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Molecular characterization of rhizobacteria isolated from walnut (*Juglans regia*) rhizosphere in Western Himalayas and assessment of their plant growth promoting activities

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Abstract. *Dar GH, Sofi S, Padder SA, Aisha Kabli A. 2018. Molecular characterization of rhizobacteria isolated from walnut (*Juglans regia*) rhizosphere in Western Himalayas and assessment of their plant growth promoting activities. Biodiversitas 19: 712-719.* The present study was aimed to isolate and characterize effective bacteria from the rhizosphere of walnut (*Juglans regia*) grown in North Western Himalayas and assess their growth promoting potential so that they may, in future, be exploited as biofertilizers. Based on preliminary screening of 98 bacterial isolates obtained from four walnut growing districts of Kashmir valley during survey in 2015, 12 isolates were characterized morpho-biochemically and molecularly basis. On the basis of 16S rDNA sequencing they were identified as *Bacillus licheniformis* WI 90, *B. tequilensis* WI 62, *B. cereus* WI 36, *B. subtilis* strains WI 63 and WI 65, *Micrococcus luteus* strains WI 12, WI 41 and WI 80; *M. yunnanensis* strains WI 60 and WI 30 and *Micrococcus* sp. strains WI 11 and WI 91. The assessment of these rhizobacteria for plant growth promoting attributes revealed that *B. licheniformis* WI 90 possessed higher phosphorus solubilization activity (312 mg/L), followed by *Micrococcus* sp. WI 91 (267 mg/L) while high siderophore was produced by *M. luteus* WI 12 (27.2% siderophore units), followed by *B. licheniformis* WI 90. *B. cereus* strains WI 36. High IAA contents (30 µg/mL) was yielded by WI 41, followed by *M. yunnanensis* WI 60 (28 µg IAA/mL) while higher and statistically at par gibberellic acid was produced by *B. licheniformis* WI 90, *Micrococcus* sp. WI 91 and *M. luteus* WI 80. Higher chitinase enzyme activity was observed in *B. subtilis* WI 63 (30.5 units/mL), followed by *B. tequilensis* WI 62 (25.3 units/mL) and *B. subtilis* WI 65 (25.1 units/mL). The study revealed high plant growth promoting potential in these rhizobacteria.

Keywords: *Bacillus, Micrococcus,* PGP attributes, rhizobacteria, walnut

INTRODUCTION

The walnut (*Juglans regia* L.), also known as Persian or English walnut, is a temperate deciduous broadleaf tree which belongs to the family Juglandaceae. The tree is native to south-east Europe to south-west China. Walnut tree is important for its prized wood and nut fruits. In Western Himalayas, walnut cultivation is generally restricted to the sites not too wet or dry and is grown at an altitude of 900-3550 m masl (Wani et al. 2014). The crop is generally grown at the sites adjacent to undulating forests and mostly no chemicals are applied for its optimal growth. The crop is, therefore, considered purely organic in the region. However, due to high fragility of soils and undulating topography in Western Himalayas, most of the nutrients are depleted fast which negatively influence the plant growth and yield. Reports reveal that the total microbial populations, root exudates, microbial biomass carbon and microbial biomass nitrogen in walnut rhizosphere soil significantly decreases because of prevailing drought and other stress conditions (Liu et al. 2014).

The rhizosphere of a plant is a microecological zone in direct proximity with plant roots. It is functionally defined as the particulate matter and microorganisms that cling to the roots after being gently shaken in water (Walker et al. 2003). The rhizosphere is a metabolically busy fast-changing competitive environment than the surrounding bulk soil. The plant roots, the component of rhizosphere, can affect the physical environment of rhizosphere. A range of interactions from beneficial symbiotic relationships to detrimental pathogenic interactions do occur in the rhizosphere (Sylvia et al. 2005). The rhizobacteria that exert beneficial effects on plant growth and development are known as plant growth promoting rhizobacteria (PGPR) (Ashrafuzzaman et al. 2009). PGPR's promote plant growth through their ability to produce either growth regulators or solubilize mineral phosphates/other nutrients or fix atmospheric nitrogen or antagonistic action against phytopathogenic microbes by the production of siderophores, antibiotics and cyanide (Sarvanakumar et al. 2007; Xuan et al. 2012; Li et al. 2014). PGPR's stimulate plant growth by one or more number of different mechanisms directly or indirectly. The experimental evidences suggest that plant growth stimulation is the net result of multiple mechanisms that may be activated simultaneously (Martinez et al. 2010). PGPR belong to diverse genera especially *Alcaligenes, Azospirillum, Arthrobacter, Acinetobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium* and *Serratia.* All of them are able to exert beneficial effects on plant growth (Tilak et al. 2005). Aravind et al. (2009) isolated 74 bacteria from black pepper which belonged to six genera *viz*., *Bacillus* sp. (22 strains), *Pseudomonas* sp. (20 strains), *Serratia* (1 strain), *Arthrobacter* sp. (15 strains), *Micrococcus* sp. (7 strains) and *Curtobacterium* sp. (1 strain). *Bacillus* species were also isolated by Figueiredo et al. (2009) from Brazilian sweet corn on the basis of their sequencing of 16S ribosomal gene and amongst the 42 isolates identified *Bacillus subtilis, B. pumilus, B. licheniformis*, *B. cereus* and *B. amyloliquefaciens* were the most frequently encountered species. Some rhizobacteria show the ability to antagonize phytopathogens through competition, antibiotics production or lytic enzymes secretion (Van Loon and Bakker 2003) that make them a potent tool for reducing damages through prevention of deleterious effects of phytopathogens.

