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Antibiotic-Resistant Rhizobacteria with Multifunctional Traits: Nutrient Solubilization, IAA Production, and Antagonism Against Phytopathogens

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Abstract

The antibiotic application reduces the microbial count of pathogenic microbes along with beneficial plant growth promoting bacteria (PGPB). This can be avoided if PGPB has antibiotic resistance. Thirty bacterial isolates were found from nutrient-rich alkaline soil, associated with phosphate and potassium solubilization and IAA production potential. Twelve isolates were shortlisted and the antibiotic sensitivity of the bacterial isolates was tested against eight antibiotics using the disc diffusion technique. Based on various physiological tests, compatibility and 16S rRNA sequence analysis, two PGPB Bacillus megaterium (M-08) and Bacillus aryabhattai (M-24) were identified. Both the isolates were positive for starch hydrolysis, catalase activity, acid and gas production, urease and gelatin liquefaction tests. Isolate (M-20) had the highest PSI of 6.0. M-26 had the highest KSI of 5.6. M-08 and M-24 were antagonistic against phytopathogenic R. solani. The present study indicated the presence of diverse P and K solubilising bacteria in the tomato rhizosphere, with IAA production potential and antibiotic resistance. These bacterial isolates can serve as potential plant growth promoters as they showed an increase in N, P and K uptake in the plant. The research tried to bring up a hypothesis of antibiotic selective plant growth promoters or biocontrol agents and their benefit in acclimatization and establishment in soil despite the microbicidal applications. This method can be used worldwide, to eradicate phytopathogens, while leaving the beneficial PGP microbes in rhizospheres.

Keywords: *Bacillus megaterium, Bacillus aryabhattai, KSB, phylogeny, PSB.*

1. Introduction

In event of plant diseases, farmers tend to avert slow-acting antagonistic microbial cultures and rely on fast-acting synthetic pesticides to safeguard the yield [42]. A single application of such a pesticide is enough to disrupt the plant probiotic microbes established in the field over years. Selective weedicides have been developed and used on a large scale, but the generation of antibiotic-selective plant growth-promoting cultures is still amidst mixed opinions by researchers despite their abundance these days [32]. It is argued that the trait of antibiotic resistance in bacteria can transmit to other bacteria through horizontal gene transfer. But it cannot be denied that there is already a larger prevalence of antibiotic resistance in many plant probiotic microbes [33]. Plant growth promoting bacteria, harnessing the nutrient solubilization and hormone production traits, alongside antibiotic resistance, can provide a failsafe approach to pesticide application, without harming the plant probiotic colonies in soil. This can limit the pesticide use, conserve the introduced plant growth promoting cultures and increase plant yield.

Isolation of growth-promoting microbe from the rhizospheric region of a healthy and mature plant of interest reduces the risk of it being pathogenic since they create a protective layer around roots, restricting pathogenic bacteria [1]. Many such bacteria have been reported to be isolated from the rhizosphere, successfully tested and marketed in plant growth promotion [2]. Nitrogen, phosphorus and potassium are crucial elements in plant growth, which are applied as basal dose fertilizers in the soil before sowing. Phosphorus and potassium are mostly present in organic and inorganic forms, but they are largely inaccessible to plants because they

are either undecomposed or insoluble forms. This creates a need for artificial fertilization [21]. The introduction of nutrient-solubilizing bacteria can assist in the uptake of already available nutrients, reducing the need for additional chemical fertilizers [3]. The fascination with higher yield has also led to the use of synthetic hormones like auxins, cytokinin or gibberellins, which doesn't suit sustainable agriculture [4]. Hence, the use of PGPB, which has the potential to convert the already available nutrients into a soluble form, and also provide phytohormone, has long been implemented for sustainable agriculture [5].

Diverse PGPB has different plant growth-promoting traits, which can augment the performance in consortia as compared to a single isolate. But antagonism of cultures, cultivar specificity, tissue specificity of isolates, fertilization concentration, crop rotation, soil characteristics, already prevalent pathogenic strains in soil, etc. impact the actual yield and complexity of consortia preparation. We have data sets of such experimental results from smaller field areas, however, the data on the successful effect of the use of such cultures in extensive trials, yield improvement, pathogenic control, etc. is drastically missing [6] and seldom studied in extensive trials. The diseases, growth conditions, plant densities and pathogenic niches in extensive fields are generally different from that of the lab trials. This, along with slow mode of action might be the reason why use of plant growth promoting microbes (PGPM) has failed to replace synthetic products in agriculture. Due to a knowledge deficit among farmers regarding such cultures, sustainable agriculture practice implementation is falling short [6].

It is well known that most of the rhizospheric microbes carry antibiotic resistance [8], but the use of antibiotics is inevitable [7]. The use of antibiotic-selective PGPM can provide assistance in plant growth promotion in case of unprecedented bacterial infections. This ensures that the PGPM is not killed, and the aggressive infections are avoided without compromising the yield. Though the hypothesis has never been researched, it is quite logical. Rhizospheric microorganisms may mediate decomposition, nutrient mobilization and mineralization, nitrogen fixation and denitrification, and plant stimulation through phytohormones [8]. Also, in case of bacterial infection, the antibiotic application can be done without disrupting the soil flora. Identification of the prevalence of antibiotic-resistant PGPB can provide insight into the prevalence of antibiotic resistance in rhizospheric bacterial samples. Assessing the nutrient solubilization potential, and consortium formation of such microorganisms may assist in plant growth promotion and sustain antibiotic treatment while selectively treating the plant bacterial disease. With this view, a survey on the soil samples from commercial tomato fields were done and the plant growth promoting isolates with IAA producing potential were isolated and their antibiotic resistance were studied.

