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In vitro antagonistic activity and phylogeny of plant growth-promoting bacteria native to Western Ghats of Karnataka, India

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Abstract
Isolates from rhizosphere and endorhizosphere niches of Western Ghats, Karnataka were assessed for antagonistic activity against major fungal and bacterial plant pathogens. The isolates exhibited varying potential of cyanogenesis, siderophorogenesis, volatile antimetabolites and indole acetic acid production. Antimetabolites *viz*., phenazine, phloroglucinol and pyrrolnitrin from endorhizospheric pseudomonads and a *Rhizobium* sp. were found active against test plant pathogens *in vitro*; indicating the involvement of multiple mechanisms and crosstalk between the antimetabolite producers. Phylogenetic analysis of isolates revealed polyphyletic separation into 3 major groups: alphaproteobacteria, gammaproteobacteria and bacteroidetus. The study sheds light on the ecology of these isolates with innate broad-spectrum antagonistic activity, thus obviating the need for introducing genetically modified organisms.

Keywords: Plant growth-promoting bacteria/ antagonistic activity/ biocontrol/ antimetabolites/ phylogeny

Introduction
Increased concerns on environmental pollution, pathogen resistance and high plant protection costs, calls for more research on identification and characterization of new disease suppressive antibiotic compounds from plant growth-promoting bacteria (PGPB).
Plant growth promoting activity of PGPB is attributed to their direct mechanisms *viz*., synthesis of plant hormones indole acetic acid (IAA) and gibberellic acid (GA), phosphate solubilisation, nitrogen fixation and indirect mechanisms by way of inhibiting soil borne pathogens by producing siderophores, hydrogen cyanide (HCN) [1] and a variety of antimicrobial compounds [2] *viz*., polyketides (2,4 diacetyl phloroglucinol; pyoluteorin; mupirocin), heterocyclic nitrogenous compounds (phenazine derivatives), phenylpyrrole (pyrrolnitrin), which have broad spectrum antimicrobial action and are synergistically acting to induce signals in the rhizosphere for systemic resistance (ISR) in plants. The growth hormone IAA and its derivatives produced by PGPB may also be involved in protecting the plants against soil-borne pathogens [3].
Although other useful traits of PGPB have been successfully exploited for sustainable agriculture, there has been little progress towards their biocontrol potential mainly due to poor understanding of the mechanism of biocontrol and rhizosphere incompetence exhibited by many PGPB. Thus it is necessary to fish out from diverse natural environments, strains which can compete with the indigenous microflora amidst heterogeneous biotic and abiotic factors prevailing in field conditions for successful application of these bacteria in agriculture in ameliorating the diseases caused by soil borne pathogens.
Present study aims to explore native PGPB having broad spectrum antagonistic activity from rhizosphere and endorhizosphere microhabitats of endemic plant and tree species belonging to the biodiversity hotspot of India, the Western Ghats. Molecular characterization and phylogeny were performed to unravel the evolutionary relationship between the strains. Attempts were made to shed light on the mechanism of biocontrol *in vitro*. The antimetabolites from such isolates were identified.

Materials and methods
PGPB strains
The PGPB strains used in this study were isolated from the soil, rhizosphere and roots of different plant species. They were maintained in the culture bank of the Department of Agricultural Microbiology, University of Agricultural Sciences (UAS), Dharwad as part of the Microbial Biodiversity project.

Screening isolates for *in vitro* antagonistic activity

A total of 133 isolates were subjected to *in vitro* screening of their antagonistic activity against four fungal plant pathogens namely, *Alternaria carthami*, (Chowdhary), causing leaf spot of safflower *Fusarium oxysporum*, f. sp., *carthami*, (Klisiewicz and Houston) causing wilt of safflower *Rhizoctonia bataticola* (Taub Buttler) causing root rot in many crops, *Sclerotium rolfsii*, Sacc. causing wilt/ root rot of a wide variety of crops, and bacterial plant pathogens *viz*., *Xanthomonas campestris* pv *malvacearum* (Smith) Dye, causing bacterial blight of cotton, *X. axonopodis* pv *punicae* (Hingorani and Singh) causing bacterial blight of pomegranate and *Rolstonia solanacearum* (Smith) Yabuuchi *et al*., causing bacterial wilt of solanaceous crops and *Xanthomonas axonopodis* pv. *citri* (Hasse) Dye, causing citrus canker. The pure cultures of these pathogens were maintained in our laboratory. Bioassay against aforementioned pathogens was performed by dual inoculation technique[4]. Out of the 133 isolates, twenty three isolates with maximum inhibitory activity were selected for further analysis.