Worldwide, amongst the temperate horticulture crops, the rhizosphere of vegetable crops has extensively been studied while the rhizosphere of fruit crops such as apple, pear, cherry, apricot, etc., has been explored to a lesser extent (Bashan 1998; Prasad and Dagar 2014; Mehta et al. 2015; Gupta et al. 2016). However, with a few exceptions, the rhizosphere of nut crops like walnut has not been explored as yet (Dar et al. 2009; Guleria et al. 2014). Since walnut trees exhibit allelopathy, therefore only specific types of microorganisms are expected to thrive under its tree canopy. Thus, the microorganisms in walnut rhizosphere may apparently be quite different from those present in the rhizosphere of other temperate fruit crops. Zang et al. (2015) identified 11 strains of phosphate-solubilizing bacteria in walnut rhizosphere by 16S rDNA which belonged to 5 genera namely *Pseudomonas*, *Staphylococcus*, *Planomicrobium*, *Microbacterium*, and *Acinetobacter*. Liu et al. (2014) have suggested that the inoculation of *B. cereus* L90 interferes with the suppression of stress conditions to the biological characteristics of walnut rhizosphere soil. The perusal of literature has revealed that with the exception of a preliminary report (Dar et al. 2009), no studies on rhizosphere microbes of walnut have been conducted in the Western Himalayas, especially in South Asian Subcontinent.

The present study was, therefore, aimed to explore the bacterial diversity existing in walnut rhizosphere under temperate Western Himalayan conditions as well as assess their plant growth promoting traits.

MATERIALS AND METHODS

Collection of soil samples

The rhizosphere soil samples along with root samples were collected from the canopy of young actively growing walnut trees from four walnut growing districts of Jammu and Kashmir state (India) *viz*., Kupwara, Baramulla, Budgam, and Shopian. Three commercially growing walnut blocks were chosen from each district, and in each block, three sites were randomly chosen. The rhizosphere soil along with root samples was collected from the canopy of young actively growing walnut trees from the selected sites. The rhizosphere soil samples were collected from all sides at root depth, thoroughly mixed and a composite sample per site drew for the isolation of rhizosphere bacteria. The sampling was done in the month of June during the year 2014-2015.

Isolation of rhizobacteria from walnut rhizosphere

For isolation of bacteria, 1 g rhizospheric soil sample from each site was serially diluted (10^3-10^7). The diluted suspensions (0.1 mL) were spread on pre-poured nutrient agar medium and incubated at 25$\pm$1°C for 24-48 hours. The isolated colonies that developed on nutrient agar medium (master plate) were replica plated (Roberts 1959) onto selective media *viz*., nitrogen-free medium for determining nitrogen-fixing ability, CAS medium (Schwyn and Neilands 1987) for assessing siderophore producing ability and Pikovskaya medium (Pikovskaya 1948) for qualitative estimation of phosphate solubilizing ability. All the colonies were transferred to the same position as the master plate with the help of a wooden block, covered with sterilized velvet cloth. At the end of incubation period, the location of colonies that appeared on replica plates was compared to the master plate. On the basis of initial screening, the best 12 isolates were chosen for morpho-biochemical and molecular characterization and assessment of their plant growth promoting attributes.

