Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(4): 1322-1328
Received: 18-05-2020
Accepted: 20-06-2020

Sunita Devi
Department of Basic Science, College of Forestry, Dr YSP University of Horticulture & Forestry Nauni, Solan, Himachal Pradesh, India

Aditi Rana
Department of Basic Science, College of Forestry, Dr YSP University of Horticulture & Forestry Nauni, Solan, Himachal Pradesh, India

Anita Kumari
Department of Tree Improvement and Genetic Resources, College of Forestry, Dr YSP University of Horticulture & Forestry, Nauni, Solan, Himachal Pradesh, India

Joginder Pal
Department of Plant Pathology, College of Horticulture, Dr YSP University of Horticulture & Forestry, Nauni, Solan, Himachal Pradesh, India

Neeraj Sankhyan
Department of Basic Science, College of Forestry, Dr YSP University of Horticulture & Forestry Nauni, Solan, Himachal Pradesh, India

Corresponding Author:
Sunita Devi
Department of Basic Science, College of Forestry, Dr YSP University of Horticulture & Forestry Nauni, Solan, Himachal Pradesh, India

Plant growth promoting potential of rhizobacteria from *Ulmus wallichiana*: An endangered plant of Western Himalayas

Sunita Devi, Aditi Rana, Anita Kumari, Joginder Pal and Neeraj Sankhyan

Abstract
This paper presents a pioneer study on the evaluation of plant growth promoting potential of bacteria associated with rhizosphere of *Ulmus wallichiana*, considering the endangered status of this taxon. *Ulmus wallichiana* (family: Ulmaceae) is a rare and endemic mountain tree of western Himalayas and is one of the richest emporiums for medical taxa. Three sites *viz*., Chansari, Jari and Naggar were selected for the collection of rhizospheric soil samples of *Ulmus wallichiana* in Kullu district of Himachal Pradesh, India. Collected samples were processed for the enumeration of rhizobacteria using Standard Plate Count Technique. In total, 21 different bacterial morphotypes were obtained from all the samples. All the isolates were screened for their multifarious plant growth promoting attributes *viz*., phosphate solubilisation, nitrogen fixation, siderophore, HCN, ammonia production and lytic enzymes production etc. Out of total 21 isolates, 9 were found to be P-solubilizers while 7 were siderophore producers. All the isolates were found to be positive for nitrogen fixing ability as well as ammonia production. Similarly, all isolates showed the production of IAA-like auxins ranging from 2.2-187.8 µg mL^{-1}. These rhizobacteria were also screened for their lytic enzymes production. Of these, only 6, 2, 3 and 1 isolates displayed considerable protease, pectinase, lipase and cellulase activities, respectively. Evaluation of their *in-vitro* PGP potential suggests their substantial role as growth promoters and biocontrol agents.

Keywords: *Ulmus wallichiana,* rhizosphere, PGP potential, PGPR

Introduction
Trees are a valuable gift of nature and the silent protector of our planet. Besides the multitudinous economic benefits, they provide us with unmatched environmental protection- they reduce soil erosion, act as sink for atmospheric carbon dioxide, release large amount of oxygen, provide shade, absorb pollutants and slow down global climate changes such as global warming [1].

Ulmus wallichiana (family: Ulmaceae) is a rare and endemic mountain tree of the western Himalayas and one of the richest emporiums for medical taxa [2]. The tree is native of the broad leaved, temperate and lower temperate forests found in moist ravines at a height of 1800 to 3000 meters mostly with species such as *Celtis tetrandra, Juglans regis, Hippophae salicifolia* and *Betula alnoides* [3, 4]. Locally known as Himalayan Elm, its distribution covers almost the entire region of the western Himalayas ranging from central Nuristan in Afghanistan, through Gilgit Baltistan Pakistan and Northern India to western Nepal [5]. Local people generally plant Elms near their houses for a sustained yield of leaves, which are dried and kept for winter-feeding of livestock. Besides, they yield good quality timber thereby providing a great source of revenue to the farmers [6].

