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Antifungal activity of some strains of plant growth-promoting rhizobacteria

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Abstract
The plant growth-promoting activities of 22 isolates of *Pseudomonas fluorescens* and *Bacillus* spp. were assess against three spore forming fungi (*Alternaria* spp., *Fusarium* spp., *Bipolaris* spp.). The bacterial isolates showed that differed in PGP range and activities, varied greatly among the isolates. The strain R-3 exhibited siderophore production, three strains (C-03, C6, R-4) solubilized phosphate, 5 strains of *Pseudomonas fluorescens* (CRM-3, R-3, C6, PSB-2, C-05) produced HCN and the *Bacillus* spp. showed antifungal activity against the spore forming fungi i.e. *Alternaria* spp., *Fusarium* spp., *Bipolaris* spp. *P. fluorescens* did not show any inhibition against the spores of the phytopathogenic fungi. *Bacillus* sp. and *P. fluorescens* are used for enhancing the growth of plants and eradicating diseases caused by bacteria and fungi. HPLC analysis showed that there was considerable amount of gallic acid present in all the three extracts of *Bacillus* spp. that confirmed the inhibitory effect showed in the dual plate assay against the tested fungi. The bacterial isolates differed in PGP activities and varied greatly among the isolates. The *Bacillus* sp. and *P. fluorescens* are used for enhancing the growth of plants and eradicating diseases caused by bacteria and fungi. The considerable amount of gallic acid present in all the three extracts of *Bacillus* spp. that confirmed the inhibitory effect showed in the dual plate assay against the tested fungi. This study provides a comprehensive assessment of 22 isolates of *Pseudomonas fluorescens* and *Bacillus* spp. The antifungal metabolites produced by *Bacillus* spp. need to be isolated, purified and tested in field for commercialization.

Keywords: *Pseudomonas fluorescens*; *Bacillus* spp; *Alternaria* spp; *Fusarium* spp; HPLC analysis.

Introduction
Biological control of plant diseases is gaining attention due to increased pollution concerns because of pesticides use for crop protection and development of pathogen resistance. The use of environmental friendly microorganisms has proved useful in plant-growth promotion and disease control in modern agriculture. Plant growth-promoting rhizobacteria (PGPR) are soil borne bacteria that colonize the roots of plants and play a great role in protection / growth of plants in several ways. PGPR are also known to induce systemic resistance (ISR) in plants and enhance the pathogen to the plant [1, 2]. *Pseudomonas* species is known generally to promote plant growth either directly by the production of hormones or indirectly by the production of antimicrobial compounds which act against pathogens viz., induced systemic resistance in the host [1,3], antibiotic production [4], growth promotion [5] and competition of nutrients [6, 7]. *Pseudomonas fluorescens* is one among them which protects host against pathogen attack through their mechanisms [1, 6, 8, 9, 10, 11]. The production of antibiotics is considered to be one of the most powerful and studied biocontrol mechanisms of plant growth promoting rhizobacteria against phytopathogens has become increasingly better understood over the past two decades [12].

Antimicrobial activities of phenolic compounds are well documented [13]. The mechanisms responsible for toxic activity of phenolic compounds against microorganisms may be attributed to adsorption and disruption of microbial membranes, interaction with vital enzymes, and deprivation of metalion [14, 15]. Among the phenolic compounds gallic acid is reported to be antimicrobial [16, 17] and its derivatives such as gallotannin along with other tannins are also known to have antibacterial and antifungal activities [18, 19]. Keeping this in view some strains of PGPR was taken up to assess their antifungal effect against pathogenic fungi.

Materials and methods
PGPR strains
Twenty two isolates of *Pseudomonas fluorescens* and a *Bacillus* species were obtained from Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras

Hindu University, Varanasi, India.

Cultural Characteristics

Shape, size, appearance and colour of the colonies (Table 1) of the isolates under study were done on the King's B (Protease peptone 20 g / L, Glycerol 15 ml / L, K2HPO4 1.8 g / L, MgSO4.7H2O 1.5 g / L) and YEME media (Yeast extract 4 g / L, Malt extract 10 g / L and Dextrose 4 g / L).

