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Isolation and Screening of Plant Growth Promoting Rhizobacteria from the Rhizospheric Soil of Wheat (*Triticum aestivum*) from Lower Western Himalayan Zone of Himachal Pradesh

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A B S T R A C T

The bacteria that colonize the plant's rhizosphere are known as PGPR. The rhizospheric region is the area under the ground surface that is linked with plant roots. PGPR bacteria are free-living bacteria that colonize plant roots and have positive impacts on plant growth. The objectives of this paper were to isolate and identify the most powerful PGPR, as well as to assess their efficacy in terms of P-solubilization, HCN generation, and lytic enzyme activity (protease). A total of 11 bacterial isolates were identified in the Hamirpur district of Himachal Pradesh. All isolates were tested for a variety of plant growth-promoting characteristics, including phosphate solubility, HCN production and protease production. On PVK agar, 8 of the 11 isolates tested positive for P-solubilization in the 5-20 mm zone. One bacterial isolate exhibited positive hydrogen cyanide activity in the event of HCN generation. In the case of lytic enzyme activity, 7 bacterial isolates were positive for protease production.

Introduction

Wheat (*Triticum aestivum*) is the most important grain for human survival (Kumar and Sharma, 2017). After rice, it is India's second most grown cereal crop. Wheat is an essential staple grain for the people of northern India. It's a Poaceae family plant that grows every year (Gramineae). Wheat flour is commonly used by humans to make Chapaties and pasta goods, while straw is utilised as animal feed and packaging material. It's utilised in the manufacturing of starch, gluten, malt, and distilled spirits. Wheat includes 70% carbohydrate, 13% protein, 1.7 percent lipids, 2.7 percent minerals, 2% fibre, and 12% moisture (Oyewole *et al.*, 2016). Wheat, when consumed as a whole grain, provides a variety

of nutrients and dietary fibre, as well as a reduced risk of a variety of illnesses, including coronary heart disease, stroke, cancer, and diabetes (Shewry P R, Hey S J, 2015).Wheat is cultivated on around 30 million hectares in India, with a yield of 106.21 million tons in 2019-20. (The Economic Times, 2020).Himachal Pradesh, a Himalayan state in India's northwestern Himalayas, is one of the country's leading wheat producers (Kumar *et al.,* 2020). Kloepper and Schroth coined the term PGPR in 1978. (Verma *et al.*, 2019). Plant growth-promoting rhizobacteria are bacteria that colonize plant roots and promote plant development by a number of processes, including phosphate solubilization, siderophore generation, HCN production, and biological nitrogen fixation (Vejan *et al.*, 2016).

The Rhizosphere was initially coined by Hiltner to describe the small zone of soil around the root where root activities increase microbe populations, commonly known as the "Rhizosphere effect" (Das *et al.,* 2013).The rhizosphere is known as a "microbe storehouse" because it is a rich source of microorganisms and microbial activity (Kundan *et al.,* 2015).These bacteria's main functions are to: (a) provide nutrients to crops; (b) stimulate plant development, for example, by producing phytohormones; (c) enhance soil structure; and (d) protect plants from diseases (Kundan *et al.,* 2015).The PGPR mechanism employs both direct and indirect approaches.

Rhizobacteria, on the other hand, are indirectly involved by decreasing the impact of diseases and developing systemic resistance (Bhattacharyya, P. N., and Jha, D. K., 2012). *Bacillus* and *Azospirillum* are two common genera participating in PGPR (Mangmang *et al.,* 2015). Phosphorous is an important plant nutrient that helps in the growth and development of plants. After nitrogen, it is the world's second-largest crop nutritional supplement (Kumar and Sharma, 2017). Microorganisms play an important role in biogeochemical processes such as soil phosphorus mineralization, solubilization, and transformation (Heijden *et al.,* 2008).

As a frequent secondary metabolite, rhizospheric pseudomonads generate hydrogen cyanide, a gas that is known to severely influence root metabolism and root development and is a viable and ecologically friendly strategy for pathogen biological control (Kumar & Sharma, 2017).

