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FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF WHEAT RHIZOSPHERE BACTERIA AND THEIR ANTAGONISTIC ACTIVITY AGAINST WHEAT FOLIAR BLIGHT PATHOGENS

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ABSTRACT

The current study aimed to explore the wheat rhizospheric bacterial community for *in vitro* plant growth-promoting (PGP) traits and antagonistic activity against foliar blight disease of wheat caused by *Alternaria triticina* and *Bipolaris sorokiniana*. The soil samples from the wheat fields across four Indian states namely Uttarakhand (2 sites), Uttar Pradesh, Madhya Pradesh, and Maharashtra were analyzed for their physicochemical properties, enzymatic activities, and bacterial population density (CFU g⁻¹). Amongst the tested soil samples, field soil from Uttar Pradesh has the highest bacterial population density (2.5x10⁷ CFU g⁻¹) while the Uttarakhand (Almora) soil has the lowest (8.5x 10⁵ CFU g⁻¹). A total of 45 bacterial isolates recovered from all the sites were morphologically identified and screened for *in-vitro* solubilization of phosphate & zinc, production of ammonia, siderophore, chitinase, protease, cellulase, amylase, lipase, and antagonistic activity. All isolates were found positive for one or more tested functional traits. Amongst 45 isolates, six showed >50% inhibition of *Alternaria triticina* and *Bipolaris sorokiniana* mycelium, and three isolates P10, UP11 & MH13 exhibited antagonistic activity against both the tested phytopathogens. Through 16S rDNA sequencing six putative biocontrol isolates, P10, UP11, MH13, MP17, MH12, and MP14 were identified as *Bacillus methylotrophicus* (MN099430.1), *Bacillus subtilis* (MN099431.1), *Bacillus sp.* (MN099432.1), *Streptomyces sp.* (MN099435.1), *Lysinibacillus sp.* (MN099433.1), and *Staphylococcus epidermidis* (MN099434.1). The selected wheat rhizobacteria exhibited PGP traits and biocontrol potential hence, may serve as putative biocontrol agents, for the management of foliar blight disease of wheat.

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1 Introduction

Wheat (*Triticum aestivum* L.) is one of the principal crops for nearly 2.5 billion population world over with a total harvested area of 220 million hectares (Ramadas et al., 2019). In India, it is the second largest food crop after rice with 30 million hectares of cultivable area (Mahajan et al., 2017). To provide food security to the ever-increasing population, there is an imminent need to increase annual wheat production from the current 642 million tonnes to 840 million tonnes by the year 2050 (Sharma et al., 2015). The production of wheat is constrained by several biotic and abiotic stresses (Ali & Baek, 2020). The warmer and humid regions of the world are mainly affected by fungal diseases. Among them, leaf blight caused by *Alternaria triticina* and spot blotch caused by *Bipolaris sorokiniana* (Sacc. in Sorok) are the two most devastating foliar blight diseases in warm and humid regions of India and other South Asian countries (Joshi & Chand, 2002). In India, it is a major biotic constraint to wheat production in the Eastern Gangetic Plains encompassing the major wheat producing regions. Occasionally, in these regions, it occurs as a complex of leaf blight (*Alternaria triticina*) and spot blotch (*Bipolaris sorokiniana*) and is collectively referred to as foliar blight complex (Verma et al., 2020). Globally an estimated 25 million hectare of the wheat area is affected by the foliar blight of which, 40 % area falls into the Indian subcontinent. The average annual crop losses are estimated to be 2-3 million tonnes (Gupta et al., 2018).

At present, the management of foliar blights is dependent on the seed treatment and foliar application of triazole group of fungicide such as tebuconazole, epoxiconazole, and propiconazole (Acharya et al., 2011). Despite the effectiveness of fungicides, these chemicals cause harmful effects, including disruption in soil microbiota, deteriorate soil quality, and environmental pollution (Li et al., 2015; Zhou et al., 2016). In this sense, biological control is a promising sustainable alternative that involves the use of plant beneficial microbes (Nafiu et al., 2016). The rhizosphere possesses a diverse group of plant beneficial microorganisms and also offers a quintessential habitat for the plant-microbe interactions. The complex plant-microbe interactions such as nutrients cycling, production of various plant growth promoting compounds, protection from phytopathogens by competing for the iron, production of antibiotics, lytic enzymes, detoxification and degradation of pathogen virulence factors improve the plant health and reduces the disease severity (Kaur et al., 2019; Kalam et al., 2020). Therefore, rhizobacteria with Plant growth promoting as well as antifungal enzyme production ability make them a suitable candidate for biocontrol agents. Many bacterial genera such as *Paenibacillus*, *Pseudomonas*, *Burkholderia*, *Lysobacter*, and *Bacillus* are considered as biocontrol agents since they produce a range of plant growth promoting compounds and antimicrobials

(Gouda et al., 2018; Villarreal-Delgado et al., 2018). Moreover, few studies have been reported the biological control of foliar blights under laboratory conditions. For instance, *Bacillus* sp. TSO2 (Villa-Rodriguez et al., 2019), and *Chaetomium* spp. (Yue et al., 2018) have been studied as potential bacterial antagonists against *B. sorokiniana* under *In vitro* conditions. Similarly, fluorescent pseudomonads and *Bacillus* spp. were found effective against *A. triticina* (Siddiqui, 2007). Besides this, till now no effective biocontrol agents have been commercialized against foliar blight pathogens. Hence, exploration of the diverse potential biocontrol strains that can sustain at different environmental conditions and soil type is a widely studied area of research for sustainable agriculture. Thus, the present study was aimed to explore the rhizospheric bacterial community for efficient bacterial antagonists against the foliar blight Pathogens/complex and to identify and characterize the putative bacterial antagonists through biochemical characteristics and 16S rDNA sequencing.

