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Research Article

Characterization of *Bacillus weihenstephanensis* AGII: A Psychrotolerant Bacteria Isolated from Rhizosphere of Medicinal Plant *Gentiana kurroo* Royle

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Abstract

The beneficial bacteria present in the area around the plant roots. These are termed as Plant Growth Promoting Rhizobacteria (PGPR). These bacteria are useful to enhance plant growth and curb plant diseases. The objectives of the present study were to isolate, identify and characterize PGPR traits in psychrotolerant bacteria native to rhizosphere of a threatened high valued medicinal plant *Gentiana kurroo* Royle. In total 10 bacterial isolates were isolated, out of which isolate AGII showed maximum antagonism against tested phytopathogens. Therefore, isolate AGII was selected further to study in vitro growth promoting attributes and for phylogenetic identification. Bacterial isolate AGII was identified as *Bacillus weihenstephanensis* by 16SrRNA gene sequence and has been registered in NCBI under the accession number MF593886. Plant growth promoting traits of this isolate were studied by performing following assays *viz*., Phosphate solubilization, Ammonia Production, HCN production, Protease production. The isolated bacterial strain was studied for its inhibitory potential against deadly plant pathogens by using a dual culture *in vitro* assay. In the present investigation, *Bacillus weihenstephanensis* MF593886 was tested against deadly plant pathogens viz., *Alternaria solani* MTCC 2101, *Botrytis cinerea* MTCC 2350, *Fusarium oxysporum* MTCC 7677, *Rhizoctonia solani* MTCC 4633, *Colletotrichum gloeosporioides* MTCC 9664 and *Sclerotinia sclerotiorum* MTCC 8785. It is capable to produce siderophore, HCN, ammonia, proteases and has a potential to solubilize phosphates. The present study proposes the potential of *Bacillus weihenstephanensis* as a PGPR in the context of cold agro-ecosystems.

Keywords: Bacillus weihenstephanensis; Biofertilizers; Psychrotolerant; PGPR; Proteolysis; Siderophore

Introduction

Gentiana kurroo Royale is a critically endangered plant of Western and North Western Himalaya. This drug plant is critically endangered and is at high risk category as far as its survival is concerned due to its over exploitation habitat destruction and unscrupulous collection [1]. To establish such plants in new environment, the study of their microflora is a must. In the present investigation, soil associated with the roots of *Gentianakurroo* has been used for isolation of PGPR. These PGPR are the beneficial bacteria that assertively inhabit in vicinity or on plant roots and provides benefit to the plants by a variety of mechanisms viz. by fixing nitrogen, making plant growth regulators, inhibiting phytopathogenic

microorganisms, solubilizing mineral phosphates and by Inducing Systematic Resistance (ISR) [2,3].

Bacillus, are among the maximum-studied rhizobacteria that has their role in plant growth promotion. *Bacillus* sp. are spore formers that can stay alive in the soil for extended period of time under adverse ecological conditions. The most commonly studied plant growth promoting bacilli are *B. megaterium*, *B. subtilis*, *B. cereus, B. amyloliquefaciens and B. licheniformis*, [4]. Though, a number of PGPR have been reported but still very little is known about psychrotolerant PGPR [5]. Among *Bacillus* sp*., Bacillus weihenstephanensis* is a cold tolerant bacterium [6,7]. In the present study, an attempt has been made to isolate psychrotolerant *Bacillus weihenstephanensis* from rhizosphere of rarely explored high valued threatened Himalayan medicinal plant *Gentiana kurroo.* The main objective of the present study was to isolate and identify potential psychrotolerant PGPR from *Gentiana kurroo* which is critically endangered medicinal plant of alpine Himalayas.

Materials and Methods

Samples collection

The rhizospheric soil of 5 altered plants of *Gentiana kurroo* growing in Sangla Valley (NW Himalaya) at an altitude range of 3885m-4205 m, District Kinnaur, Himachal Pradesh, India were collected. For isolation method, one combinedsoil sample was prepared by mixing rhizospheric soil of five different plants of *Gentiana kurroo* in an equal part insterilizedcontainer and brought to the NBPGR, Phagli, Regional Centre, Shimla, H.P. India.

Isolation and screening

Standard bacteriological procedures were used to isolate bacteria associated with rhizosphere of *Gentiana kurroo*. Soil sample was diluted by double dilution method following spread plate method on nutrient agar medium. Incubation of the plates were done at 20^0C for 72 h. Pure cultures were obtained after streaking the bacterial colonies on nutrient agar plates. Pure cultures of bacteria were preserved on nutrient agar slants (4^0) and also in 30% glycerol (-20^0C) in deep freezer. Isolated bacteria were tested for their antagonistic potential against phytopathogens.