2 Material and Methods

2.1 Collection of rhizospheric soil

Thirty distinct soil samples were collected in July 2020 in sterilized polythene bags from different plots of Hyderabad-Raichur commercial and research tomato fields (*Solanum lycopersicum* L., var. PKM-1). The region is described as Zone-VI by Telangana State Agriculture University, Hyderabad and their recommended standards of fertigation were followed for tomato cultivation. Approximately 25 t/Ha FYM was used in the fields before sowing. The plants were grown in a nursery (2 kg/m^2FYM mixed in soil and sown 1 cm deep with 10 cm row spacing and 5 cm seed-to-seed distance) until 10-15 cm height was attained. The nursery was pre-treated with 2g/lit carbendazim as a fungicidal treatment. 0.1% streptomycin and plantomycin were used to treat the roots before transplanting. A 60 x 45 cm sowing pattern was used. Earthing up was done once after 20 days of transplanting. At the fruiting stage, the 5 cm of topsoil was removed, and then soil between 6 to 10 cm below it was collected, where roots were concentrated. The rhizosphere is a zone of soil lying between 0 and 2.5 mm away from the root surface that is heavily impacted by live roots. 10 g of rhizospheric soil was collected surrounding each plant. A sample was created by combining soil samples from 10 different plants. All soil samples were stored on an icepack in sterile zip lock bags and used within 8 hours after being collected. 8 samples from Raichur (16.20466°, 77.33208°), 3 from Makthal (16.50036°,77.50719°), 7 from Medchal (17.64143°,78.5440°), 6 from Ranga Reddy farm (17.62994°,78.42845°) and 7 samples from Shaktinagar field (16.36449°,77.34519°) were collected.

2.2 Determination of soil characters

Physicochemical properties like pH, electrical conductivity, and total elemental concentration (N, P, K and Zn) of the experimental soils and their soil types were noted using standard methods mentioned by the Ministry of Agriculture, India(Basu, 2011).

2.3 Isolation of phosphate solubilising bacteria

25 ml of sterilized and molten Sperber's agar medium (10 g glucose, 0.5 g yeast extract, 0.1 g $CaCl_2$, 0.25 g $MgSO_4$.7 H2O, 2.5 g $Ca_3(PO_4)_2$ and 15 g agar per liter at pH: 7.2) was prepared (Malboobi et al., 2009). The sterile media was spread with 0.1 ml of 10^{-4} dilution of soil suspension and incubated at 28±2 °C for 4 to 7 days. The plates were observed for clear zones around the colonies. Colonies were isolated and coded based on their distinct colony characters. The phosphate solubilization index was calculated based on the ratio of the total diameter (colony + halo zone) to the colony diameter after spot inoculation and incubation time of 7 days.

2.4 Isolation of potassium solubilising bacteria

The bacteria capable of P solubilization were taken forward to test K solubilization potential.

Sterile Aleksandrov's medium plates (0.5 g $MgSO_4$.7 H2O, 0.1 g $CaCO_3$, 2.0 g $AlKO_6Si_2$, 5.0 g glucose, 0.005 g $FeCl_3$, 2.0 g $Ca_3(PO_4)_2$, 20 g agar per liter at pH: 7.2) were prepared as per the standard procedure (Muthuraja & Muthukumar, 2021). 0.1 ml of 10^{-4} dilution of the soil suspension was spread over the plates and incubated at 28 ± 2 °C for 4 to 7 days. The plates were observed for clear zones around the colonies. The rest of the bacteria were disregarded. The Potassium Solubilization Index was calculated based on the ratio of the total diameter (colony + halo zone) to the colony diameter after spot inoculation and incubation time of 7 days.

2.5 Morphological and biochemical characterisation of the bacterial isolates

IMViC test, Starch hydrolysis test, acid and gas production test, catalase test, urease test and gelatin liquefaction test were performed by standard methods for the characterization of bacterial isolates. The prediction of the bacterial isolates was done with ABIS online bacterial identification website (https://www.tgw1916.net/bacteria_logare_desktop.html). The bacterial isolates were qualitatively tested for their ability to produce polysaccharides in the plate assay by spotting 10 µl of overnight bacterial culture streaked on a glucose minimal agar medium [29]. The plates were incubated at 28 ± 2 °C for 24 to 48 h.

2.6 Antibiotic resistance tests

An antibiotic assay was performed on Muller-Hinton agar using disk diffusion method (Shakhatreh et al., 2016). 10 µL of isolated bacterial suspension (10^6 CFU/mL) was spread on the agar plate. Ampicillin (10 µg) Clindamycin (2 µg), Penicillin-G (10 µg), Vancomycin (30 µg), Polymixin-B 300 units and Kanamycin (30 µg) standard discs (HiMedia, India) were placed on this agar plate. The readings were taken in triplicates. The antibiotic resistance was recorded as zone of inhibition in millimeters against an antibiotic and interpreted as per the Centre for laboratory standard guideline.

2.7 Indole Acetic Acid (IAA) production by plant probiotic bacteria

The bacteria capable of P and K solubilization were screened for IAA production. IAA production in different isolates of PGPB was detected according to (Gordon & Weber, 1951). The active culture of each test isolate was inoculated to 25 ml of Luria Bertani broth supplemented with tryptophan (0.1 g/L) and incubated at 28 ± 2 °C for 3 to 6 days. Non-inoculated broth culture was kept as a control. After incubation, these cultures were centrifuged at 5000 rpm for 5 min. After centrifugation, 4ml of Salkowski's reagent was added to 2 ml of supernatant and incubated for 30 min in the dark to develop pink or red color (Gordon & Weber, 1951).

2.8 Compatibility studies of isolated PGPB

The bacterial isolates were tested for their compatibility with each other on nutrient agar medium (2.0 g yeast extract, 5.0 g NaCl, 5.0 g peptone, 1.0 g meat extract, 20 g agar per liter at pH: 7.2) using two cross streak method as described by [30] and tested for lysis of cells or color change at the junction of bacterial streaks. 10^6 cells/mL inoculum concentration was streaked onto an agar plate. Every isolate combination using the Punnett Square method was done to test compatibility against each other. The plates were incubated for 48 h at room temperature and observed for lysis at the juncture of the streaks.