Morpho-biochemical characterization

The isolates were characterized by some important morphological and biochemical attributes. The morphological characterization was done by observing the isolated colonies under a compound microscope (Gaynor) at 100X for colony color, form, elevation, and margin. Also, cell shape, size, endospore presence and Gram's reaction was noted. The various biochemical characterization *viz*., indole production, methyl red test, Voges-Proskauer reaction, citrate utilization test, oxidase test, catalase production, acid production, H_2S production and starch hydrolysis was carried out as per *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994).

Molecular characterization of bacterial isolates

The molecular characterization of rhizobacterial isolates was carried out on the basis of 16S rRNA sequencing. For this, the isolates were sent to Triyat Scientific 39A, Kannava Nager, Wardha, Nagpur, Maharashtra (India). As per the details shared, the total genomic DNA of isolates was extracted by N-cetyl-N-N-trimethyl-ammonium bromide (CTAB) method (Doyle and Doyle 1987; Doyle and Dickson 1987; Cullings 1992). The forward and reverse primers used for 16S rDNA amplification were: fD1 (5'AGAGTTTGATCCTGGCTCAG3') and rD1 (5'AAGGAGGTGATCCAGCCGCA3') (Luckow et al. 2000) used to amplify 1542, 1584, 1500, 1542, 1529, 1512, 1540, 1484, 1557, 1555, 1571 and 1466 bp region of 16S rRNA genes of these isolates using a thermal cycler (BioRad, USA). Amplification products were resolved by agarose-gel electrophoresis (1.5%) and visualized on a gel documentation system (Alfa Imager, Alfa Innotech Corporation, USA). The amplicons were purified using GeneiPure™ quick PCR purification kit (GeNei™, Bengaluru, India) and quantified at 260 nm using a spectrophotometer taking calf thymus DNA as control. The purified partial 16S rDNA amplicons were sequenced in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

Analysis of 16S rDNA sequences

The partial sequences of nucleotides of 16 S DNA were compared with the available sequences from National Center for Biotechnology Information (NCBI) database and the sequences showing >99 % similarity were retrieved by Nucleotide Basic Local Alignment Search Tool (BLAST-N) program available at NCBI server (www.ncbi.nlm.nih.gov/BLAST). The retrieved sequences were aligned with the sequences of isolates at http://www.ebi.ac.uk/Tools/msa/muscle/. The primer impurity was identified and unwanted sequences trimmed. The pure sequences were submitted to NCBI at https://www.ncbi.nlm.nih.gov/, and the accession numbers for each isolate was obtained. All the nucleotide sequences were aligned using CLUSTAL x 1.8 multiple alignment programme (Thompson et al. 1997) refined manually. The GENEDOC package (www.psc.edu/biomed/genedoc/gdpf.html) was used for formatting the sequences to make them compatible with the desired software. The phylogenetic tree was constructed according to Kimura 2-parameter (K2P) model using MEGA v. 5.0 (Tamura et al. 2007).

Quantitative assessment of plant growth promoting traits of bacterial isolates

Phosphate solubilization assay

The flasks containing liquid Pikovskaya's medium (PVK) were inoculated with 10% bacterial suspension (OD 1.0 at 540 nm) of each isolate separately and incubated at 35±2°C for 72 hours under shake conditions. Simultaneously, a control PVK broth without inoculum was also run. These flasks were then centrifuged at 15000 rpm for 20 minutes at 4°C. The culture supernatant was used for the estimation of soluble phosphorus as per the method of Bray and Kartz (1945).

Siderophore production

The quantitative estimation of siderophore production by isolates was performed by liquid chrome azurol-S (CAS) assay method (Schwyn and Neilands 1987). The cell-free extract of supernatant (0.1 mL) was mixed with 0.5 mL CAS assay solution along with 10 µl of shuttle solution (0.2 M 5-sulfosalicylic acid). The mixture was kept as such at room temperature for 10 minutes and absorbance noted at 630 nm using UV-VIS spectrophotometer (SL 164, Systronics). A blank reference (r) was also maintained using all above components, except cell-free extract of supernatant. The siderophore units were calculated as:

Percent siderophore unit = x 100

Where A_r is the absorbance of reference at 630 nm, and A_s is absorbance of test solution at 630 nm.

Indole-3-acetic acid (IAA) estimation

The IAA production was estimated as per the method of Gorden and Paleg (1957). The bacterial isolates were raised in Luria Bertani broth for 72 hours at 37°C under shake conditions. Then supernatant was collected by the centrifugation of cultures at 15,000 rpm for 20 minutes and stored at 4°C. The supernatant (3 mL) and Salkowski's reagent (2 mL) were mixed, and the mixture kept in dark for 30 minutes for the development of pink color, if any. The color intensity was measured at 535 nm by UV-VIS spectrophotometer. The concentration of IAA was estimated by preparing calibration curve using 10-100 µg IAA (Hi-media) per mL.