Table 1: Colony Characteristics of *Pseudomonas fluorescens* strains and *Bacillus* species.

Strains	Colony characteristics	Gram reaction
A-5	Creamy and shiny in appearance	Gram negative
CRM-3	Raised, shiny and yellowish colony, slight yellow green pigmentation	Gram negative
L3	Creamish raised colonies	Gram negative
PSB-1	Yellowish, small, shiny, convex raised colonies, yellow green pigmentation	Gram negative
L5	Creamy, convex raised colonies, mucoid in appearance, 1-2 mm in diameter	Gram negative
C3	Creamy smooth colonies giving greenish yellowish pigmentation	Gram negative
C4	Creamy smooth colonies, mucoid in appearance	Gram negative
M1	Creamy, shiny colonies	Gram negative
P1	Dark cream coloured, convex raised colonies, 3-4 mm in diameter	Gram negative
R1	Small, raised, yellowish coloured colonies, smooth round in shape	Gram negative
R-2	Dark cream coloured, raised round colonies, 2-3 mm in diameter	Gram negative
C-05	Cream coloured, raised, convex, round, 1-2 mm in diameter	Gram negative
C6	Cream, raised, shiny, small, round in shape, 1-2 mm in diameter	Gram negative
P2	Creamish shiny colonies, round in shape, 2-3 mm in diameter	Gram negative
Pf4	Creamish non shiny colonies	Gram negative
C-03	Creamish shiny raised colonies, yellow green pigmentation	Gram negative
PSB-2	Cream coloured, mucoid small, shiny colonies	Gram negative
L2	Creamish small, shiny round colonies ranging from 1- 4 mm	Gram negative
R-4	Yellowish smooth raised colonies, yellow green pigmentation	Gram negative
R-3	Creamish round colonies, 1mm in diameter	Gram negative
R2	Small whitish colonies	Gram negative
PUR-46	Creamish small colonies	Gram negative
Bacillus sp.	Creamish fluzzy colonies	Gram positive

Biochemical characteristics

Siderophore production assay

Pseudomonas fluorescens strain produces siderophore when grown on Fe^{-3} limiting conditions. Three hundred ml of sterilized nutrient agar was prepared. The medium was mixed with chromazual- S dye (Chriomazuml- S 1mM, 1mM $FeCL_3$.6H2O made in 10 mM HCL, CTAB 2nM). The plates were inoculated with the isolates separately. A nutrient agar medium without Chromazurol- S dye was similarly prepared which served as control. Inoculated plates were incubated for 4 days at 37^0C. Occurrence of orange halo against dark blue background was taken as positive test for siderophore production.

Phosphate solubilization ability test

Phosphate solubilization ability of *Pseudomonas fluorescens* strains was detected on Pikovskaya medium (Glucose 10 g / L, tricalcium phosphate 5.0 g / L, $(NH_4)2SO_4$ 0.5 g / L, KCL 0.2 g / L, $MgSO_4.7H_20$ 0.1 g / L, $MnSO_4$ trace, $FeSO_4$ trace, yeast extract 0.5 g / L). This medium was prepared, plated and plates were incubated for 48 h at 37^0C. Clear zones around the inoculated spot indicated zone of phosphate solubilization.

HCN production

HCN production by the isolates of *Pseudomonas fluorescens* was detected by using King's B medium amended with 4.4 g / L glycine. Whatman no. 1 filter paper disc soaked in 0.5 % picric acid in 2% Na_2CO_3 and placed in the lower surface of the lid of plates and individual isolates were streaked on the surface. Plates were sealed with parafilm and incubated at 28^0C for 4 days and observed for color change from yellow to orange. Uninoculated plates were kept as control.

