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Isolation and characterization of sunflower associated bacterial strain with broad spectrum plant growth promoting traits

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Abstract

Plant growth promoting rhizobacteria (PGPR) based biofertilizers act as a natural driving force, allowing crops to deliver their full potential by providing a promising alternative to chemical fertilizers and pesticides. Despite its economic importance a little is known about the response of sunflower towards inoculation with PGPR. A potential PGPR was isolated from Chamyati, Azad Jammu and Kashmir, an unexplored area towards PGPR and the bacterial mechanisms related to plant growth promotion were evaluated and characterized. The bacterium was identified as *Arthrobacter* sp. AF-163 through 16S rRNA gene sequence analysis. This bacterium was found catalase and cytochrome oxidase positive, metabolically diverse by utilizing 54 out of 93 carbon sources in Biolog microplate analysis and resistant to a number of antibiotics in intrinsic antibiotic resistance assay. AF-163 showed nitrogenase activity (107.2 nmoles mg^{-1} protein h^{-1}) in gas chromatographic analysis; produced 23.7 μgmL^{-1} indole-3-acetic acid (HPLC analysis) and solubilized 40.5 μgmL^{-1} insoluble phosphorus (spectrophotometric analysis) displaying significant decrease in pH (up to 2.3) due to the production of ascorbic acid, malic acid and gluconic acid and oxalic acid. Moreover AF-163 showed antagonistic activity against *Fusarium oxysporum* in *in vitro* dual culture assay. Inoculation with this bacterium to sunflower grown in soil-free culture showed a significant increase in sunflower growth parameters. This study concludes that *Arthrobacter* sp. strain AF-163 contains multiple plant growth promoting traits, recommended to be evaluated further under field conditions before using them as commercial bio-inoculant.

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Introduction

The rhizosphere and plant roots are aggressively colonized by the free-living bacteria called plant growth promoting rhizobacteria (PGPR), which when applied to the crop, improve its growth and yield (Kumar *et al*., 2015). A bacterium meets the criteria of plant growth promoting agent when it is capable of producing positive effect on plant growth upon inoculation, representing good quality competitive abilities over the native microbial population present in the rhizosphere (Antoun and Prevost, 2005). Beneficial rhizobacteria are reported to support the plant growth by means of synthesis or altering the concentration of plant growth hormones like indole-3-acetic acid (IAA) (Palaniappan *et al*., 2010), enhancing nitrogen fixation (Shen *et al*., 2016), disease suppression (Pérez-Montaño *et al*., 2013) by antagonism against phytopathogens (Ali *et al*., 2014), converting the organic and inorganic phosphatic forms to a soluble form (Hanif *et al.,* 2015), siderophores synthesis (Radzki *et al*., 2013), increasing photosynthetic rates (Singh *et al*., 2011), induced systemic resistance (ISR) in plants (Lugtenberg and Kamilova, 2009), antibiotic synthesis, production of enzymes and fungicidal compounds against harmful microorganisms (Lugtenberg and Kamilova, 2009).

Moreover, most of the PGPR also enhance plant tolerance against abiotic stresses like metal toxicity, salinity and drought (Dimpka *et al*., 2009; Babalola, 2010). The PGPR inoculation have the potential to increase the seedling emergence rate (Hafeez *et al*., 2004), nutrient (N, P, K, Zn, Mn, Cu) uptake (Biari, *et al*., 2008; Shen *et al*., 2016), nutrient use efficiency (Shahid *et al*., 2014) and would aid to withstand environmental health and soil productivity ultimately result into decreased crop production costs (Sharma *et al*., 2016).

Plants are well reported to affect the native soil microbial populations and each of the plant species is believed to select particular microbial community, which adds enough to their fitness by generating an appropriate and selective environment. This selective environment ultimately results in a narrow range of microbial diversity (de Weert *et al*. 2006; Berg and Smalla, 2009). Host plant specificity of PGPB have been well reported in several reviews and studies (Berg and Smalla, 2009; Buchan *et al*., 2010; Majeed *et al.,* 2015).

