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SCREENING AND CHARACTERIZATION OF POTENTIAL INDIGENOUS RHIZOBACTERIA ASSOCIATED WITH *Capsicum annuum* (chilli) IN MADHYA PRADESH INDIA

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ABSTRACT

Chilli (*Capsicum annum* L.) is an economically significant spice crop of Madhya Pradesh, as in India Madhya Pradesh is the second largest producer of chilli. Its productivity is basically restricted by anthracnose disease caused by *Colletotrichum* spp. Indigenous Plant Growth Promoting Rhizobacteria (PGPR) offers an eco-friendly approach to mitigate crop stress and improve plant growth. In this study, rhizospheric soil of chilli was collected from chilli-growing regions of Madhya Pradesh (Hoshangabad, Raisen, Bhopal and Khargone). A total of 153 morphologically diverse bacterial isolates were recovered. Isolates were evaluated for plant growth-promoting traits (IAA, Phosphate, Potassium and Zinc solubilization, Nitrogen fixation, HCN, Ammonia, Siderophore and Exopolysaccharide production), hydrolytic enzyme activities (Cellulase, Pectinase, Chitinase, Protease and Amylase) and *in vitro* antagonistic activity against four fungal phytopathogens of chilli (*Colletotrichum gloeosporioides*, *Colletotrichum capsici*, *Fusarium oxysporum and Rhizoctonia solani*) with significant biocontrol potential. Twenty (20) promising isolates showing multifunctional Plant growth-promoting (PGP) traits and antagonistic activity were shortlisted and further evaluated quantitatively for IAA, Phosphate and Siderophore production and their percentage inhibition against *C.capsici*. On the basis of this multi-trait evaluation, two isolates with composite profiles were selected for molecular identification through 16S rRNA sequencing and were identified as *Pseudomonas lini* (Isolate 08) and *Bacillus* sp. (Isolate 14); demonstrating the highest IAA (10.84 µg/mL) production and phosphate solubilisation (20.23 µg/mL) by isolate 08, and the highest inhibition of *C. capsici* 77.8% (Isolate 14) followed by 71.9% (Isolate 08), respectively. Both isolates were deposited at NCIM, Pune (accession numbers NCIM 5831 and NCIM 5832) and their sequences submitted to GenBank (OR140863 and OR140879). The results showed that indigenous rhizobacteria obtained from chilli rhizosphere of different chilli growing regions of Madhya Pradesh with multi-PGP and biocontrol potential making them a potential and promising eco-friendly bioinoculant to enhance chilli growth and disease resistance, providing sustainable alternative to chemical fertilizers and fungicides.

***Keywords*:** Chilli; PGPR; Rhizobacteria; Biocontrol; *Colletotrichum capsici*

Introduction

Chilli (*Capsicum annum* L.) is known to be a universal spice and vegetable which is being cultivated as a crop of Solanaceae family. There are various species of chilli plant being cultivated in the world and most of them are distributed in Asian countries like India, China Pakistan, Thailand and Africa. India has been found to be the world's largest producer, consumer and exporter of the red chillies (FAOSTAT, 2020). In India 0.0679 million tonnes and 1.389 million tonnes were the estimated total production of green and red chillies being cultivated in an area of 0.8 million hectares (Yadav *et al*., 2021). Chilli is grown in various part of India like Gujarat, Tamil Nadu, Maharashtra, Andhra Pradesh, Karnataka, Nagaland

and Madhya Pradesh, among them Madhya Pradesh itself contributes a total annual production of 574.80 thousand tonnes of green chilli, cultivated in 33.64 thousand hectares (Anonymous, 2017).

Chilli has been found to be rich in capsaicin, capsidiol, vitamin A, C and E, capsochrome, Folic acid, proteins and fibers (Yadav *et al*., 2023). Chilli plants are subjected to numerous abiotic stresses including heat, salinity, chilling and drought and biotic factors like bacterial, fungal, viral and nematodes causing diseases which deteriorate the quantity and economical quality of the production. The major biotic factors responsible for loss in production are due to *Fusarium* spp., *Colletotrichum* spp., *Rhizoctonia solani*, *Phytophthora capsici*, *Pythium* spp. etc. (Saxena *et al.*, 2016 and Dubey *et al.*, 2020). Among them, the major constraint for the cultivation of chilli crop is anthracnose disease (Fruit Rot) caused by *Colletotrichum* spp. (Than *et al.*, 2008) namely, *C. gloeosporioides*, *C. truncatum*, *C. acutatum*, *C. capsici* and *C. coccodes* belonging to the family Glomerellaceae of Ascomycota phylum (de Silva *et al*., 2019). In India itself an estimated loss of 10-54% in the yield of chilli crop has been reported due to dieback and fruit rot disease caused by anthracnose only (Lakshmesha *et al.*, 2005; Ramachandran and Rathnamma, 2006). For the management of anthracnose disease in chilli fruit frequent application of fungicide is recommended, which reduces the lesions progression caused by anthracnose hence increasing the crop yields, but on the other hand it leads to aggregation of fungicides ultimately affecting the quality of chilli fruits making it a major concern regarding this approach (Xavier *et al.*, 2014). The continuous use of such heavy doses of chemical compounds makes phytopathogens resistance to it and unsupportable to agricultural land and leading to environmental pollution as well. To minimize its effect, biological control method through microbial biocontrol agents (BCAs) namely PGPRs (Plant Growth Promoting Rhizobacteria)/ PGPMs (Plant Growth Promoting Microorganisms) is an alternative eco-friendly, profitable approach for the chemical control in the field (Oo and Oh, 2016) and for the management of diseases (Singh *et al*., 2012).

There are various known abiotic and biotic factors responsible for the growth of plant in any agricultural field. Co-existing presence of large number of microbes like bacteria, fungi, protozoa and algae in the immediate zone surrounding the roots of a plant i.e. Rhizosphere is the main factor responsible for many chemical and biological activities (Sivasakthi *et al*., 2014). Prolonged use of inorganic fertilizers causes extended effects like decrease/ depletion of soil fertility, soil acidification, air and water pollution (Sofyan *et al.*, 2019). To overcome with these consequences caused by the excessive use of inorganic fertilizers, alternative methods have been elicited to explore so as to reduce this concern. Plant Growth Promoting Rhizobacteria (PGPR) is an assorted group of rhizobacteria surrounding rhizospheric zone and interacting with the root of plant (Ahmad *et al*., 2008). Plant Growth Promoting Bacteria (PGPB) are the most abundant among them and mostly studied group of PGPB are PGPR presented at the root surface and in the soil associated with rhizosphere (Kloepper *et al*.,1999). PGPR's have been explored and found to be a good nitrogen fixer, potassium, phosphate and zinc solubilizer, various growth hormone like Indole-3-acetic acid (IAA) producer and siderophore producer (Othman *et al*., 2022). A diverse group of PGPR comprising *Pseudomonas, Bacillus, Azotobacter, Burkholderia, Enterobacter* and *Azospirillum* have been found to enhance the plant growth by various biological mechanisms either directly or indirectly by dealing with the stress factors for the host plant like pathogen tolerance and environmental stress tolerance (Ahemad and Kibret, 2014).