Ulmus is one among the many highly nutritious foliages with crude protein content of 20.44% and can be incorporated in the diets of ruminants [7].Traditionally, it has been used in the treatment of digestive tract diseases [3]. Bark of this tree is commonly used as folk medicine for healing of fracture among animals as well as human beings in India [4]. Bark paste of the plant is mentioned in drugs with the potential of wound healing [8]. Other uses of the plant in public domain include treatment of health related disorders with osteoporosis. Leaves are used as fodder for sheep and goats in Jammu and Kashmir, a Union territory of India [9].

There are a number of threats responsible for decrease in *U. wallichiana* number in the western Himalayas, which may include deforestation, over exploitation and climate changes. *U. wallichiana* falls under the vulnerable category of IUCN red list A1c ver 2.3 [10]. However, Walter and Gillet 1998 [11] categorized it as an endangered plant. Therefore, conservation strategies need to follow in order to improve its number.

One of the most promising conservation strategies is the use of rhizospheric microorganisms (Rhizobacteria) associated with this valuable tree as plant growth promoters.

Rhizobacteria are considered as the most important community microbiota that increase the plant growth by changing the whole microbial community structure and protect the plant roots against various phytopathogens [12]. The study of rhizospheric microorganisms associated with plants is very crucial, as they have beneficial impact on plant growth [13]. As such, there is no report on the number and composition of microorganisms present in the rhizosphere of *Ulmus wallichiana*. Moreover, the microbial diversity present in its rhizosphere may help in adaptation of plant under prevailing conditions. In this context, the present investigation was planned to evaluate the rhizobacterial diversity associated with *Ulmus wallichiana* for its plant growth promoting potential.

Materials and Methods

1. Study area and sample collection

Three sites *viz*., Chansari, Jari and Naggar were selected for the collection of rhizospheric soil samples in Kullu district of Himachal Pradesh, India. The collected samples were placed in plastic bags and stored under refrigerated conditions.

2. Isolation and enumeration of rhizobacteria:

Rhizospheric soil samples were serially diluted to 10 folds by transferring one mL of sample to 9 mL sterile dilution blank under aseptic conditions. The bacterial load present in collected samples was determined by plating serial dilutions of samples [14] on nutrient agar (NA) separately with three replicates. Plates were incubated at 28 ± 2°C for 24-48 hrs. Bacterial counts were recorded and results were expressed in terms of log CFU/g.

3. Maintenance and preservation of rhizobacteria

The bacterial isolates were grown on NA slants and preserved at 4°C in refrigerator. The glycerol stock cultures of all bacterial isolates were prepared in 40 per cent (v/v) glycerol solution and stored at -80 °C for future use.

4. Evaluation of Plant growth promoting (PGP) potential

a) Phosphate solubilization: Qualitative estimation of phosphate solubilization was determined by spot inoculating each bacterial isolate on Pikovskaya (PVK) agar plates. The P- solubilizing efficiency of the bacterial isolates was calculated using the formula: Percent solubilization efficiency = [(Z-C)/C] * 100, where Z = P solubilization zone (mm); C = Colony diameter (mm). The quantitative estimation of inorganic P solubilization was done using PVK broth supplemented with 0.5 per cent of tricalcium phosphate (TCP) by the vanadomolybdate method [15].

b) Siderophore production

The bacterial isolates were screened for siderophore production by chrome azurol sulfonate (CAS) assay as described by (Schwyn and Neilands 1987) [16]. Prior to media preparation, all the glasswares were washed in 6 M HCl to remove the traces of iron present on their surface and then rinsed thoroughly with distilled water. All bacterial isolates were spot inoculated on CAS agar plates and incubated at 28 ± 2 ºC for 3 days. The isolates producing yellow – orange coloured zone around their colonies were considered as siderophore producers. The quantitative estimation of siderophores was done by CAS-shuttle assay [17].

c) Nitrogen fixation

Each of the purified isolate was streaked on Jensen's medium plates. Plates were incubated at 28 ± 2℃ for 24-72 h. Isolates that showed growth on plates were considered as nitrogen fixers [18].