HCN will most likely block the electron transport chain and give energy to the cell, resulting in the death of the cells (Kundan *et al.,* 2015). Several PGPR inoculants are presently available for purchase, and they serve an important role in encouraging development. PGPR inoculants can be used as biofertilizers or phytopathogen antagonists, making them a viable alternative to chemical fertilizers and pesticides (Kumar *et al.,* 2020).

Materials and Methods

Collection of rhizospheric soil sample

The soil sample used for PGPR isolation was collected from rhizosphere of wheat (*Triticum aestivum*) from Lower West Central Outer Himalayan zone from Hamirpur district of Himachal Pradesh, India. Sampling was done in the month of January & February of the year 2021. Sample was stored in plastic bottles/bags loosely tied to ensure sufficient aeration and to prevent moisture loss from the soil samples(Modi *et al.*, 2017).

Isolation of potential plant growth promoting rhizobacteria

PGPR isolates were isolated from the rhizospheric soil sample by serial dilution and spread plate method using King’s B agar

medium at 28ºC (Singh & Lal, 2016). Pikovskayas agar used for phosphate solubilizing bacteria (Pikovskaya's 1948) and King's B agar for *Pseudomonas* sp. (King *et al.*, 1954).

Colony morphology and pigment production

Colony morphology (form, elevation, margin, shape & surface) and the production of pigment was checked on King's B agar at 28± 2ºC after 24 to 48 hours (Kumar *et al.*, 2020).

Characterization of selected isolates for various PGP attributes

Phosphate solubilization

The ability of bacteria to solubilize phosphorus was tested on Pikovskaya's agar plates. Each bacterial culture was spot inoculated in the centre of Pikovskaya's agar plates containing tricalcium phosphate as insoluble phosphate source (Kumar *et al.*, 2020). The plates were incubated at 28°C for 5-7 days and halozone development around the bacterial growth was observed.

HCN

All the isolates were screened out for the formation of hydrogen cyanide. Cultures were streaked on nutrient agar plates amended with glycine (1.4 g/l). Whatman No. 1 filter paper strips were soaked in 0.5% picric acid followed by 2% sodium carbonate and were placed in the lids of each petriplates (Kumar *et al.*, 2020).

Plates were sealed with parafilm and incubated at 28 degrees Celsius for four days. Plates were examined for changes in filter paper colour from yellow (-) to light brown (++) to brown (+++) to dark brown (++++) (Kumar *et al.*, 2020).

Lytic enzyme

Protease production

All isolates were tested for protease production on skim milk agar (1 percent skim milk was employed in the nutrient agar medium) and autoclaved separately before pouring, both flasks were combined, and plates were poured (Modi *et al.*, 2017). Spot inoculation of each bacterial culture in the centre of skim milk agar plates was used. We observed the clear zone around the colony after 24–48 hours of incubation at 28 ° C. The diameter of clear zones generated around the colony after 48 hours of incubation at 28 ° C is used to measure proteolytic activity (Kumar *et al.*, 2020).

Results and Discussion

Collection of rhizospheric soil sample

The rhizospheric soil sample for PGPR's isolation was collected from rhizosphere of wheat (*Triticum aestivum*) crop from Hamirpur district of Himachal Pradesh, India located at the altitude above mean sea level in Tal (300m). All the rhizospheric soil sample was collected in clean and sterilized sampling bottles and stored in refrigerator in laboratory (Nelson, 2004).

Isolation of PGPR from rhizospheric soil

Hamirpur District

PGPR isolates were isolated from the rhizospheric soil sample by serial dilution and spread plate method using King's B agar medium at 28 ºC. The total viable count of rhizobacteria of wheat (*Triticum aestivum*) from Tal site of Hamirpur is depicted in Table 1. The total rhizobacterial population on King's B agar medium (264 x 10^5) microbial population at Tal. King's B agar is a non-

selective medium generally used for the isolation of *Pseudomonas* species. The variation in the population of rhizobacteria may be attributed to location, age of plant, variety, time of sampling, physio-chemical and biological properties of the soil and environmental conditions of the locations. According to Tamilarasi *et al.*, (2008), the varied degree of population found in plant roots is due to the influence of the chemical composition of individual plant root exudates on microorganisms. In total, 11 PGPR isolates were isolated on King's B agar medium. The morphological characters of bacterial isolates, i.e. pigmentation, form, elevation, margin, shape, and gram-reaction were noted down and presented in Fig 2.