Phosphorus is involved in various metabolic activities required for the plant growth. Since it is already available in the form phosphate in soil due to use of chemical fertilizers (Vessey, 2003), but this phosphate forms complex with iron, aluminium and calcium ions making it unavailable to the plants (Havlin *et al.*, 2005). This could be converted to available form by various phosphate solubilizing rhizobacteria. Many PGPR helps in the production of phytohormone IAA (Indole-3-acetic acid), other than the host plant itself. This hormone plays vital role in lateral bud growth, vascular bundles differentiation and tropism responses (Son *et al.*,2014). Some PGPR can produce an iron chelating compound known as Siderophore which shows strong affinity for the ferric ions (Lankford, 1973), making it available to the plant roots from the adjoining environment as it can chelate unavailable form of iron (Loper and Buyer, 1991; Gyaneshwar *et al*., 1998). Along with this siderophores have the capability to restrict the growth of phytopathogens through biological control mechanism (Schippers, 1987). Many PGPRs have been found to control plant pathogens indirectly through production of hydrogen cyanide (HCN) and hydrolytic enzymes in the rhizospheric environment, thus mitigating the biotic stresses effect (Ribeiro and Cardoso, 2012).

Considering safety issues related to environmental health and restricting limits on the use of harmful

chemicals have focused the new safer and ecofriendly strategies approaches for the management of chilli plant stresses. Since last few decades, the use of PGPR as biofertilizer has been increased for the sustainable agriculture. Many studies in the different parts of world including India have shown that PGPR gives significant increase in the growth and yield of chilli plants and fruits respectively. Therefore, this study attempts to enlighten the benefits of Indigenous PGPR on isolating them from the chilli growing agro-climatic zones of Madhya Pradesh and screening the potential isolates under *in vitro* conditions so as to use them as bioinoculant for the future studies. Keeping these points in consideration, the objective of this study was aimed to (i) collection of samples from the different chilli growing zones of Madhya Pradesh (ii) isolation and screening of potential isolates from chilli plants based on Plant growth promoting traits and antagonistic activity against chilli phytopathogens under *in vitro* conditions (iii) molecular characterization and culture submission for the further studies.

Materials and Methods

Sample collection

Soil and root samples were collected from healthy and actively growing chilli fields across four chilli cultivating districts of Madhya Pradesh (Hoshangabad, Raisen, Bhopal and Khargone). Samples were collected from the rhizosphere zone at a depth of 10-15 cm using sterile tool from five randomly selected healthy plants and then pooled to form a composite sample. Samples were transported to the laboratory of Department of Microbiology, Barkatullah University in sterile polythene bags under cool conditions and were processed within 24-48 hrs.

Isolation of Rhizobacteria

Isolation of rhizobacteria was performed by serial dilution and spread plate technique. One gram of composite rhizospheric soil was suspended in 9ml sterile normal saline and then serially diluted up to 10^{-6} and aliquots of 0.1ml (100 µL) from appropriate dilutions were spread on nutrient agar (NA) plates to obtain bacterial isolates. Plates were incubated at 28±2°C for 24-48 hours. Morphologically distinct colonies were selected, purified through repeated streaking to obtain pure cultures and they were maintained on nutrient agar slants at 4°C and 20% glycerol stocks at -20°C for further analysis.

Morphological & Biochemical Characterization

Isolates were characterized on the basis of colony morphology such as colour, shape, elevation and margin. Gram staining was performed to classify them into gram-positive and gram-negative groups respectively. Biochemical characterization included Carbohydrate (Glucose, Sucrose, Fructose, Lactose, Dextrose, Mannitol, Arabinose, Mannose & Maltose) utilization test, Catalase, Oxidase were performed.

Screening for Plant-growth Promoting Traits-

IAA production

Isolates were grown in nutrient broth amended with 0.1% L-tryptophan for 48 to 72 hours. The cultural broth was centrifuged 10,000 rpm for 10 minutes. IAA was estimated colorimetrically using Salkowski's reagent (2% 0.5M $FeCl_3$ in 35% perchloric acid) in a cell-free supernatant at 530nm against a standard IAA curve (Brick *et al.*, 1991).

Phosphate solubilization

For qualitative screening, isolates were spot inoculated on Pikovskaya's agar media and incubated at 28°C for 3-7 days. Clear halo zones around the colony indicated phosphate solubilization. Semi-quantitative estimation was also done for the same by calculating Solubilization efficiency (SE). For the quantitative estimation of phosphate giving isolates, the cultures were grown in NBRIP broth at 28°C at 120rpm for 3-4 days. After centrifugation, the cell-free supernatants were subjected to stannous chloride reduction of the molybdo-phosphoric acid (600nm) (Mehta and Nautiyal, 2001).

Potassium solubilization

Isolates were spot inoculated on Aleksandrov agar media at 37°C for 7 days. Clear zones around the colony indicates solubilization (Meena *et al.*, 2015). The semi-quantitative estimation was done by calculating Solubilization Efficiency-

$$\text{Solubilization Efficiency (\%)} = \frac{Z - C}{C} \times 100$$

Z = Total diameter of the halo zone (colony + solubilization zone) in mm

C = Diameter of the bacterial colony in mm

Zinc solubilization

In modified Pikovskaya's agar media, isolates were spot inoculated and incubated at 37°C for 7 days. Clear halo zones around the colony indicates zinc solubilization. For semi-quantitative estimation Solubilization efficiency was calculated.

Nitrogen Fixation

To assess qualitatively, isolates were inoculated in Burk's N-free agar media (for free living Nitrogen Fixing Bacteria) at 28-30°C for 24-48 hours. Growth in

the media indicates that isolate can fix atmospheric nitrogen for their cell protein synthesis (Wilson and Knight, 1952).

HCN production

HCN production was assessed on nutrient broth amended with glycine (4.5g/L) and hanging a picric acid and sodium bicarbonate solution-soaked filter paper strips in the culture tubes at 28°C for 4-5 days, with a colour change from yellow to orange/brown indicates a positive result (Baker and Schippers,1987).