d) IAA production

Indole acetic acid production was measured by the colorimetric method given by (Loper and Scroth 1999) [19]. The bacterial cultures were grown for three days at 28 ± 2 ºC in nutrient broth supplemented with 0, 2 and 5 mg mL-1 DL-tryptophan. Quantitative estimations were done using Salkowski reagent spectrometric ally [20].

e) Ammonia production

The ammonia production was detected by the method given by (Dye 1962) [21]. The 24 h old bacterial cultures were inoculated in 10 mL of peptone broth and incubated at 28 ± 2ºC for 72 h. Following incubation, 1 mL of Nessler's reagent was added, and the production of varying intensity of yellow to brown in the test tubes indicated ammonia production by the bacterial isolates.

f) HCN production

The bacterial isolates were screened for the production of HCN by adopting the method given by (Bakker and Schippers 1987) [22]. The bacterial isolate was streaked on Tryptic Soya Agar amended with 4.4 g L-1 glycine. A Whatman filter paper No. 1 soaked in 2 per cent Na_2CO_3 prepared in 0.5 per cent picric acid solution was placed on the top of the plate. Plates were sealed with parafilm and incubated at 28 ± 2 ºC for 5 days. The development of orange to brown colour indicated HCN production by the bacterial isolates.

5. Morphological and biochemical characterization of potential isolates

Morphological and biochemical characterization of potential plant growth promoting isolates was done according to the standard methods described in Bergey's manual of Systematic Bacteriology [23].

Results and Discussion

Approximately, 80% of the rural population depends on tree diversity for their livelihood requirements such as timber, fuelwood, fodder, litter and compost [24].Tree fodder is particularly useful for temperate weather during the winter months [25, 26]. Fodder must be stacked to feed livestock. Other crops used throughout the year according to seasonal requirements include *Acer caesium, Aesculus indica, Alnus nitida, Quercusseme carpifoli, Celtis australis, Carpinus vimine, Rhuspunj abensi, Corylus jacquemontii* and *Ulmus wallichiana*. The rhizospheric microflora associated with these trees plays directly or indirectly an indispensable role in growth promotion and disease suppression. Henceforth, rhizosphere of *Ulmus wallichiana* was explored for the enumeration of rhizobacteria and eventually these bacteria were exploited for their multifarious plant growth promoting attributes.

Isolation and enumeration of rhizobacteria from *Ulmus wallichiana*

Rhizosphere is a combat environment where microorganisms and plant roots commu-nicate. This region of soil is much richer in bacteria than the surrounding bulk soil [27]. Rhizobacteria are considered the most significant community microbiota that essentially increases plant growth by modifying the entire microbial population structure and protecting the plant's roots from various phytopathogens [12].

Bacteria that inhabit the rhizosphere may influence plant growth by contributing to a host plant's endogenous pool of bioactive compounds such as phytohormones, antibiotics, Siderophores [28]. PGPR can exhibit a variety of characteristics i.e. indirect and direct mechanisms, responsible for influencing plant growth. The indirect effects are related to production of metabolites, such as antibiotics, siderophores, or HCN, that decrease the growth of phytopathogens and other deleterious microorganisms, whereas, the direct effects are dependent on production of plant growth regulators or improvements in plant nutrients uptake [29, 30].

Bacterial communities associated with the rhizosphere of *Ulmus wallichiana* have not yet explored for their PGP potential, hence the present investigation represents to the best of our knowledge a pioneer analysis. The perusal of data depicted in Table 1 reveals that the rhizosphere of *Ulmus wallichiana* anchorages a good population of bacteria. Maximum bacterial load (log CFU/g) of 2.21 was observed at Nagger followed by Jari (2.21) and Chansari (2.19). This disparity in bacterial density could be ascribed to the difference in the type and amount of root exudates secreted by tree roots (Grayston et al. 1997) [31] postulating the varying types and quantities of rhizodeposits as key factors that influence the density and diversity of the rhizospheric microorganisms. Under natural conditions, the rhizosphere and phyllosphere of the plants harbour a large and varied population of the microorganisms [32, 33].