2.9 Antagonistic activity of PGPB against phytopahtogenic Rhizoctonia solani strain

Phytopathogenic strains of *R. solani* were isolated from the hotspot region of Raichur. This strain was sub-cultured through serial infection to healthy plants and re-isolation to maintain the pathogenicity of the phytopathogenic fungal strain. The antifungal activity of the bacterial isolates was tested by dual culture technique. A 5 mm potato dextrose agar (4.0 g potato extract, 20.0 g glucose, 20.0 g agar per liter at pH: 7.2) plug of 15 days old mycelial culture was growtn against a streak of bacterial culture (10^6 cells/mL). The antagonistic activity of the bacteria on mycelial growth confrontation was observed. The relative growth rate inhibition was calculated as (R1-R2/R1) x 100, where R1 is radial growth of control and R2 is radial growth at dual plate confrontation.

2.10 Morphological and molecular characterisation of putative plant growth promoting bacteria

All the selected isolates were examined for cell shape gram staining and sporulation test. The isolates that showed higher nutrient solubilization and IAA production, along with the potential of compatibility to form consortia, were shortlisted for molecular characterization. Bacterial genomic DNA was isolated from overnight-grown cells using the procedure mentioned by [31]. The quality and quantity of the isolated DNA were checked spectrophotometrically (Nanodrop ND1000) as well as through agarose gel electrophoresis. The universal primers, forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-ACGGCTACCTTGTTACGA CTT-3') (Weisburg et al., 1991), were used for amplification of the 16S rRNA gene of bacterial strains. Fragment of 16S rRNA gene was amplified by 16SrRNA-F (5'- AGAGTTTGATCCTGGCTCAG-3') and 16SrRNA-R (5'-ACGGCTACCTTGTTACGA CTT-3') primers. About 25 ng of bacterial genomic DNA and 5 picomoles of each (forward and reverse) primer were used for amplification in a thermocycler programmed as 94 °C for 5 min; 34 cycles of 94 °C for 1 min, 57.4 °C for 1 min, 72 °C for 2 min; 72 °C for 10 min; 4 °C for an infinite period. On an agarose gel, a discrete PCR amplicon band was resolved. The PCR amplicons were purified to remove contaminants. The PCR amplicon was sequenced forwards and reverse using 16SrRNA-F and 16SrRNA-R primers on an ABI 3730xl Genetic Analyzer using

the BDT v3.1 Cycle sequencing kit. Using aligner software, a consensus sequence of the 16S rRNA gene was produced from forwards and reverse sequencing data. The 16S rRNA gene sequence was utilized to perform BLAST against the NCBI GenBank 'nr' database.

2.11 Phylogenetic analysis

Highly homologous accession sequences were chosen based on their highest identity score. MEGA 10 (Molecular Evolutionary Genetics Analysis) was used to create the distance matrix and phylogenetic tree [9]. The sequences were aligned using the Clustal W programme, and distances were determined using Kimura's two-parameter approach [10]. The Neighbour-joining and BioNJ algorithms were applied to a matrix of pairwise distances computed using the Maximum Composite Likelihood (MCL) technique, and the topology with the highest log likelihood value was chosen. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. This study included 11 nucleotide sequences. The following codon locations were included: 1st+2nd+3rd+Noncoding. The bootstrap analysis used 1000 resampling.

2.12 Effect of plant growth promoting isolates on tomato plant growth

Tomato variety PKM-1 from Agrotech Pvt. Ltd was grown in a nursery as per the recommended guidelines of Telangana State Agriculture University, Hyderabad. Before transplanting the plantlets, the roots were washed with sterile distilled water to remove the antimicrobial residues and then the roots were dipped in the 10^6 CFU/ml inoculum [11] and transplanted. On the next morning, 10 ml of the same inoculum of selected potent isolates were diluted up to 100 ml using sterile distilled water and then drenched at the rhizospheric region of the plants. The uninoculated plants with a recommended dose of fertilizers and with no fertilizers were used as control. The effect of inoculum with respect to N and P uptake was compared by measuring the N and P of the entire plant at the maturity stage, estimated by the standard method [12].

2.13 Statistical analysis

Each experiment was carried out in triplicate. Standard errors, ANOVA and Tukey's HSD as post hoc analysis were calculated using IBM SPSS v.24. To decrease the bias induced by soil heterogeneity, and the type of soil, replicated sampling from random locations on the same site was done.

3 Results and Discussion

3.1 Determination of soil characters

The sampling locations had predominantly red or black soil, with a basic pH range from 7.1 to 7.8. The black soil tends to have a higher pH (>7.3). The tomato plant needs a slightly acidic pH, ranging from 6.5 to 6.8. The deviation from such a range forces the plant to regulate the rhizospheric pH by releasing exudates from roots [32].

The electric conductivity of the selected samples was within a range of 0.13 to 1.06 dS/m. Higher EC (more than 1dS/m) corresponds to saline soil. There was no correlation between soil type and EC. Among the samples, only soil types of isolate M-3 and M-27 were near salinity, with higher EC values. The details of soil characteristics are shown in Fig. 1. There is a greater gap in knowledge regarding optimal EC values for the crops in fields.

Field with isolate code M-29 had the highest N and K content, whereas P was highest in the M-12 location. The concentration of nutrients in the selected soil samples ranged from 172.6 to 232.5 K/ha of N, 31.6 to 50.6 K/ha P, 144.8 to 269.9 K/ha K and 0.42 to 1.09 ppm Zn. The comparison of different nutrient contents of the soil samples are shown in Fig. 2. In general, the field had an excess of N, slightly lesser P and a very high amount of K, as compared to optimal requirements for tomato crop. The fields were not a deficit in any nutrients, and the only limiting factor was the extent of solubilization of the N, P, and K compounds and their uptake for higher yields.

Phosphorus reacts with aluminium or iron at acidic pH or calcium at basic pH, making it inaccessible to the crops. This was discovered during soil sampling in this research, where calcareous soil was identified in some plots and had a higher pH. But the best potential of most vegetable crops is with slightly acidic soil. The only self-regulated pH-controlling strategy is increasing the carbon-to-nitrogen ratio and growing nutrient-solubilizing bacteria into it [13]. The plant probiotic microbes themselves act as micro-factories, regulating the desired pH and collaborating with the plants to enhance plant growth [33]. The sampled regions were suitable for diverse microbial growth.