Dual plate assay

Screening for the antagonistic activity was carried out by dual plate assay method [20]. A loopful of culture was streaked at the periphery of the plates. Agar block of freshly grown pathogenic fungi were placed at the center of the YEME plate. The following three spore forming fungi were tested: *Alternaria* sp., *Fusarium* sp. and *Bipolaris* sp. The fungi were tested against all the 22 isolates of *Pseudomonas fluorescens* and *Bacillus* sp. The plates were incubated at 37^0C for 5 days. Development of inhibition zone around the bacterial colony was observed. The fungal cultures grown on YEME plate without any bacterial culture served as control.

Extraction of metabolites

Metabolites produced by and isolate of *Bacillus* sp. and several strains of *Pseudomonas fluorescens* were extracted using hexane chloroform and ethyl acetate. About 500 ml of YEMA was prepared for each isolate of *Pseudomonas fluorescens* and *Bacillus* sp. A loopful of the culture was inoculated in sterilized YEMA broth and kept at 30^0C for 3 days. The broth was centrifuged at 10,000 rpm at 4^0C for 10 min for separating supernatant and pellet. The supernatant obtained was partitioned with hexane, chloroform and ethyl acetate by following method. The supernatant was poured in a separating funnel and about 200 ml of hexane was added in it, shaken vigorously and left for 1 h for clear separation. The upper layer of hexane was taken and the process was repeated with the supernatant for three times. The hexane fractions thus obtained were pooled together. Similarly, the supernatants were further separated with chloroform followed by ethyl acetate. Anhydrous Na_2SO_4 was added to all the three portions for removing water molecules. These portions were filtered through Whatman filter paper and the filtrate was distilled thoroughly till the metabolite became dry.

Phenolic acid extraction

One mg of the metabolite of ethyl acetate extract of each of

the following four strains of *Pseudomonas fluorescens* were taken: R_1, M_1, P_2 and C_4 whereas the metabolite of *Bacillus* sp. fractioned from hexane, chloroform and ethyl acetate was also undertaken for the extraction of phenolic acids. All the metabolites of the given strains were diluted with 5 ml methanol separately. The supernatant of each sample was subjected to charcoal treatment to remove pigments. The solution was filtered through Whatman filter paper and dried. The dried samples were re-suspended in 1.0 ml HPLC grade methanol and filtered through membrane filters (0.05um, Millipore, Bangalore, India) before HPLC analysis.

High Performance Liquid chromatography (HPLC) analysis

HPLC analysis of the fractionated material was performed according to [21] on an HPLC system (Shimazdu Corporation, Tokyo, Japan) equipped with two LC-20 AT reciprocating pumps, a variable pumps, a variable UN-Vis detector (Shimazdu SPD- 20 A). Reverse phase chromatographic analysis was carried out under isocratic conditions using a C-18 reverse phase HPLC column (250 x 4.6 mm id.), Luna 5u C-18 (Phenomenex, Torrance, CA, USA) at 25^0C. Running conditions included a mobile phase of methanol: water / 0.5% acetic acid (80: 20 v /v), flow rate of 1.0 ml / min, an injection volume of 5 ul and detection at 290 nm. Fractionated material (1 mg / ml) and phenolic acids dissolved in HPLC grade methanol were injected into the sample loop and the means of the peak areas of individual compounds were taken for quantification. Vanillic, chlorogenic, gallic, ferulic, cinnamic and salicylic acids were used as internal and external standards. Phenolic compounds present in the sample were identified by comparing retention time (Rt) of the standards of gallic (2.86 min), vanillic (3.26 min), ferulic (3.42 min), chlorogenic (4.16 min), cinnamic (4.42 min) and salicylic acid (6.36 min). These phenolics were identified by co-injection of internal and external standards for their conformation. Amounts of individual compounds were calculated by comparing peak areas of compounds with those in the samples run under the similar elution conditions.