Decoding the mechanism of antagonistic activity against the plant pathogens

Cyanogenesis

All the 23 isolates were analyzed for their ability to produce hydrogen cyanide by following the procedure described in the literature [5]. The gaseous metabolites produced by the antagonists were allowed to react with picric acid. After incubation for a week at 30˚C, the colour change of the filter paper was noted and the antagonists assessed as weak, moderate and high HCN producers.

Siderophore production

Production of siderophore was assayed by the method described elsewhere [6]. Formation of orange colour zone around the colony on chrome azurole S (CAS) agar was taken as positive for siderophore production. The diameter of the zone was recorded.

Production of volatile antimetabolites

The isolates were examined for their ability to produce volatile antifungal metabolites by a method previously standardised by Tennakoon *et al*. [7] in the Department of Agricultural Microbiology, UAS, Dharwad. Pairs of petriplate bottoms were autoclaved. One bottom plate of each pair was poured with sterilized nutrient agar medium and streaked with the antagonist isolate. The other bottom plate of the pair was poured with potato dextrose agar (PDA), allowed to solidify, and with the help of a sterile cork borer (10 mm diameter); a disc of fungal growth from the plates was taken and placed at the centre. Later, this plate was placed in an inverted position over the bottom plate inoculated with antagonist isolate. Suitable control for each pathogen was maintained. The joints of these paired bottom plates were sealed with parafilm to avoid escape of volatile metabolites and then incubated at 30˚C for 96h. The observations on the inhibition of the growth of fungal plant pathogens were recorded in comparison with the corresponding controls. In addition the colour change of the mycelium and other variations, if any, were noted.

Extraction and identification of non-volatile antimetabolites

The non-volatile antimetabolites were extracted from the four best performing strains on the basis of above tests by using following method: Test tubes containing 5 ml nutrient broth amended with 2% glucose were inoculated with the test cultures and incubated for 48h at 30˚C. These broth cultures were spun at 10,000 rpm for 10 min and the supernatant were collected. The metabolites in the supernatants and pellets were extracted with equal volume of chloroform and then pooled. After discarding the upper aqueous layer, a pinch of sodium sulphate was added to the remaining chloroform phase to dry up the water. It was followed by spinning at 8000 rpm for 8 min to pellet sodium sulphate. The clear layer was decanted followed by removal of chloroform by flushing in air. The residue was redissolved in 200µl acetone.

Seventy µl of the crude metabolite extract was spotted on TLC plate silica gel 60 F_{254}, using chloroform: acetone (9:1) as solvent system and allowed to run for 60 min. For the detection of phloroglucinol, n-butanol: water: acetic acid (4:1:5) was used as solvent system and was run for 75 min. The solvent fronts were marked and after complete air drying, the plates were observed under UV light at 254nm. The spots were marked and the R_f values were calculated. Authentic standards of phenazine and phloroglucinol were procured from Sigma Aldrich, USA.

Antagonistic assays

The metabolites were eluted and redissolved in acetone: water (1:10). One hundred µl of the eluted portions were centrifuged to pellet the silica gel and the clear supernatants were further analyzed for toxicity against test pathogens. For fungal pathogens, the metabolites were streaked on both sides; about 1.5cm away from the fungal disc which is placed at the centre of PDA plates. For bacterial pathogens, the individual metabolite was dried on filter paper disc and placed at the centre of the agar plates over which the pathogen was spread. Suitable controls without metabolites for each pathogen were also maintained.

Production of indole acetic acid

All the 23 isolates were tested for the production of IAA by following the method described by Gordon and Paleg[8].

Molecular characterization of potent strains

The genomic DNA was extracted from twenty three best performing strains[9]. 16S rDNA gene was amplified[10] by using universal forward and reverse primers (16S F:5'-AGAAGTTTGATCMTGGCTCAG-3'and 16S R : 5'-TACGGYTACCTTGTTACGAC-3'). The single sharp amplicon of 1.45 Kb size was eluted using the Qiagen's MiniElute gel extraction kit, Germany and were sent for sequencing to Eurofins Private Ltd., Bangalore, India.