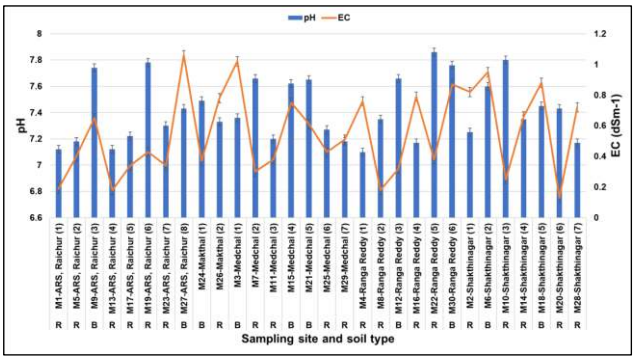

Fig. 1: pH, EC and soil type of tomato rhizospheric soil samples used for isolation of putative PGPB

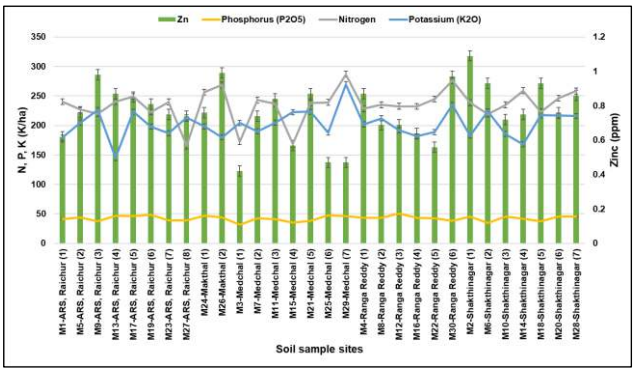

Fig. 2: Comparison of nutrients in the soil taken from different sampling sites. (Values in the parenthesis on x axis denote the sampling site from same field)

3.2 Morphological and biochemical characterization of isolated bacteria

The transplanted tomato plants were pretreated with bactericides and hence there was a lesser probability of any epiphytic bacteria being introduced into the soil through plants. All the sampled locations provided phosphate and potassium solubilizing bacteria of variable magnitudes, on respective selective media. The bacteria were identified based on biochemical characteristics as shown in Table 1. Different sporulation natures of the selective PSB and KSB isolates were found, but sporulating bacteria can be packaged and stored in wet conditions for prolonged periods [14], making it a selective characteristic in commercial PGPB formulation. The isolates created polysaccharides and also had a slimy texture on colonies that retain moisture and extend the life of the isolated bacteria in field conditions. The qualitative test of the bacteria also revealed that all of the isolates produced polysaccharides after 48 hours of incubation. The biochemical tests and IMViC results helped in the prediction of the bacterial isolates as *Lactobacillus acidophilus, Bacillus subtilis, Pseudomonas aeruginosa, Azotobacter vinelandii, Bacillus aryabhattai and Bacillus megaterium.*

Table 1: Identification of selected P and K solubilising bacterial isolates based on biochemical characterization.

Isolates	IMViC test				Biochemical characters								Identified isolate
	I	M	VP	C	GS	S	1	2	3	4	5	6	
M-01	-	-	-	-	+	-	+	-	+	+	-	+	*Lactobacillus acidophilus*
M0-2	-	-	+	+	+	+	+	+	+	-	-	+	*Bacillus subtilis*
M-03	-	-	-	+	-	-	+	-	+	-	-	+	*Pseudomonas aeruginosa*
M-04	-	-	+	+	+	+	+	+	+	-	-	+	*Bacillus subtilis*
M-05	+	+	-	+	-	+	+	+	+	+	+	+	*Azotobacter vinelandii*
M-06	-	+	+	-	+	+	+	+	+	+	+	+	*Bacillus aryabhattai*
M-07	-	-	-	+	-	-	+	-	+	-	-	+	*Pseudomonas aeruginosa*
M-08	-	+	-	+	+	+	+	+	+	+	-	+	*Bacillus megaterium*
M-09	-	-	-	-	+	-	+	-	+	+	-	+	*Lactobacillus acidophilus*
M-10	+	+	-	+	-	+	+	+	+	+	+	+	*Azotobacter vinelandii*
M-11	-	-	-	+	-	-	+	-	+	-	-	+	*Pseudomonas aeruginosa*
M-12	-	+	-	+	+	+	+	+	+	+	-	+	*Bacillus megaterium*
M-13	-	-	-	-	+	-	+	-	+	+	-	+	*Lactobacillus acidophilus*
M-14	-	+	-	+	+	+	+	+	+	+	-	+	*Bacillus megaterium*
M-15	+	+	-	+	-	+	+	+	+	+	+	+	*Azotobacter vinelandii*
M-16	-	-	+	+	+	+	+	+	+	-	-	+	*Bacillus subtilis*
M-17	-	-	-	+	-	-	+	-	+	-	-	+	*Pseudomonas aeruginosa*
M-18	-	-	+	+	+	+	+	+	+	-	-	+	*Bacillus subtilis*
M-19	-	+	+	-	+	+	+	+	+	+	+	+	*Bacillus aryabhattai*
M-20	-	+	-	+	+	+	+	+	+	+	-	+	*Bacillus megaterium*
M-21	-	+	-	+	+	+	+	+	+	+	-	+	*Bacillus megaterium*
M-22	-	-	-	-	+	-	+	-	+	+	-	+	*Lactobacillus acidophilus*
M-23	-	+	-	+	+	+	+	+	+	+	-	+	*Bacillus megaterium*
M-24	-	+	+	-	+	+	+	+	+	+	+	+	*Bacillus aryabhattai*
M-25	-	-	-	+	-	-	+	-	+	-	-	+	*Pseudomonas aeruginosa*
M-26	-	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus acidophilus*
M-27	+	+	-	+	-	+	+	+	+	+	+	+	*Azotobacter vinelandii*
M-28	-	-	-	-	+	-	+	-	+	+	-	+	*Bacillus aryabhattai*
M-29	+	+	-	+	-	+	+	+	+	+	+	+	*Azotobacter vinelandii*
M-30	-	-	+	+	+	+	+	+	+	-	-	+	*Bacillus subtilis*

I is Indole test, M is Methyl red test, VP is Voges Proskauer test, C is citrate utilization test, 1, GS is Gram staining, S is spore formation: Starch hydrolysis, 2: Catalase test, 3: Acid production, 4: Gas production, 5: Urease test, 6: Gelatin liquefaction test, +: positive test, -: Negative test.