So, it is important to search for native or region-specific microbial strains which can be used as a potential plant growth promoter and nutrient solubilizer/mobilizer to achieve desired production levels. Studies are required to prove the nature of these native isolates and to harness their potential as bio-inoculants in agriculture.

Sunflower is one of the four chief oilseed crops in the world (Škorić *et al*., 2008; Nayidu *et al*., 2013) with great potential for producing the highest oil yield per hectare. Ideal combination of saturated and poly-unsaturated fatty acids in sunflower oil helps in reduction of blood cholesterol level (Balasubramaniyan and Palaniappan, 2004). Pakistan has continual deficiency in edible oil production and is the third largest edible oil importer worldwide. One of the efforts could be the production of high yielding sunflower on large scale to shrink the gap between oil production and consumption (Ehsanullah *et al.,* 2011, Hussain *et al.,* 2010).

There have been very few studies of the microbial diversity associated with sunflower, and hence the bacterial diversity of sunflower in Pakistan soils remains largely unknown. We conducted this experiment to explore altitudes of Chamyati site of subdivision Dhirkot, Azad Jammu and Kashmir (AJK) to find efficient native PGPR, as the soils of this region are completely unexplored towards sunflower associated bacterial population.

Materials and Methods

Soil sampling and processing for bacterial isolation

For bacterial isolation, surface sterilized sunflower (cv. FH-331) seeds were sown in pots containing soil collected (0-15 cm depth) from Chamyati site of sub-division Dhirkot, Azad Jammu and Kashmir. A sub-

sample was air-dried, milled, sieved and processed for analyzing physico-chemical properties and bacterial population count (Table 1).

After 3 weeks of germination, plants were uprooted and 1 g soil (tightly bound to roots) was used in a serial dilution plating technique for the isolation of rhizobacteria as described earlier (Somasegaran & Hoben, 1994). The bacteria were purified by sub-culturing of single colonies and maintained on LB-agar/broth at 28±2 °C, preserved in 20 % glycerol and stored at -80 °C for subsequent use. Of many purified bacterial isolates (not given here), AF-163 was selected for present study on the basis of its basic plant beneficial traits.

Bacterial colony and cell morphology was studied through stereo-microscopy (BAUSCH & LOMB, ASZ30E, USA) and light microscope (Nikon LABOPHOTO-2, Japan) respectively. The Gram's reaction was performed according to the method described by Vincent and Humphrey (1970) and observed under light microscope.

Molecular characterization of isolates

Alkaline lysis method was adopted to extract total genomic DNA as described by Maniatis *et al.* (1982). The DNA was quantified by ultraspec™ 3100 ($OD_{260,\ 260/280}$). This DNA sample was used as a template in polymerase chain reaction (PCR) to amplify the 16S rRNA gene of the bacterial isolate with the help of primer set fD1 and rD1 (Weisburg *et al.*, 1991). A reaction mixture (50 µL) was used for amplification and the conditions used in a thermal cycler (Pe QLab, advanced Primus 96) were described by Hanif *et al.* (2015). The amplified product was separated on 1.5 % (w/v) agarose Tris-acetate-EDTA (TAE) gel electrophoresis. Amplified PCR product was purified using the QIA quick PCR purification kit (Qiagen, USA), and sequenced by Macrogen, Korea. The sequenced products were analyzed using sequence scanner software package and examined by NCBI BLAST against the Gen Bank database. Multiple sequence alignment was performed and phylogeny was determined by neighbor-joining using MEGA6 software package.

Determination of PGPR characteristics of AF-163

Nitrogen fixation

Acetylene reduction assay (ARA) as described by Park *et al.* (2005) was adopted to screen bacterial isolate for nitrogenase activity. Bacterium was inoculated in semisolid nitrogen free malate media vials and incubated at 28 ± 2 °C for 74 h and 10 % (v/v) air of vial was replaced with the same amount of acetylene gas. The vials were further incubated for 24 h. Acetylene reduction to ethylene was measured by gas chromatograph (Thermoquest, Trace GC, Model K, Rodon Milan, Italy) fitted with Porapak N column and flame ionization detector (FID). The experiment was conducted twice with three replicates.