Ammonia production

Ammonia production was tested in peptone water using Nessler's Reagent 30°C for 72hrs, formation of brown yellow precipitate indicates positive result (Cappuccino & Sherman, 1996).

Siderophore production

For qualitative estimation, isolates were spot inoculated in Chrome Azurol S (CAS) agar for 48-72 hours at 28°C; formation of yellow-orange halo zones around colonies indicates siderophore production (Schwyn & Neilands, 1987). Semi-quantitative estimation was done by calculating SE. For quantitative estimation (Bharucha *et al.,* 2013), microbial isolates were grown in an iron-deficient liquid medium (Succinate medium) for 24–72 hours. Cell-free culture supernatant was combined with the freshly prepared, deep-blue CAS reagent. After incubation for 20-30 mins, OD was measured at 630 nm with an uninoculated blank mixed with CAS reagent as a blank control. Siderophore production was calculated by formula-

Siderophore production % = (Ar-As)/Ar x 100

where Ar = absorbance of reference (CAS solution and uninoculated broth), and As = absorbance of sample (CAS solution and cell-free supernatant of sample)

Exopolysaccharide (EPS) production-

Isolates were spot inoculated in Solid medium ATCC no.14 at 28°C for 7 days. Thick slime (mucoid) production indicates EPS production (Santi *et al*., 2008).

Hydrolytic enzyme activity

Cellulase, Pectinase, Chitinase, Protease and Amylase activities were determined on carboxymethyl cellulose (CMC) agar, Pectin agar, Chitin agar, Skim-milk agar and Starch agar media respectively. The plates were flooded with appropriate indicator/reagent (iodine for amylase) after incubation and the results were observed by the formation of zone of hydrolysis.

***In vitro* antagonism screening against phytopathogens of Chilli**

The dual-culture plate technique was used to assess antagonistic activity of selected rhizobacterial isolates on potato dextrose agar against four fungal phytopathogens (*Colletotrichum gloeosporioides, Colletotrichum capsici, Fusarium oxysporum & Rhizoctonia solani*) of chilli. Percentage inhibition of radial mycelial growth was calculated against uninoculated control by using formula-

I = (C-T) x 100/C (Gao *et al*., 2017)

where, I = % inhibition in mycelia growth;

C= Radial growth of the target pathogen (mycelial growth) in the control plate (measured from the centre of inoculation to the edge of the colony)

T**:** Radial growth of the target pathogen in the treatment/dual culture plate (measured in the direction of the antagonistic microbe).

Characterization of promising isolates and Molecular identification

Selection criteria for Promising Isolates

There were 20 isolates selected, showing positive results in various qualitative assays for plant growth promoting attributes, enzymatic characteristics and biocontrol potential against four fungal phytopathogens of chilli. Further selection of potential isolates was done on the basis of Quantitative analysis of different plant growth promoting attributes (IAA, Phosphate & Siderophore) and isolates showing maximum inhibition for *Colletotrichum capsici,* as this fungus was the major biotic stress concern for our study responsible for causing Anthracnose disease in the Madhya Pradesh region. On the basis of our analysis, we selected two potential isolates with multi-functional abilities.

16S rRNA Gene Sequencing and Phylogenetic Analysis

Selected isolates had undergone molecular identification through 16S rRNA gene amplification and sequencing. Phylogenetic analysis reveals the evolutionary relationships among the isolates and the software used to construct phylogenetic tree was MEGA 11. The cultures were submitted to general collection of Microorganisms of the NCIM at CSIR-National Chemical Laboratory (NCL), Pune and the sequence were also submitted to GenBank of NCBI which enables tracking of strain identity.

Results and Discussion

Isolation and Morphological diversity

A total of 153 morphologically distinct rhizobacterial isolates were recovered from the chilli rhizosphere soils sampled from the Hoshangabad (38 isolates), Raisen (34 isolates), Bhopal (20 isolates) and Khargone (61 isolates) regions of Madhya Pradesh. Colony morphology was found to be diverse widely in colour like white, yellow, orange, off white/cream, brown, pink and translucent, shape, margin and elevation (Table-1 & Fig. 1-A). On the basis of Gram reaction, both Gram-positive and Gram-Negative isolates were recovered, with a relatively higher proportion of Gram-negative bacteria. Out of 153 isolates, 65 isolates were Gram-positive and 88 were Gram-Negative (Table-1).

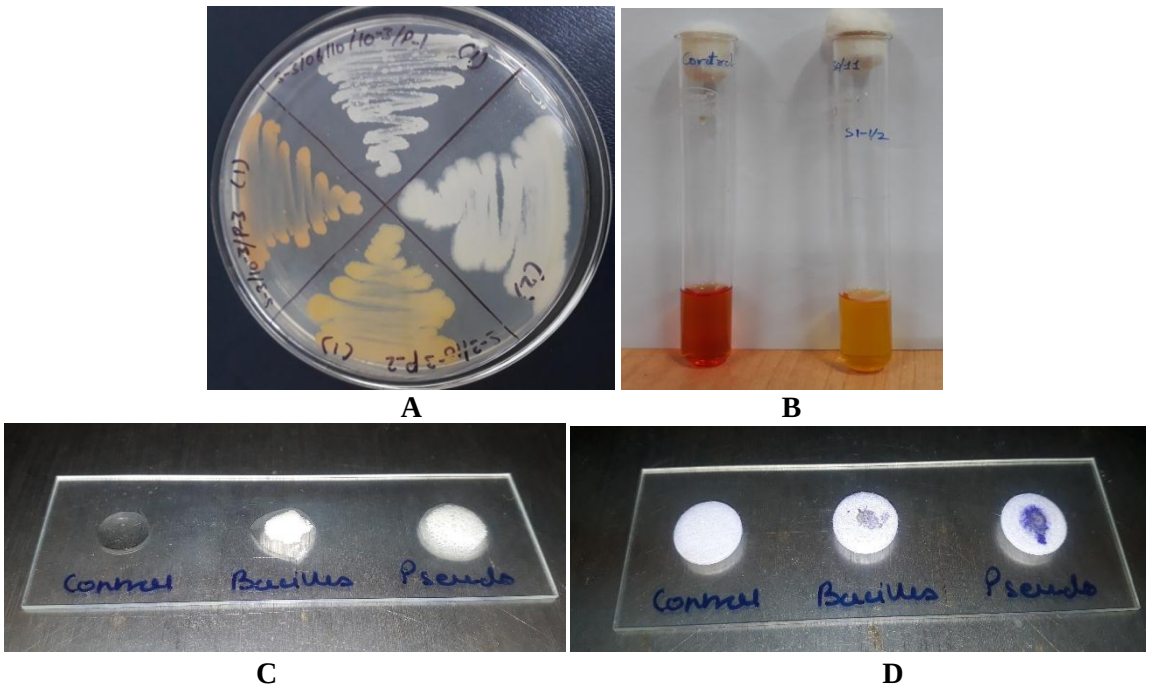


Fig. 1 : A-Rhizobacterial isolates purified colonies in Nutrient Agar Plates; B- Carbohydrate Utilization test; C- Catalase test; D- Oxidase Test