Antagonistic potential of isolates against deadly phytopathogens

Antagonistic potential was determined by Bit/disk technique as given below.

Indicators

The names of indicators selected for present study are serious plant pathogens *viz*: *A. solani* MTCC 2101, *B. cinerea* MTCC 2350,*R. solani* MTCC 4633,*F.oxysporum*MTCC 7677, *C. gloeosporioides* MTCC 9664*, S. sclerotiorum* MTCC 8785.These test strains were procured from CSIR-MTCC, IMTECH, Chandigarh, India).

Inhibitory potential of isolates against pathogenic fungus

Dual culture in vitro assay was used to evaluate inhibitory potential of AGII against phytopathogens [8]. For evaluating the inhibitory potential, bit of AGII was positioned on one region of plate and mycelial bit from 7 days old pathogenic fungus was kept on the other region of the plate (both the bits were kept on the same gap from the middle of the plate). Plates were incubated at 27±2^0C for one week. For control petri dishes were inoculated with fungal discs. This experiment was performed in 3 replicates for each isolated bacteria.

% Inhibition (I) was calculated using the formula:

Per cent inhibition (I) = C-T/C ×100

C- Growth of fungal pathogen in control.

T- Growth of fungal pathogen in test plate.

On the basis of inhibitory potential of AGII it was selected for the present study.

Identification of AGII by 16S Ribosomal RNA sequencing technique

Isolation of genomic DNA

Isolate AGII was recognized by 16S ribosomal RNA gene sequencing technique. Genomic DNA was extracted from AGII by using DNA extraction kit (ZymoBIOMICS™ Make). Quantification of DNA was performed by nanodrop spectrophotometer (Thermo Fisher Scientific make).

PCR Amplification and sequencing of 16S ribosomal RNA

Amplification of 16S ribosomal RNA region was done with following reaction mixture i.e.10 x Taq buffer - 5.0 µL; 2mM dNTP—2.5 µL; Forward primer—1.0 µL; Reverse primer -1.0 µL; Taq DNA polymerase—0.2 µL; glycerol—0.5 µL; double distilled water—12.8 µL; DNA—1 µL; 25mMMgCl2—1 µL. The program for PCR was set as follows: 35 cycles of 92^0C for 1 minute, 55^0C for 1 minute, 72 for 1 minute + 4^0C. Samples were amplified by universal prim-

ers of 16S rRNA (8F 5'AGAGTTTGATCCTGGCTCAG3) and (1492R 5'GGTTACCTTGTTACGACTT3). Amplicons were visualized by gel electrophoresis on 1% agarose gel. The sequencing process conducted by Xcelaris genomics labs, Ahmedabad, India. Sequences were edited and aligned using Sequence Navigator program (Applied Biosystems) and Clustal W (version 1.8; Infobiogene) respectively. Sequence was compared with 16S DNA sequences from reference strains by using BLAST search using the NCBI database. The 16srRNA sequence of AGII was deposited in NCBI gene Bank it nucleotide sequence database to get accession no.

Plant growth promoting characters of AGII

Siderophore production [9,10]

Siderophore production was detected on CAS (chrome azurol S) agar plates.

Regents for preparation of CAS agar plates

- **Solution A**: Mix chrome azurol (CAS) 60.5 mg/50 ml distilled water with 10 ml iron solution 1 mM $FeCl_3.6H_2O$ in 10 mMHCl. This mixture was added to hexadecyltriethyl ammonium bromide (HDTMA) (72.9 mg/40ml distilled water).
- **Solution B**: Nutrient agar (750 ml) was mixed with 1, 4 piperazinediaethane sulphonic acid (30.24 g) pH was set to 6.8. Autoclave it.

Mix Solution A with Solution B and poured in Petri plates. Bit of bacterial isolate AGII (72 h old) was kept on CAS agar plate followed by incubation at 20^0C for 72 h. The formation of yellow or orange halo around the bit indicated siderophore production by AGII.

Phosphate solubilization [11]

Isolate AGII was studied for its phosphate solubilizing activity on Pikovoskaya's agar plate. A Bit/diskof*Bacillus weihenstephanensis*AGII (72 h old)was kept on Pikovoskaya's agar plate (Hi Media make) followed by incubation at 20^0C for five days. Formation of clear zones around the culture bit after five days of incubation is a positive indication of phosphate solubilization.

