parasitism, production of lytic enzymes (cellulases, chitinases etc.) (Zahoor et al,2022), inhibiting the synthesis of toxin by pathogens and induction of induced systemic resistance in plants (Danish et al,2024). Owing to their significant role in growth promotion and stress tolerance in plants apart from biological control, PGPR have attracted considerable research interest. The present study focuses on the antagonistic potential of two groups of rhizobacteria, namely fluorescent Pseudominads and Bacillus spp. The research was undertaken to isolate and screen effective antagonistic rhizobacteria against four major phytopathogenic fungi: Bipolaris sorokiniana, Rhizoctonia solani, Colletotrichum gloeosporioides f. sp. mangiferae, and Fusarium oxysporum f. sp. lycopersici. Furthermore, the study investigated the underlying antagonistic mechanisms by evaluating the ability of the selected PGPR to produce hydrogen cyanide (HCN), chitinase, and antimicrobial secondary metabolites.

MATERIALS AND METHODS

Isolation of PGPR Isolates

Soil from rhizosphere along with some root parts were collected from various crops (wheat, tomato, onion, marigold and coriander) in the plains of Uttarakhand. Soil and root samples were processed differently to isolate PGPR. For soil, samples were dried in air for 4 hours and then serially diluted in sterile distilled water up to a 10^{-7} dilution (Krassilnikov,1950). Roots were first washed, blotted dry, and then shaken in sterile water in a rotary shaker for 20 minutes to dislodge bacteria, followed by serial dilution to a 10^{-7} dilution. King's B media (King et al., 1954) was used to selectively isolate fluorescent pseudomonads. After spreading one millilitre of the soil or root suspension on King's B agar plates, the plates were incubated for 24 hours at 28±1°C. Observation for appearance of fluorescent colonies under UV ray torch was recorded after 24 h. To isolate Bacillus spp., nutrient agar (NA) was used. For ten minutes, soil and root suspensions were heated to 80°C in a water bath to bias the selection for spore-forming bacteria (Walker et al., 1998). One ml of the suspension was spread on NA plates which were than incubated at 30°C for 24-36 hours. Individual colonies were then picked and maintained as pure cultures.

Cultural and Morphological Characterization

Selected PGPR strains were characterized based on morphology of the colony, color of pigments produced and fluorescence under ultraviolet (UV) light to assess their phenotypic profiles. Gram staining and a 3% KOH test were also performed to determine cell wall type (Anon,1957 and Barthalomew and Mittewer,1950).

Screening for Antagonistic Potential

The antagonistic activity of seventy rhizobacterial isolates against the fungal pathogens Bipolaris sorokiniana, Rhizoctonia solani, Colletotrichum gloeosporiodes f.sp. mangiferae, and Fusarium oxysporum f.sp. lycopersici was examined using the dual culture method. The study was performed in three replicates. Following formula was used to calculate percent inhibition of mycelial growth (I):

$I= [(C-T)/C] \times 100$

Where C is radial growth of pathogen in the control plate and T is radial growth of pathogen in the dual culture plate.

Elucidation of Biocontrol Mechanisms

Chitinase Activity

The antagonistic rhizobacteria were tested for their chitinase producing ability on chitin agar media (Murthy and Bleakley, 2012). The method given by Roberts et al., (1988) with some modifications was used to prepare colloidal chitin. For this 100g of chitin powder was dissolved in 600ml of concentrated HCl for approximately 3 hours. This solution was poured in double deionized water, rapid stirring was done while pouring. After centrifuging the entire mixture at 7000 g for 10 minutes at 4°C, the precipitate was gathered. To prepare the low-salt colloidal chitin, the precipitate was first washed with 200 mL of sterile distilled water until a pH of 2.0–3.0 was achieved. The suspension was then neutralized using 1M NaOH. Finally, the product was isolated via several cycles of centrifugation (7,000 × g for 10 min) and distilled water washes to remove residual salts. Acid-treated chitin was stored as a pellet at 4°C. A 2% (w/v) colloidal chitin suspension was prepared and incorporated into chitin agar. To screen for chitinolytic activity, overnight grown rhizobacterial cultures were streaked onto the chitin agar plates. The plates were than incubated for 24 hours at 37 ± 2°C and production of chitinase enzyme was confirmed by the presence of clear zones of hydrolysis surrounging the colonies (Murthy and Bleakley, 2012

HCN Production

HCN production was assessed on Tryptic Soy Agar (TSA) (Himedia, Mumbai) with 4.4 g/L of glycine added (Wei et al,1991). A solution of picric acid (2.5 g) and sodium carbonate (12.5g) in 1L of sterile distilled water was prepared and 2mL of this solution was used to soak Whatman No. 1 filter papers. These were placed on the lid of the plates (Miller and Higgins, 1970) containing TSA amended with glycine. The plates were then tightly sealed with parafilm and incubated at 28±2°C for one week. Changing color of the filter paper was observed and scored. No color change indicated no HCN production, brownish appearance on filter paper depicted mild production of HCN, brownish to orange appearance indicated moderate production of HCN and orange to reddish brown indicated strong HCN production.

Production of Secondary metabolites

Extraction of Bacterial Secondary Metabolites

Rhizobacteria were cultured in 50 mL conical flasks containing Winogradsky mineral solution, a standard medium for secondary metabolite production. Cultures were maintained at 25°C without shaking for 15 days. After 15 days they were centrifuged at 10,000 rpm for 10 minutes at 4°C. Subsequently, 25 mL of clear supernatants were carefully transferred in clean conical flasks containing 25 mL of ethyl acetate. These mixtures were than agitated on a shaker-incubator at 180 rpm and 25°C for 24 hours to facilitate extraction. After agitation, two phases were separated, the organic phase with ethyl acetate and aqueous phase. The ethyl acetate phase, containing the extracted metabolites, was collected and subjected to freeze evaporation to remove the solvent. The resulting crude extract, a thick semi-liquid, was retained for further analysis.

Separation of Secondary Metabolites Using Thin Layer Chromatography (TLC)

99% acetone @ 10µl/ µl crude extract was used for dissolving the crude metabolites. Silica gel-coated TLC plates were used as the stationary phase. Each plate was lightly marked with a pencil, and 10 µL of the acetone-dissolved extract was carefully spotted onto the marked line. The TLC plates were developed using a solvent mixture made of chloroform, methanol and benzene in a ratio of 8:1:1 (v/v/v). After migration of the solvent front to an appropriate height, the plates were removed and air-dried. Developed plates were observed under UV light to detect fluorescent spots indicative of separated secondary metabolites. Each visible spot was marked, and Rf values (Retention factors) were calculated by using the formula:

Rf value= Distance moved by the separated compound/Distance moved by the solvent system.

Some common secondary metabolites like phenazine, pyoluteorin (PLT), pyrollnitrin (PRN), 2,4-Diacetylphloroglucinol (DAPG), Indole Acetic Acid (IAA) and Salicylic Acid (SA) were also run on TLC plates under similar conditions. The Rf value of bacterial metabolites obtained were compared with Rf value of standard metabolites and result was interpreted.

Molecular Characterization

Promising antagonistic PGPR isolates were characterized by amplification of 16S rRNA gene through polymerase chain reaction (PCR). Specific primer pairs were used for different genera: BCF1 (CGGGAGGCAGCAGTAGGGAAT) /BCR2 (CTCCCCAGGCGGAGTGCTTAAT) for Bacillus spp. (yielding a 546 bp amplicon) (Cano et al., 1994; Rajendran et al., 2008) and PA-GS-F(GACGGGTGAGTAATGCCTA) /PA-GS-R (CACTGGTGTTCCTTCCTATA) for Pseudomonas spp. giving amplification of 618 bp (Spilker et al, 2004).

DNA Isolation

DNA of PGPR isolates were isolated by following the method described by Auesbel et al., (1998). Bacterial cells that were cultured overnight at 28°C in an incubator shaker with NB media were centrifuged at 12, 000 rpm for 10 min to collect the cells. Supernatant was discarded and to the cell pellets remaining in tube, 2 ml of TE buffer was added and than vortexed. Subsequently, 50 µl proteinase K (10 mg/ml) and 250 µl of 10% SDS were added to this suspension. In a water bath, the tubes were gently shaken at 37°C for 1 h. After that, 0. 4 ml of CTAB solution (10% CTAB in 0. 7 M NaCl) was added followed by incubation at 65°C for 20 min. The mixtures were then added with an equal volume of chloroform: isoamyl alcohol (24:1) and the whole was vigorously shaken for 30 min. After shaking the mixtures were subjected to centrifugation at 15, 000 rpm for 30 min at room temperature which resulted in the separation of phases. The upper aqueous phase was taken to a new tube and equal volume of cold isopropanol was added to precipitate the DNA. The isolated DNA formed a stringy, white DNA pellet, which was washed with 1 ml of 70% ethanol. After air drying, 200-400 µl TE buffer was added to dissolve the pellet. Isolated DNA was treated with 100µg/ml RNAse by incubating it at 37°C for 1h to separate RNA contamination. DNA concentration and purity were determined spectrophotometrically, and the samples were than diluted to 50 ng/µl in TE buffer and stored at -20°C for PCR.

PCR Amplification

For PCR amplification, the template DNA (50 ng) was mixed with 1x PCR reaction buffer, $MgCl_2$, dNTP mix, forward and reverse primers, triple distilled water and Taq polymerase on DNA thermocycler (Eppendorf Master Cycler) to get the desired amplicon. The total reaction size was 20µl, which contained 1µl of template DNA, 2µl of reaction buffer, 1µl of forward and 1µl of reverse primer, 0.6 µl of $MgCl_2$ 1 µl of dNTP mix and 0.3µl of Taq added in 13.1 µl of triple distilled water. Reaction mixture without template DNA was maintained simultaneously as a negative control to check for possible contamination.

PCR For amplification of 16S rRNA gene of Bacillus spp, the following protocol was performed in PCR: Initial denaturation at 94°C for 4 min, 40 cycles of denaturation at 94°C for 1min, 40 cycles of annealing at 58°C for 1min, 40 cycles of extension at 72°C for 1 min and final extension for 10 min at 72°C. Same thermal profile was used for amplification of 16S rRNA gene of Pseudomonas spp with a slight change in annealing temperature which was kept at 54°C. The final products of DNA amplification were soaked at 4°C. Amplified DNA was examined by horizontal electrophoresis in 1% agarose and were viewed in BIORAD gel documentation system for further analysis.