Fungal Spore germination

Stock solutions of metabolites of 1000 ppm (ethyl acetate extract) of *Pseudomonas fluorescens* strains and *Bacillus* sp. (hexane, chloroform and ethyl acetate extracts) were prepared by dissolving 1 mg of the compound initially in a few drops of methanol in a culture tube. After the chemical was completely dissolved, 1 ml of distilled water was added and the solvent was evaporated on a water bath (80^0C). One drop (30-40 µl) from each chemical was placed on grease free slides. With a sterile inoculation needle, fungal spores were picked up from 7-10 days old growing culture of plan pathogenic fungi i.e. *Alternaria*, *Fusarium* and *Bipolaris* species and mixed in individual solutions. The slides were late placed in the moist chamber by placing two moist filter papers, one on the lower surface and other on the lid of the plate. The slides containing spores were incubated at 25^0C for 24 h. Germination was observed after staining the spores with cotton blue and lactophenol under light microscope. Similar preparations in water served as control. All the experiments were performed in triplets.

Statistical analysis

Statistical analysis was performed by subjecting the data to ANOVA and analyzing them by the Least Significant Difference (LSD) test for statistical significance at $P \leq 0.05$.

Results and discussion

Biochemical characteristics

Twenty two strains of *Pseudomonas fluorescens* and *Bacillus* sp. were subjected to various biochemical tests.

Siderophore production

Out of 22 strains of *P. fluorescens*, only R-3 gave positive result after 96 h. In contrast to our results, Leeman *et al.* [7] demonstrated that *P. putida* WCS374 induces resistance against *Fusarium* sp. by the production of siderophores. Chipperfiled and Retyledge [22] also proved that *P. aeruginosa* 7NSK2 induces resistance by producing sideropores. Hiifte *et al.* [23] reported that majority of work on siderophores in the rhizosphere is associated with their biochemical activities due to their competitive effects with plant pathogens. According to Kumar *et al*. [24] strain JA13 of *P. fluorescens* inhibited fungal growth only in the absence of $FeCl_3$ suggesting siderophore mediation. On the contrary, Glick [25] believed that contribution of these siderophores to overall iron requirement of plants is insignificant.

Phosphate solubilization

In this experiment (Table 2), it was found that *P. fluorescens* strains C-03, C6, R-4 showed zone of solubilization in which C6 gave clear halo of 6 mm diameter after 96 h followed by C-03 and R-4 which had 4 and 3 mm zones, respectively. Other strains did not show zone of solubilization. The results of the present study corroborates with earlier findings [26]. He screened the actinobacterial isolates for plant-growth-promoting activities and underlying mechanisms of phosphate solubilization, as well as antifungal activity. According to Cattelan *et al.* [27] only two of the five rhizospheric isolates *P. chlororaphis* and *P. putida*, in association showed phosphate solubilization and actually had a positive effect of the soybean seedlings growth. de Freitas *et al.* [28] found a number of phosphate solubilization *Bacillus* sp. and *Xanthomonas meltophila* isolate from canola rhizosphere.

Table 2: Phosphorous Solubilizing Property of Some Strains of *Pseudomonas fluorescens.*

Isolate	Zone of phosphate solubilization (mm)			
	24 h	48 h	72 h	96 h
R_2	-	-	-	-
C_4	-	-	-	-
CRM – 3	-	-	-	-
Pf4	-	1.0 ± 0.21	1.5± 0.16	2.0± 0.24
R-3	-	1.0± 0.18	1.25± 0.2	1.5± 0.14
R-2	1.0± 0.09	1.75± 0.2	2.0± 0.18	2.5± 0.28
A_5	-	-	0.5± 0.08	1.0± 0.16
PSB – 1	-	-	-	-
P_1	-	-	0.5± 0.12	1.0± 0.22
P_2	1.0± 0.26	1.75± 0.32	2.0± 0.28	2.5± 0.14
L_2	-	1.0± 0.21	1.5± 0.23	2.0± 0.15
M_1	-	-	-	-
L_3	-	-	-	-
C_3	-	-	1.0± 0.14	1.5± 0.22
C-03	2.0± 0.34	3.0± 0.22	3.5± 0.19	4.0± 0.26
R_1	-	-	1.0± 0.18	1.5± 0.24
C_6	2.0± 0.215	4.0± 0.28	5.5± 0.32	6.0± 0.38
R_4	1.0± 0.22	2.0± 0.216	2.5± 0.218	3.0± 0.21
L_5	1.0± 0.12	1.5± 0.17	2.0± 0.21	2.5± 0.26
PSB-2	-	-	-	-
PUR-46	-	0.5± 0.12	1.0± 0.14	1.5± 0.22
C-05	-	-	-	-

"-" represents no phosphate solubilization.