Tal site

The pigmentation of colonies of bacterial isolates from Tal varied from white, cream, off-white and whitish-creamish. The majority of isolates were white (55%) in color, 9% were cream in color, 27% of isolates were off-white and 9% were whitish-creamish in color. 73% were circular, 18% were punctiform and 9% were round in form.

Out of total isolates, 46% isolates were raised while 45% were flat and 9% had convex elevation. 62% of isolates were entire and 38% had undulate margins. 82% of the bacterial isolates were gram-negative, while 18% were found to be gram-positive. 82% of the isolates were bacilli and 18% had a cocci shape.

Phosphate solubilization

Phosphate solubilization activity shown by bacterial isolates from the Tal site was expressed in the range of 8 to 15 mm in diameter of halozone on PVK agar plate. Out of eleven isolates, 8 isolates showed phosphate solubilization. Maximum P-solubilization was shown by the isolates Tl-1 (15 mm), followed by Tl-4 (12 mm) and Tl-10 (11mm) (Table 4). Gupta (2012) also reported that the population of phosphate-solubilizing microorganisms, in general, varied from 20-24% of the total population, but in some soils, it may be as high as 85% of the total population. In other studies, conducted by Kundu *et al.*, (2002), it was reported that about 16% of the total bacterial population in the rhizosphere of wheat was P-solubilizer.

HCN Production

Isolates of PGPR from Tal showed HCN production on nutrient agar medium amended with glycine. Out of eleven isolates, maximum production of HCN (+++) was shown by Tl-1 to change the colour of filter paper strip yellow (-) to brown (+++). No HCN production was shown by other isolates from Tal (Table 5). A similar study indicates that HCN production is established to be a common trait of *Pseudomonas* (88.89%) and *Bacillus* (50%) within the rhizospheric soil of wheat, a biocontrol metabolite in Pseudomonas species (Saharan and Nehra, 2011).

Table.1 Population density of rhizobacteria on King's B agar media from Tal site of Hamirpur District of Himachal Pradesh

Site of rhizospheric soil sample collection	Total viable count on King's B agar media		
	Dilutions		cfu/g soil x 10^5
	10^{-4}	10^{-6}	
Tal	264	34	264 x 10^5

Table.2 Morphological characteristics of bacteria isolated from site Tal

Sr. No.	Isolate	Pigment	Elevation	Margin	Form	Gram reaction	Shape
1	**Tl-1**	White	Flat	Entire	Round	-	Bacilli
2	**Tl-2**	Cream	Flat	Entire	Circular	-	Bacilli
3	**Tl-3**	White	Raised	Entire	Circular	-	Bacilli
4	**Tl-4**	White	Convex	Undulate	Punctiform	+	Bacilli
5	**Tl-5**	Whitish-cream	Flat	Entire	Circular	+	Cocci
6	**Tl-6**	Off- white	Flat	Entire	Circular	-	Bacilli
7	**Tl-7**	Off- white	Flat	Entire	Circular	-	Bacilli
8	**Tl-8**	White	Raised	Undulate	Circular	-	Cocci
9	**Tl-9**	White	Raised	Undulate	Circular	-	Bacilli
10	**Tl-10**	Off- white	Raised	Entire	Punctiform	-	Bacilli
11	**Tl-11**	white	Raised	Entire	Circular	-	Bacilli

Table.3 Purification of PGPR's isolated from rhizospheric soil of wheat (*Triticum aestivum*)

S.No.	Isolate	Site
1	Tl-1	Tal
2	Tl-2	
3	Tl-3	
4	Tl-9	

Table.4 Evaluation of different PGP attributes of rhizobacteria isolated from site Tal

Sr. No.	Isolate	Phosphate- solubilization plate (mm dia)
1	**Tl-1**	15
2	**Tl-2**	0
3	**Tl-3**	9
4	**Tl-4**	12
5	**Tl-5**	11
6	**Tl-6**	0
7	**Tl-7**	10
8	**Tl-8**	8
9	**Tl-9**	0
10	**Tl-10**	11
11	**Tl-11**	8