Table 1 : Morphological and Gram Staining Characteristics of Rhizobacterial Isolates

Characteristic	Category	Number of Isolates
Colony Colour	White	60
	Yellow	11
	Orange	10
	Off-white/Cream	35
	Brown	17
	Pink	10
	Translucent	10
Colony Shape	Filamentous	25
	Circular	38
	Irregular	90
Colony Margin	Entire	90
	Undulate	36
	Lobate	27
Colony Elevation	Convex	82
	Flat	43
	Raised	28
Gram Reaction	Gram-Positive	65
	Gram-Negative	88

Biochemical characterization of isolates

All isolates were subjected to a group of Carbohydrate utilization (Glucose, Sucrose, Fructose, Lactose, Dextrose, Mannitol, Arabinose, Mannose & Maltose) assays and biochemical tests including Catalase & Oxidase (Fig.1-B,C,D). The carbohydrate utilization profile obtained is summarized in Table-2. Oxidase and Catalase activity was observed in nearly 24% and 46% of the total isolates. These distinct traits suggested the considerable diversity within the culturable fraction of the rhizospheric community in chilli associated rhizobacteria.

Table 2 : Carbohydrate Utilization Test Result for Different Regions-

Sugar source	Regions				Percentage			
	Hoshangabad	Raisen	Bhopal	Khargone	Hoshangabad	Raisen	Bhopal	Khargone
Glucose	17	26	07	32	45%	77%	35%	52%
Sucrose	16	28	08	29	42%	82%	40%	48%
Fructose	09	21	08	18	24%	62%	40%	30%
Lactose	0	01	0	02	0	3%	0	3%
Dextrose	06	03	08	11	16%	9%	40%	18%
Mannitol	04	01	08	06	11%	3%	40%	10%
Arabinose	09	07	03	13	24%	21%	15%	21%
Mannose	17	05	14	25	45%	15%	70%	41%
Maltose	03	03	05	06	8%	9%	25%	10%

Plant growth-promoting (PGP) traits

IAA production

On qualitative screening of IAA production activity, development of pink colour indicates IAA production. Out of 153 isolates, 55 were found positive for the IAA production (Fig. 2-A) and its region wise percentage calculation is shown in Fig.3. Out of 20 selected isolates for quantitative estimation and further studies, 14 isolates were giving result for IAA quantitatively (Table-4,5 & Fig.4-A).

Phosphate solubilization

In qualitative screening for phosphate solubilization, development of clear zones surrounding bacterial colonies revealed on Pikovskaya's agar media, thereby confirming the conversion of insoluble inorganic phosphate tricalcium phosphate [$Ca_3(PO_4)_2$] into soluble available forms by the selected isolates. Out of 153 isolates, 69 were found positive for the same (Fig. 2-B) and its region wise calculation is being depicted in Fig.3. From the selected final 20 isolates, 16 isolates were calculated for phosphate solubilisation semi-quantitatively (Table-3) and quantitively (Table-4,5 & Fig. 4-B).

Potassium solubilization

The Potassium solubilization ability of rhizobacterial isolates was assessed on Aleksandrov agar media where insoluble potassium aluminium silicate converted into soluble potassium ions thus forming the clear halo zones surrounding bacterial colonies giving positive result for potassium solubilization (Fig. 2-C) and region wise percentage distribution of isolates is shown in Fig. 3. From 153 isolates, 21 were giving positive result and out of selected 20 isolates, 12 were giving potassium solubilization (Table-5), whose calculation is shown in Table-3.

Zinc solubilization

All 153 rhizobacterial isolates were assessed on modified Pikovskaya's agar media for the zinc solubilization assay (Fig. 2-D) and among them 20 isolates were found to be positive for it and its region wise assessment is shown in Fig.3. From the 20 selected isolates, 8 isolates were giving zinc solubilization (Table-5), whose SE calculation is shown in Table-3.

Nitrogen Fixation

Out of 153 isolates, 21 isolates had shown growth in the Burk's N-free agar media indicating the ability of isolates to utilize free nitrogen for the biological nitrogen fixation (Fig. 2-E), and the region wise distribution of rhizobacterial isolates giving positive result for nitrogen fixation is shown in Fig. 3. From the further 20 selected isolates, 19 isolates were showing their growth in the media (Table-5).

HCN (Hydrogen Cyanide) production

Among all 153 isolates, 9 were positive for the HCN production assay assuring the conversion of Glycine into Hydrogen cyanide and CO_2 by changing the colour of filter paper dipped in picric acid solution from yellow to orange brown (Fig. 2-F). Total of 8 isolates from the selected 20 rhizobacterial isolates were positive for HCN production (Table-5) and its distribution in all four regions of Madhya Pradesh is shown in Fig. 3.

Ammonia production

Rhizobacterial isolates produce ammonia in peptone water by enzymatic breakdown of amino acids and protein present in peptone which on reaction with Nessler's reagent produces brown yellow precipitate showing positive result for ammonia production (Fig. 2-G). Out of 153 isolates, 76 isolates were found to be positive for ammonia production and the percent calculation of distribution of these isolates in different

