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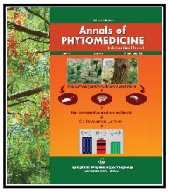

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Diversity and bioprospecting potential of PGPR from critically endangered medicinal plant, *Trillium govanianum* (Wall. ex D. Don) in North-West Himalayas

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Abstract

The present investigation aimed to study the diversity of cultivable rhizobacteria and their potential in plant growth promotion of *Trillium govanianum* (Wall. ex D. Don). The collection of rhizospheric soil samples have been done from Kinnaur district of Himachal Pradesh, India. In total, 98 bacterial isolates were isolated from rhizospheric soil of *T. govanianum*. All isolates were screened on the basis of morphological, biochemical and for the PGP traits, *viz*., phosphate solubilization, siderophores, HCN and ammonia production, protease, chitinase, cellulase activity and plant growth regulators. Among ninety eight bacterial solates, nine potential PGPR were selected and identified. The strains MR-8 showed the maximum phosphate solubilization (20 mm, 95 µg/ml), YL-12 showed maximum siderophore production (17 mm, 70.38% SU) and all isolates showed the positive result for HCN and ammonia production. The selected PGPR isolates YL-3, YL-11, YL-12, MR-6, MR-7, MR-8, BR-14, BR-16, BR-22 were identified as *Bacillus safensis, Bacillus sonorensis, Pseudomonas azotoformans, Stenotrophomonas rhizophila, Bacillus altitudinis, Rahenella aquatilis, Bacillus weihenstephanensis, Bacillus subtilis, Bacillus mycoids* by using 16S rRNA gene sequencing technique. Further, these isolates were used for preparation of liquid biofomulation to evaluate the beneficial effect of bioformulation on various plant growth parameters. Our results revealed positive influence of the inoculation with the bioformulations as compare to non-inoculated control. The consortia of *B. safensis* + *B. sonorensis* + *P. azotoformans* found to be most effective in comparison with other treatments on the different plant parameters. The study will have implications in developing bioformulations, specifically for low temperature environments, in view of environmental sustainability.

1. Introduction

High altitude medicinal plants are of great concern throughout the Himalayan region, because they are important for traditional health-care and, is well-known for its harsh ecosphere, rocky topography, and sparse vegetation that has a unique ability to biosynthesize a variety of phytochemicals (Samant *et al*., 1998; Devi *et al*., 2021) . The plant's many parts are advantageous in terms of pharmacological and biological qualities. The use of these plants, particularly subterranean stems, has been shown to have positive immunomo-dulatory effects and to modify the immune system through a variety of methods. (Naikodi *et al*., 2021) Herbal medications derived from medicinal and aromatic plants are a significant source of new antiviral treatments. Plants containing a wide range of bioactive chemicals with medicinal potential are mostly unexplored (Senthilkumar *et al*., 2021). Ancient, traditional remedies based on herbs that have successfully treated a variety of antimicrobial and antiviral diseases are expected to emerge as potent treatments in the treatment of COVID-19 (Naik *et al*., 2021).

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Trillium govanianum (Wall. ex D. Don) is a herb of the Trilliaceae family (Vidyarthi *et al*., 2013). It is indigenous to the Himalayan region (Samant *et al*., 1998) and is known as nagchhatri in India. For its profuse growth, it prefers cold, dark, wet settings (Chauhan *et al*., 2017). It is primarily found in the Himalayan region. Due to prolonged exploitation, the population of the species had plummeted in the wild environments (Vidyarthi *et al*., 2013). Prior to 2008, the plant was not listed among 960 traded medicinal plant species in India, but due to its notable health benefits and rising demand, it is now being traded illegally (Ved and Goraya, 2008) and is listed among endangered medicinal plants by the International Union for Conservation of Nature. *T. govanianum* is perennial herb which is about 30 cm tall, having stout rhizome with numerous adventitious roots. The rhizome is used traditionally in folk medicine for dysentery, healing wounds, inflammation, antiseptic, backache, boils, menstrual and sexual disorders (Sharma and Samant, 2014). The underground part of the plant, *i.e.*, rhizome is a key material of trade containing 'trillin' which upon hydrolysis yield diosgenin and extensively used in various traditional medicinal preprations of steroids and sex hormones. Phytochemical analyses of *T. govanianum* rhizome have resulted in isolation of steroides and saponins and their anti-fungal and anticancer activities have also been reported. It is native to Himalayas and distributed at an altitude of 2500-3800 m (Rahman *et al*., 2015). *T. govanianum* which exhibited good to moderate activities against *Aspergillus niger* ATCC 16888, *Aspergillus flavus* ATCC 9643,

Candida albicans ATCC 18804 and *Candida glabrata* ATCC 90030. The antibacterial activity of methanol, ethanol, acetone and distilled water rhizome extracts of *T. govanianum* was determined *in vitro* against three human pathogenic bacteria (*i.e*., *Escherichia coli*, *Yersinia pestis* and *Staphylococcus aureus)*. Methanol rhizome extract of *T. govanianum* was most effective in inhibiting the growth of *S. Aureus* (Sagar *et al*., 2017).

Exploring the functions of the native microbial population is critical for understanding the distribution and diversity of indigenous bacteria. Native and particular microbial strains can be used as plant growth promoters to achieve the required effects. Plant growth-promoting rhizobacteria (PGPR) diversity in the rhizosphere is influenced by plant species, soil type, and soil nutrient availability (Tilak *et al*., 2005). Rhizospheric bacteria are gaining in popularity as a potential new resource for promoting plant development and conserving endangered species.

The rhizosphere of medicinal plants growing in the temperate Himalayas is a rich source of helpful soil bacteria that has yet to be discovered.The presence of distinctive and highly specific microbiome in the medicinal plant is due to the unique and structurally divergent bioactive secondary metabolites which are most likely responsible for the high specificity of the associated microorganisms. A large number of bacterial species, particularly those found in the plant rhizosphere, have the ability to promote plant development and so protect plants from extinction. However, only by studying the enormous pool of indigenous soil bacteria (PGPR) and selecting the most promising strain among them for better efficacy can introduced bioinoculants function effectively. Plant growth-promoting bacteria (PGPB) colonise the rhizosphere quickly and improve growth and yield through a variety of direct and indirect mechanisms, including making macronutrients available and producing growth regulators, as well as indirect tools like biological pathogen control and increased resistance to biotic and abiotic stresses (Bulgarelli *et al*., 2013).