Table.5 Evaluation of different PGP attributes of rhizobacteria isolated from site Tal

Sr. No.	Isolate	HCN Change of color (Yellow to brown)
1	**Tl-1**	+++
2	**Tl-2**	-
3	**Tl-3**	-
4	**Tl-4**	++++
5	**Tl-5**	-
6	**Tl-6**	-
7	**Tl-7**	-
8	**Tl-8**	+++
9	**Tl-9**	-
10	**Tl-10**	-
11	**Tl-11**	-

***HCN production colour change from Yellow to brown to dark brown**
Yellow**:** - **;** Light brown**:** ++ ; Brown**:** +++; Dark brown**:** ++++

Table.6 Evaluation of different PGP attributes of rhizobacteria isolated from site Tal

Sr. No.	Isolate	Protease production plate (mm dia)
1	**Tl-1**	13
2	**Tl-2**	0
3	**Tl-3**	8
4	**Tl-4**	10
5	**Tl-5**	0
6	**Tl-6**	0
7	**Tl-7**	14
8	**Tl-8**	11
9	**Tl-9**	6
10	**Tl-10**	9
11	**Tl-11**	0

Fig.1 Collection of rhizospheric soil sample of wheat (*Triticum aestivum*) from Tal sites of Hamirpur District (H. P.)