Phylogenetic analysis

Both forward and reverse 16S rDNA sequences obtained for the individual strains were joined using CAP contig assembly program in BioEdit version 7.1.3.0, vector contamination if any were removed and submitted in BLAST Program in order to retrieve the sequences of closest NCBI strain which is hitting maximum homology with the query sequence. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 6.05 [11]. The sequences determined together with closely related sequences were then aligned using Clustal_W [12] and phylogenetic tree was constructed using neighbor joining method [13]. Reliability of the topology of tree was verified by 1000 bootstrap resamplings [14] to get a best estimated tree. In order to determine the taxonomic rank of the individual strains, all the sequences were also subjected to Hierarchy classifier in Ribosomal Database Project (RDP)

Naïve Bayesian rRNA classifier version 2.10 [15] with 80% confidence threshold level.

Results

A total of 133 isolates were obtained from rhizosphere and endorhizosphere microhabitats of different plant species. Twenty three isolates which inhibited two or more test plant pathogens *in vitro* were chosen for further analysis. Isolate numbers and corresponding strain designations for these isolates are given in table 1.

Table 1: An overview of identity and different plant growth-promoting attributes of the isolates

Isolate No.	Strain designation	HCN production	Siderophore (diameter of orange zone in mm)	Volatile metabolites produced against	Non-volatile metabolites	Closest NCBI strain
174	MDFPXXIIE 205b	+	30	Ac, Rb, Fo, Sr	ND	*Pseudomonas sp. IM4*
274	MDbc274	++	20	Ac, Rb, Fo	ND	*Pseudomonas plecoglossicida*
294	MDFPXXIVS287	++	16	NC	ND	*Pseudomonas fluorescens strain 3B*
297	MDFPXXIVR285b	+++	14	Ac, Rb, Fo, Sr		*Pseudomonas putida*
298	MDFPXXIVR285c	+	20	NC	ND	*Pseudomonas fluorescens strain HZN1*
299	MDFPXXIVR285d	-	15	NC	ND	*Pseudomonas sp.rif200872*
300	MDFPXXIVR287c	+	15	NC	ND	*Pseudomonas putida strain M35*
304	MDFPXXIVR289b	++	15	NC	ND	*Pseudomonas sp. rif200862*
354	MDFPXXVIIS315	-	30	NC	ND	*Pseudomonas monteilii strain M56*
357	MDFPXXVIIS318	+	20	NC	ND	*Pseudomonas putida strain Pp 366*
368	MDFPXXVIIR318b	_	17	NC	ND	*Acinetobacter bereziniae strain BENAB7*
371	MDFPXXVIIR319c	_	22	NC	ND	*Klebsiella pneumoniae strain S75(1)R*
373	MDFPXXVIIR320b	_	15	NC	ND	*Chrysobacterium indologenes strain WZE 87*
374	MDFPXXVIIR320c	+	45	Ac, Rb	ND	*Serratia marcescens strain MH 6*
375	MDbc375	_	14	NC	ND	*Pseudomonas putida strain RW10S2*
376	MDFPXXVIIE315c	+++	17	Ac, Rb, Fo, Sr	ND	*Pseudomonas putida*
379	MDFPXXVIIE317b	+++	30	Ac, Rb, Fo, Sr	Phenazine & Phloroglucinol	*Pseudomonas plecoglossicida strain PSG1*
380	MDbc380	+	15	NC	ND	*Pseudomonas fluorescens strain LMG 5168*
381	MDFPXXVIIE318c	++	54	Ac, Rb, Fo	Phenazine & Phloroglucinol	*Pseudomonas putida strain PD1*
382	MDFPXXVIIE319b	-	17	NC	ND	*Pseudomonas putida strain ZB-16A*
383	MDFPXXVIIE320a	-	20	NC	ND	*Pseudomonas fluorescens*
384	MDFPXXVIIE320c	++	27	NC	Phenazine, Phloroglucinol & Pyrrolnitrin	*Pseudomonas sp. JY-2*
385	MDbc385	+	32	NC	Phenazine, Phloroglucinol & Pyrrolnitrin	*Rhizobium sp.*