Since *T. govanianum* is a critically endangered species with scarce distribution in different terrains of Himalays, an attempt has been made for the first time over in the present study in view of studying the diversity of associated beneficial rhizobacteria relevant to its propagation, regeneration and conservation. In this background, the present study deals with the identification and characterization of culturable rhizobacteria from the rhizosphere of *T. govanianum* and to evaluate their PGP potential in cultivation.

2. Materials and Methods

2.1 Plant sample collection

Random samplings of rhizospheric soil and rhizome of *T. govanianum* plant grown in its natural habitat were carried out in 4 locations with 5 replications each, totaling 20 samples from district Kinnaur 31.6510º N, 78.4752º E Himachal Pradesh, India. Soil samples of 100 g each were obtained from around pineapple root system areas at 0-15 cm deep and put in labelled plastic bags for biological analysis (Figure1). The sampling was done in month of May and June. The soil samples along with rhizomes were immediately stored in at 4ºC temperature until further processing.

2.2 Isolation of plant growth promoting rhizobacteria

Rhizobacteria were isolated using a single serial dilution and spread plate technique. Microbial counts were quantified as colony forming units per gramme of soil (cfu/g soil) after 1 ml of suspension was spreaded over pre-poured solid agar medium, such as nutrient agar, Jensen medium, and King's B medium. The plates were incubated at 25°C for 24 to 48 h. Pure bacterial cultures were obtained on agar plates after subculturing.

2.3 Phenotypic and molecular characterisation

According to Bergey's manual of systematic bacteriology, preliminary identification of rhizospheric bacteria was done by evaluating morphological and biochemical features of all isolates (Sneath *et al*., 1986). The molecular characterization was carried out by Eurofins Genomic India Pvt. Ltd., Bangalore, using 16S rDNA. A single distinct PCR amplicon band of 1500 bp was visible on an agarose gel. Consensus sequences of the 16S rDNA gene were generated using aligner software using forward and reverse sequence data. The basic local alignment Search Tool was used to align the 16S rDNA gene sequences with the NCBI GenBank database (BLAST). The phylogenetic tree was inferred using the neighbor joining method, Tamura-Nei model. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). After multiple alignment of sequence data by CLUSTAL X, a phylogenetic tree was generated using the software tool MEGA 6.0 (Tamura *et al*., 2007).

2.4 Plant growth Promoting (PGP) activities

2.4.1 Phosphate solubilizing activity

Both qualitative and quantitative methods were utilised to estimate and screen rhizobacteria's phosphate solubilizing activity. Each test isolate was inoculated with 0.1 ml of 18 h old culture in 10 ml of Pikovskaya's medium with tri-calcium phosphate and cultured at 28 ± 2ºC for 48 h. Centrifugation at 12,000 rpm for 25 min yielded cell-free supernatant. The supernatant was kept at 4°C and used method described by Ambrosini and Passaglia (2017) to estimate phosphate solubilizing activity using a plate and spectrophotometric test from a standard curve of potassium dihydrogen phosphate (10-100 g/ml), the amount of soluble phosphorus was estimated.

2.4.2 Siderophore production

A plate test was used as a qualitative assay to determine siderophore production, followed by quantitative analysis. The approach provided by Schwyn and Neilands (1987) was used to estimate siderophore synthesis in rhizobacterial isolates. The appearance of orange yellow halo zone around the colonies was considered positive for the presence of siderophores. 0.5 ml of 72 h old cell free supernatant was mixed with 0.5 ml CAS assay solution (1.5 ml of 1 mM $FeCl_3$, $6H_2O$ in 10 mM HCl + 7.5 ml of 2 mM CAS stock solution dissolved 0.0219 g HDTMA in 50 ml distilled water and then mix in 100 ml cylinder, add 4.307 g piperazine in 30 ml distilled water (pH5.6) and final volume was made to 100 ml with distilled water, 10 µl shuttle solution (0.2 M 5-sulfosalicylic acid) was added for quantitative essimation of siderophore. After 10 min at room temperature, the colour intensity of the solution was measured at 630 nm against a reference. The generation of siderophores was measured in terms of a decrease in blue colour expressed as a percentage of siderophores units (% SU).

2.4.3 HCN and Ammonia Production

The isolates were streaked separately on nutrient agar medium supplemented with 4.4 g glycinel/l and filter paper discs soaked in picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3,1000 ml of distilled water) was placed in the lid of each Petri dish for determination of HCN production (Adnan *et al.*, 2016). Dishes were sealed with parafilm are incubated at 28° ± 1°C for 48 h. A change in color of the filter paper discs from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong in producing HCN by each strain, respectively. All the bacterial isolates were tested for the production of ammonia following Nessler's reagent method (Rashid *et al*., 2012).

2.4.4 Lytic enzyme activity

All rhizospheric bacteria were tested for proteolytic activity using the well plate assay method on skim milk agar plates, which included 1% individually autoclaved skim milk added to the nutrient agar medium (Chaiharn *et al*., 2008). Cellulolytic activity was determined by incubating 100 µl of 48 h old cell free culture supernatant of each bacterial isolate on Czapek mineral salt agar medium and 0.1% congo red on agar plates cells were incubated (Ghose *et al*., 1987). 1% colloidal chitin agar was made for chitinolytic activity as described by Adnan *et al*. (2016) and plates were incubated for 48 h at 28 ± 2ºC.

2.4.5 Plant growth regulators

The method of determining the amount of IAA produced by PGPR isolates was used as described by (Lamizadeh *et al*., 2016). 2-3 drops orthophosphoric acid, followed by 4 ml salper reagent, were added to 1 ml supernatant of 48-h-old broth (1 ml of 0.5 M $FeCI_3$ in 50 ml of 30 per cent $HCIO_4$: prepared fresh). For 1 h in the dark, the mixture was incubated. 535 nm was used to determine absorbance. A calibration curve with indole acetic acid (IAA) as a standard (10-100 g/ml) was used to measure the concentration of auxins generated. The quantitative colourimetric method was used for the determination of gibberellins as given by (Holbrook *et al*., 1961). Radish cotyledons expansion bioassay test was employed for estimation of cytokinins (Letham, 1971). The dose response curve (final weight-initial weight) was plotted and expressed as increase in weight of cotyledons.