RESULTS

Screening of PGPR for antagonistic potential and their morphological characterization

One hundred and twenty eight isolates of rhizobacteria were isolated from soils of rhizosphere and rhizoplane of different crops. Among these 80 isolates were of fluorescent Pseudomonads and remaining 48 belonged to Bacillus spp. Seventy

isolates were randomly selected and screened for antagonistic effect on four fungal pathogens Bipolaris sorokiniana, Rhizoctonia solani, Colletotrichum gloeosporioides f.sp. mangiferae and Fusarium oxysporum f.sp. lycopersici following the dual culture method (Nielsen et al., 1998). Among these, 30 isolates successfully controlled the growth of the mycelium of four pathogens in vitro demonstrating their antagonistic activity (Table 1, Fig. 1, 2 & 3). The screened potential antagonistic isolates varied in their morphological characters like pigment formation, fluorescence under UV, Grams test and 3% KOH test (Table: 2).

The study identified several highly effective isolates with a significant diversity in their antagonistic behavior. Pfa-50 was the most effective isolate overall, showing strong inhibition against three of the four pathogens. It demonstrated the highest inhibition against B. sorokiniana (91.46%) and C. gloeosporioides f.sp. mangiferae (68.77%), and good inhibition against R. solani (57.08%). Its inhibitory effect on Fusarium oxysporum was 39.1%. Pfa-22 was also highly effective, inhibiting all four pathogens: Bipolaris (73.12%), Fusarium (46.9%), Colletotrichum (55.42%), and Rhizoctonia (53.54%). Other notable isolates included: Pfa-24 (79.58%) and Pfa-23 (75.21%) for their strong inhibition of Bipolaris sorokiniana, Pfa-40 (64.96%) and Pfa-27 (63.91%) for their effectiveness against Colletotrichum gloeosporioides, B-36 (50.09%), B-1 (49.86%), and B-20 (49.35%) as the top performers against Fusarium oxysporum, B-24 (56.04%) and Pfa-46 (54.16%) for their good inhibition of Rhizoctonia solani. Overall, the fluorescent pseudomonad isolates, Pfa-2,Pfa-22, Pfa-23, Pfa-24, Pfa-27, Pfa-37, Pfa-40, Pfa-46, Pfa-50, Pfa-68 and Pfa-77, and certain Bacillus isolates,B-1, B-18, B-20, B-24, B-36,B-38 and B-46, proved to be the most promising biocontrol agents.

Table 1: Inhibition pattern of Bipolaris sorokiniana, Colletotrichum gloeosporiodes f.sp. mangiferae, Fusarium oxysporum f.sp. lycopersici and Rhizoctonia solani by different rhizobacterial isolates obtained by in vitro dual culture assay

Name of isolate	**Percent inhibition**			
	Bipolaris*	**Colletotrichum***	**Fusarium***	**Rhizoctonia***
Pfa-2	58.33	22.94	43.52	50.42
Pfa-11	23.54	2.12	39.36	31.66
Pfa-22	73.12	55.42	46.93	53.54
Pfa-23	75.21	52.01	41.32	47.08
Pfa-24	79.58	46.49	42.30	43.33
Pfa-26	45.42	28.44	50.34	48.33
Pfa-27	24.58	63.91	44.73	48.33
Pfa-31	58.75	39.91	44.01	51.87
Pfa-35	52.92	35.45	32.48	36.04
Pfa-37	66.46	24.81	44.24	52.92
Pfa-40	52.29	64.96	22.47	50.83
Pfa-41	24.58	37.56	27.63	33.12
Pfa-46	73.96	58.36	38.13	54.16
Pfa-47	27.5	2.32	37.39	36.04
Pfa-50	91.46	68.77	39.12	57.08
Pfa-53	25.21	5.94	37.40	42.71
Pfa-65	27.5	53.69	43.50	27.29
Pfa-68	32.5	63.48	41.04	31.87
Pfa-77	56.66	32.67	37.63	43.12
B-1	70.21	43.09	49.86	41.25
B-2	28.12	41.41	44.76	45.62
B-3	26.25	46.27	38.37	39.58
B-18	72.71	61.57	41.78	43.33
B-19	20.62	42.26	39.85	51.25
B-20	26.45	29.28	49.35	40.21
B-24	49.58	30.15	37.88	56.04
B-36	45.21	41.16	50.09	38.54
B-38	54.58	39.27	41.30	46.66
B-46	26.04	32.26	34.43	53.54
B-48	45.21	41.59	39.37	49.79
Cont	0	0	0	0
S.EM	1.65	1.40	1.29	1.28
CD at 5%	4.66	3.96	3.65	3.62

Table 2: Cultural and morphological characters of screened antagonistic rhizobacteria

Name of isolate	**Colony color on KB media**	**Fluorescence under UV Light**	**Gram reaction**	**3% Koh test**
Pfa-2	Light Green	+	-ve	+
Pfa-11	Creamy	+	-ve	+
Pfa-22	Light Green	-	-ve	+
Pfa-23	Dark Green	-	-ve	+
Pfa-24	Light Green	+	-ve	+
Pfa-26	Yellow	+	-ve	+

Pfa-27	Yellow	-	-ve	+
Pfa-31	Yellow	-	-ve	+
Pfa-35	Yellow	+	-ve	+
Pfa-37	Yellow	-	-ve	+
Pfa-40	Creamy	-	-ve	+
Pfa-41	Yellow	-	-ve	+
Pfa-46	Light Green	+	-ve	+
Pfa-47	Yellow	+	-ve	+
Pfa-50	Brown	-	-ve	+
Pfa-53	Light Brown	-	-ve	+
Pfa-65	Yellow	+	-ve	+
Pfa-68	Yellow	+	-ve	+
Pfa-77	Light Green	+	-ve	+
B-1	Grey	-	+ve	-
B-2	Grey	-	+ve	-
B-3	Light Pink	-	+ve	-
B-18	Black	-	+ve	-
B-19	Grey	-	+ve	-
B-20	White	-	+ve	-
B-24	Light Pink	-	+ve	-
B-36	Grey	-	+ve	-
B-38	Pinkish red	-	+ve	-
B-46	Pinkish red	-	+ve	-
B-48	Grey	-	+ve	-

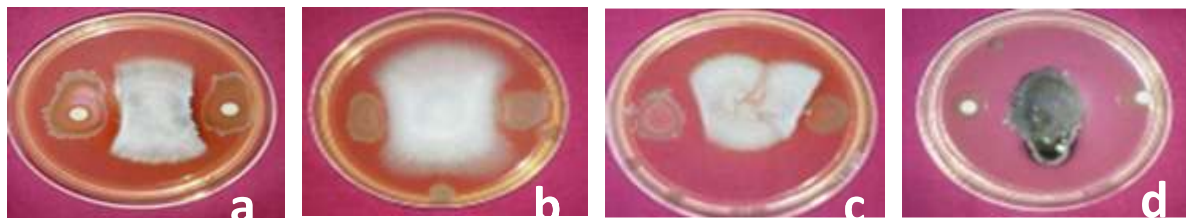

Fig 1: Inhibition pattern of (a) Rhizoctonia, (b) Colletotrichum, (c) Fusarium and (d) Bipolaris by rhizobacterial isolates in dual culture test.

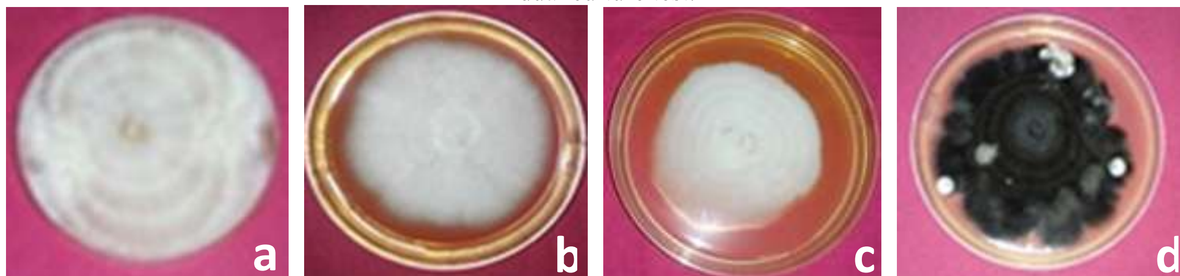

Fig 2: Growth of (a) Rhizoctonia, (b) Colletotrichum, (c)Fusarium and (d) Bipolaris in control plates

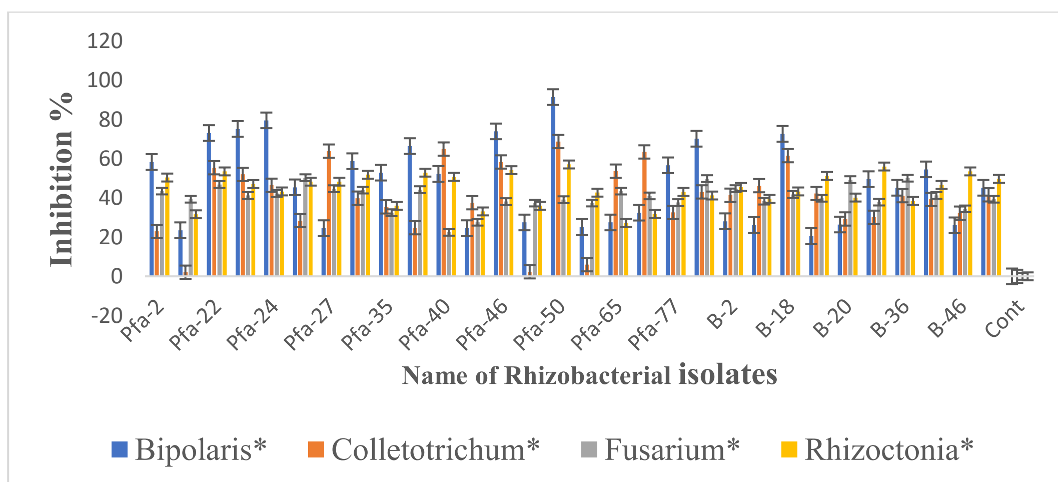

Fig 3: Inhibition percentage of Bipolaris, Colletotrichum, Fusarium and Rhizoctonia by different rhizobacterial isolates

Elucidation of mechanism of antagonism by PGPR isolates
Production of HCN and Chitinase Enzymes

Among the 30 screened antagonists only 22 isolates showed positive response for chitinase and HCN production (Table: 3). Among the 22 isolates, 16 had chitinolytic activity which was seen as clear halo zone around the bacterial colony on chitin agar media (Fig.4(a)). These isolates differed in the level of chitinase production, which was measured by the diameter of halo zone around the colony. Maximum chitinolytic activity was seen in rhizobacterial isolates Pfa-22, Pfa-35, Pfa-77 and B-24. Only 6 rhizobacterial isolates showed the ability to produce HCN which was visualized by color change of picric acid dipped filter paper (Table: 3, Fig.4(b)). Darker was the color change, more was the HCN production. Among these 6 isolates, B-38 had maximum HCN producing activity followed by Pfa-37, Pfa-31, Pfa-68 and B-48.