+, pale orange: Low HCN production; ++, Orange to brown: Moderate HCN production; +++, Deep Brown: High HCN production; - , No HCN production

Ac, *Alternaria carthami*; Fo, *Fusarium oxysporum*; Rb, *Rhizoctonia bataticola*; Sr, *Sclerotium rolfsii*; NC, No control of test pathogen

ND, not determined in this experiment

Accession numbers

The GenBank accession numbers assigned for the 16S rDNA gene sequences of the eighteen PGPB are from KJ535377 to KJ535391 and from KJ460986 to KJ460987.

Multiple mechanisms of biocontrol exert cumulative effect against plant pathogens

Moderate to high HCN production was observed in eight isolates. All the isolates tested scored positive for siderophore production on CAS media but with varying potential (Fig. 1). Of the twenty three isolates tested for volatile antimetabolite production, isolates 381, 379, 174 and 274 demonstrated maximum inhibition of growth and sporulation of test fungal pathogens *in vitro* (Table 2). Isolates 379 and 385 produced 30.5 and 33.2 µg $ml^{-1}$of IAA. Table 1 gives an overview of the different plant beneficial attributes of isolates and their identity by 16S rDNA sequencing.

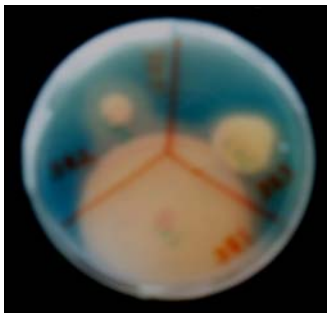

Fig 1: Siderophore assay – demonstrating orange halo around the colonies on CAS media

Table 2: Inhibition of test fungal pathogens by volatile antimicrobial metabolites produced by the isolates

Isolate No.	Zone of inhibition (mm) of pathogen vs. control			
	Alternaria carthami	***Rhizoctonia bataticola***	***Fusarium oxysporum***	***Sclerotium rolfsii***
381	70	70	13	No inhibition
379	40	40	38	63
376	Normal mycelial growth but no sporulation	Normal mycelial growth but no sporulation	42	10
274	45	50	38	Normal mycelial growth but no sporulation
174	20	Normal mycelial growth but no sporulation	28	34
297	Normal mycelial growth but no sporulation	10	25	30
374	Normal mycelial growth but no sporulation	20	No inhibition	No inhibition

Investigation of antimetabolites from outperforming endorhizospheric strains

Based on the above results, four endorhizospheric isolates were chosen for investigation of antimetabolites in action. Thin layer chromatography separation of the antimetabolites from isolates 379, 381, 384 and 385, with chloroform: acetone (9:1) as solvent system, revealed production of phenazine in all four isolates with R_f value 0.85. In addition, as can be seen in Fig. 2, in lanes for isolates 384 and 385 another spot with R_f 0.5 was observed, which was identified as pyrrolnitrin[16]. On the other hand, when TLC was run using n-butanol: water: acetic acid (4:1:5) as solvent system, only one spot with R_f 0.78 was observed in all four isolates and this compound was identified as phloroglucinol (Fig. 3).

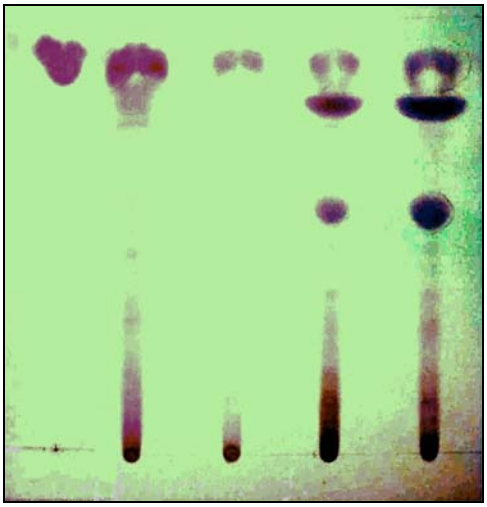

Fig 2: Identification of non-volatile antimicrobial metabolites. Metabolites extracted from the isolates in chloroform were analyzed by using thin-layer Silica Gel chromatography plates in an ascending Chloroform: Acetone (9:1), vol /vol) solvent system. Individual metabolites were visualized by UV irradiation at 254 nm (Lane 1, standard phenazine; lane 2, antimetabolite from isolate 379; lane 3, antimetabolite from isolate 381; lane 4, antimetabolites from isolate 384; lane 5, antimetabolites from isolate 385).