2 Materials and methods

2.1 Collection of soil Samples

The rhizospheric soil from wheat plants grown in farmer's fields were collected in November, 2018 from two sites at Uttarakhand i.e Almora (29° 35' 39.08" N and 79° 39' 14.01" E, elevation 1,610 m) and Pantnagar (29°1'15.74"N and 79°29'23.06"E, elevation 236.54 m) and one site at Uttar Pradesh (27° 54' 1.37" N and 78° 4' 20.21" E, elevation 191 m), Madhya Pradesh (24° 38' 60.00" N and 77° 19' 12.00" E, elevation 479 m) and Maharashtra (19° 59' 50.83" N and 73° 47' 23.29" E, elevation 583 m). The soil was collected from the rhizospheric zone at about 0-15 cm depth from ten randomly selected wheat plants. Subsequently, the soil from all plants was pooled to make a composite sample, put into sterile poly bags, and transferred to the laboratory in an icebox to isolate the bacteria.

2.2 Estimation of soil physicochemical properties and enzyme activities

The soil was dissolved in distilled water (DW) in the ratio, 1:5, and pH determined with century pH meter (model CP 931) (Miller & Donochue 1990). Electrical conductivity (EC) of soil samples was assessed in 1:25 soil to water solution at 25°C with a digital microprocessor-based conductivity meter (Systronic Model 306). Organic carbon (OC) was determined by the Walkley-Black titration method (Walkley & Black 1934) based on the oxidation of dichromate ion. Excess dichromate ion is back titrated with ferrous ion. Total N (TN) was estimated by the Kjeldahl digestion method (TKN). Available phosphorus (AP) content was measured colorimetrically after extraction with 0.5 mol l⁻¹ NaHCO₃ (pH 8.5) for 30 minutes (Olsen et al., 1954). Available potassium (AK) content was determined by flame photometer after extraction with

1 mol NH₄Ac (pH 7.0) for 30 minutes (Yuan, 1983). Minor trace elements like Fe, Zn, Ca, Mn, and Mg were measured by atomic absorption spectrometry (AAS) (Yao et al., 2003).

Also, the functional potential of the soil microbial communities inhabiting wheat rhizosphere was monitored through soil enzyme activities such as dehydrogenase (EC 1.1.1.) (DHA), fluorescein diacetate (FDA), acid phosphatase (EC 3.1.3.2) (ACP), alkaline phosphatase (EC 3.1.3.1) (ALP) and urease (EC 3.5.1.5) (URE). These enzymes activity was estimated through a spectrophotometer and compared with a standard curve. All assays were conducted in triplicates. DHA activity was assessed by Thalmann (1968). FDA activity was measured as described by Inbar et al. (1991). ACP and ALP activities were estimated by following the method of Tabatabai & Bremner (1969). URE activity was assayed by following the method given by Kandeler & Gerber (1988).

2.3 Isolation of rhizobacteria

Isolation of bacteria was carried through the conventional serial dilution method using Nutrient agar (NA) (composition g⁻¹: Peptone 5g, beef extract 1g, yeast extract 2g, sodium chloride 5g, and agar 15g) (Majeed et al., 2015). Soil samples were serially diluted in normal saline and plated on NA. Plates were incubated at 30±2°C for 12h. Bacterial colonies were counted and represented as colony forming unit (CFU g⁻¹) per gram of soil. A total of forty five isolates with distinct colony morphology were purified by re-streaking on NA plates.

2.4 *In vitro* antagonism against *A. trititica* and *B. sorokiniana*

A total of 45 rhizobacterial isolates were screened against two foliar blight pathogens through dual culture plate assay. Test fungal pathogens *A. trititica* (ITCC 1186) and *B. sorokiniana* (ITCC 4869) were collected from the Indian Type Culture Collection (ITCC), IARI, New Delhi, India. Fungal pathogens were cultured in potato dextrose agar (PDA) at 25±2°C for 5-7 days. A dual culture assay was performed against *A. trititica* and *B. sorokiniana* strain separately in half-strength NA-PDA medium. Agar plugs (0.5cm diameter) containing *A. trititica* and *B. sorokiniana* mycelia separately were placed at the center of half-strength NA-PDA plates. Bacterial strains were streaked at equidistant points from the corners and incubated at 28°C for 5-7 days. The experiment was conducted in triplicates. The antagonistic activity was estimated according to the formula given by Hazarika et al. (2019)

$$\% \text{ MGI} = [(A*B)/A] \times 100.$$

Where A is the fungal colony diameter on the control plate and B is the fungal colony diameter on the test plate.