2.5 Field experiment

2.5.1 Preparation of liquid bioformulation

The PGPR strains were grown in shaker at 28°C, 120 rpm for 48 h and checked for culture density (cfu/ml) by using serial dilution method on nutrient agar for preparation of liquid bioformulation. 8 x 10^8 cfu/ml concentrations of each inoculum were used for treatment of plants.

2.5.2 Evaluation of liquid biofomulation on plant growth parameters

Field experiments were carried out at three separate climatic areas in district Kinnaur (31.5361°-31.5657°N, 77.9240°-78.1208°E) Himachal Pradeshs, India, during the month of February to July. The field experiment conducted with five treatments, five replications and analysed by the randomised block design. At their various sites, the PGPR strain was utilised individually and in combination. The rhizome cuttings of *T. govanianum* were pretreated with liquid bioformulation by dipping the rhizome for 10 min and sown in uniform order of 2 cm deapth and 30 × 30 spacing. The cyclic treatment of basin for successive five months was done at one month and 30 days interval in rhizosphere of plants by adding 20 ml of inoculum to the basin of plants. Results were compared with control plants.

2.6 Statistical analysis

The data were analyzed as per analysis of variance technique, one way classification, performed in triplicate. Statistical significance was determined using ANOVA. A $p<0.05$ was considered as significant.

Figure 1: Collection of rhizospheric soil samples from *T. govanianum* from Kinnaur district of Himachal Pradesh.

3. Results

3.1 Isolation of rhizobacteria

These findings revealed that the bacterial population in rhizospheric soil varied in growth on different media, including nutrient agar, King's B medium, and Nitrogen free medium (Figure 2). The highest culturable count (95×10^5 cfu/g soil) was observed on the nutrient agar (NA) medium whereas, lowest count (80×10^5 cfu/g soil) on King's B medium. In total, 98 bacterial strains were isolated on the different media and all the isolates were evaluated for *in vitro* plant

growth promoting traits. The diversity of rhizobacteria according to their plant growth promoting traits was calculated by the Shannon-Weaver algorithm and Simpson's index of dominance (1.79) was observed (Figure 3).

3.1.1 Identification of rhizobacteria

Table 1 shows the colony shape and microscopic features, as well as their physiological and biochemical characteristics. The optimal growth temperature for all nine PGPR strains was found to be 28°C for 48-52 h. The PGPR isolates were further characterized on the basis of 16S rRNA gene sequencing. Using BLAST, 16S rDNA sequences were compared to the sequences of other bacteria. The results were then compared to GenBank sequences based on partial 16S rDNA sequences to see if, they were related to the rhizobaterial isolate. The accession numbers for each sample's sequences were obtained from GenBank. On the basis of % similarity isolates have been identified as YL-3 (*Bacillus safensi*) MK124971, YL-11 (*Bacillus sonorensis*) MK124965, Yl-12 (*Pseudomonas azotoformans*) MK124966, MR-6 (*Stenotrophomonas rhizophila*) MK124963, MR-7 (*Bacillus altitudinis*) MK124970, MR-8 (*Rahenella aquatilis*) MK124964, BR-14 (*Bacillus weihenstephanensis*) MK124968, BR-16 (*Bacillus subtilis*) MK124969 and BR-22 (*Bacillus mycoids*) MK124967. The phylogenetic tree constructed for these bacteria, on the basis of NCBI US BLAST search analysis (Figure 6).

Table 1: Morphological and biochemical characterization PGPR isolates

Tests	YL-3	YL-11	YL-12	MR-6	MR-7	MR-8	BR-14	BR-16	BR-22
Colour	Cream	Cream	Cream	Cream	Cream	White	Cream	Cream	Cream
Elevation	Flate	Raised	Flat	Raised	Flat	Flat	Flat	Raised	Raised
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Form	Punctiform	Punctiform	Circular	Punctiform	Circular	Circular	Irregular	Circular	Irregular
Gram's reaction	+	+	-	-	+	-	+	+	+
Shape	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Indole production	-	-	-	-	-	-	-	-	-
Urease test	-	+	-	+	-	-	+	-	-
Citrate utilization	-	-	+	+	+	+	+	+	+
Methyl red	-	+	-	+	-	-	-	-	-
VP test	+	-	-	-	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+	+
Hydrogen sulphide production	-	-	-	-	-	-	-	-	-
Motility	+	+	-	+	-	-	+	+	+
Fermentation of carbohydrate									
Glucose	A- G-	A- G-	A- G-	A- G-	A+G-	A- G-	A- G-	A+G-	A- G-

(-) Indicates negativity of test (+) indicates positivity of test.

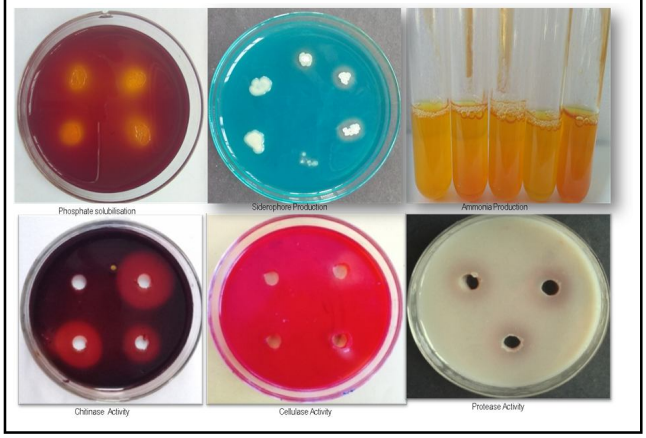

Figure 2: Various PGP traits shown by bacterial isolates.

3.2 PGP traits of the potential PGPR

3.2.1 Qualitative and quantitative phosphate solubilisation

Phosphate solubilization was expressed by all PGPR isolates in the range of 5 to 20 mm diameter of yellow zone on PVK agar plates by qualitative method as shown in Table 2 and Figure 2. Maximum SI and solubilized phosphorus were shown by isolates such as *Rahenella aquatilis* MR-8 (20 mm ± 0.12 and 95.0 µg/ml).

3.2.2 Siderophpre production

CAS agar assay confirmed the production of siderophore. The siderophore chelated to the iron of medium resulted in change of colour from blue to yellow-pink.The PGPR isolates were capable for production of siderophore in the range of 5 to 20 mm diameter of yellow zone on chrome azurol-S (CAS) agar plates by qualitative method. The maximum zone was recorded in *Pseudomonas azotoformans* YL-12 (17 ± 0.01). Quantitatively, it was observed that bacterial isolates showed siderophore production in the range of 47-70.70 % SU (Table 2, Figure 2).