Fig 3: Detection of Phloroglucinol by thin-layer Silica Gel chromatography using n-Butanol: water: acetic acid (4:1:5), vol/vol) solvent system, visualized by UV irradiation at 254 nm (Lane 1, standard Phloroglucinol; lane2, antimetabolite from isolate 379; lane 3, antimetabolite from isolate 381; lane 4, antimetabolite from isolate 384; lane 5, antimetabolite from isolate 385)

The toxicity of each of the antimetabolites was tested against the test bacterial and fungal plant pathogens in question and it was observed that the individual metabolites only checked the sporulation of different fungi, although the growth was

normal as compared to control plates. Phenazine produced by the isolate 381 showed inhibition of bacterial pathogens (zone of inhibition; 2mm). These results are summarized in table 3.

Table 3: R_f values and toxicity of antimicrobial metabolites produced by effective isolates against plant pathogenic fungi and bacteria

Isolate No.	Antimicrobial metabolite	R_f value	Toxicity against
379	Phenazine	0.85	*Alternaria carthami, Rhizoctonia bataticola and Fusarium oxysporum*
381	Phenazine	0.85	*Alternaria carthami, Rhizoctonia bataticola, Fusarium oxysporum and bacterial pathogens tested*
384	Phenazine	0.85	*Rhizoctonia bataticola and Fusarium oxysporum*
	Phloroglucinol	0.79	*Rhizoctonia bataticola, Fusarium oxysporum and Sclerotium rolfsii*
	Pyrrolnitrin	0.5	*Alternaria carthami and Fusarium oxysporum*
385	Phenazine	0.85	*Alternaria carthami and Fusarium oxysporum*
	Phloroglucinol	0.79	*Sclerotium rolfsii, Alternaria carthami and Rhizoctonia bataticola*
	Pyrrolnitrin	0.52	*Sclerotium rolfsii and Rhizoctonia bataticola*

Taxonomic affiliation of isolates and phylogenetic analysis

A phylogenetic tree depicting the evolutionary relationship between the twenty three strains under study and their closely related NCBI strains is presented in Fig. 4. The number of base substitutions/site from averaging overall sequence pairs was 0.164 which is the estimate of average evolutionary divergence determined by maximum composite likelihood method.