Table. 3: Chitinolytic activity and HCN production ability of potential antagonistic isolates

Sl. No.	Treatment	Chitinase activity	HCN production activity
1	Pfa-2	-	-
2	Pfa-11	-	-
3	Pfa-22	++++	-
4	Pfa-23	+++	-
5	Pfa-24	-	-
6	Pfa-26	-	+
7	Pfa-27	+++	-
8	Pfa-31	-	++
9	Pfa-35	++++	-
10	Pfa-37	-	++
11	Pfa-40	+	-
12	Pfa-41	+	-
13	Pfa-46	+	-
14	Pfa-47	+	-
15	Pfa-50	-	-
16	Pfa-53	+++	-
17	Pfa-65	-	-
18	Pfa-68	-	+
19	Pfa-77	++++	-
20	B-1	+	-
21	B-2	+++	_
22	B-3	++	-
23	B-18	-	-
24	B-19	-	-
25	B-20	-	-
26	B-24	++++	
27	B-36	+	-
28	B-38	-	++++
29	B-46	++	-
30	B-48	-	+

- = no chitinase/ HCN production
+ = very low level of chitinase/ HCN production
++ = moderate level of chitinase/ HCN production
+++ = high level of chitinase/ HCN production
++++ = very high level of chitinase/ HCN production

Fig. 4 (a): Clear halo zone around bacterial colonies grown over chitin agar due to chitinolytic activity of potential antagonistic PGPR isolates

Fig 4 (b): Color changes (Brownish to reddish brown) observed in picric acid soaked filter paper due to HCN producing ability of potential antagonistic PGPR isolates

Production of Secondary Metabolite

TLC analysis revealed that ethyl acetate extract of fluorescent pseudomonas isolates separated into different compounds with Rf values of 0.26, 0.35, 0.41, 0.57, 0.66, 0.75, and 0.80. Some isolates like Pfa-50 and Pfa-2 each separated into six distinct compounds with Rf values of 0.26, 0.35, 0.41, 0.57, 0.66 and 0.80 (Fig 5(a)). Extracts of isolate Pfa-24 showed three compounds at Rf 0.26, 0.35, and 0.57 (Fig.5(b)), while of Pfa-11 exhibited the same three bands along with an additional compound at Rf 0.66 (Fig 5(b)). The extracts from Pfa-65 separated into 4 compounds at Rf 0.26, 0.35, 0.57 and 0.80 (Fig 5(d)). The separation of compounds like PLT, DAPG, phenazine, IAA, and PRN on TLC under similar conditions showed separation at 0.26, 0.35, 0.57, 0.66 and 0.80 Rf values. These Rf values are similar to those observed in metabolites separated by Pfa-50 and Pfa-2 confirming their production by these isolates. Isolate Pfa-24 was found to produce PLT, DAPG, and phenazine while Pfa-11 additionally produced IAA. The isolate Pfa-65 produced PLT, DAPG, Phenazines and PRN.

The bacillus isolates B-20 and B-18 showed separation into different compounds with Rf values of 0.34, 0.48, 0.50, 0.55, 0.57, 0.66, 0.75, 0.875 and 0.91 (Fig. 5(c)). Compounds at Rf 0.34 and 0.48 are indicative of macrolactins and amicoumacins, 0.50–0.55 corresponds to surfactins. The Rf value 0.57 is similar to standard phenazine, 0.66 to IAA, and 0.75 to SA.

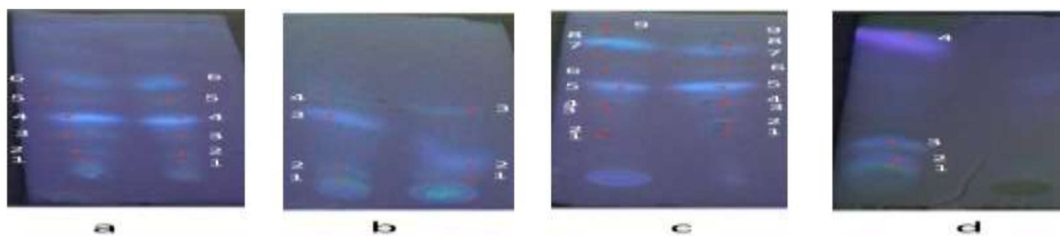

Fig 5 (a): Separation of metabolites of Pfa-50 and Pfa-2 on TLC plates at different Rf values 0.26 (1), 0.35(2),0.41 (3) 0.57(4), 0.66(5) and 0.8(6)

Fig 5(b) Separation of metabolites of Pfa-11 and Pfa-24 on TLC plates at different Rf values0.26 (1), 0.35(2), 0.57(3) and 0.66(4)

Fig 5 (c): Separation of metabolites of B-18 and B-20 at Rf values of 0.34 (1), 0.48(2), 0.50(3), 0.55(4), 0.57(5), 0.66(6), 0.74(7), 0.875(8) and 0.91(9)

Fig 5 (d): Separation of metabolites of Pfa-65 on TLC plates at Rf values of 0.26 (1), 0.35(2), 0.57(3) and 0.8(4)

Molecular identification of PGPR

Screened antagonistic PGPR isolates were subjected to molecular identification using gene specific primers. PCR amplification with 16S rRNA gene specific primers BCF1 and BCR2 of all gram positive isolates gave amplification at 546 bp (Fig.7). This amplification is specific for Bacillus spp., thus confirming that all the PGPR isolates which were gram positive belong to Bacillus spp. Similarly, PCR amplification with 16SrRNA gene specific primers for Pseudomonas spp. PA-GS-F and PA-GS-R gave an amplicon at 618 bp. Among 28 fluorescent pseudomonad isolates subjected to PCR amplification, DNA of 17 isolates showed amplification at 618bp confirming their identity as Pseudomonas spp. Other isolates whose DNA did not show amplification may belong to other groups (Fig.6 a & b)

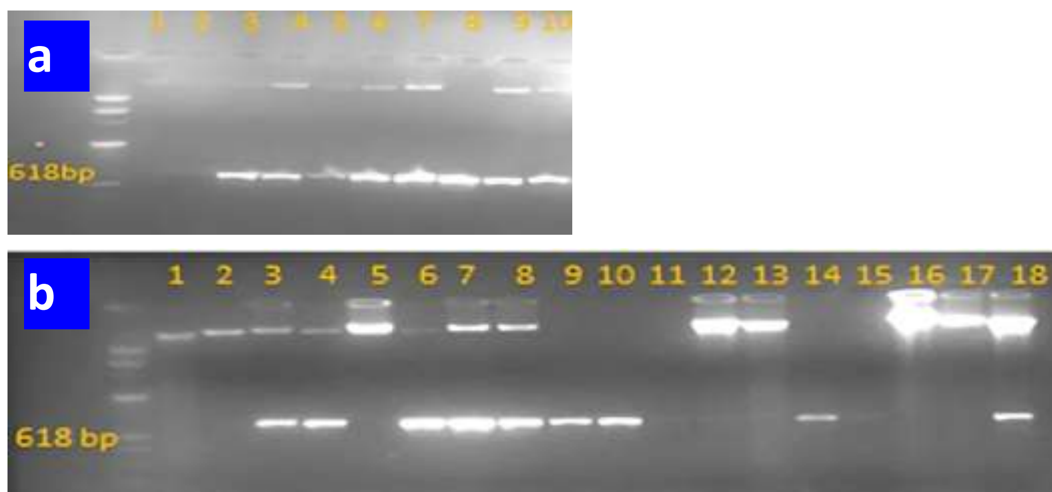

Fig 6 (a). 16s rRNA gene amplification of Pseudomonas sp. with gene specific primers PA-GS-F and PA- GS-R. Isolates from 1-10: Pfa-12, Pfa-28, Pfa-37, Pfa-40, Pfa-41, Pfa-46, Pfa-47, Pfa-68, Pfa-77, Pfa-27

Fig 6 (b). 16s rRNA gene amplification of Pseudomonas sp. with gene specific primers PA-GS-F and PA- GS-R. Isolates from 1-18: Pfa-1, Pfa-5, Pfa-2, Pfa-3, Pfa-10, Pfa-11, Pfa-22, Pfa-23, Pfa-24, Pfa-26, Pfa-81,Pfa-78, Pfa-31 Pfa-35, Pfa-36, Pfa-50,Pfa-53,Pfa-65.

Fig 7. 16s rRNA gene amplification of Bacillus sp. with gene specific primers BCF1 and BCR2 using PCR. Isolates from 1-14: B-1, B-2, B-3, B-4, B-18. B-19, B-20, B-21, B-24, B-36, B-38, B-39, B-46, B-48

DISCUSSION

Microbial biocontrol agents (MBAs) are beneficial microorganisms used to control plant diseases and pests to limit the reliance on chemical pesticides. MBAs usually target specific pathogens or pests so that beneficial organisms are less damaged. The risk of developing resistance against chemical pesticides is also reduced. These are a promising tool for integrated pest management (IPM) strategies necessary for sustainable and eco-friendly agricultural practices. Integrating them with other disease management strategies reduce chemical pesticide use, leading to lower environmental contamination.

The present research work was focused on biocontrol traits of two groups of microbial biocontrol agents: the fluorescent pseudomonads and the Bacillus spp. Under field conditions, Bacillus spp. and fluorescent Pseudomonas spp. have shown significant potential for disease control (Miljaković et al., 2020; Panpatte et al., 2016). The current investigation focused on antimicrobial traits in these two groups of microbes. As a preliminary step for bioagent development, fluorescent Pseudomonas and Bacillus strains were isolated from diverse habitats and subsequently characterized. Antagonistic trait of seventy rhizobacterial isolates was checked following dual culture technique against four fungal pathogens Bipolaris sorokiniana, Rhizoctonia solani, Colletotrichum gloeosporioides f.sp. mangiferae and Fusarium oxysporum f.sp. lycopersici. The interaction of thirty biocontrol agents with all tested pathogens was recorded negative or antagonistic. 16S rRNA gene amplification confirmed that majority of Gram-negative isolates belonged to the genus Pseudomonas, whereas Gram-positive isolates belonged to the genus Bacillus.