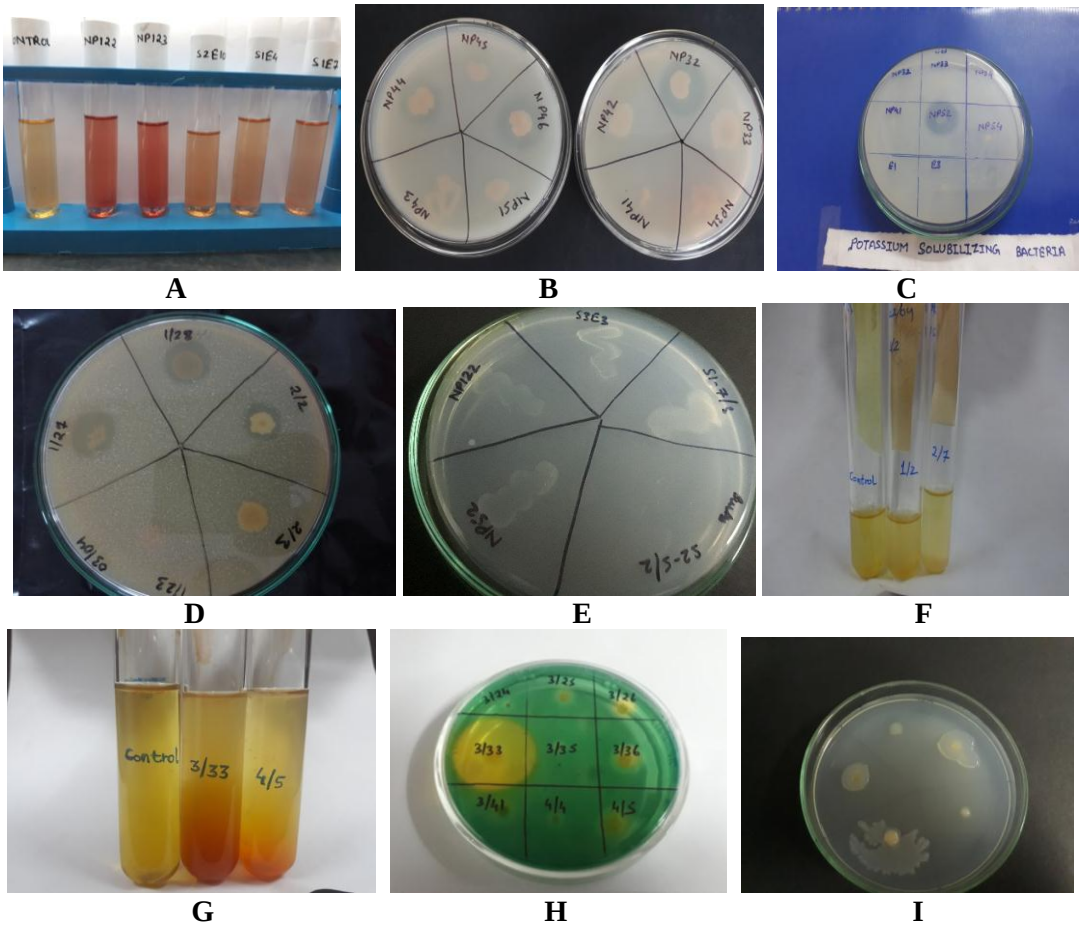
region is shown in Fig. 3. Among all 20 selected isolates, 14 were giving positive result for the same (Table-5).

Siderophore production

In qualitative screening, 118 isolates from all 153 isolates were giving positive test for Siderophore production (Fig. 2-H) where siderophore produced by isolates chelates a pre-existing dye complex and converting its colour from blue to orange or yellow zone around the bacterial colony. Among selected 20 isolates, 19 were positive for siderophore production and their semi-quantitative and quantitative estimation is calculated and is shown in Table 3,4 and Fig. 4-C respectively. The distribution of siderophore producing isolates in different regions is shown in Fig. 3.

Exopolysaccharide production (EPS)

Out of 153 isolates, 16 isolates had potential to produce EPS in Solid medium ATCC no.14 media by the development of thick slime (mucoid) indicating positives test for the same (Fig. 2-I). From the selected 20 isolates, 13 rhizobacteria were showing mucoidal growth in the media confirming the assay (Table-5). Percent distribution of all 16 positive isolates in different region is shown in Fig. 3.



A **B** **C**

D **E** **F**

G **H** **I**

Fig. 2 : Plant Growth Promoting attributes (A-IAA; B-Phosphate; C-Potassium; D-Zinc; E-Nitrogen fixation; F-HCN; G-Ammonia; H-Siderophore; I-EPS)

Table 3 : Solubilization Efficiency of selected 20 isolates for the Semi-Quantitative estimation

Isolates / PGP Attributes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Phosphate (%)	91.7	84.6	88.7	72.7	93.4	101.3	99.7	160.6	140	74	-	102	-	79.6	72	88	-	109.8	134.5	-
Potassium (%)	188	256	-	-	187	223.4	204.7	243.6	274	57.8	-	229.8	-	-	-	221	-	111.1	273.2	-
Zinc (%)	-	-	80.4	70.6	56.6	53	40	79.8	44.2	-	-	-	-	-	-	-	-	56.8	-	-
Siderophore (%)	85.7	88.9	89.1	-	94.8	84.9	86.9	98	86.6	96.6	80.3	82.4	77.6	95.6	81	85.2	75	82.8	83	81

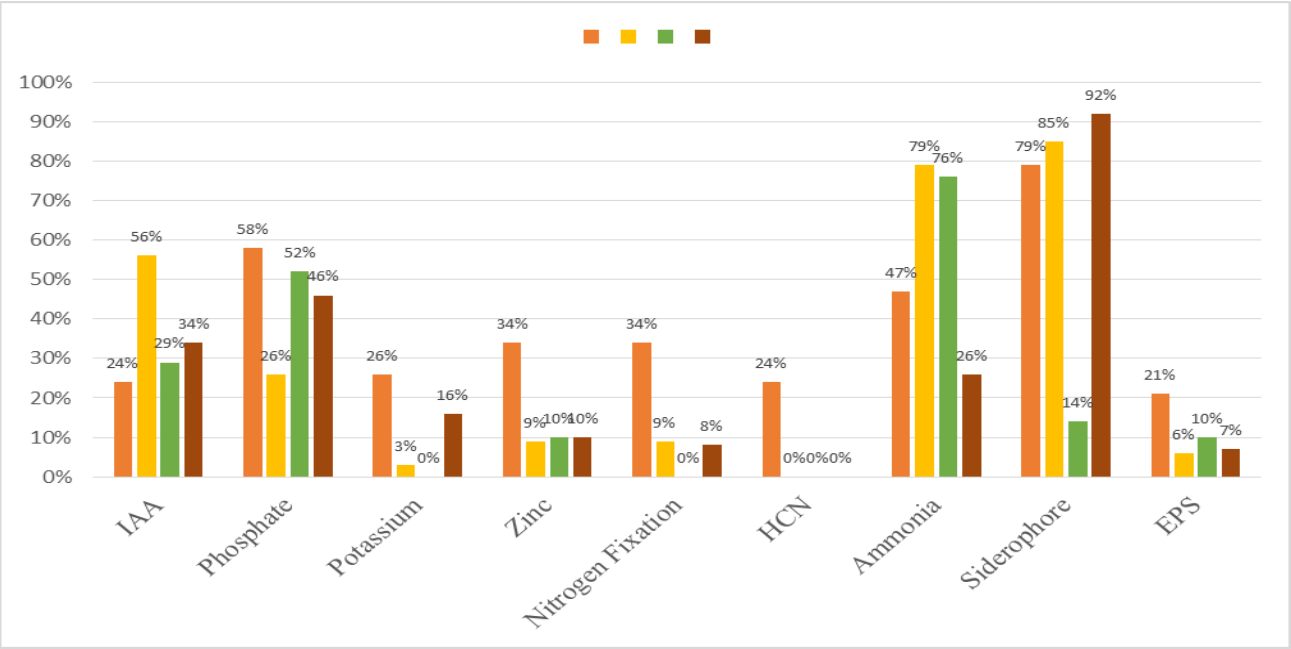
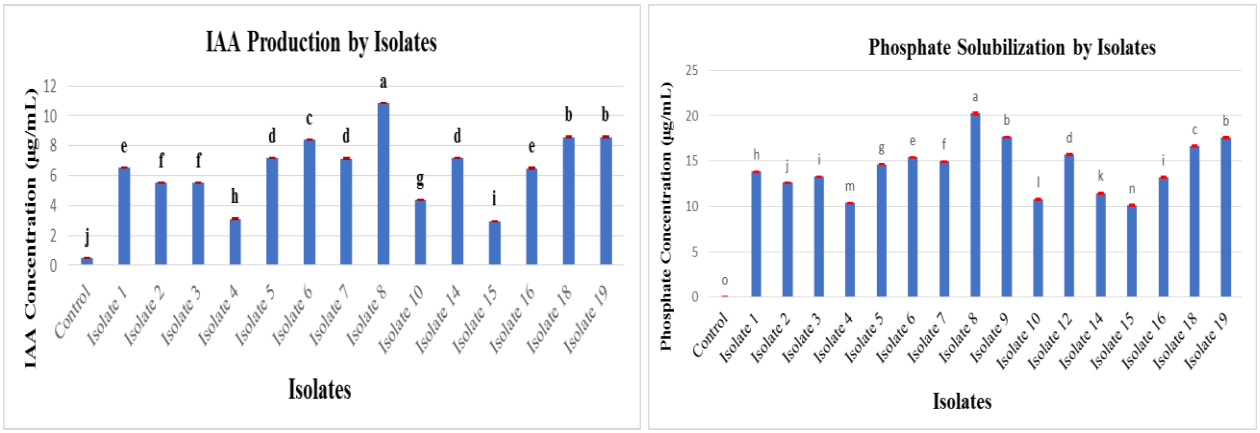