Indole-3-acetic Acid Production (IAA)

AF-163 was grown in LB broth supplemented with 100 mgL^{-1} tryptophan as IAA-precursor. Indole-3-acetic Acid production ability of bacteria was qualitatively checked by spot test as described by Gordon and Weber, (1951). For the quantification of IAA produced, ethyl acetate oxidation method was adopted (Tien *et al.*, 1979). Bacterial cells were harvested and supernatant was acidified with hydrochloric acid up to pH 2.8 and extracted twice with equal volume of ethyl acetate (Tien *et al.*, 1979). The extract was evaporated to dryness and dissolved in ethanol and passed through 0.2 µm nylon filters (Millipore, USA). Samples were analyzed by high-performance liquid chromatography (HPLC, λ = 260 nm) equipped with Turbochrom software (Perkin Elmer, USA) and C-18 column at a constant flow rate of 0.5 mL min^{-1} using 30:70 (v/v) methanol: water as mobile phase. The experiment was conducted twice with three replicates.

Phosphate solubilization and production of extracellular organic acids

Phosphate solubilizing ability of AF-163 was determined by the methods of Pikovskaya, (1948). For qualitative screening, plates containing Pikovskaya's agar (Sigma, USA) supplemented with

tricalcium phosphate as insoluble P source were inoculated with aliquots (10 µL) of pure bacterial culture grown in LB broth. Plates were incubated at 28±2 ºC and observed daily for 7-10 days until formation of transparent "halos". The solubilization index was determined by the method of Edi-Premono *et al.* (1996).

For quantitative analysis, AF-163 was grown in Pikovskaya's broth for 10 days on continuous shaking. The cells were separated by centrifugation at 12000 rpm for 10 min and cell free supernatant was collected. Phospho-molybdate blue colour method as described by Murphy and Riley (1962) was adopted to quantify solubilized P using spectrophotometer (Camspec, M350-Double Beam UV-Visible Spectrophotometer, UK) at 882 nm.

For the detection of organic acid, the cell-free supernatant was filtered through 0.2 µm nylon filters (Millipore, USA) and 20 µL was injected to HPLC equipped with Turbo chrom software (Perkin Elmer, USA) and C-18 column at a flow rate of 0.6 mL min^{-1} using 30:1:70 (v/v/v) methanol: acetic acid: water as mobile phase. Signals were detected at 210 nm. The organic acids including gluconic, malic, lactic, oxalic, tartaric, and ascorbic acid (Sigma-Aldrich) were used as standard. Experiments were performed in triplicate.

Intrinsic antibiotic resistance

To access intrinsic antibiotic resistance pattern by AF-163, disc diffusion method was adopted as described by Valverde *et al.* (2005). Fresh bacterial culture was spreaded on solid Antibiotic Sensitivity Sulphonamide agar (Merck, Germany) and ready-to-use antibiotic discs (Bioanalyse®, Turkey) were placed on these inoculated plates. Antibiogram (clear zone formation around the antibiotic disc) was observed after 24-48 h of incubation at 28±2 ºC. Experiment was conducted in triplicate.

Biocontrol activity

AF-163 was tested for *in vitro* antagonistic activity against fungal phytopathogen *Fusarium oxysporium* by using dual-culture assay as proposed by Sakthivel and Gnanamanickam, (1987) on potato dextrose agar plates. About 5 mm fungal disc placed in the center and bacterial colony was streaked 3 cm away from the fungal plug on the plate, while control plates were kept without bacterial streaking. The plates were incubated at 28 ±2 ºC for 3-5 days and observed for antifungal activity. This experiment was replicated thrice.