Table 1: Bacteria load in the rhizosphere of *Ulmus wallichiana*

Sr. No.	Sites	Bacterial load (Log CFU/g of soil)
1.	Chansari	2.19
2.	Jari	2.21
3.	Naggar	2.24

Plant growth promoting (PGP) potential of rhizobacteria from *Ulmus wallichiana*

Phosphate solubilization

Phosphate-solubilizing bacteria (PSB) have been considered as one of the possible alternatives for mediating inorganic phosphate solubilization and increasing its availability to the plants Thus, P-solubilization is considered as one of the most important attributes of the PGPR [34, 35, 36]. A total of 21 rhizospheric bacteria were screened for phosphate solubilization qualitatively. Out of 21 isolates, 9 isolates were found to be positive out of which, only 2 isolates showed halo zone size ≥4 mm. For quantification, the P-liberation that that ranged from 10.09-25.51 µg/mL from TCP was recorded. Among all isolates, U21 showed maximum P- solubilization (25.51µg/mL) with 150 per cent P- Solubilization efficiency. The isolates U7, U11, U13 and U20 were found to be statistically at par in terms of P- solubilization (Table 2). Phosphate solubilizing bacteria convert the insoluble form of phosphorus to soluble form through acidification, secretion of organic acids or protons [37].

A decline in the pH of the medium was also recorded which is attributed mainly to the production of low molecular weight organic acids like gluconic, α-ketogluconic, glycolic, oxalic, lactic, acetic, formic, malonic, malic and succinic acids which dissolves the insoluble P at low Ph [38, 39].

Table 2: P-solubilization efficiency of rhizobacteria from *Ulmus wallichiana*

S. No.	Isolates	Halo zone diameter (HZD) = Zone diameter (ZD) – Colony diameter(CD)	P-solubilization (µg/mL)	P-solubilizationefficiency (%SE) (SE= ZD-CD/CD× 100)	Pikovskaya's broth	
					Initial pH	Final pH
1.	U7	3	11.40 ^{b}	67	7.0	6.22
2.	U8	2	10.25^{c}	50	7.0	6.76
3.	U11	3	11.38 ^{b}	75	7.0	5.76
4.	U12	2	10.34 ^{c}	67	7.0	6.40
5.	U13	4	13.20^{b}	80	7.0	5.70
6.	U18	2	10.21^{c}	40	7.0	6.50
7.	U19	2	10.09^{c}	50	7.0	6.53
8.	U20	3	11.40 ^{b}	60	7.0	5.72
9.	U21	6	25.51^{a}	150	7.0	5.42
	CD(0.05)		1.85			

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

Nitrogen fixation, Ammonia and HCN production:

All rhizospheric bacteria were found to possess nitrogen-fixing ability and also recorded positive for ammonia and HCN production (Table 3). However, all isolates displayed difference in their abilities to fix nitrogen and produce HCN, and ammonia.

Nitrogen (N) is an essential nutrient for improved growth and yield in plants. Nitrogen fixing bacteria convert unavailable atmospheric nitrogen (N_2) into available form (NH^{4+}) for plants. Ammonia production by the plant growth promoting bacteria helps to influence plant growth indirectly. This accumulation of ammonia in soil may lead to an increase in pH creating alkaline condition of soil at pH 9-9.5. It suppresses the growth of certain fungi and nitrobacteria due to its potent inhibition effect. It also upsets the microbial community and inhibits germination of spores of many fungi [40]. The HCN production by PGPR helps in disease suppression [41].