Fig.2 Morphological characteristics of rhizobacterial isolates from site Tal

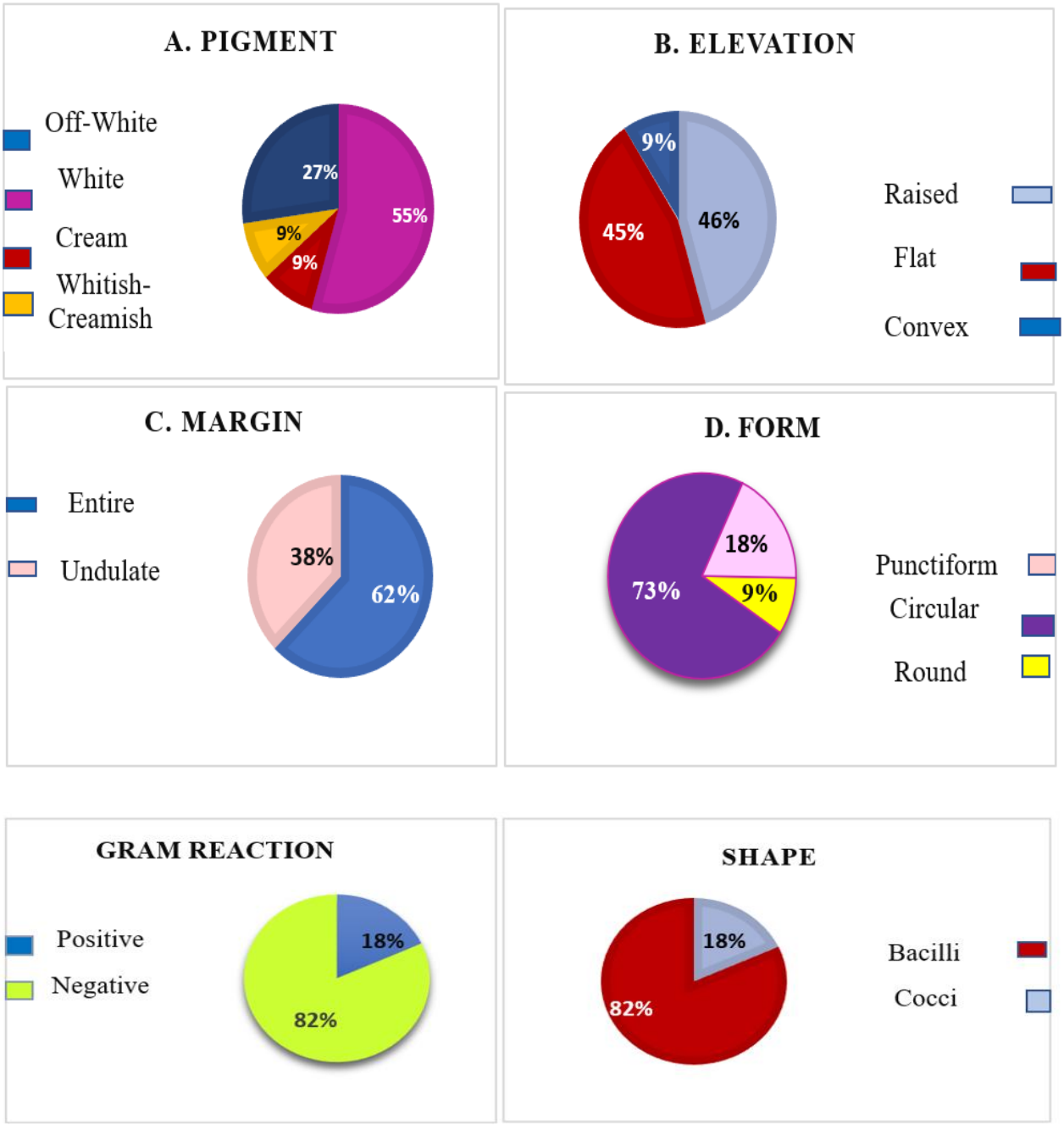


Fig.3 Total viable count of rhizobacteria from the rhizospheric soil of wheat on King's B agar medium from Hamirpur district