A great diversity was seen in the antagonistic behavior of these screened isolates. Maximum inhibition of Bipolaris sorokiniana was noted by Pfa-50 (91.46%) followed by Pfa-24 (79.58%) and Pfa-23 (75.21%). Earlier Yi et al, (2021) reported mycelial growth inhibition of Bipolaris sorokiniana by 53.52% by Bacillus amyloliquefaciens (XZ34-1). Bae et al, (2013) reported mycelial inhibition of Bipolaris cactivora by B. amyloliquefaciens (GA4-4) and Bacillus subtilis (GA1-23) by 67.9% and 66.6% respectively. Behdani et al. (2012) found five isolates of P. fluorescens to have antagonistic potential against Bipolaris spicifera, the causal agent of wheat root rot. Volatile compounds and antibiotic production were associated with inhibition. Soleimani et al. (2005) screened nine isolates of Pseudomonas and Bacillus spp as potential antagonist of Bipolaris autraliensis and Bipolaris sacchari. On testing for antifungal substances, volatiles, siderophores and lytic enzymes it was found that the effect of antifungal substance was more effective than volatiles.

On screening the isolates against Colletotrichum gloeosporioides f.sp. mangiferae, Pfa-50(68.77%) followed by Pfa-40 (64.96%) and Pfa-27 (63.91%) were found to be best. Gowtham et al (2018) studied 59 PGPR isolates and found fourteen of them to inhibit the mycelial growth of the pathogen Colletotrichum truncatum. Parthiban and Kavitha, 2014 screened five Pseudomonas fluorescens isolates and four Bacillus subtilis isolates against C. lindemuthianum and an inhibition of 33.33% of mycelial growth was reported. Similarly, Akinbode and Ikotun (2008) also reported significant inhibition of mycelial growth of Collectotrichum destructivum by Pseudomonas fluorescens.

Many rhizobacteria species having antagonistic potential against Rhizoctonia solani and also against Fusarium oxysporum f.sp. lycopersici have been reported. These species include Fluorescent pseudomonads, Bacillus sp., Burkholderia (Pseudomonas) cepacia, Streptomyces sp., Pseudomonas fluorescens and Erwinia sp. (Donmez et al., 2015; Huang et al., 2012; Tariq et al., 2010).In vitro and in-vivo antagonistic potential of six isolates of Pseudomonas and six isolates of Bacillus genera, isolated from chickpea rhizosphere, was observed against Fusarium oxysporum f. sp. ciceris (Karimi et al., 2012). Under greenhouse conditions, isolates of B. amyloliquefaciens CS–1 and PCfS significantly inhibited Fusarium wilt disease by 31% and 28% respectively (Gowtham et al., 2016). Huang, et al (2012) observed the inhibitory effect of Bacillus pumilus on mycelial growth of Rhizoctonia solani. Observations included hyphal deformation, swelling of cytoplasmic vacuoles, and leakage of cytoplasmic contents. An inhibition of 83% and 48% of Rhizoctonia solani and also 40 % and 41% of Fusarium oxysporum by P. fluorescens and B. subtilis respectively was reported by Singh et al, (2021). In the present study, the isolates B-36(50.09%) followed by B-1 (49.86%) and B-20 (49.35) were most effective in mycelial growth inhibition of Fusarium oxysporum f.sp. lycopersici. All the isolates showed inhibition of mycelial growth of Rhizoctonia solani in vitro with maximum inhibition observed in Pfa-50 (57.08%) followed by B-24 (56.04%) and Pfa-46 (54.16%) confirming their biocontrol potential against this pathogen.

Elucidation of the mechanism of biocontrol by the screened 30 isolates was done by their ability to produce HCN, chitinases and other secondary metabolites. HCN is an antimicrobial secondary metabolite formed through the oxidation

of glycine to HCN and carbon dioxide in the presence of electron acceptors. It exerts its antimicrobial effect by binding to essential metal ions such as Cu^{2+}, Fe^{2+}, and Mn^{2+}, thereby inhibiting cytochrome oxidase and other metalloenzymes critical for the electron transport chain (Nandi et al., 2017). Energy supply to the cells are thus blocked and the bacteria dies (Shehrawat et al, 2022).

A diverse group of soil-dwelling bacteria, including Gram-positive and Gram-negative species from genera like Pseudomonas, Bacillus, Streptomyces, Chromobacterium, and Rhizobium, produce cyanogenic compounds as secondary metabolite (Anderson & Kim, 2018; Anwar et al., 2016).Among these Pseudomonas sp. and Bacillus sp. have great potential to produce hydrogen cyanide HCN (Ahmad and Kibret, 2014). The HCN produced by these two group of rhizobacteria play an important role in inhibiting a wide range of plant pathogens (Mishra et al, 2018; Prasad et al, 2023: Shehrawat et al, 2022). In agriculture, this HCN production is one of the key mechanism for biocontrol, helping to suppression of a number of fungal diseases like root rot, collar rot, stem rot, charcoal rot, damping off, wilt and seedling blight (Sehrawat et al, 2022; Sendi et al, 2020; Reetha et al., 2014; Defago et al., 1990). Two HCN producing strains of Pseudomonas, P. putida R32 and P. chlororaphis R47, were able to suppress growth of Phytophthora infestans mycelium in vitro (Anand et al, 2020). The rhizobacterial strain Pseudomonas frederiksbergensis PVB9 and Pseudomonas lini PVB19 showed good inhibition of root rot pathogens, Alternaria sp. PVF1and Macrophomina sp. PVF32, in common bean. These two isolates were found to be good producers of HCN (Sendi et al, 2020). Reduction in growth of pathogen Macrophomina phaseolina by cyanide-producing Pseudomonas spp. was earlier also reported (Reetha et al, 2014). Suppression of crown gall pathogen Agrobacterium tumefaciens and the root-knot nematodes (Meloidogyne incognita) by HCN produced by rhizobacteria was reported by Abd El-Rahman et al., 2019. The Pseudomonas fluorescens strain CHAO produces HCN that is not inhibited by fusaric acid, making it effective in suppressing Fusarium wilt of tomatoes (Duffy et al., 2003). P. koreensis WA15 which is an HCN producing strain of Pseudomonas has the ability to supress many phytopathogens like Fusarium oxysporum, Rhizoctonia solani and Botrytis cinerea and (Yoo et al,2018).

Chitinases are a type of PR-protein (pathogenesis-related protein) that break down chitin, a key component of fungal cell walls (Akocak et al,2015). When present in high concentrations, chitinolytic enzymes compromise the fungal cell wall resulting in its degradation. This action can also inhibit the germination of fungal spores by disrupting the synthesis essential cell wall components such as chitin and β-1,3-glucans (Liu et al., 2010) This makes chitinases an effective defense against pathogenic fungi.

Chitinase-excreting microorganisms are considered efficient biocontrol agents because of their ability to degrade chitin, a major structural component of fungal cell walls. Many bacterial species like Pseudomonas fluorescrence, B. subtilis, B. cereus, B. thuringiensis, S. marcescens, Serratia plymuthica, Paenibacillus sp., Achromobacter mucicolens have been reported to produce chitin (Vijay Karuppiah et al, 2025; Panicker and Sayyed, 2022; Wang et al, 2021; Toua et al, 2013). Maiden et al, 2017 reported the biocontrol potential of nine chitinolytic isolates of rhizobacteria and reported them to belong to Pseudomonas, Burkholderia and Streptomyces. Dukare et al, 2020 identified Pseudomonas spp. NS-1 and Bacillus spp. NS-22 as potential chitinolytic rhizobacteria which were effective in reducing disease severity of pigeon pea wilt caused by Fusarium udum. The chitinolytic activity was confirmed both qualitatively in colloidal chitin agar plates and quantitatively. The mycolytic action of the two strains was observed in scanning electron microscope. Rathod et al, 2023 reported control of Fusarium wilt of chickpea by a marine Bacillus spp. Gowtham et al., 2016 reported involvement of chitinase along with cellulases, ACC deaminases and antifungal metabolites in inhibition of Fusarium oxysporum growth by Bacillus amyloliquifaciens. Among 13 screened antagonistic and chitinase producing CRB isolates, Bacillus subtilis isolate CRB20 led to efficient root colonization in tomato plants and contributed to a reduction in Fusarium wilt incidence statistically lower level when its application was combined with crude fungal cell wall (CFCW) (Hariprasad et al, 2011). B. subtillis TD11 was found to control some phytopathogens like Aspergillus, Fusarium, Rhizoctonia and Colletotrichum and this biocontrol activity was corelated to production of chitinase by this bacillus strain (Prasad et al, 2023). The presence of chitin in the soil signals B. subtilis to ramp up its production of chitinase and other fungal cell wall-degrading enzymes. These enzymes directly attack and degrade the cell wall of the pathogenic Fusarium oxysporum fungus, inhibiting its growth and reducing disease severity.

Viswanathan & Samiyappan (2001), reported antifungal activity of two fluorescent Pseudomonads strains ARR1 and KKM1 against Colletotrichum falcatum of sugarcane. The two isolates produced higher content of chitinase in liquid cultures than other strains. Role of microbial chitinase against Colletotrichum falcatum has been well demonstrated under in vitro and in vivo conditions. In vitro studies have confirmed the antibiosis activity and chitinase production of antagonistic Pseudomonas strains (Malathi & Viswanathan, 2013). Nielson et al. (1998) demonstrated that fluorescent pseudomonads suppress Rhizoctonia solani in the sugar beet rhizosphere through endochitinase production. The suppression of growth of many plant pathogenic fungi like Rhizoctonia solani, Sclerotinia sclerotiorum, Fusarium oxysporum, F. solani, Colletotricum sp. and Macrophomina phaseolina, in vitro. by Bacillus sp. BPR7 was reported by Kumar et al, 2012. The antagonism reported in-vitro was found to be due to production by these rhizobacteria chitinase and β-1,4-glucanase besides production of other compounds like siderophores. Inhibition of pathogens like Aspergillus, Fusarium, Rhizoctonia and Colletotrichum which have chitin in their cell wall by chitinase producing Bacillus subtillis TD11 has also been reported (Malik et al, 2023). Akocak et al, 2015 studied the effect of chitinase producing strains Pseudomonas fluorescens PB27, Bacillus cereus B1, and Bacillus thuringiensis K1 on aflatoxin producing Aspergillus flavus which was significantly controlled by the three isolates. SEM of enzyme sprayed fungus showed ultrastructural changes in spores and mycelium of the fungus. The complete genome sequence of S. plymuthica, S. marcescens, S. nematodiphila and P. aeruginosa have shown the presence of chitinase and protease genes (Adam et al., 2016; Basharat et al., 2018; Folders et al., 2001; Su et al., 2017). ChiC gene is responsible for chitinase secretion in Pseudomonas aeruginosa and Serratia marcescens and ChiD is chitinase expressing gene in Bacillus circulans (Folders et al., 2001).