Table 3: Nitrogen fixation, HCN and ammonia production shown by rhizobacteria from *Ulmus wallichiana*

Sr. No.	Isolate	Nitrogen fixation	Ammonia production	HCN production
1.	U1	++	+++	+
2.	U2	+	++	+
3.	U3	+	+	+
4.	U4	++	+	++
5.	U5	+	+++	+
6.	U6	+	++	+
7.	U7	+++	++++	++
8.	U8	+++	+	++
9.	U9	+	+	+
10.	U10	++	+++	+
11.	U11	++	++	+
12.	U12	+	+	+++
13.	U13	+++	+++	+++
14.	U14	++	++++	+
15.	U15	+	++	++
16.	U16	+	+	+
17.	U17	+	++	++
18.	U18	++	+	+
19.	U19	+++	++	
20.	U20	++	+	
21.	U21	+++	++++	+++

+ += Weak activity, + + += Moderate activity, + + + += Strong activity

Siderophore production

In the present study, out of a total of 21 isolates, only seven isolates were observed to be positive for siderophorogenesis. The differential ability of isolates to produce siderophores as determined by the orange halo around the colonies is outlined in Table 4. Statistically, the isolate U21 displayed maximum siderophore production (59.0% SU) followed by U20 (40.9% SU), U17 (31.4% SU) and U13 (28.3% SU). However, isolates U7, U9 and U10 were observed to be statistically at par in terms of siderophore production. Siderophores are usually produced by the various soil microbes to bind Fe^{3+} from the surrounding environment and make it available for its own growth and for the plant. The production of siderophores is also a mechanism used by PGPR for rhizosphere colonization competence. Besides, competition for iron plays a vital role in controlling the phytopathogens [42].

The potential to produce siderophore by microorganisms in improving iron availability to plants and sequestering it from pathogens has been reported by many workers [43]. Siderophore producing microorganisms protects plants at two levels: first, limiting growth of plant pathogens and secondly triggering plants defensive mechanism [44].

Indole-3-acetic acid (IAA) production:

As depicted in Table 5, a statistical difference was observed in IAA production by rhizobacterial isolates that ranged from 2.2 - 187.8 µg/mL. Isolate U6 produced the highest amount of 187.8 µg of IAA per mL of culture filtrate followed by U21 (40.8 µg/mL) and U2 (29.2 µg/mL). Minimum IAA production of 2.2µg/mL was displayed by U2.

Microorganisms which produce IAA are known to promote plant growth and root elongation [45]. Tryptophan is believed to be the primary precursor for the formation of IAA in plants and microorganisms. By the production of plant hormones, microorganisms stimulate plant growth in order to increase the production of plant metabolites which can be beneficial for their growth (Gracia de Salamone 2001) [46] reported that IAA is one of the physiologically most active auxins. The bacterial IAA stimulates the root development of host plant, which results in better absorption of water and nutrients from the soil [47, 36].

Table 4: Siderophore production shown by rhizobacteria from *Ulmus wallichiana*

Sr. No.	Isolate	Halo zone Diameter (HZ) in mm	% Siderophore units (%SU)
1.	U7	9	24.6^{e}
2.	U9	10	25.4^{e}
3.	U10	10	25.2^{e}
4.	U13	14	28.3^{d}
5.	U17	19	31.4^{c}
6.	U20	17	40.9^{b}
7.	U21	31	59.0^{a}
	CD(0.05)		1.94

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

Table 5: IAA production shown by rhizobacteria from *Ulmus wallichiana*

Sr. No.	Isolate	IAA production (µg/mL)
1.	U1	12.0^{d}
2.	U2	29.2^{c}
3.	U3	2.2^{e}
4.	U4	10.6^{d}
5.	U5	8.4^{d}
6.	U6	187.8^{a}
7.	U7	11.2^{d}
8.	U8	12.0^{d}

9.	U9	10.8[d]
10.	U10	6.0[e]
11.	U11	4.8[e]
12.	U12	11.4[d]
13.	U13	6.8[e]
I14.	U14	12.8[d]
15.	U15	5.2 [e]
16.	U16	9.2[d]
17.	U17	11.2[d]
18.	U18	10.0[d]
19.	U19	10.8[d]
20.	U20	10.6[d]
21.	U21	40.8[b]
	CD(0.05)	5.95

Each value represents mean of three replicates. According to one way ANOVA, different letters indicate significant differences. Same letters represent that their values are statistically at par.