% Solubilization efficiency (SE) was calculated as follows:

% Solubilization efficiency (SE) = Solubilization diameter/Growth diameter x 100.

HCN production [12]

Bacillus weihenstephanensis AGII was streaked on King's medium (Hi Media Make) modified with glycine (4g/L). Whatman filter paper No 1 soaked in sodium carbonate (2 %) and then in picric acid (0.5 %) inside the apex of the plate. HCN production was assessed in terms of change of colour of the whatman filter paper from deep yellow to orange brown to dark brown after incubation at 20^0C for 5 days. Control was set aside for comparison.

Ammonia production [13]

Bacillus weihenstephanensis AGII was inoculated in peptone water (5 ml) and incubated at 20^0C for 5 days. Nessler's reagent (1 ml) was added to these incubated cultures to check ammonia production by bacterial isolate which was indicated in terms of change in colour of the culture from light yellow to brown (++ to +++++).

Protease production [14]

A bit of *Bacillus weihenstephanensis* AGII (72 h old) was kept on skim agar plate followed by incubation at 20^0C for 72 h. Proteolytic activity of AGII was expressed in terms of clear zone (in mm) formation around thesbit.

Results

Isolation of bacterial isolate

Psychrotrophic bacterial isolate AGII was isolated from the mixed samples of the rhizospheres of *Gentiana kurroo*of Sangla valley (from altitude of 3885m to 4205m amsl) in District Kinnaur of Himachal Pradesh, India which is located in northwestern Himalaya. *Gentiana kurroo* is a threatened high value Ayurvedic medicinal plant found in Himalaya and the root of this plant has a long history of use in the treatment of digestive disorders, skin diseases, leucoderma, leprosy, bronchial asthma, dyspepsia, flatulence, colic, anorexia, helminthiosis, inflammations, amenorrhea, dysmenorrheal, and urinary infections and is an ingredient of many proprietary medicines [15]. In total, 10 different bacterial isolates were isolated on nutrient agar medium after incubation at 20^0C.

Screening and identification of bacterial isolate AGII

Out of 10 isolated bacterial isolates, the best one was selected on the basis of its inhibitory potential against deadly phytopathogens. Bit/disk method was used to check antagonistic potential. Bacterial isolate AGII was found to inhibit 4 selected pathogens out of 6 (Table 1). The isolate AGII displayed maximum inhibitory activities against *Alternaria solani* MTCC 2101 (62.5%), *Botrytis cinerea* MTCC 2350 (50%) and *Fusarium oxysporum* MTCC 7677 (56.25%), *Collectotrichum gloeosporioides* MTCC 9664 (87%). Although isolate did not show antagonism against other selected in-

dicators viz *Rhizoctonia solani* MTCC 4633, *Sclerotinia sclerotiorum* MTCC 8785 but the antibiotic potential of isolate was quite good against four deadly phytopathogens which are the major cause of plant diseases.

Name of plant pathogen	Inhibition Percentage
Alternaria solani MTCC 2101	62.5%
Botrytis cinerea MTCC2350	50%
Fusarium oxysporum MTCC 7677	56.25%
Colletotrichum gloeosporioides MTCC 9664	87%
Rhizoctonia solani MTCC 4633	-
Sclerotinia sclerotiorum MTCC 8785	-

Table 1: Antagonistic activity of isolate AGII in terms of inhibition percentage against serious plant pathogens.

The isolate AGII was identified by 16Sribosomal RNA gene sequence technique and it was identified as *Bacillus weihenstephanensis* and has been deposited in NCBI gene Bankit nucleotide sequence database. It is submitted under the accession number MF593886.Phylogenetic tree for the organism is displayed in (Figure 1).

Figure 1: Phylogenetic relationship between AGII and representative.

Plant growth promoting traits of *Bacillus weihenstephanensis* AGII

Antagonistic activity

Bacillus weihenstephanensis AGII inhibited four deadly plant-pathogens out of six tested (Figure 2). The antagonistic potential of this strain could be useful as biological control of plant disease which may be an alternative approach to the use of hazardous chemical fungicides. Several microbial antagonists have been accounted so far to have antagonistic activities against phytopathogens. Authors [16] screened *Bacillus* strains isolated from the rice rhizosphere on the basis of their antifungal activity towards to *M. oryzae*. Similarly, authors [3] reported that *V. arenosi* PH15 inhibited *Alternaria solani* (87%), *Botrytis cinerea* (62.5%) and *Fusarium oxysporum* (87.5%) [17] observed antagonistic activity of *Bacillus velezensis* against *Ralstonia solanacearum* and *Fusarium oxysporum*.