Phenotypic microarrays

BIOLOG GN2 micro-plate system was employed to access the metabolic potential of the isolate as reported by Müller and Ehlers, (2007). Bacterial culture was starved by inoculating to Eppendorf tubes containing 1 mL DEPC H_2O and incubated at room temperature for 3 h. It was mixed with inoculation fluid (IF-0a) and redox indicators as instructed by the manufacturer. Then 100 mL mixture was added to each well micro-plate PM2A (Biolog, Hayward, CA) and incubated at 28±2 ºC for 24 h. VERSA max micro-plate reader (Molecular Devices) with Softmax pro-software was used for qualitative analysis as described by Line *et al.* (2011).

Catalase and oxidase activity

Commercially synthesized strips (Merck, Darmstadt, Germany) were used for cytochrome oxidase test. For catalase production, single bacterial colony was transferred to glass slide and one drop of H_2O_2 was added. Bubble production was considered as positive reaction for catalase.

Acid or alkali production

Acid/alkali production was tested on LB agar plates containing 0.025% (w/v) bromothymol blue as pH indicator.

Plant inoculation test

The experiment was conducted to evaluate the effectiveness of *Arthrobacter* sp. AF-163 on the respective crop Sunflower (cv. FH-331) under controlled conditions.

Bacterial growth and seed inoculation

Surface sterilized (Shahid *et al.* 2014) seeds were germinated on water agar plates and seedlings were aseptically transferred to the autoclaved growth pouches (Waver and Frederick, 1982). Bacterial inoculum was adjusted to logarithmic phase (~10^9 viable cells /mL) obtained at exponential growth phase in LB broth as described by Majeed *et al.* (2015). Inoculation doses were adjusted to 1mL per seedling.

The un-inoculated pouches were watered with full strength Hoagland while inoculated pouches were provided by phosphorus deficient Hoagland. Tri-calcium phosphate (TCP, Sigma) @ 1.239 mL (g)/pouch was used as an insoluble form of P. An efficient phosphate solubilizing strain Fs-11 (*Enterobacter* sp.) obtained from the BIRCERN culture collection NIBGE, Faisalabad, Pakistan was used as positive control in this experiment. Seedlings were maintained for 30 days in a growth chamber with a photoperiod of 16 h light and 8 h darkness at day/night temperature 25/20 ^oC.

Four treatments were used: (1) non-inoculated seeds (control) in P-deficient Hoagland (2) seeds inoculated with *Arthrobacter* sp. AF-163 in P-deficient Hoagland; (3) seeds inoculated with *Enterobacter* sp. Fs-11 in P-deficient Hoagland (4)) non-inoculated seeds in Hoagland with 100% recommended P. The following parameters were measured as indicator of growth promotion: (a) shoot and root length; (b) shoot and root fresh weight (c); shoot and root dry weight.

Data analysis

To compare the difference between treatment means 'least significant difference (Fisher's LSD) test was used at 5 % probability. Analysis of variance (ANOVA) technique (Steel *et al.,* 1997) was used to analyze data regarding plant inoculation experiment using Statistix (version 8.1) software.

Results

Bacterial isolation and characterization

Identification of a putative plant growth-promoting bacterial strain isolated from sunflower rhizosphere through 16S rRNA sequence analysis indicated that AF-163 has high homology (99%) with *Arthrobacter* sp. strain M18-2. The microscopic examination revealed that bacterial strain AF-163 was a medium rod shaped motile bacterium with small round brown colored colony having smooth margins (Table 2).

Biochemical assays

Arthrobacter sp. AF-163 cells were Gram negative and positive for catalase and cytochrome oxidase activity and showed neutral reaction when screened for acid/alkali tested (Table 2).

Additionally, this isolate was positive for phosphate solubilization as discernible by the formation of halo zone on Pikoviskaya's agar plates (Fig. 1A) with solubilization index of 2.2.

Table 1. Meteorological and soil physicochemical properties of sampling site Chamyati.

Meteorological Properties							
Altitude	Air Temp ^oC	Soil Temp. ^oC. (20 cm)	Heat Index ^oC	Humidity (%)	Barometric pressure (kpa)		
1565	26.4	23	25.8	51.7	839.4		
Physicochemical properties							
Textural class	O.M (%)	Total N (%)	Available K(mg/kg)	Available P (mg/kg)	ECe (dsm^{-1})	Soil pH	CFU
Silt loam	2.36	0.181	72.19	13.48	0.64	6.59	9×10^6

ECe= electrical conductivity; OM=organic matter; CFU=colony formation unit.