3.3 Shortlisting of IAA, Potassium and Phosphorous solubilizing bacteria

Among the 30 isolates, the *in vitro* screening of bacteria with better phosphate and potassium solubilizing traits resulted in 12 promising bacterial isolates (Table 2). The phosphate solubilization index (PSI) of the bacteria ranged from 2.0 to 5.0. whereas that of KSB ranged from 1.7 to 5.6. *Bacillus megaterium* (M-20) had the highest PSI of 6.0. PSB dissolves the insoluble calcium phosphate in the medium and the bacteria form a clean zone surrounding the colony [15-16]. The capacity of the strain to lower the pH of its surroundings, either by releasing organic acids or protons, has been linked to its phosphorus solubilization capability [17]. PSB-secretes organic acids such as gluconic acid, oxalic acid, formic acid, and citric acid that plausibly solubilize mineral phosphate through anion exchange or indirectly chelate both Fe and Al ions associated with phosphate [18]. This increases P availability, in turn, promotes plant P absorption.

A similar phenomenon occurs with K uptake [19]. *Bacillus aryabhattai* (M-19, M24), and *Azotobacter vinelandii* (M-29) had the highest KSI. The Potassium solubilization potential of the isolates was better in general as compared to phosphate solubilization. The isolated bacterial cultures in this research showed acid and gas production, which supports the nutrient solubilization traits of the isolated cultures. The PSI and KSI of these isolates were quite promising as compared to other isolates [20].

The zone of solubilization of P and K in the respective media is shown in Fig. 3. The IAA production of these isolates was found to be in the range of 0.24 µg/100 ml to 0.34 µg/100 ml, which is quite competitive as compared to other studies [21], M-17 had the highest IAA production potential (0.346 µg/100 ml). The results of IAA, PSI and KSI show that the respective isolates have quite promising potential in plant growth promotion.

The rhizospheric region increases the probability of occurrence of PSB and KSB as compared to the non-rhizospheric region [22]. The present research found 30 rhizospheric regions with PSB and KSB potential, with a varied magnitude of P and K solubilization. The availability of P and K are worthless if the plant roots are unable to absorb them adequately. As a result, the study aimed at discovering and shortlisting PSB and KSB with IAA production traits, contributing to the isolates' ecological value. IAA promotes plant roots by increasing root surface area, allowing better nutrient and water absorption. The present study discovered 12 interesting isolates with high PSB, KSB, and IAA production potential. M-08 and M-24 had the average best IAA production but the best PSI and KSI potential. PSB, KSB and IAA media optimization study needs to be performed to check the supraoptimal performance of the isolates. Though, the PSI and KSI were quite commendable as compared to other findings [23].

Table 2: Indole acetic acid production, phosphorous and potassium solubilization ability of plant probiotic isolates

Isolates	IAA (µM/ml)	PSB			KSB		
		HD (cm)	CD (cm)	PSI	HD (cm)	CD (cm)	KSI
M-08	151±0.01	0.70±0.02	0.25±0.01	2.8	0.50±0.0	0.30±0.00	1.7
M-12	142±0.01	1.30±0.01	0.40±0.01	3.3	1.60±0.01	0.35±0.01	4.6
M-15	190±0.01	1.00±0.01	0.20±0.01	5.0	0.95±0.01	0.25±0.01	3.8
M-17	198±0.02	0.65±0.01	0.35±0.01	1.9	0.70±0.00	0.25±0.00	2.8
M-19	164±0.01	1.00±0.01	0.30±0.01	3.3	1.50±0.00	0.30±0.00	5.0
M-20	156±0.01	1.80±0.01	0.30±0.01	6.0	1.45±0.00	0.30±0.00	4.8
M-23	183±0.02	1.00±0.01	0.35±0.01	2.9	1.25±0.00	0.35±0.00	3.6
M-24	147±0.01	0.90±0.01	0.25±0.01	3.6	1.80±0.00	0.36±0.00	5.0
M-26	152±0.01	1.20±0.01	0.35±0.01	3.4	1.60±0.01	0.40±0.01	4.0
M-27	150±0.01	0.70±0.01	0.25±0.01	2.8	1.40±0.04	0.25±0.01	5.6
M-29	172±0.02	0.85±0.01	0.35±0.01	2.4	1.50±0.01	0.30±0.01	5.0
M-30	139±0.02	0.90±0.01	0.50±0.01	2.0	1.50±0.00	0.40±0.00	3.8

HD is Halo diameter; CD is colony diameter; PSI is Phosphate solubilization index; KSI is Potassium solubilization index. SE is mentioned as any mean followed by '±'.

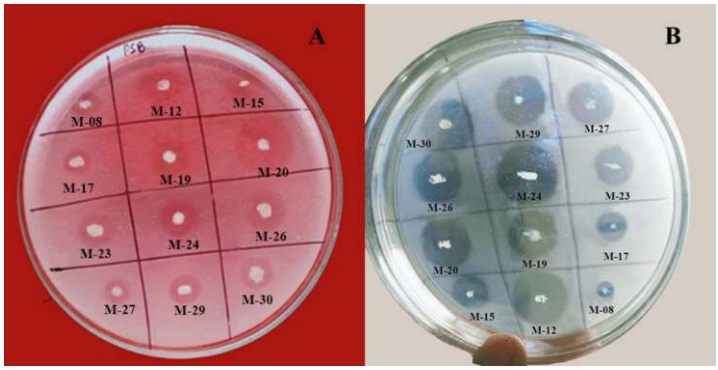

Fig. 3 A) Phosphate and B) Potassium solubilization performance of selected isolates as shown by a clear halo of solubilization around the bacterial colony

3.4 Antibiotic resistance in shortlisted isolates

The shortlisted potassium and phosphorous solubilizing bacteria were tested for antibiotic resistance. All putative shortlisted PGPB isolates were resistant to at least one of the antibiotics in the test. 5 bacteria (about 42%) were resistant to at least 2 antibiotics. The rest of the isolates exhibited varied responses, as shown inTable 3.