Bacillus and Pseudomonas spp showing good biocontrol potential are also known to produce a wide array of antimicrobial compounds such as lipopeptides, phenazines, polyketides and ribosomally synthesized peptides. Fluorescent Pseudomonas spp. are known to produce phenazines (PHZ), 2,4-diacetylphloroglucinol (DAPG), and pyrrolnitrin (PRN) pyoluteorin (PLT). Bacillus species predominantly produce cyclic lipopeptides (CLPs), ribosomally synthesized peptides (bacteriocins) and polyketides. These compounds work through various mechanisms, including direct inhibition of microbial growth and induction of a plant's systemic resistance. Members of the genera Pseudomonas, Bacillus, Paenibacillus, Brevibacillus and Streptomyces are well known for their lipopeptide producing ability (Manhas and Kaur, 2016; Grady et al., 2016). Lipopeptides and glycopeptides, classified as antimicrobial peptides (AMPs), are extensively investigated for their antibacterial and antifungal potential (Lei et al., 2021).
Phenazines are naturally occurring nitrogenous molecules with redox activity and notable environmental stability (Dar et al., 2020; Huasong et al., 2020; Biessy and Filion, 2018; Mavrodi et al., 2013). Phenazines and their derivatives are an important group of antimicrobial metabolites produced by different bacterial genera like fluorescent pseudomonads, Bacillus, Burkholderia, Streptomyces, Lactococcus, Nocardiopsis, Alcaligenes, Rahnell and Rhizobium showing effective biocontrol potential against fungi and oomycetes (Wang et al, 2021; Mishra and Arora, 2018; Anderson and Kim, 2018; Guttenberger et al., 2017; Zhao et al., 2016). In their study, Karmegham et al. (2020) verified the presence of phenazine derivatives in fluorescent Pseudomonas isolates through thin-layer chromatography, as the observed spots corresponded with authentic phenazine and shared an identical Rf value of 0.57. Many antimicrobial rhizobacteria especially Pseudomonas fluorescens are known to produce DAPG which is a natural, broad-spectrum antibiotic. DAPG has been reported to impair mitochondrial functions and calcium homeostasis in filamentous fungi, thereby being toxic to them (Troppens et al., 2013). DAPG producing Pseudomonas fluorescens has been used for biological control in agriculture to suppress plant diseases by inhibiting various fungi, oomycetes and bacteria like Gaeumannomyces tritici (Cook, 2003), Thielaviopsis basicola (Almario et al, 2014), Pythium ultimum (Fenton et al, 1992), Erwinia carotovora subsp.atroseptica (Cronin, D. et al.1997). Separation of metabolites at Rf value 0.57 & 0.35 in TLC experiment corresponding to phenazine and 2,4-Diacetylphloroglucinol (DAPG) confirmed the production of these metabolites by the isolates Pfa-50, Pfa-2, Pfa-24, pfa-11 and Pfa-65.
Another antibiotic Pyoluteorin (PLT)which is produced by some antagonistic Pseudomonas sp. is chlorinated polyketide antibiotic characterized by a dichlorinated pyrrole moiety and a resorcinol ring. Pyoluteorin is synthesized via a polyketide synthase (PKS)-non-ribosomal peptide synthetase (NRPS) hybrid pathway and this biosynthesis is directed by the plt gene cluster (Balthazar et al, 2022). A retardation factor (Rf) value 0.26 was reported for PLT (Abd El-Azeem and Ramadan, 2015) produced by rhizobacterial isolates which was confirmed by comigration of standards. Compounds at Rf 0.26 produced by Pfa-50 and Pfa-65 can be due to production of PLT. PLT has been found to be of antimicrobial component responsible for the control of Pantoea ananatis on Maize by Pseudomonas protegens Pf-5 (Gu et al, 2022). Pseudomonas strain Pf-5 produces the antibiotics DAPG and PLT, both of which exhibit significant antifungal activity against Botrytis cinerea (Balthazar et al., 2022). Their critical roles in disease suppression by beneficial Pseudomonas strains have been clearly established through studies using mutants impaired in DAPG and PLT biosynthesis (Zhang et al., 2020). An Rf value 0.8 has been reported for Pyrollnitrin (PRN) produced by fluorescent pseudomonad's metabolites extracted in ethyl acetate by Abd El-Azeem and Ramadan (2015). The production of PRN was confirmed by comigration of standard PRN at Rf value 0.8. The metabolic extract of fluorescent pseudomonads isolates Pfa-2, Pfa-50 and Pfa-65 showed a compound at Rf 0.8 which is similar to PRN. Pyrollnitrin is reported to have antifungal effect on a wide range of significant plant pathogens, including Rhizoctonia solani (which causes damping-off and root rot in cotton and cucumber), Fusarium oxysporum, Botrytis cinerea, and Gaeumannomyces graminis, Alternaria sp., Pythium aphanidermatum, F. graminearum, Penicillium expansum, Sclerotium rolfsii, and others (Ligon et al, 2000; Pawar et al, 2019)
Arbsuwan et al, 2017 identified macrolactins and amicoumacins as the antimicrobial compounds against Salmonella typhi ATCC 5784 produced by Bacillus spp. These compounds were separated at Rf value of 0.35 and 0.48 on TLC plates. In their experiments Rf value of 0.5 to 0.55 corresponded to surfactins which are a group of powerful cyclic lipopeptide biosurfactants primarily produced by various species of the Gram-positive bacterium Bacillus, most notably Bacillus subtilis. The metabolic extracts of rhizobacterial isolate B-20 and B-18 showed separation at 0.35, 048, 0.5 and 0.55 similar to macrolactins, amicoumacins and surfactins. Some lipopeptides secreted by Bacillus spp. like surfactins, iturins, and fengycins are well known for their damaging action on the cell wall of phytopathogens. While B. subtilis is the "gold standard" producer of surfactins, several other species in the genus produce surfactin or its structural variants like Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus pumilus, Bacillus mojavensis. Surfactins are important antimicrobial compounds as they insert into the lipid bilayer of cell membranes, causing "leaks" (pore formation) or complete membrane disintegration of the pathogens. Hazarika et al., 2019, reported the antagonistic effect of B.subtils SCB1 on Alternaria, Fusarium and Cochliobolus was due to production of antifungal metabolites like surfactins and other volatiles.
Macrolactins and amicoumacins (often referred to as amicoumerins) are non-ribosomal polyketides produced by Bacillus spp. Macrolactins are mostly produced by marine derived strains like Bacillus amyloliquefaciens, and Bacillus licheniformis. The antimicrobial properties of these compounds are due to their ability to inhibit protein synthesis or fatty acid synthesis. They also have the ability to inhibit replication in viruses. Macrolactin isolated from B. amyloliquefaciens have shown to exhibit significant antagonistic effects against Fusarium oxysporum and Ralstonia solanacearum (Yuan et al, 2012). In nature, Bacillus species often produce surfactins, macrolactins, and amicoumacins simultaneously. This "chemical cocktail" provides a multi-pronged attack as surfactin softens or permeabilizes the competitor's cell membrane, macrolactins and amicoumacins then enter the cell more easily to shut down internal machinery (DNA/Protein synthesis). The metabolites of bacillus strain B. licheniformis MML2501 showed blue bands at Rf value of 0.66 corresponding to authentic Indole Acetic Acid (IAA) bands (Prashanth and Mathivanan, 2010). This reveals the production of IAA by all

test isolates as they too gave compounds with Rf value 0.66. Earlier IAA production by these isolates was confirmed by Salkowski's reagent test calorimetrically (Arzoo et al 2024). The metabolites of two rhizobacterial isolates studied by Pravin et al, 2015 showed blue bands at Rf value 0.74 corresponding to that of authentic salicylic acid (SA). As all the Bacillus isolates showed a compound at Rf value 0.74 the production of SA by these isolates can be inferred. IAA are well known for their effect on plant growth promotion. SA provides the plant stress tolerance and plays active role in activating plant's defense mechanism besides improving various physiological traits and modulation of phytohormones.

The experiments on in vitro antagonistic potential of isolated rhizobacteria and elucidation of the mechanism involved in growth inhibition of the pathogen revealed that the isolates in this study, particularly Pfa-50 exhibit a high level of antagonism. Out of the 30 screened antagonistic isolates only 22 isolates showed positive response for HCN and chitinase production tests. Six of them had the ability to produce HCN and sixteen had chitinolytic activity. The antagonistic effect of other eight isolates which were neither HCN nor chitinase producer was expected to be due to synthesis of antimicrobial secondary metabolites. Extraction of secondary metabolites and their separation on TLC plates confirmed production of many antimicrobial secondary metabolites and also growth promoting metabolites. These in vitro tests provide strong evidence of isolated rhizobacterium's potential as a biocontrol agent. This initial screening helps select the most effective strains for further development into a viable and sustainable alternative to chemical fungicides.