It solubilized insoluble phosphorus (P) up to 40.5 μgmL^{-1} in the culture medium with concomitant decrease in pH 7 to 4.7 after 10 days of inoculation (Table 2). High performance liquid chromatographic analysis of the cell-free supernatant showed gluconic acid (12.4 μgmL^{-1}), ascorbic acid (1.69 μgmL^{-1}), malic acid (10.6 μgmL^{-1}) and oxalic acid (3.25 μgmL^{-1}) production by AF-163 (Table 2).

This bacterial strain was also able to produce another known phytohormone indole-3-acetic acid up to 12.77 μgmL^{-1} along with the ability to exhibit nitrogenase activity in acetylene reduction assay (ARA) up to 107.24 nmoles mg^{-1} protein h^{-1}, as conformed by Gas chromatographic analysis (Table 2).

Table 2. Morphological, biochemical and plant beneficial traits of *Arthrobacter* sp. AF-163.

Morphological characters					
Colony morphology	Round, Smooth, Small, Brown	*Cell morphology*	Short Rods, Motile		
Biochemical Characters					
Reaction/Test	Values	Reaction/Test	Values		
Gram's reaction	-	Catalase	+		
Cytochrome oxidase	+	Acid/Alkali reaction	Neutral		
Plant beneficial traits					
Phosphate solubilization (μg mL^{-1}) *(Solubilization index)*	40.50±1.6 (2.2±0.3)	*Nitrogenase activity*(nmoles mg^{-1} protein h^{-1})	107.24±12.38		
		Indole-3-acetic acid production(μg mL^{-1})	12.77±1.11		
Organic Acids production (μg mL^{-1})					
Malic Acid	10.6±1.96	*Ascorbic Acid*	1.69±0.02	*Change in pH	2.3
Gluconic Acid	12.4±1.4	*Oxalic Acid*		3.25±0.21	

- Shows the reaction/test is negative, + shows that reaction is positive, ± shows standard deviation.

**pH of medium was adjusted at 7 initially and pH decrease represents the difference between initial and final pH.*

Arthrobacter sp. AF-163 showed *in vitro* antifungal activity against a known phytopathogen *Fusarium oxysporum* on PDA plates after 5 days of inoculation in a dual cultural assay (Fig. 1C). *Arthrobacter* sp. AF-163 when screened for intrinsic antibiotic activity, showed resistance to Cephradine (30 μg), Erthromycin (15 μg), Streptomycin (10 μg), Ciprofloxacin (5 μg), Gentamicin (10 μg), Nalidixic acid (30 μg), Kanamycin (30 μg), Tetracycline (30 μg) but it was found highly sensitive to Aztreonam (30 μg), Carbenicillin (100 μg) Amikacin (10 μg), Cefixime(5 μg), Rifampicin (5 μg) and Chloramphenicol (30 μg), (Fig. 2B). Moreover, AF-163 was able to metabolize 54 out of 93 carbon sources revealed by phenotypic microarray analyses done using BIOLOG GNII micro plates system (Table 3).

Inoculation studies

After physiological and biochemical screening of plant growth promoting traits, *Arthrobacter* sp. AF-163 was evaluated in soil-less culture (growth pouches) for its plant growth promoting potential.

Comparisons were made with sunflower associated potential PGPR strain Fs-11 (*Enterobacter* sp.) used as positive control along with an un-inoculated positive control with recommended dose of nutrients, and a non-inoculated negative control.

Results revealed that *Arthrobacter* sp. AF-163 inoculation significantly ($P < 0.05$) enhanced sunflower growth characteristics in soil-less culture including plant height, plant fresh and dry weight, root length and root fresh and dry weight over un-inoculated control treatments (data given in Fig. 3).

In case of plant height AF-163 inoculation resulted in shoot length was statically same as that of reference strain used as positive inoculated treatment (Fig. 3A).