HCN production

The experiment conducted on the HCN production by several strains of *P. fluorescens* indicated that CRM-3 gave the best result showing dark orange colour after 96 h. CRM-3, C6 and C-05 gave positive results after 72 h of incubation while light orange color was observed in R2, R-3 and PSB-2 after 96 h. The primary mechanism of biocontrol by fluorescent pseudomonads is production of HCN [29]. HCN is known to inhibit the electron transport, disrupting the energy supply to the cells [30].

Dual plate assay technique

The dual plate assay of *P. fluorescens* strains and *Bacillus* species gave antagonistic results against the spore forming fungi after five days of incubation. Using this technique, all the 22 strains of *Pseudomonas fluorescens* were examined against some pathogenic fungi. None of the bacterial strains showed antifungal activity against any of these fungi. P2 and M1 strains of *P. fluorescens* did not show any antifungal activity while Kumar *et al.* [24] reported that among 40 isolates that were confirmed as *P. fluorescens*, 18 exhibited strong antifungal activity against *Fusarium oxysporium* and *Rhizoctonia bataticola*, mainly through production of antifungal metabolites. Strains PAON2, PUJA1, PUJA3 and SOC06 strongly inhibited growth of both fungal pathogens. Strains MKU3 andV35 inhibited growth of *Rhizoctonia bataticola*, while TVM2 andJO5 inhibited growth of *F. oxysporum*.

Bacillus sp. gave positive results against *Alternaria*, *Fusarium* and *Bipolaris* species (Table 3). Zone of inhibition was observed after 96 h of incubation and was quite clear up to 120 h. Yoshida *et al*. [31] reported that *B. amyloliquifaciens* strain RC-2 inhibited anthracnose disease of mulberry leaves. In the same year, Sadfi *et al.* [32] observed that when *B. cereus* X16 was inoculated with *F. roseum* in dual culture, fungal hyphae were unable to reach the bacteria culture after 2 days. Basha and Ulaganthan [33] also reported about the inhibitory effect of *Bacillus* sp. stain BC121 against *Curvularia lunata*. A clear inhibition zone of 0.5-1.0 cm was observed in the dual plate assay. After 10 days of incubation, the bacterial strain grew over the fungal mycelium surface and multiplied extensively on it. Scanning electron microscopic observations showed clear hyphal lysis and degradation of cell wall. Mari *et al.* [34] reported that *B. amyloliquifaciens* 2TOE reduced the severity of gray mold caused by *Botrytis cinerea* in pears. They suggested that the antifungal activity of the bacterium was due to competition for nutrients.

Table 3: Antagonistic activity (mycelia inhibition) of *Bacillus* sp. against some fungi.

Duration (h)	*Alternaria* sp.	*Fusarium* sp.	*Bipolaris* sp.
24 h	-	-	-
48 h	-	-	-
72 h	-	-	-
96 h	+	+	+
120 h	+	+	+

"+" represents positive antagonistic activity; "-" represents negative antagonistic activity