Fig. 3 : Comparative Analysis of PGP Attributes given by the total isolates from different regions of Madhya Pradesh



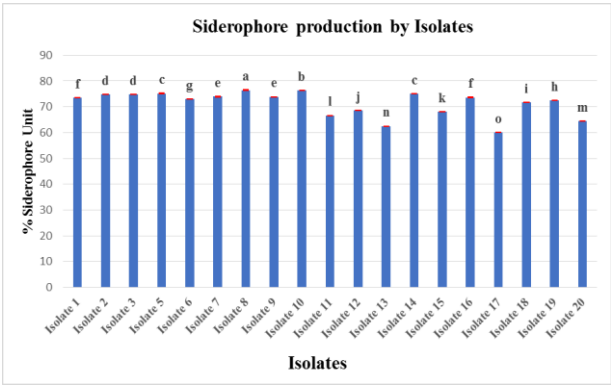
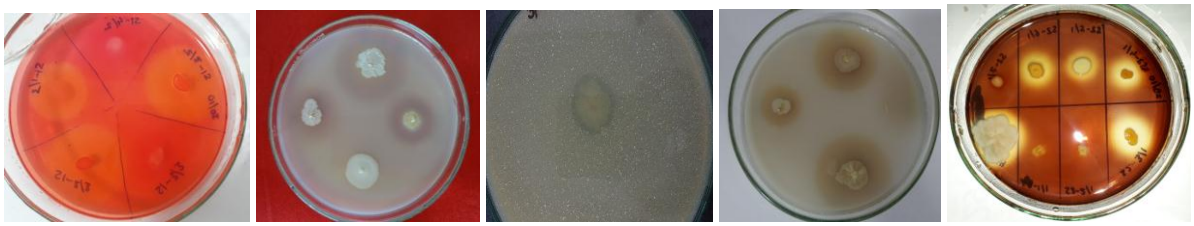


Fig. 4 : Quantitative estimation (A-IAA; B-Phosphate; C-Siderophore) by selected isolates. Values in each column are the means of three replications ± standard error (SE). Statistical analyses were performed using the SPSS version 32.0 statistical package (IBM SPSS, USA) where, different letters indicate significant difference p≤0.05

Hydrolytic enzyme activity

On qualitative screening of Cellulase, Pectinase, Chitinase, Protease and Amylase, development of clear zones surrounding the bacterial colonies indicates positive test for cellulase activity in CMC agar where zone appears on addition of Congo red stain, pectinase

activity by clear zone formation in pectin agar, clear zone around colony appeared due to chitin degradation in Chitin agar, protease activity by the formation of clear zone in Skim- milk agar and amylase activity by the formation of colourless zone around colony, which appears on addition of iodine solution on the starch agar media. Out of 153 rhizobacteria 35, 20, 08, 102 and 94 were positive for Cellulase, Pectinase, Chitinase, Protease and Amylase respectively (Fig.5). From 20 selected isolates 06, 06, 07, 11 and 06 were positive for Cellulase, Pectinase, Chitinase, Protease and Amylase (Table-5).



A **B** **C** **D** **E**

Fig. 5 : Enzymatic activity (A-Cellulase; B-Pectinase; C-Chitinase; D-Protease; E-Amylase)

Table 4 : Quantitative estimation of IAA, Phosphate & Siderophore. Values in each column are the means of three replications ± standard error (SE).

Isolates PGP Attributes	Con-trol	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
IAA (µg/mL)	0.48± 0.005j	6.54± 0.145e	5.53± 0.03f	5.51± 0.01f	3.13± 0.011h	7.16± 0.01d	8.39± 0.032c	7.15± 0.015 d	10.84 ± 0.041a	-	4.37± 0.047 g	-	-	-	7.16± 0.01d	2.96± 0.006i	6.5± 0.02e	-	8.58± 0.049 b	8.59± 0.026 b	-
Phosphate (µg/mL)	0.03± 0.025 o	13.82 ± 0.056 h	12.61 ± 0.061j	13.28 ± 0.062i	10.38± 0.068 m	14.63 ± 0.07g	15.41 ± 0.047e	14.93 ± 0.053f	20.23 ± 0.095a	17.63 ± 0.078 b	10.76 ± 0.090l	-	15.7± 0.07d	-	11.48 ± 0.091 k	10.14 ± 0.076 n	13.21 ± 0.090i	-	16.65 ± 0.082c	17.58 ± 0.092 b	-
Siderophore (%)	-	73.57 ± 0.021f	74.73 ± 0.021 d	74.74 ± 0.031 d	-	75.19 ± 0.03c	72.93 ± 0.03g	73.94 ± 0.021e	76.50 ± 0.035a	73.86 ± 0.02e	76.36 ± 0.04b	66.65 ± 0.03l	68.56 ± 0.021j	62.48 ± 0.015 n	75.11 ± 0.021c	68.05 ± 0.025 k	73.65 ± 0.12f	73.65 ± 0.12o	71.77 ± 0.064i	72.44 ± 0.005 h	64.54 ± 0.01m