Estimation of gibberellins

The gibberellin producing ability of bacterial isolates were estimated as per Holbrook et al. (1961). For this, bacterial isolates were grown in nutrient broth for 72 hours at 37°C under shake conditions. The supernatant was then centrifuged at 15,000 rpm for 20 minutes and stored at 4°C till use. The supernatant (15 mL) was taken and 2 mL zinc acetate reagent added to it. After 2 minutes, 2 mL potassium ferrocyanide was added and the contents centrifuged at low speed (2000 rpm) for 15 minutes. Then, to 5 mL supernatant 5 mL of 30% HCI was added and the mixture incubated at 20ºC for 75 minutes. For blank, 5 mL of 5% HCI was used. The absorbance was noted at 254 nm using UV-VIS spectrophotometer. The concentration of gibberellins was calculated by preparing standard curve using gibberellic acid (GA_3) as standard (100-1000 µg/mL).

HCN production

The method of Baker and Schippers (1987) was adopted for the estimation of HCN production by rhizobacteria. The test cultures were streaked on pre-poured plates of King's medium B. The Whatman No.1 filter paper strips were soaked in 0.5% picric acid in 0.2% sodium carbonate and was placed in between the petri-plates. The petri-plates were sealed with parafilm and then incubated at 37°C for 1-4 days. Uninoculated control was also maintained for comparison. The plates were observed for color change in filter paper from yellow to orange-brown to dark brown.

Chitinase enzyme assay

For chitinase assay, the bacterial isolates were grown in 100 mL fresh medium (3% w/v chitin; 0.1% KH_2PO_4; 0.05% $MgSO_4.7H_2O$; 50 mM sodium phosphate buffer, pH 6.0) in 250 mL Erlenmeyer flasks for three days at 30°C. After incubation, the supernatant (enzyme solution) was collected by centrifuging the mixture at 12,000 rpm for 20 minutes. For the estimation of chitinase activity, the method of Berger and Reynolds (1958) was followed.

Statistical analysis

All the data were analyzed statistically using analysis of variance (Narayanan and Adorisio 1983). The significance of treatments was tested at 5% level of probability and the treatment mean values compared using Duncan's multiple range test (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

Overall 98 morphologically dissimilar bacterial isolates were isolated from the walnut rhizosphere soils of Kashmir. The twelve best bacterial isolates WI 11, WI 12, WI 30, WI 36, WI 41, WI 60, WI 62, WI 63, WI 65, WI 80, WI 90 and WI 91 were selected on the basis of preliminary screening based on qualitative tests for phosphorus solubilization and production of siderophore, ammonia, hydrogen cyanide, indole acetic acid and gibberellic acid (Shakeela Sofi 2017). The morpho-biochemical characterization of these isolates revealed that seven isolates *viz.,* WI 11, WI 12, WI 30, WI 41, WI 60, WI 80 and WI 91 tentatively belonged to genus *Micrococcus* and five isolates *viz.,* WI 36, WI 62, WI 63, WI 65 and WI 90 tentatively belonged to genus *Bacillus* (Table 1). Our findings are supported by Naveed et al. (2014) who found rods, cocci and coco-bacillus dominant in rhizosphere, and confirmed by Vega et al. (2005) who isolated high number of bacteria from coffee rhizosphere which belonged to genera *Bacillus*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Escherichia*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Serratia* and *Stenotrophomonas*.

Molecular characterization

The isolates were identified by amplifying their 16S rRNA gene sequences of different lengths. The partial sequences of nucleotides were compared with the available sequences from NCBI database and the sequences showing >99% similarity were retrieved by BLAST-N program (NCBI; www.ncbi.nlm.nih.gov/BLAST). The sequences submitted to NCBI and their accession numbers and number of base pairs amplified are indicated in Table 2. Phylogenetic analysis revealed that the bacterial isolates resembled with many reference sequences existing in the global bacterial gene pool and accordingly were identified on the basis of maximum sequence homology and phylogeny with the global reference sequences (Figure 1). The bacterial isolates belonged to two genera namely *Bacillus* and *Micrococcus*. The three isolates were identified as *Micrococcus luteus* (strains WI 12, WI 41 and WI 80) while two isolates each were identified as *Micrococcus yunnanensis* (strains WI 30 and WI 60), *Micrococcus* sp. (strains WI 11 and WI 91) and *Bacillus subtilis* (strains WI 63 and WI 65). One isolate each was identified as *Bacillus tequilensis* (strain WI 62), *Bacillus cereus* (strain WI 36) and *Bacillus licheniformis* (strain WI 90). All the above rhizobacterial species are reported for the first time from walnut rhizosphere although previously Dar et al. (2009) have reported the presence of genera *Azotobacter, Azospirillum, Bacillus, Pseudomonas, Aspergillus* and *Penicillium* in walnut rhizosphere but they did not identify them upto species level. Perusal of literature revealed that no work has been conducted on the rhizobacteria of walnut based on molecular characterization, especially in India and North-Western Himalayan region and the present work appears first of its kind conducted in Himalayan mountainous region (Jammaludin et al. 2004).