2.5 Screening for multifarious plant growth promoting (PGP) attributes and antifungal enzymes

All isolates were screened for a wide array of PGP and antifungal traits. To check for ammonia production, actively growing bacterial isolates were inoculated in 10 ml peptone water and incubated at 28±1°C at 120 rpm for 72 h on a rotatory shaker. The Nessler's reagent (1 ml) was added to the bacterial culture and observed for the production of ammonia. The colour change from yellow to brown indicates the production of ammonia (Cappuccino & Sherman 1996). Phosphate solubilization was determined by spot inoculation of log-phase bacterial cultures on Pikovaskya's agar plates (composition: g⁻¹ Dextrose 10, Yeast extract 0.5, Calcium phosphate 5, Ammonium sulphate 0.5, Potassium chloride 0.2, Magnesium sulphate 0.1, ferrous sulphate 0.0001, Manganese sulphate 0.0001, Agar 15). Thereafter, plates were incubated at 30°C for 48 h and observed for the clearance zone around bacterial spots, which indicated inorganic phosphate solubilization (Dutta & Thakur 2017). Zinc solubilization ability was detected by spot inoculating log-phase bacterial cultures on minimal medium supplemented with zinc carbonate (0.1%). The inoculated plates were incubated at 28±2°C for five days and were observed for the clearing zone around the colonies (Saravanan et al., 2004). Siderophore production was detected by spot inoculating log-phase bacterial cultures on chrome azurol S' (CAS) agar plates. Plates were incubated at 30°C for 5-6 days. The development of a yellow-orange halo zone around bacterial colonies against a dark blue background indicates siderophore production (Schwyn & Neilands, 1987). To determine antifungal traits, firstly the chitinolytic activity of isolates was determined on a minimal agar medium containing (g/L) (NH₄)₂SO₄ 1g, K₂HPO₄ 1g, NaCl 0.1g, MgSO₄·7H₂O 0.5g, yeast extract 0.5g, agar and 15g supplemented with 0.5% colloidal chitin. The minimal agar plates, spot inoculated with log-phase bacterial culture were incubated at 30°C for 5-6 days and observed for the halo zone around colonies (Sadfi et al., 2001). Amylase production by bacterial isolates was tested by growing them on glucose yeast extract peptone agar (GYP) pH 6.0 supplemented with 0.2% soluble starch (Kasana et al., 2008). The inoculated plates were incubated at 30°C for two days. Thereafter, plates were flooded with 1% iodine in 2% potassium iodide to visualize the zone of clearance. Protease production was determined by spotting active bacterial cultures on skim milk agar plates (Skim milk powder 28.0, Tryptone 5.0, Yeast extract 2.5, Dextrose 1.0, Agar 15.0 g⁻¹, pH 7.0±0.2) and incubated at 28-30°C for 2 days. The clear halo zone around bacterial growth indicated the protease production (Shaheen et al., 2008). To detect lipase production, log-phase bacterial cultures were inoculated on nutrient agar plates containing 1% (w/v) tween 80. The deposition of precipitate around the colony or halos indicated the positive test. The pectinolytic activity was assessed by spot inoculating the bacterial cultures on agar plates containing 1% pectin. The plates

were incubated at 28°C for 48 h. Thereafter, plates were flooded with iodine solution (1% iodine in 2% potassium iodide). A clear halo zone around the bacterial colony indicated pectinolytic activity (Joshi et al., 2019).

2.6 Ranking of bacterial isolates

All 45 rhizobacterial isolates were ranked based on the bonitur scale (Krechel et al., 2002). Bonitur score included the points (Σ =29) for all functional attributes i.e. PGP, antifungal traits, and antagonistic activity. Each property is assigned 1 to 3 points. The distributions of the points are as follows: three points each for P solubilization, Zn solubilization, Siderophore production, protease, pectinase, chitinase, and amylase activities (Σ =21). Whereas, one point each for ammonia production and lipase activity (Σ =2) and 3 points each to *in-vitro* antagonism against *A. triticina* and *B. sorokiniana* (Σ = 6).

2.7 Morphological and biochemical characterization of potent bacterial antagonists

For the primary identification and characterization, morphological and biochemical tests are still one of the key criterions utilized (Rai et al., 2014). Morphological characterization was done to determine the colony characteristics of the bacterial isolates on NA plates and through Gram's reaction. The biochemical tests viz., catalase, urease, voges-proskauer, and methyl red were performed as per the standard procedures (Jain et al., 2020).

Also, carbon source utilization patterns of efficient bacterial antagonists were analyzed using TM HiCarbo Kit (HiMedia Laboratories) containing 35 carbon sources (Table 6). This is a standardized colorimetric identification system based on the principle of pH change and substrate utilization. To test carbohydrate utilization, the test strips were pre-inoculated with 100 μ l of bacterial culture and incubated at 30°C for 12-48 h. On incubation, organisms undergo metabolic changes which are indicated by a spontaneous colour change (red to yellow) in the medium. The test provides a "metabolic fingerprint" of the microorganism that can be used to identify the metabolic diversity among the microorganism.

2.8 DNA isolation and molecular identification of potent bacterial antagonists

The total genomic DNA isolation of bacterial isolates was carried out following the method outlined by Joshi et al. (2019). Thereafter, the 16S rDNA gene was amplified using the forward GM3f (5'TACCTTGTTGTTACGACTT3') and reverse GM4r (5'TACCTTGTTACGACTT3') universal primers set (Rajwar & Sahgal, 2016). The PCR reaction was carried out in 50 μ l reaction mixture containing the following components: 1 $\times$ PCR buffer,

50 ng DNA template, 0.5 mM MgCl₂, 400 μ M dNTP mixture and 0.25 μ M each of primer, and one unit of *Taq* Polymerase (Bangalore Genie, India) in a thermal cycler (Applied Biosystems GeneAmp PCR 9700) under the following cycling conditions (initial denaturation 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C with the final extension of 5 min at 72°C). Amplified products were resolved in 1.0% agarose gel using 1 $\times$ TAE buffer till the bands resolved. The gel was visualized under the UV documentation imaging system GelDocMega (Biosystematica).

The PCR product was purified using a GeneiPure gel extraction kit (Bangalore Genei, India) following the manufacturer's instruction. Subsequently, it was sequenced directly on 3730 DNA sequencer using ABI big dye terminator technology at Central Instrumental Facility, Biotech Centre, UDSC, New Delhi. The obtained DNA sequences were edited using the software, Genomics Workbench 20.0. Similarity search for the sequences was carried out and compared with the 16S rDNA sequences of related species available in GenBank databases using the BLAST search program of the National Center for Biotechnology Information (NCBI) at <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> (Shrestha et al., 2016). The BLAST results revealed the identity of the query sequences based on their percentage query coverage and sequence similarity. Thereafter, sequences were submitted to the NCBI GenBank database and accession numbers were obtained. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 7.0 (Tamura et al., 2013). Bootstrap replication (1000 replications) was used as statistical support for the nodes in the phylogenetic tree.