The primary goal of the research was to test the antibiotic resistance prevalence in commercial fields and its control using antibiotics on lab scale. Hence, the nature of antibiotic resistance, resistance transfer among other isolates, resistance by mutation, etc. was not studied. It need not be proven that antibiotics can kill the sensitive microbes in the field, since extensive data is currently available on antibiotic applications to control bacterial diseases in plants [24].

The isolated bacteria, except *P. aeruginosa* are not recorded to be human pathogens, and hence, the antibiotic selection, interpretation and standardization for non-clinical isolates are yet not available in CLSI guidelines. There is no reference zone of inhibition to interpret the resistance and susceptibility of *Lactobacillus acidophilus, Bacillus subtilis*, *Azotobacter vinelandii, Bacillus megaterium and Bacillus aryabhattai*. But the antibiotic performance of these isolates has been recorded in Table 3. The reference antibiotic performance of the representative isolates is shown inFig. 4.

The result clarifies that antibiotic resistance is a fact in PGPB from field soils, and not many have reported it.

The number of small landholders in any country is more, partially relying on subsistence farming. Many of them can't withstand the pressure of crop loss in case of biotic stress. Hence, they use fungicides or bactericides and kill most of the microbes in the phyllosphere as well as the rhizosphere. To replenish the beneficial microbial flora, additional expenses are incurred, which reduces the economic viability of farming. It is a fact that organic farming has not been successful in practical yield improvement to the extent of modern synthetic chemical-based farming [25;35]. Surprisingly, there is a knowledge gap in data regarding pathogenic microbial count reduction using antagonistic cultures in extensive trials at the field. Many scientific reports are from lab-scale performances, performed on smaller areas, which are different from actual field performances. Hence, the use of microbicidal chemicals has been an eminent practice. The concept of selective weedicide has been implemented, but the production of antibiotic-selective PGPB has many different opinions. [26;34] has provided insight into the use of antibiotic resistance traits in agriculture, but no such products are on market yet. Organic farming relies more on disease preventive modes and most of the products are lesser effective and costlier as compared to modern chemical-based remedies. The number of applications of organic products, efficiency, reliability and non-selective death of microbes by microbicidal chemicals use limits the practical applicability of farming with plant growth promoting bacteria.

In the current scenario, the uncertainty and heavy rains accompanied by water lodging force the farmers to perform antimicrobial chemical drenching. This practice is prevalent every year, and the introduced plant growth promoting microbes die and fail to compete with phytopathogenic microbes in the soil. Prolonged practices of such chemicals are not sustainable. The true establishment of chemical-free farming can prevail only when the soil health is regulated by antagonistic PGPB, which prevents the entry of other plant pathogenic microbes. This can be made possible only when PGP bacteria or fungi are made to survive in the soil containing comparatively fastidious pathogenic microbes. In the competing environment, the antibiotic-selective PGPB provide a hope to selectively survive microbicidal applications, leaving only the PGPB in the soil, to associate with the plants and regulate the soil health. In consecutive applications, the colony count of pathogenic microbes would reduce, and the PGP microbes would increase. There are several debates on resistance gene transfer to other bacteria, but the same is already present in PGP microbes as proven in this research. Harnessing this trait can provide an opportunity to reduce the phytopathogenic microbial count and raise the PGPB count in soil. This hypothesis of selective pathogenic microbe control is very similar to selective herbicide activity in controlling weeds, yet needs to be proven in the field with an extensive trial.

Table 3: Antibiotic assay of plant probiotic isolates

Isolate	Zone of inhibition diameter against antibiotic (mm)					
	AMP 10	K 30	VA 30	PB 300	CD 2	P 10
M-08	0	14	17	12	22	0
M-12	0	13	16	11	23	0
M-15	0	13	19	12	19	10
M-17	0	13	19	12	19	10
M-19	0	15	20	11	20	11
M-20	0	13	17	12	22	0
M-23	0	13	16	13	23	0
M-24	0	16	19	12	19	12
M-26	23	13	19	20	16	0
M-27	0	14	18	13	19	10
M-29	0	14	19	12	18	11
M-30	0	12	19	12	20	12

AMP 10 is ampicillin, K 30 is Kanamycin, VA 30 is Vancomycin, PB 300 is Polymyxin-B, CD 2 is Clindamycin, P 10 is Penicillin-G The zone of inhibitions is in centimeters. The number followed by antibiotic abbreviation corresponds to the microgram concentration of that antibiotic. The standard error of all the means was found to be 0.33mm.

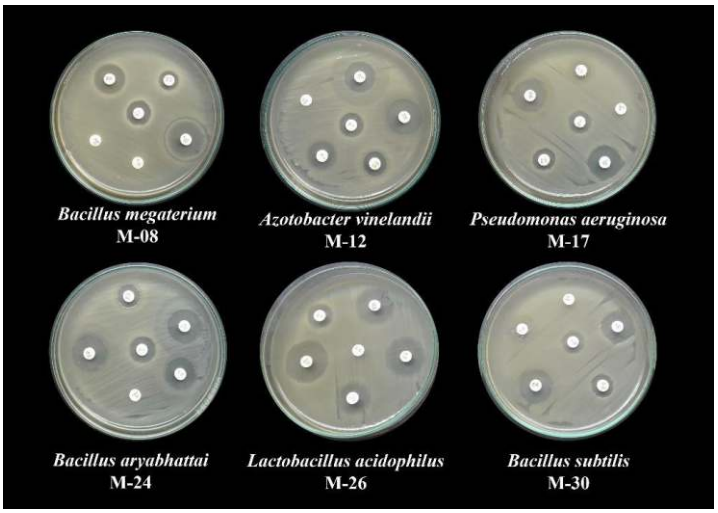

Fig. 4: Antibiotic resistance and susceptibility test of representative isolates against antibiotics

3.5 Bacterial selection based on compatibility

The shortlisted bacteria were quite antagonistic to each other and the only combination showing non-antagonistic growth was between *Bacillus megaterium* (M-08) and *Bacillus aryabhattai* (M-24) (Fig. 5). The rest of the bacteria provided nutrient competition, colour change or lytic zone at the junction of streaks on agar plates. Such antagonistic isolates were ruled out despite their high PSI or KSI. M-08 and M24 were both resistant to at least one antibiotic tested in this research and had considerable PSI and KSI along with IAA production potential. Most approaches in the preparation of biological control agents against plant pathogens include one antagonistic microbe [27;36]. Though antagonism is necessary for survival, it might probably hamper the synergy of an inoculum. This research encompassed the selection of cultures that maintain the traits to associate with the soil flora and thrive mutually, but also possess at least one antibiotic-selective trait.