Table 5 : Plant Growth Promoting attributes, Biochemical and Enzymatic activity by Selected Isolates

Isolates / PGP/ Biochemical & Enzymatic Attribute	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
IAA	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	+	-	+	+	-
Phosphate	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-
Potassium	+	+	-	-	+	+	+	+	+	+	-	+	-	-	-	+	-	+	+	-
Zinc	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-
Nitrogen fixation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
HCN	+	-	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Ammonia	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-
Siderophore	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EPS	+	-	-	+	+	-	-	+	-	+	+	-	+	+	-	+	+	+	+	+
Catalase	+	+	+	-	+	-	-	+	-	+	-	+	+	+	-	+	-	+	+	+
Oxidase	+	+	+	+	+	-	+	+	-	-	-	-	-	+	-	+	-	-	+	-
Cellulase	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	+	-	+
Pectinase	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	+	-	-	+
Chitinase	+	-	-	-	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+
Amylase	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	+

Antagonistic activity against fungal pathogens of Chilli

Dual culture assay identified potential rhizobacterial isolates inhibiting major four fungal phytopathogens of chilli (*Colletotrichum gloeosporioides, Colletotrichum capsici, Fusarium oxysporum & Rhizoctonia solani*) (Fig.6-A,B,C). Out of 153 isolates, 37 isolates were found to be antagonistic against *Colletotrichum gloeosporioides*, 25 against *Colletotrichum capsici*, 24 against *Fusarium oxysporum* and 27 against *Rhizoctonia solani* respectively. Percentage inhibition of radial mycelial growth was calculated for our concerned phytopathogen i.e. *Colletotrichum capsici* (Fig.6-D)

and out of selected 20 isolates 08 isolates were giving inhibition against *C.capsici* (Table-6).

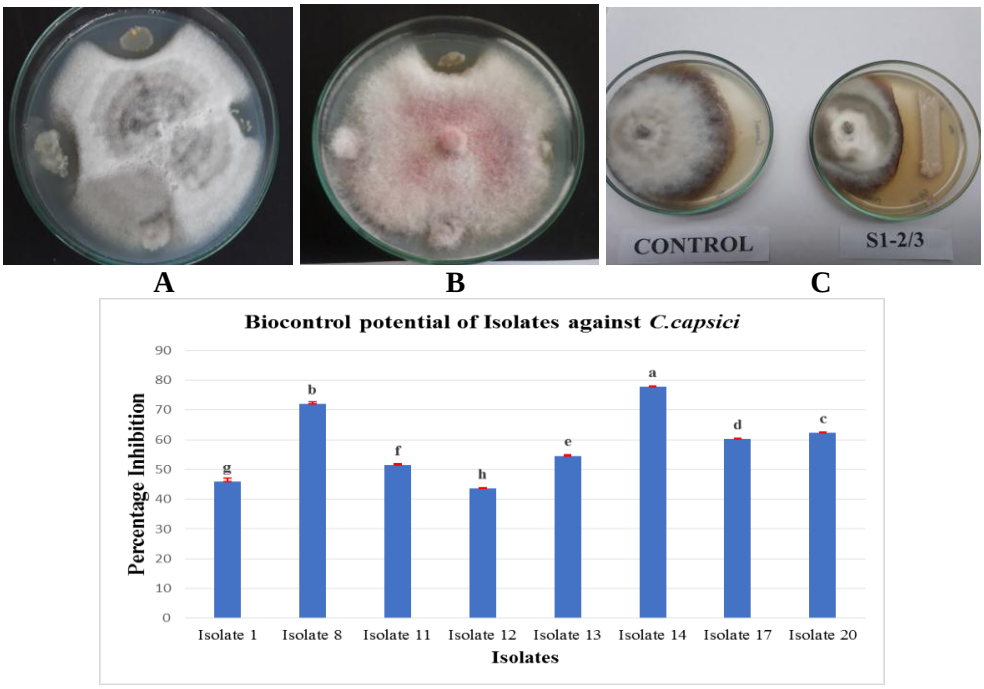


Fig. 6 : Antagonistic activity of Isolates against Phytopathogens (A-*C.capsici*; B-*F.oxysporum*), Dual culture assay (C- Inhibition of *C.capsici* by Rhizobacterial Isolate) & D-Percentage inhibition of *C.capsici* by selected isolates. Values in each column are the means of three replications ± standard error (SE) where, different letters indicate significant difference p≤0.05

Table 6 : Percentage (%) inhibition of *C. Capsici* by selected isolates. Values in each column are the means of three replications ± standard error (SE)

Isolates	1	8	11	12	13	14	17	20
% Inhibition for *C. capsici*	45.9± 1.159g	71.9± 0.833b	51.4± 0.503f	43.5± 0.208h	54.5± 0.503e	77.8± 0.208a	60.2± 0.252d	62.3± 0.3c

Molecular Identification and Phylogenetic analysis

Out of 20 isolates, 02 isolates (Isolate no. 08 & 14) were selected for the molecular identification. Molecular identification of the selected two isolates was done by amplification and sequencing of 16S ribosomal DNA. Two isolates were identified as, Isolate No.8- *Pseudomonas lini* and Isolate No. 14- *Bacillus sp.* respectively. The cultures were submitted to general collection of Microorganisms of the NCIM, Pune with accession number NCIM 5831 and NCIM 5832. The sequences were also submitted to Genbank with accession number OR140863 and OR140879 for *Pseudomonas lini* and *Bacillus sp.* respectively. Phylogenetic tree of both selected isolate 08 and 14 based on its 16S rRNA Sequencing is given in Fig. 7.