REFERENCES

1. Abd El-Azeem M and Ramadan E. 2015.Characterization of antifungal metabolites from antagonistic fluorescent pseudomonads. Journal of Applied Plant Protection, 4 (1): 13-21.
2. Abd El-Mageed, S.A. (2020). Residual acidified biochar modulates growth, physiological responses, and water relations of maize (Zea mays) under heavy metal–contaminated irrigation water. Environ. Sci. Pollut. Res. 27: 22956–22966.
3. Abd El-Rahman, A.F., Shaheen, H.A., Abd El-Aziz, R.M. et al. Influence of hydrogen cyanide-producing rhizobacteria in controlling the crown gall and root-knot nematode, Meloidogyne incognita. Egypt J Biol Pest Control **29**, 41 (2019). https://doi.org/10.1186/s41938-019-0143-7
4. Abriouel, H., Franz, C. M. A. P., Omar, N. B., and Gálvez, A. (2011). Diversity and applications of bacillus bacteriocins. FEMS Microbiol. Rev. 35: 201–232. doi: 10.1111/j.1574 6976.2010.00244.x
5. Adam E, Müller H, Erlacher A,G. Berg G. (2016), Complete genome sequences of the Serratia plymuthica strains 3Rp8 and 3Re4-18, two rhizosphere bacteria with antagonistic activity towards fungal phytopathogens and plant growth promoting abilities.Stand. Genom. Sci., 11 p. 61, 10.1186/s40793-016-0185-3
6. Ahemad M & Kibret M. 2014, Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. Journal of King Saud University - Science,26(1): 1-20, https://doi.org/10.1016/j.jksus.2013.05.001
7. Arbsuwan, N., Payoungkiattikun, W., Sirithorn, P., Daduang, S., Jangpromma, N., Dhiravisit, A. Klaynongsruang, S. (2017). Purification and characterization of macrolactins and amicoumacins from Bacillus licheniformis BFP011: a new source of food antimicrobial substances. CyTA - Journal of Food, 16(1), 50–60. https://doi.org/10.1080/19476337.2017.1337047
8. Arzoo K, Balodi R, Erayya , Kalmesh M and J. Kumar. 2024. Indole Acetic Acid (IAA) Mediated Plant Growth Promotion Activity of Fluorescent Pseudomonads and Bacillus spp. Journal of Advances in Biology & Biotechnology, 27(7):162-171 https://doi.org/10.9734/jabb/2024/v27i7976
9. Akocak P B, Churey J J, Worobo, R W. 2015. Antagonistic effect of chitinolytic Pseudomonas and Bacillus on growth of fungal hyphae and spores of aflatoxigenic Aspergillus flavus, Food Bioscience: 10: 48-58, ISSN 2212-4292, https://doi.org/10.1016/j.fbio.2015.01.005
10. Akinbode, O.A. and Ikotun, T. (2008) Evaluation of Some Bio-Agents and Botanicals in in Vitro Control of Colletotrichuwm destructivum. African Journal of Biotechnology, 7, 868-872.
11. Almario, J., Muller, D., Défago, G. & Moënne-Loccoz, Y. Rhizosphere ecology and phytoprotection in soils naturally suppressive to Thielaviopsis black root rot of tobacco. Environ. Microbiol. **16**, 1949–1960. https://doi.org/10.1111/1462-2920.12459 (2014).
12. Alwahshi, K. J., Purayil, G. P., Saeed, E. E., Abufarajallah, H. A., Aldhaheri, S. J., AbuQamar, S. F. and El-Tarabily, K. A. (2022) The 1-aminocyclopropane-1-carboxylic acid deaminase-producing Streptomyces violaceoruber UAE1 can provide protection from sudden decline syndrome on date palm. Front. Plant Sci. 13:904166. doi: 10.3389/fpls.2022.904166
13. Anand, D Chinchilla, C Tan, L Mène-Saffrané, F L'Haridon, L Weisskopf. Contribution of hydrogen cyanide to the antagonistic activity of Pseudomonas strains against Phytophthora infestans. Microorganisms, 8 (2020), Article 1144 https://doi.org/10.3390/microorganisms8081144
14. Anderson AJ & Kim YC. (2018). Biopesticides produced by plant-probiotic Pseudomonas chlororaphis isolates, Crop Protection,105: 62-69 https://doi.org/10.1016/j.cropro.2017.11.009
15. Anon, 1957. Mannual of microbiological methods. Mc-Graw Hill Book Co.,New York. 127p
16. Antoun, H., and Kloepper, J. (2001). "Plant growth promoting rhizobacteria" in Encyclopedia of genetics. eds. S. Brenner and J. H. Miller (New York: Academic Press), 1477–1480
17. Anwar S., Ali B., Sajid I. (2016). Screening of rhizospheric actinomycetes for various in-vitro and in-vivo plant growth promoting (PGP) traits and for agroactive compounds. Front. Microbiol. 7. doi: 10.3389/fmicb.2016.01334
18. Arbsuwan, N., Payoungkiattikun, W., Sirithorn, P., Daduang, S., Jangpromma, N., Dhiravisit, A., Klaynongsruang, S. (2018). Purification and characterization of macrolactins and amicoumacins from Bacillus licheniformis BFP011: a new source of food antimicrobial substances. CyTA - Journal of Food, 16(1), 50–60. https://doi.org/10.1080/19476337.2017.1337047

19. Auesbel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1992. Current protocols in molecular biology. New York, Greene Publishing Association; Wiley-Interscience.
20. Bae S, Kim SG, Kim YH. 2013. Biocontrol Characteristics of Bacillus Species in Suppressing Stem Rot of Grafted Cactus Caused by Bipolaris cactivora. Plant Pathol J. (1):42-51. doi: 10.5423/PPJ.OA.07.2012.0116 .
21. Barthalomew, J. W. and Mittewer, T. 1950. A simplified bacterial strain. Stain Technology. **25:** 153
22. Balthazar C, St-Onge R, Léger G, Lamarre SG, Joly DL and Filion M (2022) Pyoluteorin and 2,4-diacetylphloroglucinol are major contributors to Pseudomonas protegens Pf-5 biocontrol against Botrytis cinerea in cannabis. Front. Microbiol. 13:945498. https://doi.org/10.3389/fmicb.2022.945498
23. Behdani, M., Etebarian, H. R., Khodakaramian, G. and Mohammadifar, M. 2012. Biological control of Bipolaris spicifera, the causal agent of wheat root rot by Pseudomonas fluorescens isolates. International Journal of Agriculture and Crop Sciences. 4 (8): 483-488
24. Basharat Z, F. Tanveer, A. Yasmin, Z.K. Shinwari, T. He, Y. Tong. (2018). Genome of Serratia nematodiphila MB307 offers unique insights into its diverse traits. Genome, 61: 469-476. https://doi.org/10.1139/gen-2017-0250
25. Biessy A, Filion M. 2018. Phenazines in plant-beneficial Pseudomonas spp.: biosynthesis, regulation, function, and genomics. Environ Microbiol 20:3905–3917. https://doi.org/10.1111/1462-2920.14395
26. Cano, R.J., Borucki, M.K., Highby- Schweitzer, M., Poinar, H.N., Jr. G.O. and Pollard, K.J. 1994. Bacillus DNA in Fossil Bees: an Ancient Symbiosis? Applied Environment Microbiology.60 (6): 2164-2167
27. Chita Ranjan Deb CR Mum Tatung. Siderophore producing bacteria as biocontrol agent against phytopathogens for a better environment: A review, South African Journal of Botany, Volume 165, 2024,Pages 153-162, ISSN 0254-6299, https://doi.org/10.1016/j.sajb.2023.12.031 .
28. Cook, J. R. Take-all of wheat. Physiol. Mol. Plant Pathol. 62, 73–86. https://doi.org/10.1016/S0885-5765(03)00042-0 (2003).
29. Cronin, D. et al. 1997. Ecological interaction of a biocontrol Pseudomonas fluorescens strain producing 2,4-diacetylphloroglucinol with the soft rot potato pathogen Erwinia carotovora subsp. atroseptica. FEMS Microbiol. Ecol. 23, 95–106. https://doi.org/10.1111/j.1574-6941.1997.tb00394.x
30. Danish, M, Mohammad Shahid, Mohammad Altaf, Anshika Tyagi, Sajad Ali,(2024) Plant growth-promoting rhizobacteria and biocontrol agents triggered plant defence responses against phytopathogenic fungi and improved rice growth, Physiological and Molecular Plant Pathology, 133. ISSN 0885-5765,
https://doi.org/10.1016/j.pmpp.2024.102337.
31. Dar D, Thomashow LS, Weller DM, Newman DK. Global landscape of phenazine biosynthesis and biodegradation reveals species-specific colonization patterns in agricultural soils and crop microbiomes. Elife. 2020 Sep 15;9:e59726. https://doi.org/10.7554/elife.59726
32. Deb CR, Tatung, M.(2024) Siderophore producing bacteria as biocontrol agent against phytopathogens for a better environment: A review, South African Journal of Botany,165: 153-162, https://doi.org/10.1016/j.sajb.2023.12.031
33. Defago, G., Berling, C.H., Burger, U., Haas, D., Kahr, G., Keel, C., Voisard, C., Wirthner, P. and Wuthrich, B. 1990. Suppression of black root rot of tobacco and other root diseases by strains of Pseudomonas fluorescnes: Potential applications and mechanisms. In: Hornby, D. ed. Biological Control of Soil Borne Plant Pathogens .CAB International, Wallingford, Oxon, UK. pp.93-108
34. Dönmez, M.F., Uysal, B., Demirci, E., Ercişli, S., Çakmakçı, R., 2015. Biological control of root rot disease caused by Rhizoctonia solani Kühn. on potato and bean using antagonist bacteria. Acta Scientiarum Polonorum Hortorum Cultus, 14 (5), 29-40.
35. Duffy, B., Schouten, A. and Raajimakers, J. 2003. Pathogen self-defense: mechanisms to counteract microbial antagonism. Annu. Rev. Phytopathol. **45:** 501-538
36. Dukare, A., Paul, S. & Arambam, A. Isolation and efficacy of native chitinolytic rhizobacteria for biocontrol activities against Fusarium wilt and plant growth promotion in pigeon pea (Cajanus cajan L.). Egypt J Biol Pest Control **30**, 56 (2020). https://doi.org/10.1186/s41938-020-00256-7
37. El-Saadony Mohamed T. , Saad Ahmed M. , Soliman Soliman M. , Salem Heba M. , Ahmed Alshaymaa I. , Mahmood Mohsin , El-Tahan Amira M. , Ebrahim Alia A. M. , Abd El-Mageed Taia A. , Negm Shaimaa H. , Selim Samy , Babalghith Ahmad O. , Elrys Ahmed S. , El-Tarabily Khaled A. , AbuQamar Synan F. (2022). Plant growth-promoting microorganisms as biocontrol agents of plant diseases: Mechanisms, challenges and future perspectives. Frontiers in Plant Science, 13. https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2022.923880 . ISSN=1664-462X
38. Fenton, A. M., Stephens, P. M., Crowley, J., O'Callaghan, M. & O'Gara, F. Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a Pseudomonas strain. Appl. Environ. Microbiol. **58**, 3873–3878. https://doi.org/10.1128/aem.58.12.3873-3878.1992 (1992).
39. Folders J, Algr J, Roelofs MS, van Loon LC, Tommassen J and Bitter W. 2001, Characterization of Pseudomonas aeruginosa Chitinase, a Gradually Secreted Protein. Journal of Bacteriology, 183 (24): 7044–7052 DOI: 10.1128/JB.183.24.7044–7052..,
40. Gowtham et al (2018) H.G. Gowtham HG, M. Murali, S. Brijesh Singh, T.R. Lakshmeesha, K. Narasimha Murthy, K.N. Amruthesh, S.R. Niranjana, 2018. Plant growth promoting rhizobacteria- Bacillus amyloliquefaciens improves plant growth and induces resistance in chilli against anthracnose disease, Biological Control,126: 209-217,
https://doi.org/10.1016/j.biocontrol.2018.05.022
41. Gu Q, Qiao J, Wang R, Lu J, Wang Z, Li P, Zhang L, Ali Q, Khan AR, Gao X, Wu H. The Role of Pyoluteorin from Pseudomonas protegens Pf-5 in Suppressing the Growth and Pathogenicity of Pantoea ananatis on Maize. Int J Mol Sci. 2022 Jun 9;23(12):6431. https://doi.org/10.3390/ijms23126431