Table 2: Potential of bacterial isolates for production plant growth promoting activity

Isolates	PGP activities								
	Phosphate solubilization		Siderophore production			Lytic enzymes clear zone (mm dia)*			
	Qualitative estimation zone size (mm dia)*	Quantitative estimation (Pi µg/ml)**	Qualitative estimation zone size (mm dia)*	Quantitative estimation %SU***	Protease	Cellulase	Chitinase	Ammon-ia**	HCN **
YL3	5.00 ± 0.058	84.00	5.00 ± 0.031	64.96	25.00 ± 0.28	13.00 ± 0.15	-	+++	+
YL11	11.00 ± 0.183	64.00	-	-	25.00 ± 0.35	19.00 ± 0.14	24.00 ± 08	+++	++
YL12	18.00 ± 0.024	92.00	17.00 ± 0.018	70.38	27.00 ± 0.34	20.00 ± 0.30	-	++++	+
MR6	12.00 ± 0.020	75.00	-	-	25.00 ± 0.31	23.00 ± 0.34	-	++++	+
MR7	16.00 ± 0.223	64.00	7.00 ± 0.110	53.0	15.00 ± 0.19	15.00 ± 0.21	-	++++	++
MR8	20.00 ± 0.129	95.00	8.00 ± 0.041	47.0	14.00 ± 0.05	25.00 ± 0.14	10.00 ± 0.13	++++	+
BR14	12.00 ± 0.024	70.52	6.00 ± 0.084	67.51	20.00 ± 27	20.00 ± 0.24	30.00 ± 0.29	+++	+
BR16	11.00 ± 0.131	56.00	14.00 ± 0.195	70.70	-	18.00 ± 0.06	30.00 ± 0.31	++	+
BR22	8 ± 0.005	63.00	12.00 ± 0.143	64.46	22.00 ± 0.32	-	28.00 ± 0.09	++	+
CD	0.340	2.360	0.276	1.490	0.805	0.609	0.458	NA	NA

Table 3: Effect of bacterial liquid bioformulation on plant growth parameters of *T. govanianum*

Sites	Treatments	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Size of rhizome (cm)	Root length (cm)	Weight of rhizome (g)	
Site-1	Control	1.50 ± 0.001	2.00 ± 0.001	1.93±0.026	1.67±0.020	2.18±0.021	1.52 ±0.019	0.14±0.001
	B. safensis YL3	2.80 ± 0.003	2.06 ± 0.015	1.33±0.006	1.00±0.001	2.25±0.050	1.34 ±0.002	0.14±0.001
	B. sonorensis YL11	0.80 ± 0.001	1.43 ± 0.006	1.13±0.007	1.00±0.016	2.44±0.030	1.62± 0.014	0.14±0.002
	P.azotoformans YL12	1.00 ± 0.004	2.03 ± 0.001	1.26±0.001	1.28±0.003	2.56±0.030	1.56 ±0.014	0.18±0.003
	B. safensis +*B. sonorensis* + *P.azotoformans*	4.50 ± 0.012	2.80 ± 0.004	2.20±0.022	3.00±0.017	3.25±0.034	3.58 ±0.041	0.67±0.003
	CD	0.065	0.022	0.051	0.043	0.112	0.071	0.006
Site-2	Control	0.80 ± 0.001	0.66 ± 0.014	0.70 ± 0.016	1.00± 0.012	3.06 ±0.043	1.02 ±0.001	0.28 ±0.002
	S. rhizophil	1.20 ± 0.003	1.73 ± 0.003	0.90 ± 0.014	1.50 ±0.007	4.00 ±0.015	1.29 ±0.006	0.19 ±0.003
	B. altitudinis	1.00 ± 0.008	1.03 ± 0.003	1.30 ± 0.010	1.00 ±0.011	5.00 ±0.024	1.24± 0.019	0.18 ± 0.003
	R. aquatilis	1.18 ± 0.005	0.93 ± 0.007	1.13 ± 0.008	1.80 ±0.005	3.12 ±0.035	1.56 ±0.002	0.11 ± 0.001
	S. rhizophil+*B. altitudinis*+ *R. aquatilis*	2.00 ± 0.001	1.50 ± 0.024	1.90 ± 0.004	3.50 ±0.001	5.38 ±0.020	2.93 ±0.023	0.64 ±0.009
	CD	0.014	0.039	0.032	0.027	0.088	0.042	0.014
Site-3	Control	-	-	-	-	-	-	-
	B. weihenstephanensi	1.52 ± 0.012	1.10 ± 0.002	0.96 ± 0.012	0.32±0.025	4.30 ±0.042	1.52±0.008	0.45±0.003
	B. subtili	-	-	-	-	-	-	-
	B. mycoids	-	-	-	-	-	-	-
	B. weihenstephanensi+ *B. subtili* + *B. mycoids*	3.50 ± 0.044	1.70 ± 0.021	1.50 ± 012	3.50 ±0.040	7.55±0.092	3.44±0.009	0.84±0.003
	CD	0.062	0.021	0.017	0.056	0.129	0.013	0.004

3.2.3 HCN and ammonia production

In present study, production of ammonia was observed for the change of colour of 48 h old culture broth. All the bacterial isolates showed ammonia production and maximum production (++++) was shown by five isolates, moderate production of ammonia (+++) was observed in six isolates and rest of the isolates of showed minimum (++), (+) production of ammonia. All the nine isolates showed the moderate HCN production (Table 2, Figure 2).

3.2.4 Lytic enzyme activity

The presence of proteolytic activity of bacterial isolates was shown by 8 isolates amongst 9 isolates in the range of 12 to 27 mm diameter of clear zone. The maximum proteolytic activity was recorded in

isolate *Pseudomonas azotoformans* YL-12 (27 ± 0.34 mm). Cellulolytic activity was expressed by 8 isolates in the range of 13-25 mm diameter of clear zone. The maximum cellulolytic activity was shown by *Rahenella aquatilis* MR-8 (25 ± 0.14 mm) The production of chitinolytic enzyme was shown by 5 isolates amongst 9 isolates in the range of 10-30 mm diameter of clear zone (Table 2 and Figure 2). The maximum chitinolytic activity was shown by *Bacillus weihenstephanensis* BR-14 and *Bacillus mycoids* BR-22 (30± 0.29 mm).