Lytic enzymes production

In the present study, a total of 21 bacterial isolates were screened for their lytic enzymes *viz.*, protease, pectinase, cellulase and lipase production qualitatively. Of these, only 6, 2, 3 and 1 isolates showed halo zone size between 10-15 mm for protease, pectinase, lipase and cellulase activities, respectively. Henceforth, these were subjected for quantitative estimation of enzymatic activities (Table 6). Results revealed that values of protease activity varied from 40.2 to 88.2 (µg/mL) being highest for U8 (88.2µg/mL) and lowest for U14 (40.2µg/mL). Maximum and minimum pectinase activities of 62.2 and 30.25 µg/mL were shown by U2 and U6 respectively. The lipase activity values varied from 20.0 to 80.0(IU/mL) being highest for U8 (80.0 IU/mL) and lowest for U2 (20 IU/mL). However, only U2 showed cellulase production of 44.4 IU/mL. Extracellular productions of lytic enzymes such as chitinases, glucanases, proteases and lipases by rhizobacteria explain their role as growth promoters and biocontrol agents [48].

Table 6: Lytic enzymes production shown by rhizobacteria from *Ulmus wallichiana*

Sr. No	Isolate	Lytic enzymes production							
		Protease		Cellulase		Pectinase		Lipase	
		Halo Zone (mm)	Protease activity (IU/mL)	Halo Zone (mm)	Cellulase activity (IU/mL)	Halo Zone (mm)	Pectinase activity (IU/mL)	Halo Zone (mm)	Lipase activity (IU/mL)
1.	U2	30	80.2	22	44.4	19	62.2	12	20.0
2.	U5	21	51.2	-	-	-	-	-	-
3.	U6	25	70.2	-	-	10	30.2	16	60.0
4.	U8	32	88.2	-	-	-	-	21	80.0
5.	U10	20	56.2	-	-	-	-	-	-
6.	U14	20	40.2	-	-	-	-	-	-

Morphological and biochemical characterization

Based on PGP potential, six isolates i.e. U2, U6, U8, U17, U20 and U21 were found to be efficient and hence were characterized on the basis of their morphological and biochemical characteristics (Table 7). While being relevant to a limited number of bacterial organisms, the use of colony morphotype with marked characteristics and biochemical tests is of ecological significance to obtain information on rhizosphere distribution in the environment. On the basis of morphological and biochemical characteristics, three isolates out of six were tentatively identified as *Bacillus* sp. one as *Pseudomonas* sp. while two remained unidentified. The predominance of *Bacillus* is due to its ability to efficiently use the nutrients provided by plants through exudates, including the fact that root exudates exert a selective pressure on the proliferation of specific group of bacteria [49]. Type of plant species has had insightful effects on the dynamics of microbial populations, usually exceeding that of plant type [50].

Table 7: Morphological and biochemical characterization of rhizobacteria from *Ulmus wallichiana*

Isolate	Colony characteristics	Biochemical tests							Probable identification
		Gram reaction	MR	VP	Indole	Citrate	Oxidase	Catalase	
U2	Small, circular, raised	+	-	+	+	-	+	+	*Bacillus* sp.
U6	Large, rhizoidal, raised	-	-	+	+	+	+	+	*Pseudomonas* sp.
U8	Large, glistening, mucoid	+	-	+	+	-	+	+	*Bacillus* sp.
U17	Small, circular, flat	-	+	-	+	-	-	+	Unidentified
U20	Large, rough, raised	+	+	-	+	+	+	+	*Bacillus* sp.
U21	Large, irregular, lobate	+	+	-	+	+	-	+	Unidentified

Conclusion:

The present study evinces that rhizobacteria from *Ulmus wallichiana* exhibited considerable *in-vitro* plant growth potential. Hence, it can be efficiently utilized for the preparation of bioinoculants and further evaluated for their response under field conditions as growth promoters and biocontrol agents.

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