2.9 Statistical analysis

All the experiments were conducted in triplicates. The data of soil physicochemical properties, enzyme activities, and antagonistic assay were statistically analyzed through one-way analysis of variance (ANOVA) via Duncan's multiple range tests at the $P \leq 0.05$ significance level using SPSS (IBM SPSS Statistics version 16) software package and presented as means $\pm$ standard error (SE). Pearson's correlation analysis and graphing were performed using R 3.4.2 software.

3 Results and Discussion

3.1 Soil physicochemical properties and enzyme activities

The soil physicochemical characteristics revealed soil nutrient status and health. The soil texture of Almora and Pantnagar was silty clay loam, Uttar Pradesh was clay loam and that of Madhya Pradesh and Maharashtra was sandy clay loam (Table 1). The soil pH of Almora was lowest i.e. 6.2, this was followed by Pantnagar (7.1), Uttar Pradesh (7.8), Madhya Pradesh (8.0), and Maharashtra

Table 1 Geographical locations of sampling sites, soil type and bacterial density (Cfu g⁻¹)

Sampling sites		Location	Soil type (USDA)	No of isolates	Cfu g ⁻¹ soil
Uttarakhand	Almora	29° 35' 39.08" N 79° 39' 14.01" E	Silty clay loam	9	8.5x10 ⁵
	Pantnagar	29°1'15.74"N 79°29'23.06"E	Silty clay loam	9	1.3x10 ⁷
Uttar Pradesh		27° 54' 1.37" N 78° 4' 20.21" E	Clay loam	10	2.5x10 ⁷
Madhya Pradesh		24° 38' 60.00" N 77° 19' 12.00" E	Sandy clay loam	8	1.26x10 ⁷
Maharashtra		19° 59' 50.83" N 73° 47' 23.29" E	Sandy clay loam	9	1.38x10 ⁷

Table 2 Soil Physicochemical properties and enzyme activities of rhizospheric soil samples collected from wheat growing regions of India

Parameters	Sampling sites				
	Almora	Pantnagar	Uttar Pradesh	Madhya Pradesh	Maharashtra
OC %	0.45±0.011 ^a	0.84±0.013 ^a	1.4±0.011 ^a	0.97±0.012 ^a	0.98±0.006 ^a
OM%	0.7±0.02 ^a	1.43±0.03 ^a	2.4±0.02 ^a	1.56±0.18 ^b	1.69±0.02 ^a
soil pH	6±0.11 ^a	7.23±0.08 ^b	7.53±0.14 ^b	7.96±0.14 ^b	8.26±0.14 ^b
EC (µs/cm)	33.91±0.02 ^c	27.28±0.01 ^c	31.27±0.02 ^c	43.6±0.01 ^c	151.6±0.80 ^h
AV P (kg/hectare)	41±6.83 ^d	55.66±0.66 ^e	68±2.88 ^f	62.66±0.88 ^e	50.66±2.08 ^e
AV K (kg/hectare)	264.66±2.72 ^f	293.66±1.45 ^h	389.33±1.76 ^g	315.66±2.33 ^g	364±3.05 ⁱ
NH ₄ -N (kg/hectare)	34.66±2.72 ^c	50.33±1.76 ^{gf}	22±1.73 ^h	40.33±1.76 ^c	32.66±2.90 ^d
NO ₃ -N (kg/hectare)	21.33±2.33 ^a	47.66±1.20 ^f	12±1.73 ^d	41.66±2.02 ^c	16.33±2.02 ^c
Zn (µg ml ⁻¹)	0.84±0.02 ^a	0.74±0.02 ^a	1.74±0.02 ^{bc}	0.95±0.02 ^a	1.23±0.02 ^a
Fe (µg ml ⁻¹)	0.23±0.01 ^a	4.44±0.03 ^b	14.21±0.02 ^a	0.233±0.02 ^a	1.16±0.01 ^a

(8.5). Electrical conductivity for Almora, Pantnagar, Uttar Pradesh, Madhya Pradesh, and Maharashtra was 33.96, 27.29, 31.31, 43.63, 152.4 µs cm⁻¹ respectively. Uttar Pradesh soil had the highest percentage of organic carbon (%OC) (1.4%) while the lowest was reported for Almora soil (0.43%). The macro and micronutrients (available phosphorous, Potassium, iron, and zinc) were highest in Uttar Pradesh and lowest in Almora (Table 2). The study is supported by several reports, for instance, Maurya et al., 2011 stated that the soil of Almora is acidic and deficit in available phosphorous content. Baqir et al., 2018 reported that the pH of soil from Uttar Pradesh was slightly alkaline (6.78 to 8.21) with higher organic matter, nitrogen, phosphorus, and potassium content.

Soil nutrient status and soil enzyme activities are correlated. Moreover, soil enzymes are well known to play a significant role in soil health and their environment. DHA, FDA, and ALP enzyme activities were significantly ($p < 0.05$) higher in soil samples of Uttar Pradesh as compared to samples from other regions whereas, ACP and URE activities were higher in soil samples from Almora and Pantnagar respectively. These findings indicated a vast difference in soil enzyme activities concerning the sampling site. The difference may be due to the agro-ecological variations between the sampling regions. Also, the soil enzyme activities vary with soil type in quantity and level of

activity (Veeraragavan et al., 2018). Moreover, the soil enzyme activities have broad-scale spatial variability at any location and are expected to vary to an extent according to the direction and spacing of sampling points (Tan et al., 2014).