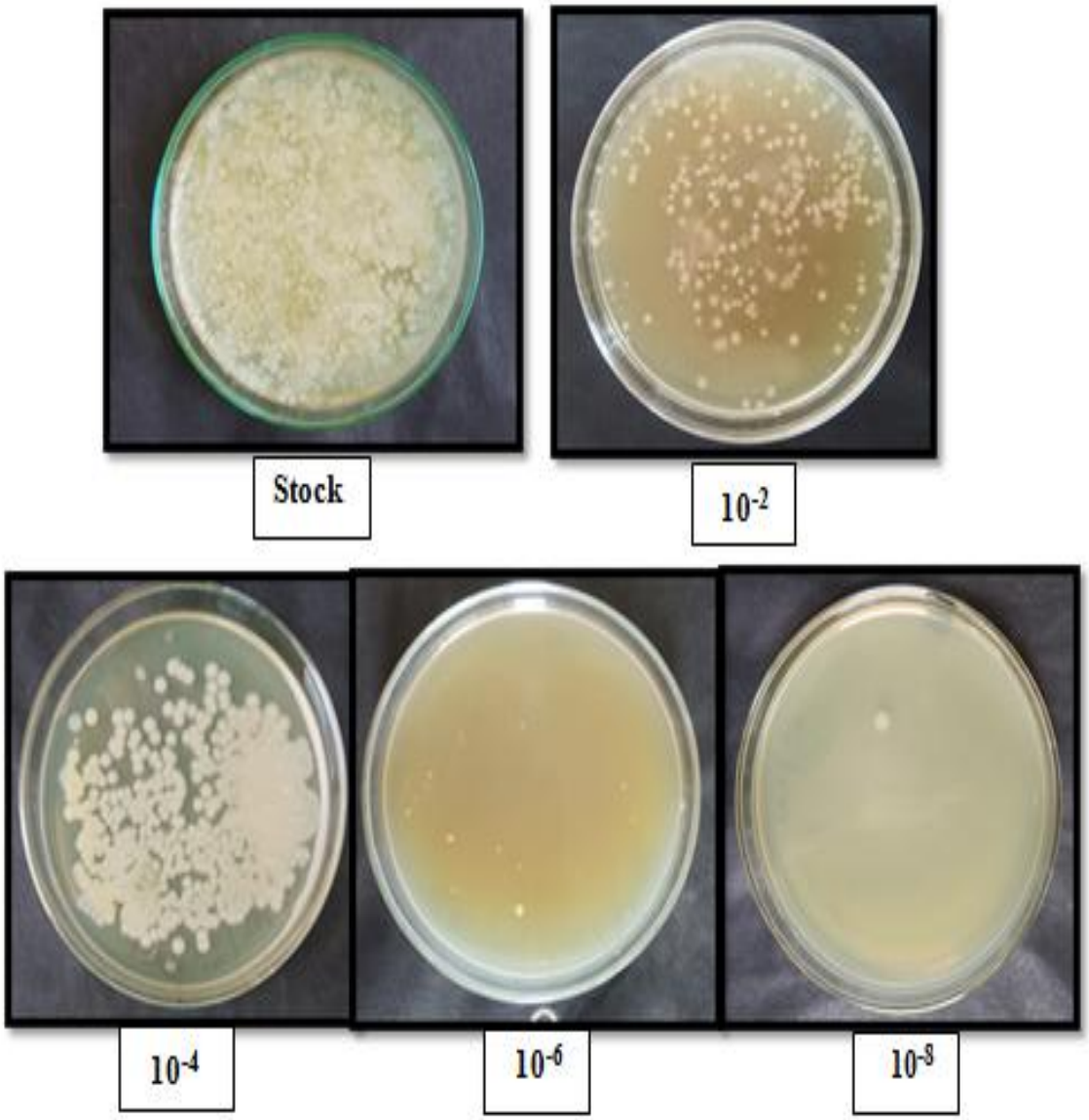


Fig.4 Purified bacterial isolates from Tal site of Hamirpur District (H.P.)

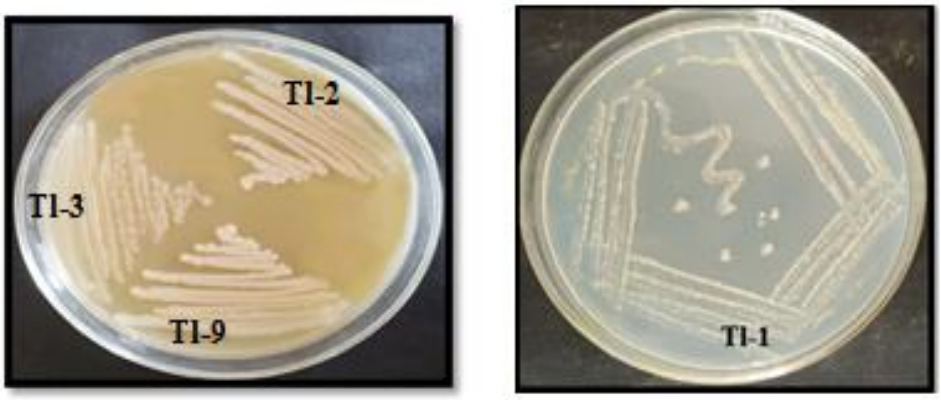


Fig.5 Phosphate solubilization activity shown by bacterial isolates from Tal site of Hamirpur District

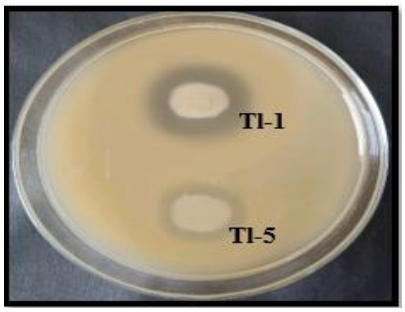


Fig.6 (A) Show the negative control of HCN; (B) Show the positive control of HCN

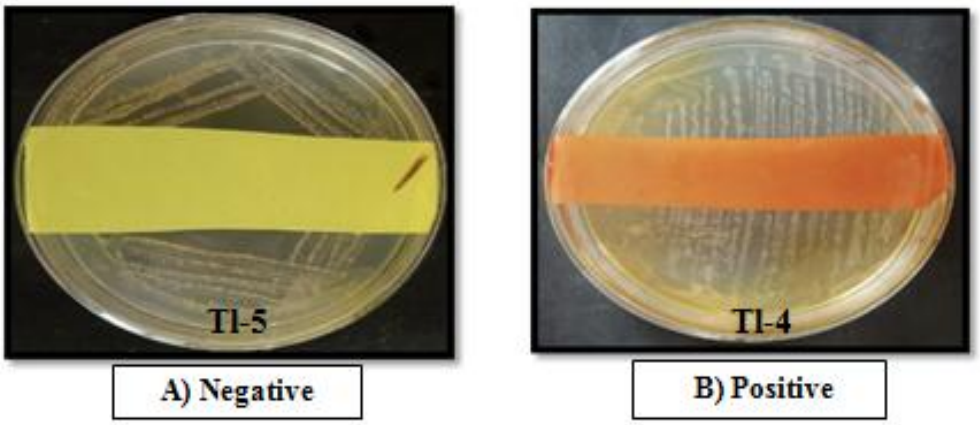
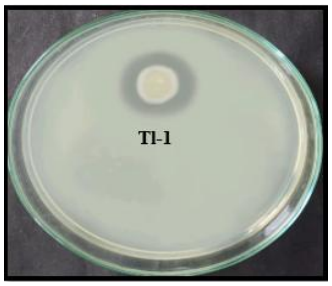