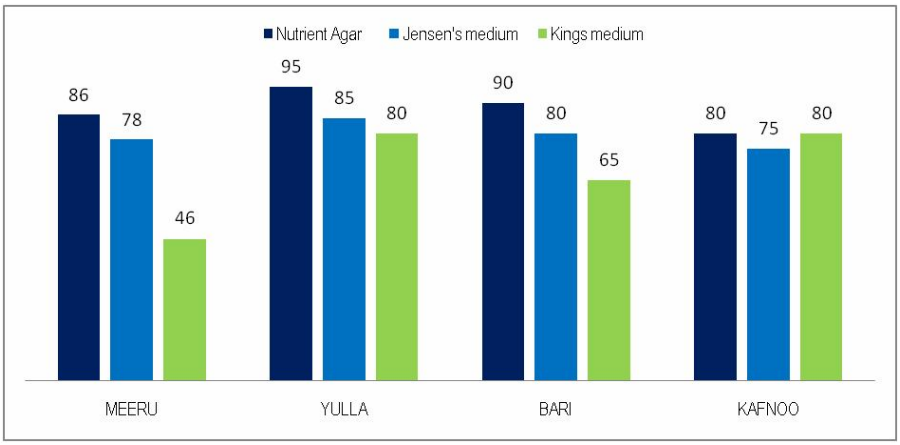

Figure 3: Total culturable count cfu/g of rhizobacteria on three different medium.

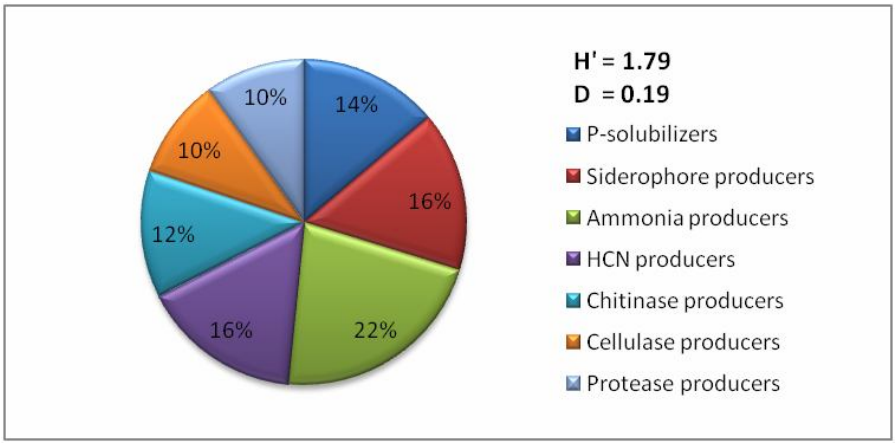

Figure 4: Representation of abundance of bacterial isolates with multifarious PGPTs in the rhizpsphere of *T. govanianum* according to the Shannon-Weaver index and Simpson's index of dominance.

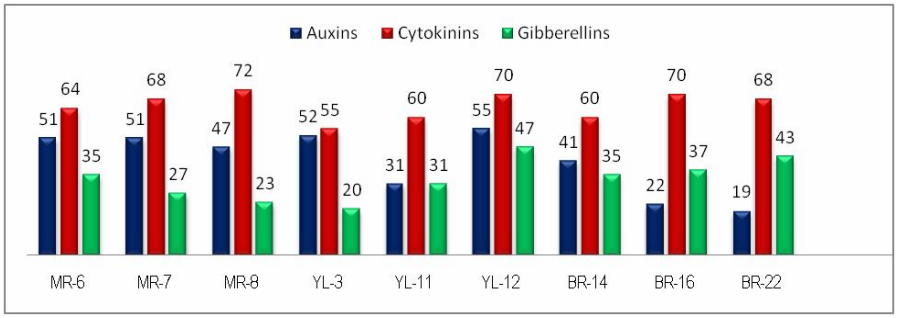

Figure 5: Potential of selected bacterial isolates for the production of plant growth regulators, *i.e.*, auxins, cytokinins and gibberellins.

3.2.5 Production of plant growth regulators

All the selected PGPR isolates were characterized for the production of plant growth regulators, *viz.*, auxins, gibberellins and cytokinins by their respective spectrophotometric and bioassay methods. Production of auxins was recorded in the isolates in range of 19 to 55 µg/ml. Maximum production of auxins was recorded in the isolates *Pseudomonas azotoformans* YL-12 (55 µg/ml). Among all the PGPR isolates, the production of cytokinins was recorded in the range of 48 to 70 µg/ml. Maximum production of cytokinin was recorded in

Pseudomonas azotoformans YL-12 and *Bacillus weihenstephanensis* BR-16 (70 µg/ml). All the strains of PGPR showed the production of gibberellins. Maximum production was shown by *Pseudomonas azotoformans* YL-12 (47 µg/ml) as shown in Figure 4.