42. Grady, E. N., MacDonald, J., Liu, L., Richman, A., and Yuan, Z. C. (2016). Current knowledge and perspectives of Paenibacillus: a review. Microb. Cell Fact. 15, 1–18. doi: 10.1186/s12934-016-0603-7
43. Guttenberger N., W. Blankenfeldt, R. Breinbauer. 2017. Recent developments in the isolation, biological function, biosynthesis, and synthesis of phenazine natural products. Bioorg. Med. Chem., 25 (22): 6149-616610.
44. Hariprasad P., S.T. Divakara, S.R. Niranjana. 2011. Isolation and characterization of chitinolytic rhizobacteria for the management of Fusarium wilt in tomato. Crop Protection, 30(12): 1606-1612, https://doi.org/10.1016/j.cropro.2011.02.032
45. Hazarika, D.J., Goswami, G., Gautom, T. et al. Lipopeptide mediated biocontrol activity of endophytic Bacillus subtilis against fungal phytopathogens. BMC Microbiol **19**, 71 (2019). https://doi.org/10.1186/s12866-019-1440-8
46. Hong SY, Lee DH, Lee JH, Haque MA, Cho KM. Five Surfactin Isomers Produced during Cheonggukjang Fermentation by Bacillus pumilus HY1 and Their Properties. Molecules. 2021 Jul 24;26(15):4478. doi: 10.3390/molecules26154478. PMID: 34361631; PMCID: PMC8346962.
47. Huang, X., Zhang, N., Yong, X., Yang, X., and Shen, Q. (2012). Biocontrol of Rhizoctonia Solani damping-off disease in cucumber with Bacillus pumilus SQR-N43. Microbiol. Res. 167, 135–143. doi: 10.1016/j.micres.2011.06.002
48. Huasong, P, H. Qingwen, M. Bilal, W. Wang, X. Zhang. 2020. Kinetics, mechanism and identification of photodegradation products of phenazine-1-carboxylic acid. Environ. Technol., 41: 1848-185
49. Karimi et al., Karimi K, J. Amini, B. Harighi, B. Bahramnejad. 2012. Evaluation of biocontrol potential of Pseudomonas and Bacillus spp. against Fusarium wilt of chickpea Aust. J of Crop Sci. 6(4):695-703
50. Karmegham N, Vellasamy S, Balasubramanian Natesan B, Sharma MP, Dunia A. Al Farraj, Mohamed S. Elshikh. 2020. Characterization of antifungal metabolite phenazine from rice rhizosphere fluorescent pseudomonads (FPs) and their effect on sheath blight of rice. Saudi Journal of Biological Sciences,27 (12): 3313-3326 https://doi.org/10.1016/j.sjbs.2020.10.007
51. Kumar P., Dubey R.C., Maheshwari D.K. 2012. Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. Microbiol. Res. 167:493–499. https://doi.org/10.1016/j.micres.2012.05.002
52. King, E.O., Ward, M.K. and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44**:** 301–307.
53. Krassilnikov, N.A. 1950. Antagonistic Actinomycetes and Antibiotic Substances. Academic Press of the USSR, Moscow (in Russian). pp. 303
54. Lei M, Jayaraman A James A. Van Deventer, JA and Lee, K. 2019. Engineering Selectively Targeting Antimicrobial Peptides, Annual Review of Biomedical Engineering, 23: 339-357 https://doi.org/10.1146/annurev-bioeng-010220-095711
55. Li, Y., Li, Z., Yamanaka, K. et al. Directed natural product biosynthesis gene cluster capture and expression in the model bacterium Bacillus subtilis. Sci Rep **5**, 9383 (2015). https://doi.org/10.1038/srep09383
56. Ligon, J. M., D. S. Hill, P. E. Hammer, N. R. Torkewitz, D. Hofmann, H. J. Kempf and K. H. v. Pée. 2000. Natural products with antifungal activity from Pseudomonas biocontrol bacteria. Pest Management Science: formerly Pesticide Science. 56, 688-695.
57. Liu et al. (2010). Isolation and partial characterization of an antifungal protein from the endophytic Bacillus subtilis strain EDR4. Pesticide Biochemistry and Physiology
58. Liu et al. Purification and partial characterization of a 36-kDa chitinase from Bacillus thuringiensis subsp. colmeri, and its biocontrol potential. (2010) Enzyme and Microbial Technology
59. Lucas, J. (2011). Advance in plant disease and pest management. J. Agric. Sci. 149, 91–114. doi: 10.1017/S0021859610000997
60. Maiden, N.A., Noran, A.S., Ahmad Fauzi, M.A.F. et al. Screening and Characterisation of Chitinolytic Microorganisms with Potential to Control White Root Disease of Hevea brasiliensis. J Rubber Res **20**, 182–202 (2017). https://doi.org/10.1007/BF03449151
61. Malathi P , Viswanathan R (2013). Role of microbial chitinase in the biocontrol of sugarcane red rot caused by Colletotrichum falcatum Went . Electronic Journal of Plant Breeding 6 : 17 - 23 .
62. Malik, M. S., Rehman, A., Khan, I. U., Khan, T. A., Jamil, M., Rha, E. S., & Anees, M. (2023). Thermo-neutrophilic cellulases and chitinases characterized from a novel putative antifungal biocontrol agent: Bacillus subtilis TD11. PLoS One, 18(1), e0281102.
63. Manhas, R. K., and Kaur, T. (2016). Biocontrol potential of Streptomyces hydrogenans strain DH16 toward Alternaria brassicicola to control damping off and black leaf spot of Raphanus sativus. Front. Plant Sci. 7:1869. doi: 10.3389/fpls.2016.01869
64. Mavrodi, D.V., Parejko, J.A., Mavrodi, O.V., Kwak, Y.-S., Weller, D.M., Blankenfeldt, W. and Thomashow, L.S. (2013), Recent insights into the diversity, frequency and ecological roles of phenazines in fluorescent Pseudomonas spp.. Environmental Microbiology, 15: 675-686. https://doi.org/10.1111/j.1462-2920.2012.02846.x
65. Miljaković D, Marinković J, Balešević-Tubić S. The Significance of Bacillus spp. in Disease Suppression and Growth Promotion of Field and Vegetable Crops. Microorganisms. 2020 Jul 13;8(7):1037. https://doi.org/10.3390/microorganisms8071037
66. Miller, R. L. and Higgins, V. J. 1970. Association of cyanide with infection of birdsfoot trefoil by Stemphyluim loti. Phytopathology. **60:** 104-110
67. Mishra,J, Arora, NK, 2018. Secondary metabolites of fluorescent pseudomonads in biocontrol of phytopathogens for sustainable agriculture, Applied Soil Ecology,125, 35-45, https://doi.org/10.1016/j.apsoil.2017.12.004

68. Murthy, N and Bleakley, B. 2012. Simplified Method of Preparing Colloidal Chitin Used For Screening of Chitinase-Producing Microorganisms. The Internet Journal of Microbiology. 10 (2):
69. Nandi M, Selin C, Brawerman G, Fernando WGD, de Kievit T. 2017. Hydrogen cyanide which contributes to Pseudomonas chlororaphis strain PA23 biocontrol is upregulated in the presence of glycine. Biol Control. 108:47–54.
70. Nazari M and Smith DL (2020) A PGPR-Produced Bacteriocin for Sustainable Agriculture: A Review of Thuricin 17 Characteristics and Applications. Front. Plant Sci. 11:916. doi: 10.3389/fpls.2020.00916
71. Nielsen, M. N., Sorensen, J., Fels, J., and Pedersen, H. C. 1998. Secondary metabolite and endochitinase dependent antagonism toward plant-pathogenic microfungi of Pseudomonas fluorescens isolates from sugar beet rhizosphere. Appl. Environ. Microbiol. **64**: 3563- 3569
72. Panicker, S., Sayyed, R.Z. (2022). Hydrolytic Enzymes from PGPR Against Plant Fungal Pathogens. In: Sayyed, R., Singh, A., Ilyas, N. (eds) Antifungal Metabolites of Rhizobacteria for Sustainable Agriculture. Fungal Biology. Springer, Cham. https://doi.org/10.1007/978-3-031-04805-0_10
73. Park, G. K., Lim, J. H., Kim, S. D. & Shim, S. H. Elucidation of antifungal metabolites produced by Pseudomonas aurantiaca IB5-10 with broad-spectrum antifungal activity. J. Microbiol. Biotechnol. **22**, 326–330. https://doi.org/10.4014/jmb.1106.06042 (2012).
74. Panpatte, D.G., Jhala, Y.K., Shelat, H.N., Vyas, R.V. (2016). Pseudomonas fluorescens: A Promising Biocontrol Agent and PGPR for Sustainable Agriculture. In: Singh, D., Singh, H., Prabha, R. (eds) Microbial Inoculants in Sustainable Agricultural Productivity. Springer, New Delhi. https://doi.org/10.1007/978-81-322-2647-5_15
75. Pawar,S., A. Chaudhari,A., R. Prabha,R., Shukla,R and D.P. Singh. Microbial pyrrolnitrin: natural metabolite with immense practical utility. Biomolecules, 9 (2019), p. 443
76. Parthiban, V. K. and Kavitha, R. (2014). In vitro screening of effective biocontrol agents against bean anthracnose pathogen, Colletotrichum lindemuthianum. Internat. J. Pharma. Screening Methods 4:32-35
77. Prasad B**,** Sharma,D, Kumar,P, Dubey,RC. 2023. Biocontrol potential of Bacillus spp. for resilient and sustainable agricultural systems, Physiological and Molecular Plant Pathology, 128: 102173, https://doi.org/10.1016/j.pmpp.2023.102173
78. Prashanth S and Mathivanan, N. 2010. Growth promotion of groundnut by IAA producing rhizobacteria Bacillus licheniformis MML2501. Archives of Phytopathology and Plant Protection 43(2):191-208
79. Parvin W. and Md. Mahbubur Rahman. In vitro Detection and Optimization of Salicylic Acid from the Rhizobacterial Strains Pseudomonas aeruginosa UPMP3 and Burkholderia cepacia UPMB3 for Plant Defense. Bangladesh Journal of Forest Science. Vol 37 (2), 2021: 1-14.
80. Qiao, J., Yu, X., Liang, X., Liu, Y., Borriss, R., and Liu, Y. (2017). Addition of plant-growth-promoting Bacillus subtilis pts-394 on tomato rhizosphere has no durable impact on composition of root microbiome. BMC Microbiol. 17:131. doi: 10.1186/s12866-017-1039-x
81. Raaijmakers, J. M., Vlami, M., and de Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek 81:537. doi: 10.1023/A:1020501420831
82. Radzki, W., Mañero, F., Algar, E., Lucas, J., García-Villaraco, A., and Ramos, B. (2013). Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. Antonie van Leeuwenhoek 104, 321–330. doi: 10.1007/s10482-013-9954-9
83. Rajendran, L., Karthikeyan, G., Raguchander, T. and Samiyappan, R. 2008. Cloning and sequencing of novel endophytic Bacillus subtilis from coconut for the management of basal stem rot disease. Asian Journal of Plant pathology. **2(1):** 1-14
84. Rathod, K, Rana, S, Dhandhukia, P. Thakker. J.N. Marine Bacillus subtilis as an effective biocontrol agent against Fusarium oxysporum f. sp. ciceris. Eur. J. Plant Pathol. (2023), pp. 1-12
85. Reetha, A. K., Pavani, S. L., & Mohan, S. (2014). Hydrogen cyanide production ability by bacterial antagonist and their antibiotics inhibition potential on Macrophomina phaseolina (Tassi.) Goid. Int. J. Curr. Microbiol. Appl. Sci, 3(5), 172-178.
86. Rijavec T, Lapanje A. Hydrogen Cyanide in the Rhizosphere: Not Suppressing Plant Pathogens, but Rather Regulating Availability of Phosphate. Front Microbiol. 2016 Nov 18;7:1785. doi: 10.3389/fmicb.2016.01785. PMID: 27917154; PMCID: PMC5114478.
87. Roberts, A., and Selitrenkoff, N., (1988). Colloidal chitin preparations. J. Gen. Microbiol. 134: 169 – 176
88. Salomon, M. V., Funes Pinter, I., Piccoli, P., and Bottini, R. (2017). "Use of plant growth-promoting rhizobacteria as biocontrol agents: induced systemic resistance against biotic stress in plants" in Microbial applications vol. 2: Biomedicine, agriculture and industry. ed. V. C. Kalia (Cham: Springer International Publishing), 133–152.
89. Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. Nat. Ecol. Evol. 3, 430–439. doi: 10.1038/s41559-018-0793-y
90. Singh, S., Balodi, R., Meena, P.N. and Sonali Singhal. Biocontrol activity of Trichoderma harzianum, Bacillus subtilis and Pseudomonas fluorescens against Meloidogyne incognita, Fusarium oxysporum and Rhizoctonia solani. Indian Phytopathology **74**, 703–714 (2021). https://doi.org/10.1007/s42360-021-00368-62021.
91. Sehrawat,A, Satyavir S. Sindhu, Bernard R. Glick, 2022, Hydrogen cyanide production by soil bacteria: Biological control of pests and promotion of plant growth in sustainable agriculture, Pedosphere,32(1): 15-38, ISSN: 1002-0160, https://doi.org/10.1016/S1002-0160(21)60058-9 .
92. Sendi, Y., Pfeiffer, T., Koch, E. et al. Potential of common bean (Phaseolus vulgaris L.) root microbiome in the biocontrol of root rot disease and traits of performance. J Plant Dis Prot 127, 453–462 (2020). https://doi.org/10.1007/s41348-020-00338-6