HPLC analysis

HPLC analysis of four strains of *P. fluorescens* and *Bacillus* sp. was carried out for detection of phenolic acids. Five phenolic acids were observed by HPLC analysis of the ethyl acetate extract of four strains of *P. fluorescens*. Gallic acid was present in the maximum amount in the C_4 (7.84 µg / ml) followed by M_1 (2.53 µg / ml). Vanillic acid was absent in R_1 and P_2 but C_4 and M_1 showed 0.49 and 0.16 µg / ml, respectively, 0.26 ug / ml cinnamic acid was found in R_1. Ferulic acid was present in the amount of 1.73, 0.143, 0.156 and 0.05 µg / ml in R_1, P_2, C_4 and M_1 respectively. Cholorogenic acid was absent in R_1 but present as 0.32 and 0.05 µg / ml in P_2 and M_1, respectively. The highest amount (0.491 µg / ml) of chlorogenic acid was recorded in C_4. Galllic acid was noticed as 1.89, 2.53 and 1.87 µg / ml in hexane, chloroform and ethyl acetate extract of *Bacillus* sp., respectively. Vanillic acid was found only in ethyl acetate extract (0.096 µg / ml). Cinnamic acid was absent in all the cases. High amount of ferulic acid (3.65 µg / ml) was observed in hexane extract. Chloroform and ethyl acetate extract showed 0.19 and 0.14 µg / ml ferulic acid. 0.20 µg / ml of chlorogenic acid were detected in hexane extract. Trace amounts (0.078 and 0.09 µg / ml) were detected in chloroform and ethyl acetate extract. In the present study, it was observed that there was considerable amount of gallic acid in all the three extracts of *Bacillus* sp., thus confirming the results of inhibitory effect in dual plate assay against some fungi and also against spore germination. High content of gallic acid in *P. fluorescens* strains indicates that it can be inhibitory against some other fungi also. Vanillic and cinnamic acids present in traces also show that they can act as antifungal agents against some other fungi. High amount of ferulic acid in hexane extract of *Bacillus* sp. confirms its antifungal activity. In comparison to our results, several authors [35-39] reported that phenolic acids play an important role inducing resistance against various pathogens. According to Maurya *et al.* [40] high amount of phenolic acid in chickpea (*C. arietinum*) was observed when challenged with against *Sclerotium rolfsii*. They also reported accumulation of high amounts of phenolic acids in leaves while reduction in collar and root region. They conducted analysis at 24 h interval of both inoculated and uninoculated plants and recorded that the amount of phenolic acids increased in leaves, collar and root region of chickpea as compared to uninoculated plants. They observed that gallic and tannic acids were maximum but others were in traces, gallic acid being synthesized maximum.

Spore germination

The spore germination activity of *Alternaria* sp., *Fusarium* sp. and *Bipolaris* sp. were found to be variable against different solvents extracts of test organisms used in the present study. Hexane extract of *Bacillus* sp. on spore germination (Table 4) of three fungi was found to be effective in inhibiting spore germination in *Fusarium* sp. (11.35%) but increased the germination of spores in *Altlernaria* sp. (90%) and *Bipolaris* sp. (64.78%). The sensitivity of different fungi considerable varied when treated with chloroform extract of *Bacillus* sp., *Alternaria* sp. and *Fusarium* sp. showed considerable inhibitory effect by 48.2 per cent. *Bipolaris* sp. was not sensitive to the extract (Table 5).

With ethyl acetate extract, *Alternaria* sp. was not significantly affected while there was germination of *Fusarium* sp. and *Bipolaris* sp. by 45 and 27.71 per cent, respectively. When overall comparison was done, it was found that the *Bacillus* sp. did not only inhibit spore germination of *Fusarium* and *Alternaria* species but also inhibited the mycelia growth of *Alternaria*, *Fusarium* and *Bipolaris* species. Hence it may be used in control of some plant pathogenic fungi under field conditions also but it needs further research. Basha and Ulaganathan [33] also performed spore germination study. They reported inhibition in spore germination when the metabolite extract of *Bacillus* strain BC121 was tested against

Curvularia lunata. Similar results were obtained when Podile *et al.* [41] performed experiments with crude protein extract of *B. subtilis* strain AF1 inhibiting *A. niger* and was successful in the control of crown rot disease in groundnut.

Table 4: Effect of hexane extract of *Bacillus* sp. on spore germination of some fungi.

Name of Fungi	Control† (% germination)	Treated† (% germination)
Alternaria sp.	63.04 ± 4.6[a]	90.00 ± 4.8[c]
Fusarium sp.	45.00 ± 6.2[b]	11.35 ± 2.1[d]
Bipolaris sp.	62.20 ± 7.6[a]	64.78 ± 4.6[a]

†Column data superscript with different letters varies significantly by Least Significant Difference (LSD) test at P ≤ 0.05.

Table 5: Effect of chloroform extract of *Bacillus* sp. on spore germination of some fungi.