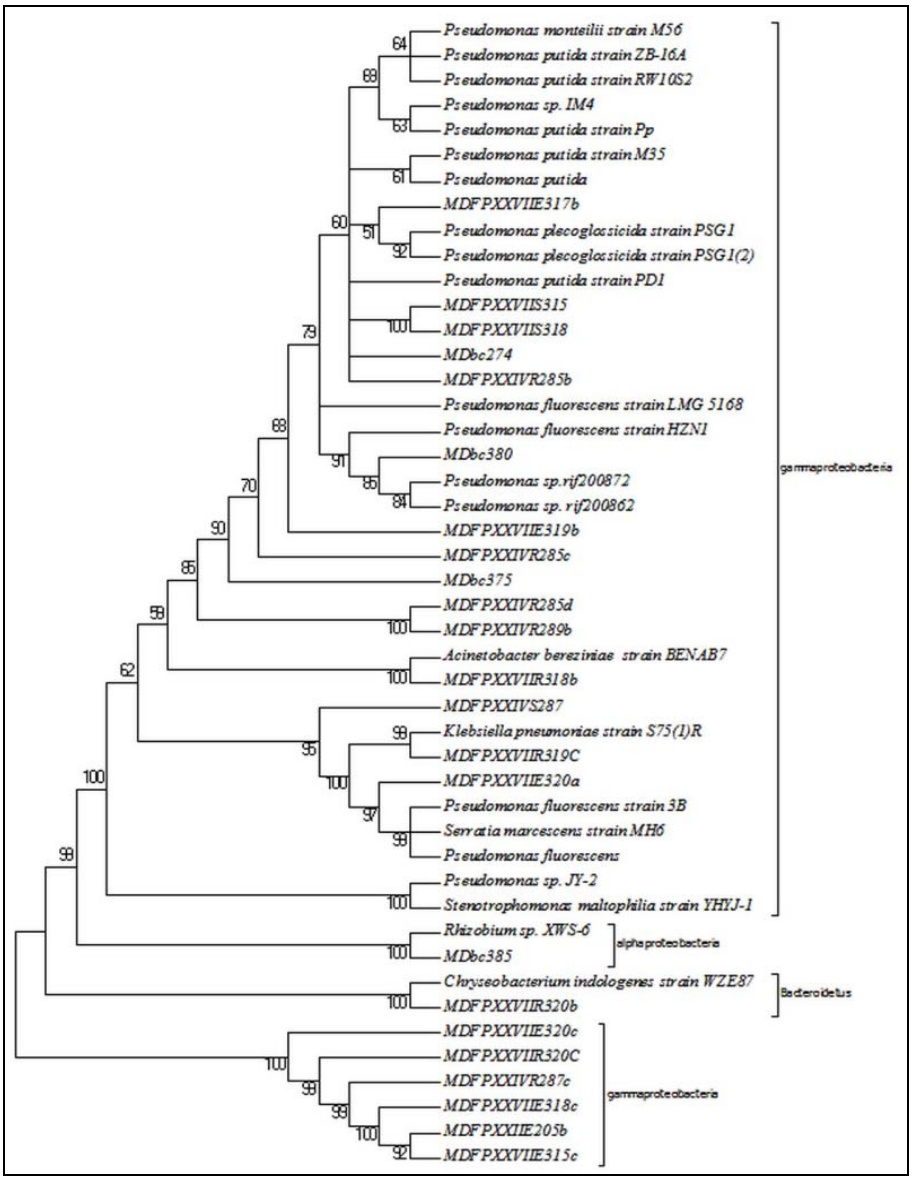

Fig 4: Phylogenetic tree based on 16S rDNA sequencing constructed using neighbour-joining method showing relationships of the PGPR and their closest NCBI strains. Bootstrap values greater than 50% are indicated at nodes. Scale bar 0.05 substitutions /nucleotide positions

Polyphyletic separation of taxa into three major groups namely alphaproteobacteria, gammaproteobacteria and Bacteroidetus can be observed in the dendrogram. The cluster of gammaproteobacteria formed two distinct clades showing

sufficient diversity within them (d= 0.364). The mean interpopulational diversity among all the isolates was found to be 0.24.

Analysis of sequences using Hierrarchy classifier indicated that the strains isolated and showing antagonistic activity in this study are more or less closely related, with a major branch of gammaproteobacteria in the order Pseudomonadales, Xanthomonadales and Enterobacteriales. As seen in the reconstructed phylogenetic tree, some strains showing affiliation to Enterobacteriales, Pseudomonadales and Xanthomonadales do not form a clade but rather represent an evolutionary grade, in fact paraphyletic separation from some of their descendents. For instance, strain MDFPXXVIIR318b which showed 99% affiliation to *Acinetobacter* belonged to the same order Pseudomonadales but to a different family Moraxellaceae.

Strain MDFPXXVIIE320c also belonging to cluster gammaproteobacteria excluded from the other Pseudomonadales showing 100% affiliation to genus *Stenotrophomonas* in the family Xanthomonadales.

Strain MDFPXXVIIR320b in this analysis showed total phyletic separation from the rest of the isolates, showing taxonomic affiliation to genus *Chrysobacterium* (100%) in class Flavobacteria of Bacteroidetus.

Discussion

Apart from the ubiquitous pseudomonads, strains of *Rhizobium, Chrysobacterium indologenes, Acinetobacter berezianae, Serratia marcessens* and *K. pneumoniae* were also obtained in our study from the small pool of effective PGPB; showing the diversity and heterogeneity of root environments. Beneficial effects of fluorescent and other pseudomonads [17, 18], Rhizobium [19, 20], *Serratia* sp. [21], *Acinetobater* sp., *Chrysobacterium indologenes* [22], and *K. pneumonia* [23] as biocontrol agents is exemplified in several studies, thus showing common trends in predominant bacterial types.