Bacillus subtilis, *B. cereus* and *B. licheniformis* have earlier been reported as culturable bacterial endophytes of saffron in Kashmir (Sharma et al. 2015a,b) while *B. tequilensis* has been isolated from the water samples of Manasbal lake of Kashmir by Sana Shafi et al. (2017). There is no report of *Micrococcus luteus, Micrococcus* sp. and *M. yunnanensis* from Jammu and Kashmir. *M. luteus* has been reported from Kerala (India) as being associated with the rhizosphere of black pepper (Dinesh et al. 2014) while *Micrococcus* sp. NII-0909 has been found as novel plant growth promoting rhizobacteria associated with cowpea (Dastager et al. 2010) from Trivandrum (India). There is no report of *M. yunnanensis* as rhizobacteria from India, although it has been reported as an endophyte of *Catharanthus roseus* wherein it has been evaluated for production of antibiotics against antibiotic resistant pathogens (Rajan and Jadeja 2017) and not for plant growth promoting traits. *M. yunnanensis*as PGPR microbe is reported for the first time from South Asian subcontinent. Worldwide, there are two reports of *M. yunnanensis* being PGPR, one is from Iran by Ghavami et al. (2017) who amongst the 45 isolates from mustard (*Brassica napus*) rhizosphere molecularly characterized highest siderophore producing isolates on the basis of 16S rRNA sequence analysis and identified them as *M. yunnanensis* YIM 65004 (T) and *Stenotrophomonas chelatiphaga* LPM-5 (T). These two rhizobacteria showed growth promoting effect on canola and maize in terms of increased grain weight and iron content of roots and shoots. Another report from Korea by Siddikee et al. (2010) showed that of the 36 halotolerant bacterial strains isolated from the rhizosphere of six naturally growing halophytic plants in the vicinity of Yellow Sea and identified on the basis of 16S rRNA gene sequence belonged to 10 different bacterial genera which included *Micrococcus yunnanensis* RS222 and *Bacillus aryabhattai* RS341. Zhang et al. (2015) isolated 54 strains of phosphate-solubilizing bacteria from walnut rhizosphere soils in Xinjiang province (China) and the best 11 strains identified by 16 S rDNA belonged to 5 bacterial genera *viz*., *Pseudomonas, Staphylococcus, Planomicrobium, Microbacterium,* and *Acinetobacter.*

Plant growth promoting activities of rhizobacterial isolates

The isolates were screened for their multifarious plant growth promoting activities quantitatively wherein a significant variation in phosphorus solubilization and siderophore production was noticed in different rhizobacteria (Table 3). The bacterial isolate *B. licheniformis* strain WI 90 showed maximum phosphorus solubilization activity (312 mg/L), followed by *Micrococcus* sp. isolate WI 91 (267 mg/L) and *B. subtilis* strain WI 65 (242 mg/L). Phosphorus solubilization is considered as one of the most important attributes of plant growth promoting rhizobacteria (Patel et al. 2008; Yasmin et al. 2012). Siderophore was produced maximum by *M. luteus* strain WI 12 (27.2% siderophore units), followed by *B. licheniformis* WI 90 (25% siderophore units) and *M. luteus* strain WI 41 (22.5% siderophore units). Shobha and Kumudhini (2012) reported that *Bacillus* isolate JUMB7 produced 10% siderophore units while Pal and Gokarn (2010) found that *Klebsiella* sp. were able to produce 3.22 and 11.99% siderophore units which fall within our observed range.