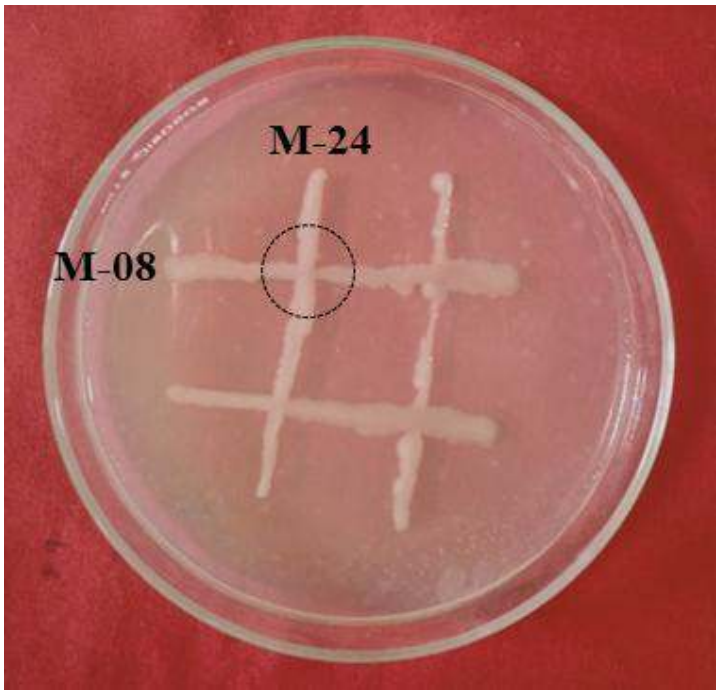

Fig. 5: Compatibility of M-08 and M-24, as shown by no zone of inhibition or cell death at the junction of two streaks shown in the dotted circle

3.6 Antifungal characteristic of the isolates against *Rhizoctonia solani*

The isolates M-08 and M-24 were found to show antibiosis and inhibited the growth of phytopathogenic *R. solani*. M-08 showed 36.36 % relative growth inhibition, whereas M-24 showed 40.0 %. Both the isolates restricted the pathogenic fungal growth. There was no mycoparasitism observed against the phytopathogen, nor did the fungus show any lytic activity against the bacterial isolates. [37-38] was quite fastidious and did matrix competition against the culture. This trait is quite common among pathogenic fungal isolates. Despite, the isolated bacterial cultures showed biocontrol activity.

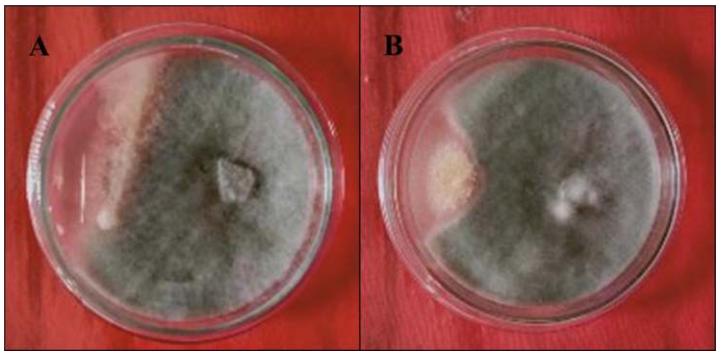

Fig. 6: Antagonistic activity of M-08 (in plate A) and M-24 (in plate 2) against phytopathogenic R. solani

3.7 Genetic identification of isolates M-08 and M-24 and evolutionary homology

The bacterial genomic DNA and 16S rRNA amplicon was found to be of optimal quality, as evident from Fig. 2. The amplicons were approximately 1500 bp in size. The BLAST homology of the top 10 sequences with that of the 16S rRNA amplicon of M-08 and M-24 is shown in Figure 7 and Figure 8 respectively.

The bacterial strain M-08 amplicon was registered to NCBI Genbank with accession number OP256573.1 The E value of both the sequences was 0, when compared to BLAST hits. The evolutionary history by the Maximum likelihood method shows a close evolutionary relationship of the query sequence with Accession number NR115953.1, registered as *Bacillus aryabhattai*. The evolutionary divergence between the sequences performed gave a low divergence (0.001) between NR115953.1and M-08. This supported the M-08 isolate to be *Bacillus aryabhattai*. A similar study with that of the 16S rRNA amplicon of M-24 is shown in figure 8. The evolutionary history shows evolutionary homology to accession number MT605458.1, registered as *Bacillus megaterium*. The BLAST hits for M-24 were more ascertaining the identification of the bacterial isolate.

A previous whole-genome study on [28;39-42] discovered a 5,403,026 bp chromosome, 5226 putative protein-coding sequences, 120 tRNA, 16 rRNA,58 non-protein coding genes,8 ncRNAs, and 11 prophage areas. The majority of the common genes linked with plant growth promotion capabilities were found to be present within core genes in all of the genomes utilised for comparison, suggesting that all *B. aryabhattai* strains may have comparable plant growth-stimulating characteristics. Sequencing of diverse *B. aryabhattai* and *B. megaterium* and its functional annotation is necessary to predict the predominance of mineral phosphate solubilization, siderophores, acetoin, butanediol, exopolysaccharides, and indole acetic acid production within this species. Based on the results of PSI and KSI data, isolate M-08 can be considered a novel strain for P and K solubilisation. In addition, the remaining isolates also had the promising potential for use as soil inoculants when compared to other isolates, though their antagonistic nature restricts their use in a consortium. These PSB isolates have promising future possibilities for sustainable agricultural practices with low chemical inputs and for organic farming. The production and use of biofertiliser formulations based on these rhizobacterial isolates in agricultural areas may improve soil fertility and crop productivity.