Name of Fungi	Control† (% germination)	Treated† (% germination)
Alternaria sp.	81.10 ± 11.6[a]	53.30 ± 7.4[c]
Fusarium sp.	78.00 ± 5.4[a]	29.80 ± 3.6[d]
Bipolaris sp.	100.00 ± 0.0[b]	100.00 ± 0.0[b]

†Column data superscript with different letters varies significantly by Least Significant Difference (LSD) test at P ≤ 0.05.

Conclusion

In view of present investigation, it can be concluded that *in vitro* studies of microbial agents show considerable promises for achieving good performance in the field where there is heterogeneity of abiotic and biotic factors and competition with organism because chemical fungicides and synthetic fertilizers affect human health and environment. Effective strategies are now in use for biological fertilizers like *Rhoizobium*, *Bacillus*, *P. fluorescens*, *Trichoderma* etc. for enhancing the growth of plants and eradicating disease caused by bacteria and fungi. On the basis of biochemical characteristics, siderophore production was exhibited by R-3 strain of *P. fluorescens*, phosphate solubilisation by C-03, C6 and R-4 strains and HCN productions was examined in strains CRM-3, R-3, C6, PSB-2 and C-05 of *P. fluorescens*. On the contrary, dual plate assay technique was opted for both the bacterial strains. The metabolites were used for HPLC analysis. Lastly, effects of different solvents extracts were examined against some phytopathogenic fungi. From the study, 1) All the 22 strains of *P. fluorescens* did not exhibit antagonistic activity against spore forming fungi in dual plate assay. On the other hand, *Bacillus* sp. was found to exert antifungal activity. 2) HPLC analysis of 4 strains of *P. fluorescens* and *Bacillus* sp. was carried out for the presence of phenolic acids. It showed considerable amount of gallic, ferulic and chlorogenic acid whereas vanillc and cinnamic acids were present in traces 3) Among the 3 solvents extract, *P. fluorescens* did not show any inhibition against the spores of phytopathogenic fungi using ethyl acetate extract whereas *Bacillus* sp. was found to be highly potent against the three fungi using hexanes and chloroform extracts (Table 6). The antifungal metabolites produced by *Bacillus* sp. need to isolated, purified and tested in fields for commercialization. Biological control measures can become a boon for agriculture because of their eco-friendly nature of organisms and shelf life.

Table 6: High Performance Liquid Chromatography analysis of four strains of *Pseudomonas fluorescens* in Ethyl acetate extract and one *Bacillus* sp. in Hexane, Chloroform and Ethyl acetate extract.

Name of Strains of *Pseudomonas fluorescens*	Phenolic Acid (µg / ml)†				
	Gallic acid	**Vanillic acid**	**Cinnamic acid**	**Ferulic acid**	**Chlorogenic acid**
C4	7.84 ± 1.6[a]	0.49 ± 0.03[c]	-	0.156 ± 0.03[c]	0.491 ± 0.16[c]
M1	2.53 ± 0.84[b]	0.16 ± 0.05[c]	-	0.05 ± 0.01[c]	0.05 ± 0.01[c]
R1	-	-	0.26 ± 0.11[c]	1.73 ±0.26[b]	-
P2	-	-	-	0.143 ±0.02[c]	0.32 ± 0.04[c]
***Bacillus* sp.**					
Name of different extracts	**Gallic acid**	**Vanillic acid**	**Cinnamic acid**	**Ferulic Acid**	**Chlorogenic acid**
Hexane	1.89 ± 0.32[b]	-	-	3.65 ± 0.67[b]	0.20 ± 0.12[c]
Chloroform	2.53 ± 0.66[b]	-	-	0.19 ± 0.03[c]	0.078 ± 0.02[c]
Ethyl acetate extract	1.87 ± 0.23[b]	0.096 ± 0.02[c]	-	0.14 ± 0.06[c]	0.09 ± 0.01[c]

"-" represents non-detectable limit; †Column data superscript with different letters varies significantly by Least Significant Difference (LSD) test at P ≤ 0.05.

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