Table 1. Morpho-biochemical characterization of the rhizobacterial isolates

Isolates	Indole test	Methyl red test	Voges Proskauer test	Starch hydrolysis	Citrate utilization test	H_2S production	Oxidase test	Catalase test	Gram's reaction	Cell shape	Endospore position	Probable genus
WI 11	-	-	-	+	-	-	+	+	+	Minute cocci	-	*Micrococcus*
WI 12	-	-	-	+	-	-	+	+	+	Minute cocci	-	*Micrococcus*
WI 30	-	-	-	+	-	-	+	+	+	Minute cocci	-	*Micrococcus*
WI 36	-	-	+	+	+	-	-	+	+	Long rods	Central	*Bacillus*
WI 41	-	-	-	+	-	-	+	+	+	Minute cocci	-	*Micrococcus*
WI 60	-	-	-	+	-	-	+	+	+	Minute cocci	-	*Micrococcus*
WI 62	-	-	+	+	+	-	-	+	+	Long rods	Central	*Bacillus*
WI 63	-	-	+	+	+	-	-	+	+	Long rods	Central	*Bacillus*
WI 65	-	-	+	+	+	-	-	+	+	Medium rods	Central	*Bacillus*
WI 80	-	-	-	+	-	-	+	+	+	Minute cocci	-	*Micrococcus*
WI 90	-	-	+	+	+	-	-	+	+	Long rods	-	*Bacillus*
WI 91	-	-	-	+	-	-	+	+	+	Minute cocci	-	*Micrococcus*

Table 2. The molecular characterization of some selected rhizobacterial isolates based on16s rRNA sequencing (submitted to NCBI, USA)

Rhizobacterial isolates	Accession number	No. of base pairs amplified	Isolates identified
WI 91	KY777463	1542	*Micrococcus* sp. strain WI91
WI 60	KY777460	1584	*Micrococcus yunnanensis* strain WI60
WI 11	KY777456	1500	*Micrococcus* sp. strain WI11
WI 80	KY777455	1542	*Micrococcus luteus* strain WI80
WI 12	KY777454	1529	*Micrococcus luteus* strain WI12
WI 30	KY777453	1512	*Micrococcus yunnanensis* strain WI30
WI 41	KY777452	1540	*Micrococcus luteus* strain WI41
WI 36	KY777451	1484	*Bacillus cereus* strain WI36
WI 90	KY777445	1557	*Bacillus licheniformis* strain WI90
WI 65	KY777444	1555	*Bacillus subtilis* strain WI65
WI 62	KY777443	1571	*Bacillus tequilensis* strain WI62
WI 63	KY777442	1466	*Bacillus subtilis* strain WI63

Table 3. Multiple plant growth promoting activities of rhizobacteria isolated from walnut rhizospehere

Rhizobacteria	Phosphorus solubilization (mg/L)	Siderophore production (% siderophore unit)	IAA production (µg/mL)	Gibberellic acid production (µg/mL)	Chitinase activity (units/mL)	HCN production
Micrococcus luteus strain WI 12	205	27.2	21.0	51.0	18.2	++++
M. luteus strain WI 41	180	22.5	30.0	45.0	21.0	+++
M. luteus strain WI 80	162	16.0	21.0	64.7	17.7	+++
M. yunnanensis strain WI 30	185	17.7	21.0	46.0	16.5	++
M. yunnanensis strain WI 60	172	16.5	28.0	49.0	19.8	++++
Micrococcus sp. strain WI 11	164	18.8	24.0	60.0	24.1	++
Micrococcus sp. strain WI 91	267	15.0	16.0	65.0	17.5	++++
Bacillus cereus strain WI 36	180	17.5	30.0	59.0	18.3	++++
B. subtilis strain WI 63	152	16.6	22.0	62.0	30.5	+++
B. subtilis strain WI 65	242	18.8	22.5	48.0	25.1	+++
B. licheniformis strain WI 90	312	25.0	19.0	65.3	23.2	++++
B. tequilensis strain WI 62	222	22.3	21.0	60.0	25.3	++++
C.D at 5%	4.28	2.91	3.03	3.59	2.79	-
SEM±	1.47	0.99	1.04	1.23	0.96	-
CV	1.25	1.86	1.35	1.79	1.74	-