Fig.7 Protease activity shown by bacterial isolates from Tal site of Hamirpur District



Lytic enzyme

Protease activity

Protease activity shown by bacterial isolates from the Tal site was expressed in the range of 6 to 14 mm in diameter of the clear zone around the bacterial growth on skim milk agar plate. Out of eleven isolates, seven isolates showed protease activity. Maximum activity was shown by Tl-7 (14 mm), followed by Tl-1 (13mm) and Tl-8 (11mm). From Tal, most of the isolates were found positive for protease activity (Table 5).

Protease is a hydrolytic enzyme which suppresses or inhibits the growth of the bacterial and fungal pathogens present in the rhizosphere of the plant. So, the protease production by rhizobacteria is an important attribute. All the isolates produced protease in good quantity. A similar study was reported by Upadyay *et al.*, (2013). Some proteolytic enzymes, especially elastase and subtilisin, also possess bacteriolytic properties against different gram-positive and gram-negative bacteria.

In conclusion, a total of eleven isolates were recovered from the Tal site in the Hamirpur district of Himachal Pradesh. These isolates were isolated on King's B agar media. The pigmentation of colonies of bacterial isolates from Tal varied from white, cream, off-white and whitish-creamish. The majority of isolates were white (55%) in color, 9% were cream in color, 27% of isolates were off-white and 9% were whitish-creamish in color. 73% were circular, 18% were punctiform and 9% were round in form. Out of total isolates, 46% of isolates were raised, 45% were flat, and 9% had convex elevation. 62% of isolates were entire, and 38% had undulating margins.82% of the bacterial isolates were gram- negative, while 18% were found to be gram-positive. 82% of the isolates were bacilli and 18% had a cocci shape. Our results are in agreement with those from other investigations that found gram-negative bacteria as the main composition of the rhizosphere and root-associated microbial communities in many plant species. P-solubilization was observed in eight isolates from the Tal site in Hamirpur district.Phosphorus is one of the most important elements for plant biological growth and development. The capacity of rhizobacteria to convert insoluble phosphorus (P) to an accessible form, such as orthophosphate, is a critical characteristic for improving plant development and production during a PGPB (Saharan and Nehra, 2011).Chemical fertilizers are the primary source of phosphorus in agricultural systems, although Fe, Ca2+ complexes fix around 75-90 percent of the phosphorus applied to the soil (Kumar *et al*., 2020).The most common mechanism of action implicated by PGPR for increasing nutrient availability to host plants is phosphate solubilization (Thakur *et al*., 2014).One isolate was discovered to have

HCN activity, out of a total of eleven. Other isolates were less efficient in producing HCN. The colour of the filter paper strip changed from brown to (+++) when Tl-1 was applied. Rhizobacterial species are involved in the synthesis of HCN and play an important role in biological pathogen control.

In the case of the lytic enzyme, all isolates were tested for protease activity, which has previously been shown in several isolates. Seven of the 11 isolates tested positive for protease activity. On skim milk agar, protease activity was measured in the range of 5-20 mm.

In vitro, this work shows that a functional PGPR plays a critical role in protease activity. It is stated that this is a fundamental study that has offered insight into the bacterial community found in Himachal Pradesh, India's mid-zone. In the present study discovered P-solubilizing, HCN-producing, and lytic enzyme (protease)-producing bacteria in the natural population.

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