Soil enzymes produced by microorganisms play a substantial role in nutrient cycling. The results from this and numerous other studies have indicated that the level of macro and micronutrient status in soil affects enzyme activities (Jian et al., 2016; Wang et al., 2018; Ullah et al., 2019). Pearson's correlation matrix among soil physicochemical properties and enzyme activities revealed a positive as well as a negative correlation between soil properties (Figure 1). For example, soil organic matter (OM) content was significantly correlated with DHA, FDA, and URE activities. However, there was a negative correlation between available phosphorous (AVP) and nitrogen content (NH₄-N and NO₃-N) to the acid and alkaline phosphatase and urease activity respectively. These results are supported by findings of several earlier studies that showed the positive correlation among enzyme activities and soil organic matter, the major energy source for microorganisms (Liu et al., 2020). Earlier Tan et al. (2014) reported that soil organic matter influences soil properties and nutrient cycling. In contrast Nannipieri et al., 2017 observed a negative correlation

Figure1. Correlation coefficient matrix of Pearson's correlation analysis of soil physicochemical properties and enzyme activities. The circle size and color scale and both represent the correlation coefficient. It ranged from -1 (red color) negative correlation to +1 (blue color) strong positive correlation.

between P availability and phosphatase activity. In P-deficient soils, phosphatase secretion from plant roots increases to enhance phosphate availability to plants (Bueis et al., 2018). Similarly, Urease activity was also negatively correlated with $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ content of soil (Wang et al., 2014; Xu et al., 2015). The findings of this study proved that biological (microbial and biochemical) activity in soil plays a key role in nutrient cycling and is responsible for the wide ecological functions of soil.

3.2 Isolation of bacteria from rhizospheric soil

The bacterial population in wheat rhizospheric soil at five sites Almora, Pantnagar, Uttar Pradesh, Madhya Pradesh, and Maharashtra, India varied from 8×10^5 to 2.5×10^7 cfu g^{-1} . The population was highest at Uttar Pradesh (2.5×10^7 cfu g^{-1}) and lowest at Almora (8.5×10^5 cfu g^{-1}). A total of 45 bacterial isolates, 18 from Uttarakhand (2 sites, 9 from each site), 10 from Uttar Pradesh, 9 from Madhya Pradesh, and 8 from Maharashtra were recovered and identified based on distinct morphological characteristics (Table 1). Further, they were screened for *in-vitro* antagonistic and plant growth-promoting activity. The rhizosphere is considered a hot spot of bacterial diversity due to the rhizodeposition. Rhizobacteria associated with cereals especially wheat under different ecological conditions have been isolated and studied for PGP and antifungal traits in several studies (Zhang et al., 2012; Majeed et al., 2015). Verma et al. (2016) found that average bacteria populations in soil collected from wheat fields in Uttar Pradesh ranged from 6×10^6 to 1×10^8 cfu g^{-1} .

3.3 *In-vitro* antagonism against *A. triticina* and *B. sorokiniana*

All forty-five rhizobacterial isolates showed varying degrees of antagonism against both the pathogens. The degree of antagonism ranged from 12.22 to 65 % against *A. triticina* whereas 12.59 to 69.14% against *B. sorokiniana* (Table 3). Out of the forty-five isolates,

Table 3 *In vitro* antagonistic activity of selected bacterial isolates against *B. sorokiniana* and *A. triticina*

Bacterial Isolates	% MGI (<i>B. sorokiniana</i>)	% MGI (<i>A. triticina</i>)
A1	32.96 ± 0.97 ^a	20.37 ± 0.97 ^{ab}
A2	21.11 ± 1.28 ^c	29.62 ± 0.97 ^{ab}
A3	20 ± 1.69 ^{bc}	26.29 ± 0.74 ^{ab}
A4	28.88 ± 1.11 ^c	30.74 ± 1.61 ^{ef}
A6	32.222 ± 1.11 ^a	20.37 ± 1.33 ^{ef}
A12	27.03 ± 0.74 ^c	31.11 ± 1.11 ^{cde}
A15	18.14 ± 1.48 ^a	17.03 ± 1.33 ^a
A19	23.33 ± 0.64 ^a	19.25 ± 1.33 ^{abc}
A21	24.81 ± 0.37 ^{ab}	22.22 ± 1.11 ^{bcd}
P1	33.33 ± 1.11 ^b	40 ± 2.31 ^b
P2	37.4 ± 1.33 ^{bc}	32.22 ± 2.93 ^{bc}
P3	38.14 ± 1.33 ^{bc}	28.51 ± 1.48 ^b
P4	18.51 ± 0.97 ^{bc}	38.88 ± 1.69 ^{ab}
P5	12.59 ± 0.97 ^c	32.96 ± 1.61 ^{ab}
P6	18.51 ± 2.67 ^a	40 ± 1.69 ^{bc}
P7	39.62 ± 2.59 ^a	22.22 ± 1.69 ^b
P9	27.4 ± 1.48 ^{bcd}	15.55 ± 1.11 ^a
P10	69.14 ± 2.02 ^c	65 ± 1.60 ^{bcd}
UP1	18.88 ± 1.28 ^d	18.14 ± 1.85 ^c
UP2	26.66 ± 1.11 ^c	12.59 ± 2.06 ^a
UP3	37.77 ± 1.11 ^b	41.11 ± 1.92 ^{bc}
UP4	42.59 ± 0.74 ^a	28.88 ± 1.92 ^{ab}
UP5	37.03 ± 0.97 ^e	37.77 ± 1.11 ^{ab}
UP6	25.92 ± 0.97 ^b	23.33 ± 1.69 ^e
UP7	16.66 ± 0.64 ^a	23.33 ± 2.22 ^b
UP9	31.85 ± 0.97 ^a	27.4 ± 1.61 ^a
UP10	14.81 ± 0.97 ^e	31.48 ± 1.85 ^a
UP11	66.92 ± 0.87 ^c	62.37 ± 1.93 ^{ef}
MP4	17.4 ± 0.74 ^b	29.62 ± 2.06 ^e
MP5	32.59 ± 0.74 ^b	34.81 ± 1.33 ^a
MP12	27.03 ± 1.48 ^{cd}	12.22 ± 1.69 ^a
MP14	51.88 ± 0.33 ^{de}	21.48 ± 1.95 ^a
MP16	46.29 ± 0.37 ^{de}	24.44 ± 2.31 ^a
MP17	56.33 ± 0.83 ^e	24.07 ± 0.74 ^d
MP20	37.77 ± 1.11 ^a	27.77 ± 1.69 ^a
MP22	31.11 ± 1.69 ^{ab}	21.48 ± 1.33 ^a
MH6	39.25 ± 2.25 ^{ab}	26.66 ± 2.22 ^b
MH10	29.62 ± 2.25 ^{ab}	25.55 ± 1.28 ^a
MH11	34.44 ± 1.11 ^{ab}	29.25 ± 1.33 ^b
MH12	43.7 ± 0.74 ^b	55.25 ± 1.40 ^{def}
MH13	63.40 ± 2.95 ^d	58.37 ± 1.73 ^{def}
MH14	38.88 ± 1.69 ^d	19.62 ± 1.61 ^a
MH16	27.77 ± 1.69 ^a	41.11 ± 1.69 ^{bc}
MH17	31.11 ± 1.69 ^e	26.29 ± 1.33 ^{ab}
MH20	28.14 ± 0.74 ^{bc}	24.81 ± 1.95 ^c