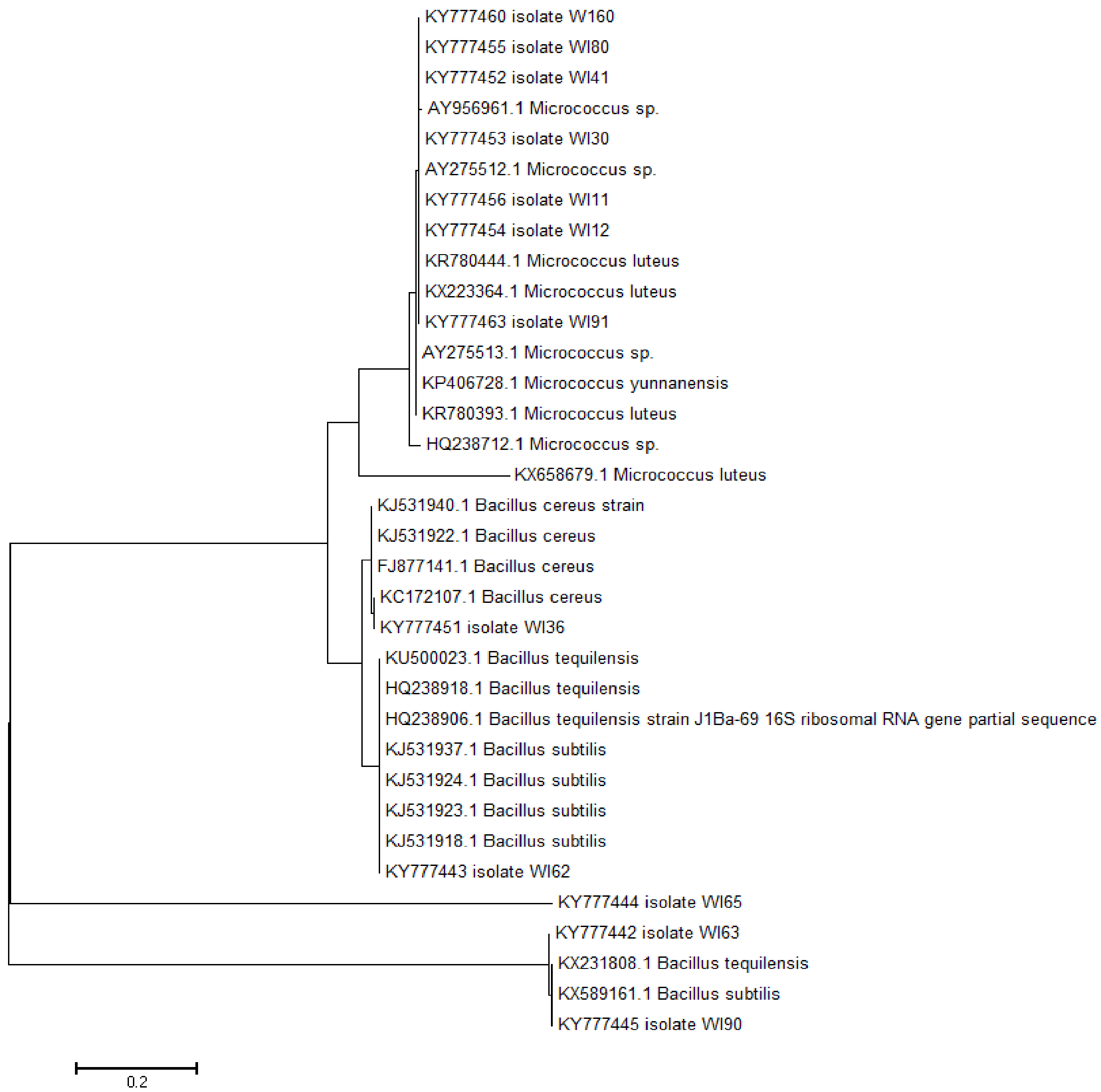

Figure 1. Phylogenetic tree of rhizobacterial isolates from walnut

The production of IAA is an important plant growth promoting trait in PGPR's as well as it is a signal molecule in the regulation of plant development. Higher auxin level impairs plant defense mechanisms making colonization easier and stimulates both rapid (increase in cell elongation) and long-term (cell division and differentiation) responses in plants. IAA production is widespread among soil and plant-associated bacteria, and its biosynthesis in an integral core trait of symbiotic species within genera of *Rhizobium*, *Bradyrhizobium* and *Nostoc* and other plant-associated PGPR (Shah et al. 2013). In the present study, the bacterial isolates varied in their ability to produce IAA. *Bacillus cereus* strains WI 36 and WI 41 yielded maximum IAA (30 µg/mL), followed by statistically at par *M. yunnanensis* WI 60 (28 µg IAA/mL) (Table 3). Our results are in agreement with Beneduzi et al. (2008) who reported that many *Bacillus* sp. and *Paenibacillus* sp. produce IAA in Luria Bertani broth. Similar to our observations Khin et al. (2012) and Kaur and Sharma (2013) reported IAA production by rhizobacteria in the range of 53.1 to 71.1 µg/mL under optimum growth conditions while Husain (2003) reported a lesser range of IAA production (2.09 to 33.28 µg/mL) in bacteria. Shobha and Kumudini (2012) reported that *Bacillus* isolates produced IAA in varying quantities from 35 to 217 µg/mL. IAA production varies in different PGPR species and strains and is influenced by the organism involved, cultural conditions, growth stage and substrate availability (Ashrafuzzaman et al. 2009).