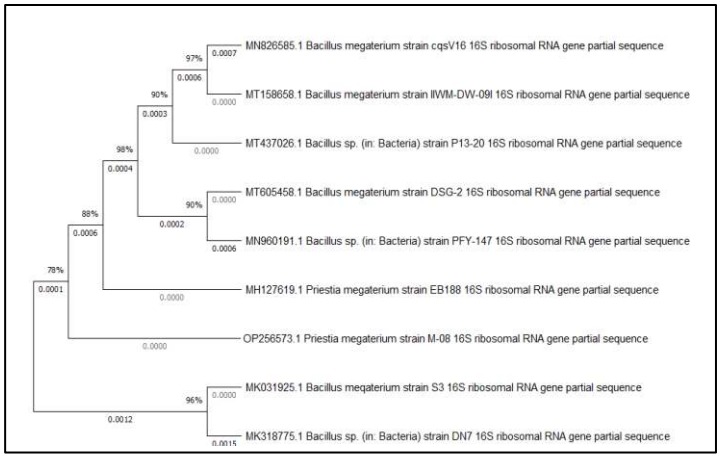

Fig.7: The evolutionary history of M-08 was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The 16S rRNA amplicon query sequence of M-24 (Accession number OP256573.1) shows close homology to to the clade identified as Bacillus megaterium)

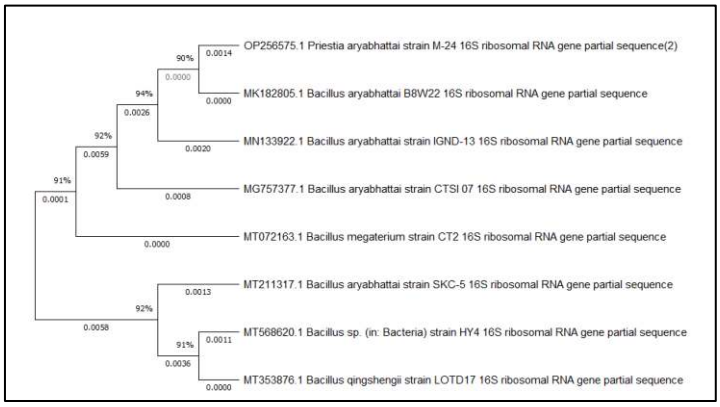

Fig.8: The evolutionary history of M-24 was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The 16S rRNA amplicon query sequence of M-08 (Accession number OP256575.1) shows close homology to clade which is identified as Bacillus aryabhattai.

3.8 Effect of inoculation of isolate M-08 and M-24 on N, P and uptake

The comparative analysis of macronutrient uptake with and without inoculation of the isolates M-08 and M-24 showed significant differences. The macronutrient uptake was least with the control plants, slightly higher with fertilized soil and best when treated with bacterial inoculum. The Tukey's HSD analysis proved that all the mean of N, P and K of different sets of control, RDF and inoculated plants were significantly different from each other. The nitrogen and potassium uptake were the highest with plants treated with M-24 (Figure 9). There was not much difference in phosphorus uptake of M-24 and M-08 treated plants as compared to plants grown on RDF. Yet, the difference was statistically significant. The field trial results proved the micronutrient solubilization and the potential to localize it in the plants.

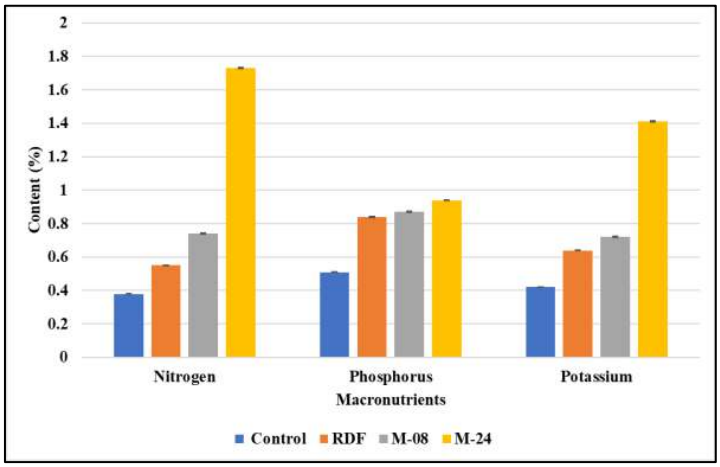

Fig. 9: Comparison of macronutrient uptake of plants treated with isolate M-08 and M-24, compared to control (no fertilizers used in soil) and RDF (Control with a recommended dose of fertilizer)tai.

4 Conclusions

The sample sites in Raichur regions showed bacterial isolates with quite promising phosphate and potassium solarization indices. *B. aryabhattai* and *B. megaterium* were discovered to be promising P and K solubilizing microorganisms with IAA production potential along with antibiotic resistance and compatibility with each other.

The isolates proved macronutrient solubilization potential, as well as on-field macronutrient uptake increase in plants. The research proved the prevalence of antibiotic resistance in most of the plant growth promoting bacteria from soil samples against one or two antibiotics selected in this research. Alongside, isolates showed promising traits to be biocontrol agents against pathogenic fungi. *B. aryabhattai* and *B. megaterium* showed antibiosis against phytopathogenic *R. solani*. The concept of antibiotic-selective plant growth promoting bacteria can help farmers to establish novel isolates in fields containing wild, fastidious and antagonistic pathogenic microbes. Antibiotic treatment of such soil would kill the pathogenic cultures, keeping our introduced isolates undamaged. This would eventually let only PGPB proliferate and reduce pathogenic microbial count from the soil. The effect of isolates on plant growth promotion or endophytism needs to be studied to evaluate its practical applicability. The study of differential expression of mRNA regulating the high PSI and KSI in these isolates, the sequences regulating the antibiotic resistance, eventual horizontal gene transfer of resistant traits in undesired microbes can provide greater depth in understanding of genetic regulation, evolution, and bacterial selections.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dr Madhavi Lunavath. The first draft of the manuscript was written by Dr. Madhavi Lunavath and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Preprint available on Research Square:
https://doi.org/10.21203/rs.3.rs-2364433/v1

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