four showed >50% antagonism against *A. triticina* and five against *B. sorokiniana* (Figure 2). The three isolates P10, UP11, and MH13 showed more than 50% antagonism against both the test pathogens. Several earlier studies have reported the *in vitro* inhibition of *A. triticina* and *B. sorokiniana* through diverse microbial antagonists. For instance, *Actinomyces* sp. (Monterio *et al.*, 2017), *Trichoderma* spp. (Dal Bello *et al.*, 2008) and *Pseudomonas fluorescens* UBSPF-10 (Singh *et al.*, 2019a) suppressed the mycelia growth of *B. sorokiniana* by 44%, 51%, and 67.96% respectively. Similarly, *Bacillus subtilis* TE3, able to inhibit the mycelial growth of *B. sorokiniana* by 55% *in-vitro* (Villa-Rodriguez *et al.*, 2019). Sood *et al.* (2020), reported that rhizobacterial isolate *Serratia marcescens* B2 effective *in vitro* inhibition of *B. sorokiniana* mycelia. A year later the same group of workers found that ten tested *Bacillus* sp. showed broad-spectrum antagonistic activity against *Alternaria triticina* and *Fusarium graminearum* *in-vitro*. The bacterial production of antifungal traits is reported as reason of antagonism (Tsegaye *et al.*, 2019).

3.4 Multifarious Plant growth promoting (PGP) attributes and antifungal enzymes

All 45 rhizobacteria exhibited PGP traits like the production of ammonia and siderophore, phosphate and Zn-solubilization (Figure 3A). Out of 45 isolates, 51.11% were positive for ammonia, and 62.22% for siderophore production. Likewise, 26.66% were positive for P-solubilization and 60% for Zn-solubilization. Besides, these isolates also exhibited the production of chitinase, amylase,

protease, lipase, and pectinase enzymes. Out of these, 64.44% were considered to be positive for amylase, 57.77% for pectinase, 35.55% for protease, 26.66% for chitinase, and 17.77% for lipase activity (Figure 3B). Many microorganisms capable of solubilizing insoluble phosphate and zinc have been isolated from the rhizosphere of cereal crops (Rana *et al.*, 2011). Sood *et al.* (2019) recovered three bacterial isolates (B2, SIR1, and BIS2) positive for phosphate solubilization, siderophore production, and ammonia production for wheat rhizosphere. Application of rhizobacteria with PGP traits and hydrolytic enzyme production enhance the plant growth and reduce the growth of phytopathogenic fungi in the plant rhizosphere. For example, wheat rhizospheric bacteria PRP-6 can produce ammonia, solubilize phosphate, and excrete lytic enzyme. The use of these bacteria as biofertilizers reduced the growth of the fungal pathogen, *Fusarium oxysporum* resulting in increased plant growth (Jadoon *et al.*, 2019). Similarly, rhizobacterial isolate *Pseudomonas* sp M2 that can produce siderophore and hydrolytic enzymes (protease, cellulase, and chitinase) is a putative biofertilizer (Joshi & Dhingani, 2020). These examples support the hypothesis that bacteria producing hydrolytic enzymes act as agents for the prevention of plant diseases by causing lysis of pathogenic microbes found in the close vicinity of the plants (Tsegaye *et al.*, 2019).

The bacterial isolates were ranked based on points they scored through the bonitur scale ($\Sigma=29$ points) (Table 4). The isolates scoring more than 12 points on the bonitur scale ($\Sigma>12$) were selected as potential bacterial isolates and further processed for characterization. In this study, six bacterial isolates scored >12 points. Among them, P10

Figure 2 Antagonistic activity against test fungus in a dual plate assay; fig (a-f) showing the results of *in-vitro* antagonism against *B. sorokiniana*, Control, P10, UP11, MH13, MP17 and MP14 isolates and fig (g-k) against *A. triticina*, control, P10, UP11, MH13 and MH12 respectively.