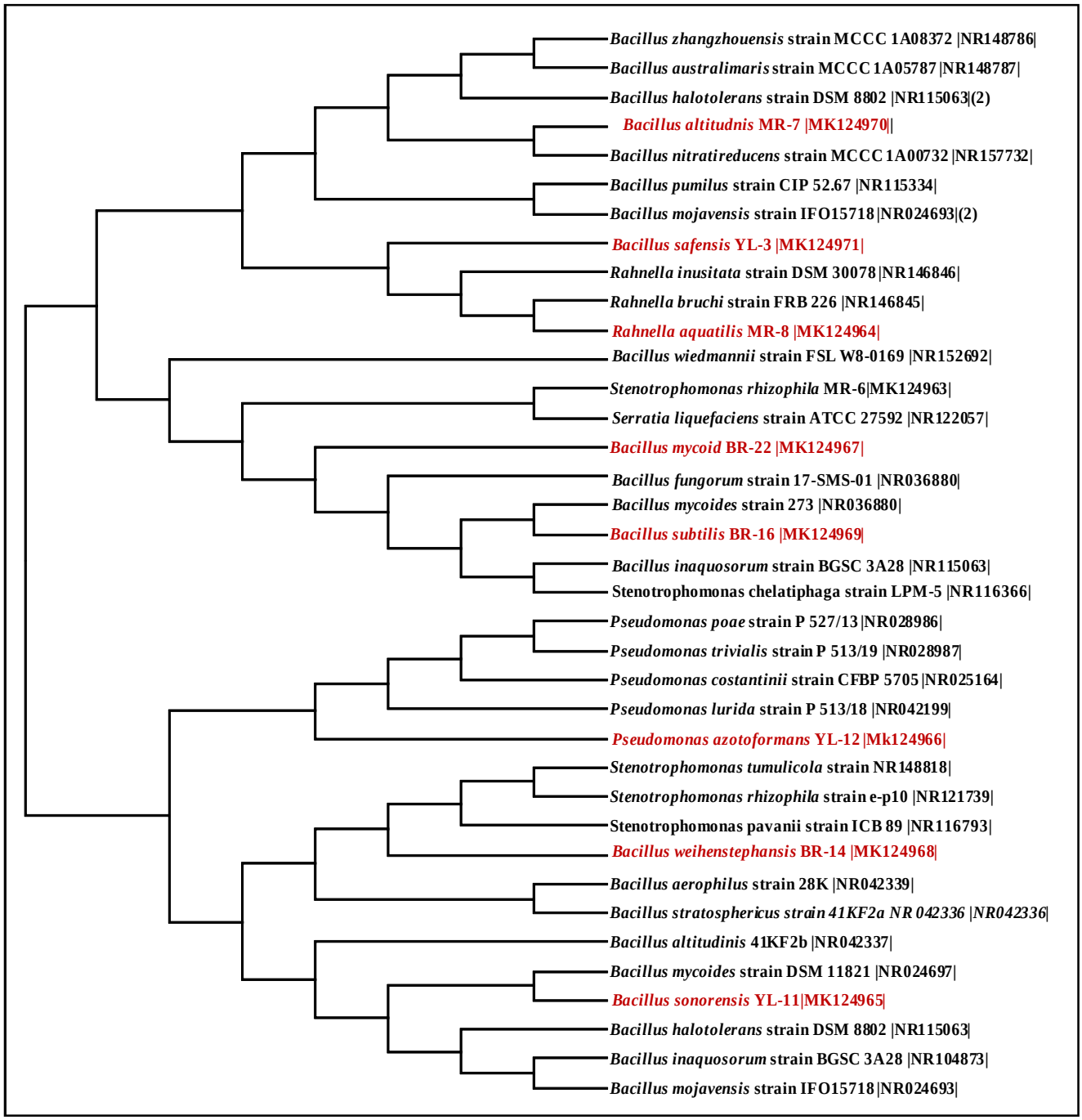

Figure 6: Phylogenetic relationship based on a neighbour joining analysis of 16S rRNNA sequences By MEGA 6.0

3.3 Field evaluation

The inoculation with PGPR, influenced various plant growth parameters. 100 % germination was recorded in consortia of *B. safensis* + *B. sonorensis* + *P.azotoformans* at Site-1, 66.6% germination was observed in consortia of *S. Rhizophil* + *B. altitudinis*+ *R. Aquatilis* at Site-2 and 66.6% germination was observed in consortia of *B. weihenstephanensi* + *B. subtili* + *B. mycoids* which is otherwise not possible and no germination in control. The survival % of germinated plants was similar like that of germination % in all treatments. The plant survived for 95 days at site-1, 66 days at site-2 and 84 days at site-3. Plants inoculated with consortium of these three PGPR strain showed a noteworthy boost in plant height, leaf length, leaf width, rhizome size and rhizome weight of *T. govanianum*.

Maximum plant height (4.50 ± 0.01cm) and leaf length (2.80 ± 00 cm), leaf width (2.20 ± 0.02 cm), rhizome size (3.00 ± 0.01 cm), rhizome weight (fresh weight 3.58 ± 0.01 g /dry weight 0.67 ± 0.00 g) and root length ((Table 3, Figures 7, 8) was recorded in consortia of consortia of *B. safensis* + *B. sonorensis* + *P. azotoformans* at site Site-1 as compared to control. Plants inoculated with consortium *S. rhizophil* + *B. altitudinis* + *R. aquatilis* showed maximum plant height (2.00 ± 0.001 cm), leaf length (1.50 ± 0.024 cm), leaf width (1.90 ± 0.004 cm) weight of rhizome (fresh weight 2.93 ± 0.02 g/dry weight 0.64 ± 0.009), root length (5.38 ± 0.020 cm) and size of rhizome (3.50 ± 0.001g) (Table 3, Figures 7,8) as compared to control at site Site-2. The plants inoculated with consortium *B. weihenstephanensi* + *B. subtili* + *B. mycoids* showed maximum plant height (3.50 ± 0.044 cm), leaf length (1.70 ± 0.021 cm), leaf width (1.50 ± 0.012 cm) weight of rhizome (fresh weight 3.44 ± g 0.009/dry weight 0.84 ± 0.003), average root length (7.55 ± 0.092 cm) and size of rhizome (3.50 ± 0.040 cm) (Table 3, Figures 7,8) as compared to control where no germination occurred at site Site-3.