Maximum gibberellic acid (GA) production was observed in *B. licheniformis* WI 90 (65.3 µg/mL), followed by *Micrococcus* sp. strains WI 91 (65.0 µg/mL) and *M. luteus* WI 80 (64.7 µg/mL). Karakoc and Aksoz (2006) optimized cultural parameters for GA production by *Pseudomonas* sp., isolated from wastes of processed olive. In their study, the highest GA production (250.1 mg/L) was obtained in nutrient broth (pH 7.0) incubated at 30ºC for 72 hours on a rotatory shaker and in dark conditions.

Hydrogen cyanide is a secondary metabolite produced commonly by rhizobacteria. HCN is postulated to play a role in biological control of pathogens (Defago et al. 1990). Though all of the twelve rhizobacterial isolates exhibited HCN production but six isolates *viz., M. luteus* strain WI 12, *B. cereus* strain WI 36, *M. yunnanensis* strain WI 60, *B. tequilensis* strain WI 62, *B. licheniformis* strain WI 90 and *Micrococcus* sp. strain WI 91 showed very high HCN production and in these cases the colour of filter paper changed from pale yellow to dark brown. In rest six isolates, four isolates *viz*., *M. luteus* strain WI 41, *B. subtilis* strain WI 63, *B. subtilis* strain WI 65 and *M. luteus* strain WI 80 showed HCN production wherein the colour of filter paper changed from pale yellow to light brown and in two isolates *viz*., *Micrococcus* sp. strain WI 11 and *M. yunnanensis* strain WI 30, the colour of the filter paper changed from pale yellow to deep orange. The HCN production reportedly is a common trait of *Bacillus* (88.9%) and *Pseudomonas* (50%) in rhizospheric soil (Heydari et al. 2008). Noumavo et al. (2015) reported that of the 15 rhizobacterial isolates from maize 86.7, 80.0 and 60.0% produced ammonia, HCN and IAA, respectively.

Among different lytic enzymes, chitinases are particularly useful in agriculture as biocontrol agents against various fungal pathogens owing to their ability to hydrolyze chitinous fungal cell wall (Chaiharn and Lumyong 2009; Suresh et al. 2010; Wahyudi et al. 2011). In the present study, a significantly high chitinase enzyme activity was exhibited by *B. subtilis* WI 63 (30.5 units/mL), followed by *B. tequilensis* WI 62 (25.3 units/mL) and *B. subtilis* WI 65 (25.1 units/mL), *Micrococcus* sp. WI 11 (24.1 units/mL) and *M. yunnanensis* WI 90 (23.2 units/mL). Dhar and Kaur (2010) observed a high chitinase activity of 2.64-35.08 IU/mL in 17 bacterial isolates after 120 hours of incubation. Chitinase production is induced in a colloidal chitin containing environment (Gupta et al. 1995; Mahadevan and Crawford 1997).

Conclusion

The microbially unexplored horticultural plants like walnut possess diverse and potential microbial association which can be exploited for use as microbial inoculants for improving the growth of a wide variety of plants growing under nutrient-stress conditions. The present work for the first time reports the presence of a high diverse PGPR's from walnut rhizosphere which possesses the ability to solubilize phosphates and produce siderophore, IAA, gibberellic acid, chitinase enzyme, ammonia, and HCN. The study elucidates the multifarious role of rhizobacterial isolates, especially *Bacillus licheniformis*, *B. tequilensis*, *B. cereus*, *B. subtilis*, *Micrococcus luteus*, *Micrococcus* sp. and *M. yunnanensis*. Thus, the use of these PGPR's can be successfully exploited as biofertilizers for sustainable crop production. However, there is need to develop suitable microbial consortium to offset the microclimatic unfavorable effects on individual species. Also, suitable delivery system needs to be evaluated so that these consortia reach target site without any intense competition for niche and nutrients as well as they remain viable and effective for longer periods.

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