(A)

(B)

Figure 3A Production of PGP and Hydrolytic enzymes by isolates. (a-d) PGP Traits: Ammonia production, P- solubilization, Zn- solubilization and Siderophore production. (e-i) Hydrolytic enzymes: Amylase, Protease, Lipase, Pectinase and chitinase production.

Figure 3B Percentage of bacterial isolates for positive reactions with (i) PGP traits, (ii) Hydrolytic enzymes.

Table 4 Assessment and ranking of rhizobacterial isolates based on *in-vitro* multifarious plant growth promoting, antifungal and antagonistic activities through bonitur scale

antagonistic and antagonistic activities through bioassay scale															
Region	Bacterial isolates	Multifarious plant growth promoting traits ^a					Hydrolytic enzymes ^a					Antagonistic activity ^b		Total assessment points (Σ=29)	Rank
		NH ₃ production	Phosphate solubilization	Zn solubilization	Siderophore production	Chitinolytic activity	Amylase production	Protease production	Lipase production	Pectinase activity	F1	F2			
Almora	A1	1	0	2	0	0	1	1	1	2	2	1	11	6	
	A2	0	0	0	3	0	1	1	0	2	1	1	9	7	
	A3	0	0	1	0	0	1	0	1	2	1	1	7	9	
	A4	1	0	0	1	1	1	0	0	1	1	1	7	10	
	A6	1	1	1	0	0	1	2	1	1	1	1	10	7	
	A12	0	0		2	0	0	0	0	1	1	1	6	10	
	A15	1	3	0	0	0	0	0	1	1	1	1	8	8	
	A19	1	0	1	0	0	1	0	0	1	1	1	6	10	
	A21	0	0	1	0	1	1	0	0	1	1	1	6	10	
Pantnagar	P1	1	0	1	0	0	1	1	0	0	2	2	8	8	
	P2	1	0	1	1	0	1	0	0	3	2	2	11	6	
	P3	0	0	1	2	0	1	0	1	0	2	1	8	8	
	P4	0	0	1	0	1	0	0	0	0	1	2	5	11	
	P5	0	0	0	1	0	0	0	0	0	1	2	4	12	
	P6	1	1	1	2	0	0	0	0	2	1	2	10	7	
	P7	0	0	0	0	0	1	1	0	0	2	1	5	11	
	P9	1	2	1	1	0	1	1	0	0	2	1	10	6	
	P10	1	2	2	2	3	2	3	0	3	3	3	24	1	
Uttar Pradesh	UP1	1	0	1	0	0	2	2	0	1	1	1	9	8	
	UP2	1	0	3	0	1	1	1	0	1	1	1	10	7	
	UP3	0	0	2	2	1	0	0	1	0	2	2	10	7	
	UP4	1	0	1	1	0	1	1	0	0	2	1	8	8	
	UP5	0	0	1	1	0	0	0	0	0	2	2	6	10	
	UP6	0	0	1	2	0	0	0	0	1	1	1	6	10	
	UP7	1	0	0	0	0	0	0	0	1	1	1	4	12	
	UP9	0	0	0	0	0	1	1	0	1	1	1	5	11	
	UP10	0	2	2	1	0	1	1	0	0	1	2	10	7	
	UP11	1	3	2	3	2	1	1	1	1	3	3	21	2	
	Madhya Pradesh	MP4	1	0	1	1	0	0	0	0	2	1	1	7	9
MP5		1	1	1	2	0	1	1	0	1	1	2	11	6	
MP12		0	1	0	0	0	1	1	0	1	1	1	6	10	
MP14		1	2	0	1	1	3	2	0	0	2	1	13	5	
MP16		1	0	1	2	0	1	1	0	1	2	1	10	6	
MP17		0	2	2	0	3	3	3	1	0	3	1	18	3	
MP20		0	0	1	0	0	1	1	0	1	2	1	7	9	
MP22		0	0	0	2	0	0	0	0	1	1	1	5	11	
Maharashtra	MH6	0	0	0	1	0	0	0	0	1	2	1	5	11	
	MH10	1	0	0	0	0	1	1	0	0	1	1	5	11	
	MH11	1	0	0	1	0	1	1	0	0	2	1	7	9	
	MH12	0	2	0	1	1	3	3	1	0	2	3	16	4	
	MH13	1	2	2	2	1	3	3	0	0	3	3	21	2	
	MH14	0	1	1	0	0	1	1	0	0	2	1	7	9	
	MH16	0	2	0	1	0	0	0	0	0	1	2	6	10	
	MH17	1	0	1	1	0	1	1	0	0	2	1	8	8	
	MH20	1	0	0	1	0	0	0	0	0	1	1	4	12	

^a PGP and hydrolytic enzymes traits points specification (+ = 1; ++ = 2; +++ = 3)F1 = test fungus *B. sorokiniana*; F2 = test fungus *A. tritici*^b Antagonistic activity = (1 = 10-30%; 2 = 31-50%; 3 = 51-70%)

scored maximum points ($\Sigma = 24$) followed by UP11 ($\Sigma = 21$), MH13 ($\Sigma = 21$), MP17 ($\Sigma = 18$), MH12 ($\Sigma = 16$) and MP14 ($\Sigma = 13$). Bonitur scale has been used to select elite strains with high plant growth promotion potential and antifungal traits *in vitro* (El-Sayed et al., 2014; Dheeman et al., 2020). Therefore, the production of lytic enzymes and multiple PGP traits could be a prime reason for the plant growth promotion and disease suppressing ability of bacterial antagonists.