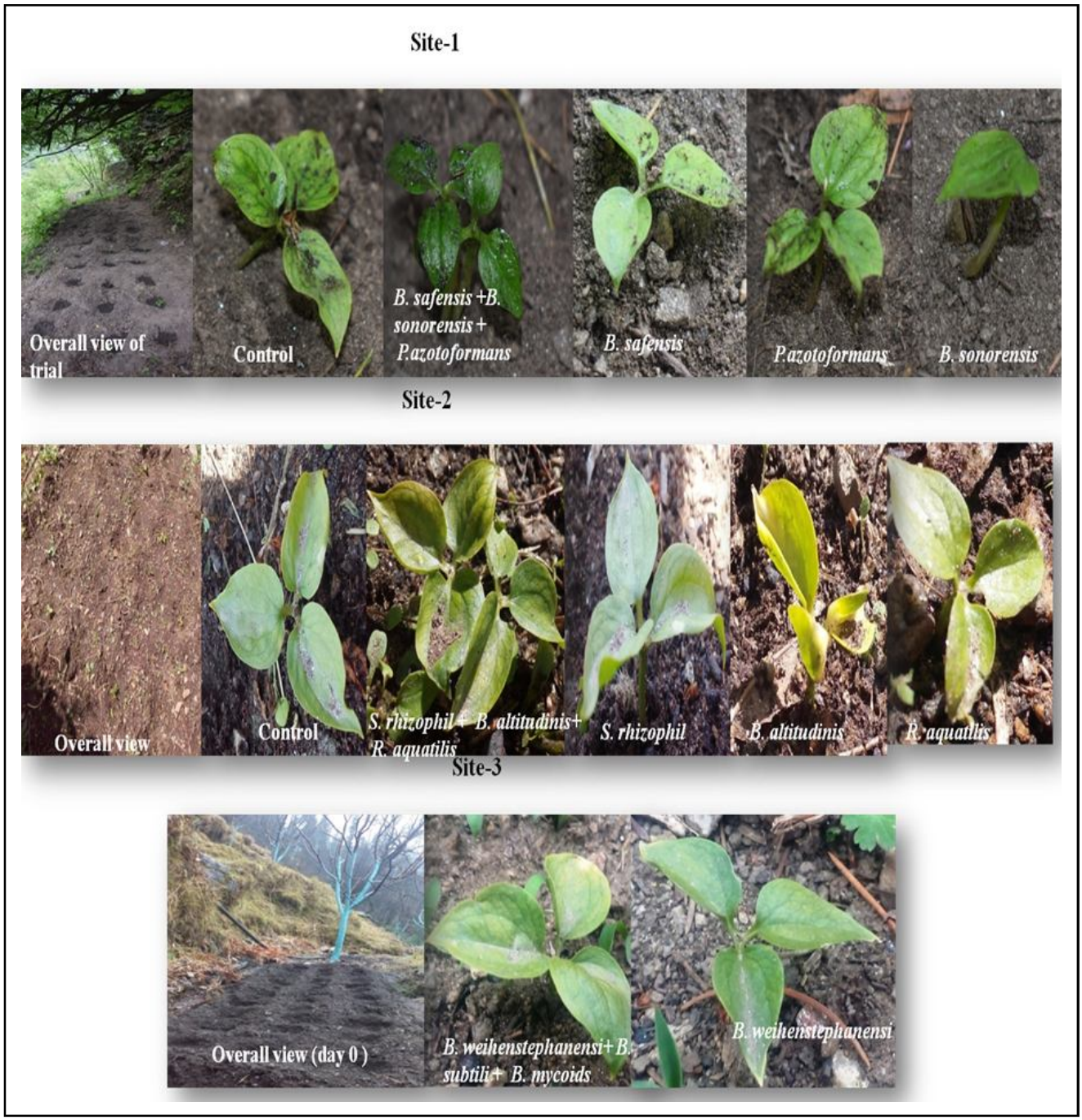

Figure 7: Effect of cyclic application of liquid bioformulation on *T. govanianum*.

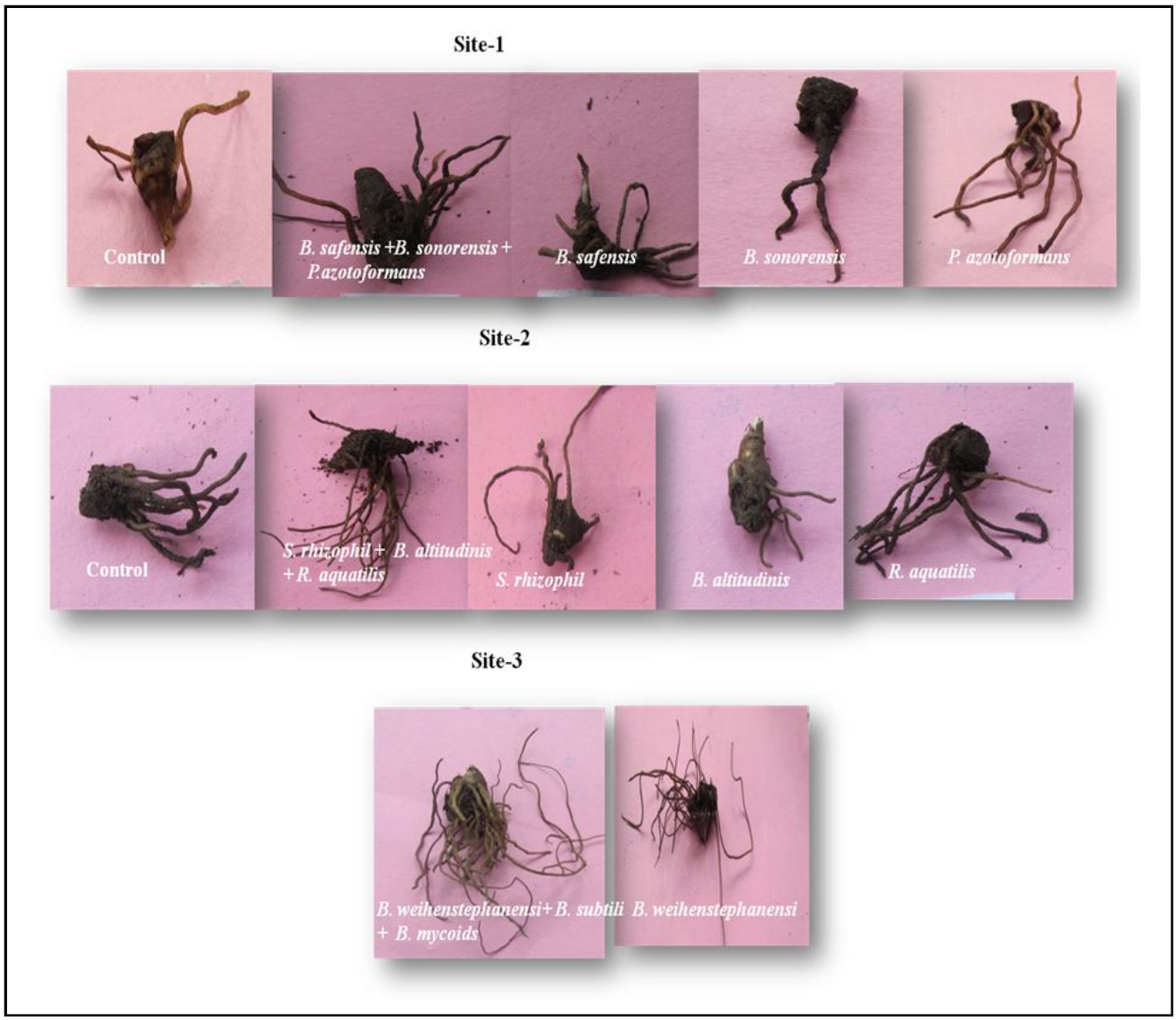

Figure 8: Effect of cyclic application of liquid bioformulation on rhizome size of *T. govanianum*.