93. Singh, S., Balodi, R., Meena, P.N. et al. Biocontrol activity of Trichoderma harzianum, Bacillus subtilis and Pseudomonas fluorescens against Meloidogyne incognita, Fusarium oxysporum and Rhizoctonia solani. Indian Phytopathology **74**, 703–714 (2021). https://doi.org/10.1007/s42360-021-00368-6
94. Soleimani, M. J., Shamsbakhsh, M., & Taghavi, M. (2005). Biological Control of Stem and Root-rot of Wheat Caused by Bipolaris spp. by using Antagonistic Bacteria, Fluorescent Pseudomonads and Bacillus spp. Journal of Biological Sciences, 5(3), 347-353 https://doi.org/10.3923/jbs.2005.347.353
95. Spilker, T., Coenye, T., Vandamme, P. and Li Puma, J.J. 2004. PCR-based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fibrosis patients. J Clin Microbiol. **42:** 2074–2079
96. Srivastava P, Sahgal M, Sharma K, Enshasy HAE, Gafur A, Alfarraj S, Ansari MJ and Sayyed RZ (2022) Optimization and identification of siderophores produced by Pseudomonas monteilii strain MN759447 and its antagonism toward fungi associated with mortality in Dalbergia sissoo plantation forests. Front. Plant Sci. 13:984522. https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2022.984522.
97. Srivastava, P., Jaggi, V., Dasilva, H., and Sahgal, M. (2020). Identification and characterization of siderophore positive Pseudomonas from north Indian Rosewood (Dalbergia sissoo) Roxb. Forest ecosystem. Int. J. Agric. 10, 239–256. doi: 10.24247/ijasraug202032
98. Subramanian, S., and Smith, D. L. (2015). Bacteriocins from the rhizosphere microbiome: from an agriculture perspective. Front. Plant Sci. 6:909. doi: 10.3389/fpls.2015.00909
99. Su, Y. C., Xu, L. P., Wang, S. S., Wang, Z. Q., Yang, Y. T., Chen, Y., et al. (2015). Identification, phylogeny, and transcript of chitinase family genes in sugarcane. Sci. Rep. 5:10708. doi: 10.1038/srep10708
100. Tariq M, Yasmin S, Hafeez FY. 2010. Biological Control of Potato Black Scurf by Rhizosphere Associated Bacteria. Braz J Microbiol. 41: 439-451. https://goo.gl/t7hSpg
101. D. Toua, D., M. Benchabane, F. Bensaid, R. Bakour. (2013), Evaluation of Pseudomonas fluorescens for the biocontrol of fusarium wilt in tomato and flax. African Journal of Microbiology Research, 7(48): 5449-5458, 10.5897/AJMR12.2019
102. Troppens, D. M., Chu, M., Holcombe, L. J., Gleeson, O., O'Gara, F., Read, N. D., et al. (2013). The bacterial secondary metabolite 2, 4-diacetylphloroglucinol impairs mitochondrial function and affects calcium homeostasis in Neurospora crassa. Fungal Genet. Biol. 56, 135–146. doi: 10.1016/j.fgb.2013.04.006
103. Vijay Karuppiah, PK Senthil Kumar, Nagamani Kathiresan, Balasubramani Ravindran, Soon Woong Chang, Ravishankar Ram Mani, Karthikeyan Ravi, Kavitha Thangavel, 2025, Affinity of rhizobacterial chitinases towards chitin polymers from shrimp exoskeleton and fungal cell wall: Optimization for effective biocontrol potentials, Carbohydrate Polymer Technologies and Applications 11: 100959. https://doi.org/10.1016/j.carpta.2025.100959
104. Viswanathan R & Samiyappan R. 2001 Antifungal activity of chitinases produced by some fluorescent pseudomonads against Colletotrichum falcatum Went causing red rot disease in sugarcane. Microbiological Research, 155 (4): 309-314. ISSN 0944-5013, https://doi.org/10.1016/S0944-5013(01)80009-4
105. Wang Y, Yongfu Pei, Xin Wang, Xiongfeng Dai, Manlu Zhu. (2024) Antimicrobial metabolites produced by the plant growth-promoting rhizobacteria (PGPR): Bacillus and Pseudomonas,Advanced Agrochem, 3(3): 206-221. ISSN 2773-2371, https://doi.org/10.1016/j.aac.2024.07.007
106. Wang, S.-Y. et al. Biocontrol ability of phenazine-producing strains for the management of fungal plant pathogens: A review. Biol. Control **155**, 104548. https://doi.org/10.1016/j.biocontrol.2021.104548 (2021).
107. Wang H, Liu R, You MP, Barbetti MJ, Chen Y. 2021; Pathogen Biocontrol Using Plant Growth-Promoting Bacteria (PGPR): Role of Bacterial Diversity. Microorganisms. 9(9):1988. https://doi.org/10.3390/microorganisms9091988
108. Wei, G., Kloepper, J. W. and Tuzun, S. 1991. Induction of systemic resistance of cucumber to Colletotrichum orbiculare by select strains of plant growth-promoting rhizobacteria. Phytopathology. **81:** 1508-1512
109. Wei Ge, Linxuan Zhang, Fanli Meng, Chengming Tian (2025). Study on biocontrol potential of volatile organic compounds produced by Pseudomonas atacamensis GZ-3 on poplar anthracnose, Industrial Crops and Products,224:120402, ISSN 0926-6690, https://doi.org/10.1016/j.indcrop.2024.120402
110. Walker, Powell, Seddon, Bacillus isolates from the spermosphere of peas and dwarf French beans with antifungal activity against Botrytis cinerea and Pythium species, Journal of Applied Microbiology, Volume 84, Issue 5, 1 June 1998, Pages 791–801, https://doi.org/10.1046/j.1365-2672.1998.00411.x
111. Yi Y, Shan Y, Liu S, Yang Y, Liu Y, Yin Y, Hou Z, Luan P, Li R. Antagonistic Strain Bacillus amyloliquefaciens XZ34-1 for Controlling Bipolaris sorokiniana and Promoting Growth in Wheat. Pathogens. 2021; 10(11):1526. https://doi.org/10.3390/pathogens10111526
112. Yoo,J.Y., Jang, E J, Park, S Y, Son, H J. Production of HCN, weed control substance, by Pseudomonas koreensis and its plant growth-promoting and termiticidal activities J Environ Sci Int, 27 (2018), pp. 771-780
113. Yuan et al, 2012, Zahoor, S., Naz, R., Keyani, R. et al. Rhizosphere bacteria associated with Chenopodium quinoa promote resistance to Alternaria alternata in tomato. Sci Rep **12**, 19027 (2022). https://doi.org/10.1038/s41598-022-21857-2
114. Zhang, Q., Kong, X., Li, S., Chen, X. J., and Chen, X. J. (2020). Antibiotics of pseudomonas protegens FD6 are essential for biocontrol activity. Australas. Plant Pathol. 49, 307–317. doi: 10.1007/s13313-020-00696-7
115. Zhao et al., 2016; Zhou, Y. et al. Management of the soybean cyst nematode Heterodera glycines with combinations of different rhizobacterial strains on soybean. PLoS ONE https ://doi.org/10.1371/journ al.pone.01826 54 (2017).