3.5 Morphological and biochemical characterization of potent bacterial antagonists

Morphology and *in vitro* biochemical characteristics are the primary step in bacterial identification and gives presumptive identification (Saqib et al., 2020). The morphological and biochemical characteristics of the six potent bacterial antagonists were determined (Table 5). The variation was observed in the color, margin, and shape of the isolates. Colonies were creamy and white; rough to smooth; with regular to irregular edges. Gram's reaction differentiates the six strains. Four isolates P10, UP11, MH13 & MH12 were gram positive rods, isolate MP14 was gram positive cocci present in bunches and MP17 was gram positive filamentous. Based on morphological properties and gram's reaction isolates P10, UP11, MH13 & MH12 were belonging to genera *Bacillus*, MP14 *Staphylococcus* and MP17 *Streptomyces*. The bacterial isolates differed in their biochemical characteristics. Four *Bacillus* strains were catalase and voges-proskauer positive, whereas urease and methyl red negative. The finding is supported by the results of Prajakta et al., (2019). *Staphylococcus* strain was catalase, methyl red, and urease positive whereas voges-proskauer negative. The biochemical test for the strain of genera *Staphylococcus* is VP negative and MR positive (Mutmainnah et al., 2020). *Streptomyces* strain

was catalase and urease positive whereas methyl red and voges-proskauer negative. The genus, *Streptomyces*, can be distinguished from other bacterial groups based on their distinctive morphological, physiological, and biochemical features. Isolate VITAKN showed positive for Gram's staining test, methyl red test while, negative for mannitol test, indole, and Voges Proskauer's test (Ishaque et al., 2020).

The carbohydrate utilization profile was indicative of metabolic diversity among six potent isolates. The isolates MP17 utilized a maximum of 20 substrates, followed by P10 and UP11 (17 substrates), MH13 (16 substrates), MP14 (13 substrates), and MH12 (9 substrates) from a total of 35 substrates (Table 6). This test provides a "metabolic fingerprint" of the potent antagonist and was used to group the isolates into two distinct clusters (Figure 4). Cluster I, included two isolates P10 and MP17 placed at a distance in between of 0.49-0.64 on Jaccard's scale. Cluster II consisted of three isolates UP11, MH13, MP14. UP11 and MH13 were 100% similar, isolate MP12 was on a separate lineage showing the least similarity to all the other isolates. The C substrate utilization pattern is strain specific. The strains showing similar C utilization patterns were closely placed in the cluster (Singh et al., 2019b).

3.6 Molecular identification of potent bacterial antagonists

The 16S rDNA sequencing is still the most reliable and frequently used technique for the identification of microorganisms (Pomastowski et al., 2019). Through 16S rDNA sequencing, six potent antagonistic isolates P10, UP11, MH13 and MH12, MP17 & MP14 were identified as *Bacillus methylotrophicus* (MN099430.1), *Bacillus subtilis* (MN099431.1), *Bacillus sp.*

Table 5 Morphological and biochemical characteristics of potent bacterial antagonists

Isolates	Colony morphology	Gram's reaction observation	Biochemical activities			
			Catalase	Urease	Methyl red	Voges Proskauer
P10	creamy with irregular edges	Gram positive, rods	+	-	-	+
UP11	creamy with irregular edges	Gram positive, rods	+	-	-	+
MH13	creamy with irregular edges	Gram positive, rods	+	-	-	+
MP17	Discrete, granular, powdery white colonies with regular edges	Gram positive, rods	+	+	-	-
MP14	round, usually golden-yellow colonies	Gram positive, coccus	+	+	+	-
MH12	Spherical White color colonies	Gram positive, filamentous	+	-	-	+

Figure 4 UPGMA-dendrogram based on the carbohydrate utilization profile of six potential bacterial antagonists. The tree is constructed using NTSYSpc version 2.0 software by calculating jaccard coefficient.

(MN099432.1), *Lysinibacillus* sp. (MN099433.1), *Streptomyces* sp. (MN099435.1) *Staphylococcus epidermidis* (MN099434.1) respectively. The neighbor joining tree reflected the phylogenetic relationships within six isolates (Figure 5). *Bacillus* and *Streptomyces* have been extensively studied for its antagonistic PGPR activities (Hossain & Rahman, 2014; Lee et al., 2017; Thennaras et al., 2019; Ali et al., 2020).

Conclusion

The present study underlines the screening of rhizobacteria for multiple PGP and biocontrol traits and molecular identification of the promising isolates. Based on *in vitro* dual plate assay six isolates viz., *Bacillus methylotrophicus* (MN099430.1), *Bacillus subtilis* (MN099431.1), *Bacillus* sp. (MN099432.1) *Streptomyces* sp. (MN099435.1), *Lysinibacillus* sp. (MN099433.1), and *Staphylococcus epidermidis* (MN099434.1) (out of the 45 rhizobacteria) markedly suppress the mycelia

growth of tested fungus. Among them, three isolates *Bacillus methylotrophicus*, *Bacillus subtilis*, and *Bacillus* sp. showed *in-vitro* antagonism against both foliar blight pathogens. Therefore, this is the first report that identified the bacterial antagonists against the foliar blight complex of wheat. Findings suggested that selected isolates have the potential to be used as a biocontrol agent against the foliar blight complex. Nevertheless, to determine the disease suppression efficacy of the isolates in the natural soil environment field trial must be conducted in the future.

The submitted manuscript is original, unpublished and not under simultaneous consideration with other journals.

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Figure 5 Phylogenetic tree of six potent bacterial antagonists (Black dot) recovered from wheat rhizosphere. Genetic relatedness between the six isolates was inferred from Neighbor-Joining tree based evolutionary analyses with a Bootstrap value of 1000. The numbers given over branches indicate bootstrap coefficient. The phylogenetic tree was constructed using MEGA 7.0 software.

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