Siderophore production

Siderophore production by *Bacillus weihenstephanensis* AGII was tested by growing it on CAS agar plates at 20^0C for 72 h. Siderophore production by AGII was predicted in a form of yellow to orange colored zone of 8 mm on CAS agar plate. The Iron is an essential element that plays a crucial role in metabolic processes of microbes. Though, the concentration of free iron in the environment is lower than 10^{-7} mol which is not sufficient for the microbial growth [18]. Therefore, to acquire iron microbes secrete special siderophores.

Phosphate Solubilization

In the present study, *Bacillus weihenstephanensis* AGII was recognized as phosphate solubilizer as it is forming yellow zone around the bit on Pikovoskaya' sagar medium following incubation (Figure 2). Phosphate solubilization was determined in terms ofsolubilization efficiency which was found 62.2 % for AGII. The yellow coloured zone is formed as a result ofpresence of phosphatase enzyme in bacterial isolates [19]. The normal Phosphorus amount in soil is 0.05% (w/w). Yet, only 0.1% of Phosphorus can be taken up by plants, rendering existing Phosphorus a limiting factor for plant growth [20]. Phosphate solubilizing bacteria (PSBs) alter unusable inorganic and organic phosphorus into usable phosphorus to meet the necessities of plants through dissolution and absorption. Phosphate solubilizing bacteria can be divided into two classes: (1) Pi-solubilizing bacteria that dissolve Pi compounds by releasing organic acids. (2) Po-mineralizing bacteria that mineralize Po compounds by secreting phosphatase enzymes. Both the classes of Phosphorus solubilizing bacteria in soil reduced the pH of the soil and forms a P-offering microareain the region of the plant rhizosphere, thus making the phosphorus accessible to the plants [21].

HCN production

In the present study *Bacillus weihenstephanensis* AGII was assessed to produce HCN which was observed in terms of change in

colour of Whatman filter paper No 1 from deep yellow to orange brown to dark brown. The hydrogen cyanide is one of the dominant antifungal compounds produced by PGPR's that plays a crucial role in biological control [22]. Many authors have reported production of HCN *Bacillus* sp. isolated from rhizosphere bacteria. Researchers [23] have observed 6 isolates viz. *Pseudomonas japonica*, *Bacillus megaterium*, *Pseudomonas* sp., *P. tolaasii*, *P. chlororaphis* and *P. mosselii* producing Hydrogen Cyanide (HCN).

Ammonia production

Bacterial ammonia and amine production were observed using Nessler's reagent. *Bacillus weihenstephanensis* AGII was illustrated to release ammonia at considerable levels. Ammonia production is an important feature of PGPR, which persuade plants growth by indirect means. Ammonia production weaken the colonization of plant pathogens and also accomplish the nitrogen requirement of the host plant. Microorganisms make ammonia by hydrolyzing urea to$NH_3$and CO_2 [24]. In one of the research paper [25] confirmed ammonia production by isolates *E. cloacae*, *P. fuscovaginae*, and *K. oxytoca* and authors concluded that ammonia production by bacteria is an hint for ammonification process that takes place in the rhizosphere than non-rhizosphere soil.

Protease production

Isolate *Bacillus weihenstephanensis* AGII was detected positive for protease production as it forms a clear zone (5mm) around the bit placed on skim milk agar plates following incubation (Figure 2). Protease producing microorganisms has potential to destroy some fungal and bacterial pathogens as this enzyme hydrolyze proteins [26]. Therefore, recognized under the class of biocontrol agent.

Figure 2: Plant Growth Promoting Traits of *Bacillus weihenstephanensis* AGII.

A= Antagonistic activity, B= Phosphate solubilization, C= Proteolytic activity.

Conclusion

In the present study bacterial isolate *Bacillus weihenstephanensis* AGII has been isolated from critically endangered plant grown in District Kinnaur Himachal Pradesh, India. Strain AGII was selected for the present study on the basis of its inhibitory potential against phytopathogens and was identified by 16S ribosomal RNA gene sequence technique. This cold tolerant bacterial isolate exhibited varied plant growth promoting traits viz., siderophore production, phosphate solubilization, HCN production, ammonia and protease production. Hence, it is concluded that *Bacillus weihenstephanensis* AGII can be deployed as A potential PGPR. However, different field trials and the interaction of this PGPR with other native soil microflora have to be evaluated in future.

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