Endorhizospheric bacteria owing to their capability of self-perpetuation and root colonization prove potential biocontrol agents, as compared to the non symbiotic and free living rhizobacteria [24]. Barring one *Rhizobium* sp., in our study endorhizospheric niches were predominated by pseudomonads *viz*., *Pseudomonas putida and P. plecoglosscida*. Endorhizospheric isolates 379, 381, 384 and 385, emerged as potent biocontrol agents with multiple PGP traits and an ability to excrete one - three antimetabolites that showed antagonistic activity against two or more test fungal pathogens. In addition, all the effective strains isolated in our study produced siderophores under the iron stressed conditions and moderate to high HCN production by most of them, besides production of notable amount of indole acetic acid (a possible determinant of biocontrol), substantiate for the underlying biocontrol mechanism. The inhibition of growth and sporulation of fungal pathogens through the production of volatile antimetabolites was remarkable in the present study with a zone of inhibition of 70 mm as compared to control. Such volatile organic compounds have been demonstrated to act as determinants of ISR and to promote growth in *Arabidopsis* [25]. Besides, we report a *Rhizobium* strain producing phenazine. The fact that phenazine producers have an added advantage of long term survival and ecological competence in rhizosphere [26], further obviates the need to genetically engineer rhizobia to produce phenazine which could result in loss of symbiotic performance [27].

Di acetyl phloroglucinol (DAPG) production is a conserved trait identified in pseudomonads from diverse environments [28]. Its' derivatives act as a diffusible signal molecule inducing cross talk between DAPG producers and inducing its own biosynthesis [29]. All four effective isolates tested in our study reported phloroglucinol production. The production of pyrronitrin by the isolates 384 and 385, also suggests their broad-spectrum biocontrol potential.

As to phylogenetic and molecular evolutionary analysis, results of RDP analysis were contradictory to BLAST results, in that some strains of gamaproteobacteria which showed 98-99% affiliation to genus *Pseudomonas* in blast analysis have actually showed taxonomic affiliation to genus *Serpens* (36% to 52%) and *Azomonas* (54 to 78%) in the unclassified Pseudomonadaceae when subjected to analysis using hierarchy classifier. This is reflected in the polyphyletic separation of taxa within the genus *Pseudomonas* in the reconstructed phylogenetic tree. For example, two distinct clades of gammaproteobacteria can be observed; one showing paraphyletic separation of Pseudomonadales, Enterobacteriales and some in the unclassified Pseudomonadaceae forming several subclusters. Despite 100% taxonomic affiliation of isolate 384 to *Stenotrophomonas maltophila* in the order Xathomonadales, the strain clustered with other Pseudomaonadales forming a second clade of gammaproteobacteria and its homologous NCBI strain *Stenotrophomonas maltophila* excluded from it as though they are distantly related. This cluster has been arbitrarily outgrouped and its separation from the other gammaproteobacteria was supported by a bootstrap value of 100%. Phylogenetically polyphyletic genus *Pseudomonas* was reported in several studies [30, 31]. Overall, the phylogeny inferred deflects that the strains studied here are closely related; however, did not fetch higher resolution of evolutionary relationships between the strains so as to draw taxonomic conclusions and to correlate with multiple PGP traits exhibited by the strains. Application of *rpoB* gene as a potential phylogenetic marker or a polyphasic approach could eliminate such biases in the classification of gammaproteobacteria [32].

We have tried to piece out the mechanism of biocontrol based on the previous reviews where in various traits of PGPB *viz*., IAA production, cyanogenesis, siderophorogenesis, volatile and non volatile antimetabolite production have been proposed to work holistically in an isolate, ultimately bestowing it an overall biocontrol potential [2]. As revealed in several reports, our results also indicate that multiple mechanisms exert cumulative effect. A further study on genes conferring the traits is warranted to ascertain the operational mechanism of biocontrol in these isolates.

Nevertheless, the study is significant in its own right adding to the list of new and novel multi-trait PGPB; pressing the need to conserve them. An insight into the genetic control of the biocontrol mechanism and optimizing inoculation strategies by activating the defense gene by prior application of PGPB against the challenge pathogen, novel formulation strategies and effects of coinoculation of bacterial and fungal antagonists, should be worked out in order to provide full fetched plant protection.

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