4. Discussion

Several microbial species invade plants; however, the majority of them are not cultivable. Rhizospheric bacteria have attracted more and more attention as novel resource to be explored for plant growth promotion. Considerable number of bacterial species mostly those associated with the plant rhizosphere, are able to exert beneficial effect upon plant growth (Müller *et al*., 2016). Since there are no other relevant report available on *T. govaninaum*, but similar studies on rhizoshpere had been conducted on *Valeriana jatamansi* by Chauhan *et al*. (2014), they collected samples from five distinct sites in the Chamba district of Himachal Pradesh, yielded a diversified community of plant growth-promoting rhizobacteria. Molecular methods are a powerful tool for identifying microorganisms in biological samples. The methods make it possible to screen for a wide range of compounds in a single test (Rahmoune *et al*., 2017).

Phosphorus (P) is an essential plant nutrient that plays a key role in photosynthesis, energy transmission, signal transduction, macromolecular biosynthesis, and respiration. Phosphate solubilizing bacteria are capable of solubilizing the insoluble phosphate; enhance soil quality and plant growth and development of different plants (Khan *et al*., 2010). Thakur *et al*.(2016) recovered phosphate solubilizing *Bacillus* and *Pseudomonas* spp. (AvNB-1, AvSB-2, and AvSB-5) and *Pseudomonas* spp. (AvHP-1, AvSP-1, and AvSP-7) from the rhizosphere of the major medicinal plant *Aloe vera* from diverse agroclimatic areas in Himachal Pradesh.

Iron-chelating chemicals released by PGPR isolates are known to boost the availability of iron to plants in iron-deficient soils. Plant growth-promoting rhizobacteria produce siderophores that bind the majority of the Fe III in the area around the plant root, preventing the spread of fungal and other diseases. The absence of iron that results inhibits germs from growing in this area (Radzki *et al*., 2013). Under iron-limiting conditions, *Bacillus*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhodococcus* and other rhizobacteria produced siderophores, small iron chelator molecules that enable the transport

of iron to the root cells and this process helps to maintain plant growth and creates an unfavourable environment for phytopathogens (Raza and Shen, 2010).

Microbial production of ammonia and HCN have been suggested as an important biofertilizer and biocontrol feature to enhance the plant growth. Devi *et al*. (2020) identified 21 distinct bacterial morphotypes from *Ulmus wallichiana*'s rhizosphere. All of the isolates were tested for a variety of plant growth-promoting properties, including phosphate solubilization, nitrogen fixation, siderophore, HCN, ammonia synthesis, and lytic enzyme production, and all of them tested positive for ammonia production. The capacity of bacteria isolated from *Valeriana officinalis* to reduce fungal infections was attributed to extracellular enzyme synthesis such as chitinase, lipase, protease, and amylase, demonstrating the potential of PGPR for biological control (Ghodsalavi *et al*., 2013). Jabborova *et al*. (2020) screened nine endophytic bacterial isolates (GS1, GS2, GS3, GS4, GS6, GS7, GS8, GS9, and GS11) for the production of enzymes. The results of the study revealed that maximum isolates have positive protease activity and this was followed by lipase and cellulase activities.

Plant Growth-Promoting Rhizobacteria (PGPR) are bacteria that live in the soil surrounding plant roots or in plant tissues and promote plant growth either directly or indirectly. One of the primary impacts of PGPR on plant growth is the synthesis of the phytohormone auxin indole-3-acetic acid (IAA) (Yousef, 2018). Cytokinins are important class of phytohormones which are adenine derivatives. Phytostimulatory effects of PGPR may be initiated by several ways but cytokinin production by such bacteria is the direct mechanism to improve plant growth (Remans *et al*., 2008).

T. govanianum is found only in wild pockets of Himalays and their number is decreasing gradually with time. Cultivation of *T. govanianum* using PGPRs is not reported but there are reports on other medicinal plants such as *Rauvolfia tetraphylla*. Kademani *et al*. (2017) investigated the effects of various propagation media and PGPRs on seed germination and seedling vigour in *Rauvolfia tetraphylla* and discovered a germination rate of 16.5 per cent (0.29). The investigations on bacterial community in the rhizosphere soil and roots of many plant species especially the medicinal plants have shown the beneficial effects of microbial communities on plant growth and health (Malleswari and Bagyanarayana, 2021).

Since there is no study has been reported so far on *T. govanianum* cultivation but Karthikeyan *et al*. (2015) studied the effect of plant growth promoting rhizobacteria such as *Azotobacter, Bacillus and Pseudomonas* which were tested separately or in combination in *Catharanthus roseus* for two consecutive years (2005 and 2006). The combinations of above mentioned PGPR strains significantly increased plant height, root length and root girth in this medicinal plant in comparison to the control. Anuroopa and Bagyaraj (2017) isolated and screened nine different PGPR from *Withania somnifera* and observed that plants inoculated with PGPR showed significantly improved growth and yield as compared to uninoculated plants and *Bacillus sonorensis* has a great potential to increase its growth and yield. Jaleel *et al*. (2017) studied the *Pseudomonas fluorescens* on growth parameters and the production of ajmalicine in *Catharanthus roseus* under drought stress.

5. Conclusion

The first time ever novel approach of exploring PGPR from its respective habitat and application of three selected potential PGPR isolates *Bacillus safensis, Bacillus sonorensis* and *Pseudomonas azotoformans* and their consortium for cultivation, growth and establishment of *T. govanianum* in field trial showed significant results with 100% germination and survival of *T. govanianum* in consortium. The inoculation of PGPRs is a sustainable technology and attractive ecofriendly alternative to chemicals to enhance the quality and quantity of medicinal compounds. Thus, open up the possibilities for the utilization of these potential PGPRs in cultivation, growth increase and subsequent boost of yield for *T. govanianum.* Remarkably, use of these novel and potential PGPR isolates have opened a gateway of possibility for cultivation in field and conservation of this rare, most valuable and critically endangered medicinal plant in Indian Himalayan region, and thus can be protected from extinction.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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