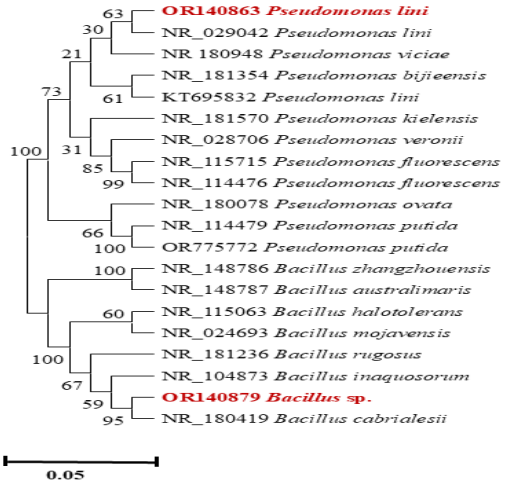


Fig. 7 : Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of bacterial isolates with closely related species retrieved from NCBI database. The tree was constructed using the neighbour-joining method with bootstrap analysis (1000 replicates). Bootstrap values (>50%) are indicated at branch nodes. Isolates OR140863 and OR140879 are highlighted in the phylogenetic tree.

Pseudomonas lini and *Bacillus* species form distinct clusters indicating their evolutionary divergence.

Discussion

The present study successfully surveyed, isolated and characterised indigenous rhizobacterial strains from the chilli rhizosphere across four chilli growing zones of Madhya Pradesh and recovered a culturable community of 153 morphologically, biochemically and functionally diverse rhizobacterial isolates. The predominance of Gram-negative isolates over Gram-positive is consistent with reports that Gram negative genera such as *Pseudomonas* and *Enterobacter* likely to dominate the rhizosphere fraction of vegetables and spice crops, making them grow faster and with greater adaptability on standard nutrient media (Sarma *et al.*,2014). The broad diversity in colour, shape, margin and elevation, along with the different types of carbohydrate utilization and biochemical profiling of catalase, oxidase listed among the four regions, indicates a heterogeneous rhizobacterial community shaped by local soil-based conditions but not by a single dominant taxon.

The screening of plant growth-promoting traits demonstrated that a large proportion of isolates possess multifunctional potential including IAA production, phosphate, potassium solubilization, nitrogen fixation, siderophore production and hydrolytic enzyme activity. Among them, siderophore production (118/153) and ammonia production (76/153) were the widespread activities, followed by phosphate solubilisation (69/153) and IAA production (55/153), while nitrogen fixation, HCN production, zinc solubilisation, potassium solubilisation and EPS production were comparatively less frequent. These traits all together contribute to enhance nutrient availability and reformed root development. The co-existence of various PGP traits within the same isolates, as in isolates 8 and 14 in Table-5, supports the point that multifunctional rhizobacteria are likely to translate into consistent field-level growth promotion than isolates possessing only a single activity, since the different mechanisms (nutrient solubilisation, phytohormone synthesis, iron scavenging and pathogen suppression) are expected to act complementarily on the host plant (Eshaghi *et al.*, 2024). Quantitative estimation confirmed that the highest amount of IAA (10.84 µg/mL) and phosphate solubilisation (20.23 µg/mL) is produced by Isolate 8 along with the highest siderophore production (76.50%).

In hydrolytic enzymes assays, protease (102/153) and amylase (94/153) activities were the most prevalent, whereas chitinase (8/153) was less, even though chitinase is one of the important enzymes most directly involved in the lysis of fungal cell walls. Therefore, it suggests that antifungal activity in the isolate collection is likely to be driven by combination of siderophore-mediated iron competition, ammonia /HCN production and cell-wall-degrading enzymes acting together, rather than by chitinase alone (Ashwini and Srividya, 2014). In the dual-culture assay, isolates varied in their ability to suppress the four chilli phytopathogens tested, with the highest inhibition of *Colletotrichum capsici* recorded for isolate 14 (77.8%) followed by isolate 8 (71.9%), the same two isolates that also ranked highest for the combined PGP and enzymatic profile in Table-5. This convergence between plant growth-promoting potential and antagonistic activity in the same isolates is a desirable outcome for bioinoculant development, since it indicates that a single strain could, in principle, deliver both a nutritional/hormonal benefit to the chilli plant and a measure of protection against anthracnose, rather than requiring separate strains for each function (Ashwini and Srividya, 2014; Jayapala *et al.*, 2019).

Molecular identification placed isolate 8 as *Pseudomonas lini* and isolate 14 as a *Bacillus* sp., both genera that are very widely reported as effective plant growth-promoting rhizobacteria and biocontrol agents in a range of crops. The recovery of these genera from the chilli rhizosphere of Madhya Pradesh, coupled with their comparatively strong performance across the PGP, enzymatic and antagonism assays used here, is in line with the broader literature identifying *Pseudomonas* and *Bacillus* as two of the most consistently useful genera for PGPR-based biofertilizer and biocontrol formulations (Guzmán Guzmán and Santoyo, 2022). Depositing these two isolates in the NCIM culture collection and depositing their sequences in GenBank provides a traceable reference point for these strains and a basis on which they, or other isolates from this collection, can be taken forward for pot and field-level validation.

The results collectively support the application of indigenous PGPR as bioinoculants specifically adapted to the agro-climatic conditions for chilli cultivation in Madhya Pradesh. The chosen rhizobacteria possess multifunctional characteristics that not only boost plant growth but also offer sustainable control of phytopathogens, aligning with the objectives of environmentally friendly and sustainable agriculture.

Conclusion

This study successfully isolated and characterized a diversified population of native rhizobacterial isolates from rhizosphere of chilli from Hoshangabad,

Raisen, Bhopal and Khargone regions of Madhya Pradesh. Most of them were having combined PGP traits like IAA, Phosphate, Potassium and Zinc solubilization, Nitrogen Fixation, Siderophore and Ammonia production, while some of them were possessing capability of HCN production, along with hydrolytic enzymes production. Isolates were found to have good antagonistic activity against four major fungal pathogens of chilli, including *Colletotrichum gloeosporioides*, *Colletotrichum capsici*, *Fusarium oxysporum* and *Rhizoctonia solani*. Screening of all 153 isolates compressed to 20 promising isolates and finally to two multi-trait isolates, identified as *Pseudomonas lini* (isolate 8) and a *Bacillus* sp. (isolate 14) respectively. Both of them were recorded with strong IAA and Phosphate solubilization along with highest inhibition of anthracnose causal organism *C. capsici* in this study.

The present study demonstrates that integration of such indigenous rhizobacteria from the chilli growing regions of Madhya Pradesh can reduce dependency on chemical fertilizers and could be a resource for developing eco-friendly approaches like biofertilizers thus reducing environmental pollution. Deposition of both isolates 8 and 14 in NCIM, Pune culture collection and submission of their 16S rRNA sequences to GenBank makes it available for further studies. Future work will be focused on field and greenhouse evaluations of these isolates along with development of formulations suitable for their use as a practical bioinoculant for cultivation of chilli.

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Conflicts of Interest

The authors declare no conflict of interest.

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