While, in case of plant dry matter, *Arthrobacter* sp. AF-163 inoculation resulted in statically improved shoot and root dry weight over reference strain.

The relative increase in shoot and root dry weight due to AF-163 inoculation over reference strain was 41 % and 45 % respectively, and root dry matter was statically at par with un-inoculated positive control supplemented with full dose recommended nutrients.

Table 3. Metabolic profiling of *Arthrobacter* sp. AF-163 (Biolog PM2A Microplate analysis).

Carbon Source	AF-163	Carbon Source	AF-163	Carbon Source	AF-163	Carbon Source	AF-163	Carbon Source	AF-163
Glycine	-	Arbutin	+	L-Sorbose	-	γ-Hydroxy Butyric Acid	+	L-Tartaric Acid	-
α-Cyclodextrin	+	2-Deoxy-D-Ribose	-	Stachyose	-	α-Keto-Valeric Acid	+	L-Alaninamide	+
β-Cyclodextrin	+	i-Erythritol	+	D-Tagatose	-	Itaconic Acid	+	N-Acetyl-L-Glutamic Acid	+
γ-Cyclodextrin	-	D-Fucose	-	Turanose	-	5-Keto-D-Gluconic Acid	+	3-o-β-D-Galacto-pyranosyl-D-Arabinose	+
Dextrin	+	L-Arginine	+	Xylitol	+	D-Lactic Acid Methyl Ester	+	Chondroitin Sulfate C	-
Gelatin	+	Gentiobiose	-	Acetamide	-	Malonic Acid	+	L-Histidine	+
Glycogen	-	L-Glucose	-	γ-Amino Butyric Acid	+	Melibionic Acid	+	L-Homoserine	-
Inulin	-	Lactitol	-	δ-Amino Valeric Acid	+	Oxalic Acid	+	Hydroxy-L-Proline	-
Laminarin	+	D-Melezitose	+	Butyric Acid	-	Oxalomalic Acid	+	L-Isoleucine	+
Mannan	-	Maltitol	-	Capric Acid	+	Quinic Acid	+	L-Leucine	+
Pectin	-	α-Methyl-D-Glucoside	-	Caproic Acid	-	D-Ribono-1,4-Lactone	+	L-Lysine	+
D-Raffinose	-	β-Methyl-D-Galactoside	+	Citraconic Acid	+	Sebacic Acid	-	L-Methionine	-
Salicin	+	3-Methyl Glucose	-	Citramalic Acid	+	Sorbic Acid	+	Glucosaminitol	-
β-D-Allose	+	2,3-Butanediol	+	D-Glucosamine	-	Succinamic Acid	+	N-Acetyl-D-Galactosamine	-
Amygdalin	+	α-Methyl-D-Mannoside	-	2-Hydroxy Benzoic Acid	+	D-Tartaric Acid	+	N-Acetyl-Neuraminic Acid	-
D-Arabinose	-	β-Methyl-D-Xyloside	+	4-Hydroxy Benzoic Acid	+	L-Ornithine	+	3-Hydroxy 2-Butanone	+
D-Arabitol	+	D.L-Octopamine	-	β-Hydroxy Butyric Acid	+	L-Phenylalanine	-	N-Acetyl-D-	-
D,L-Carnitine	-	Putrescine	+	Dihydroxy Acetone	+	L-Pyroglutamic Acid	+	β-Methyl-D-Glucuronic Acid	
Sec-Butylamine	+	2,3-Butanone	+	Sedoheptulosan	+	L-Valine	-		

+ = Substrate metabolized; – = substrate not metabolized

**Water used as control.*

Discussion

Bacterial isolation, characterization and screening of plant beneficial traits

In present study, we have isolated a sunflower associated rhizobacteria (AF-163) from an altitude of 1565 m, demonstrated its beneficial plant traits and its likely contribution in promoting growth of host crop. As the prevailing agriculture is largely dependent on extensive chemicals (fertilizers and pesticides), causing serious threats to soil and environment resulting in a significant decline in the organic matter and productivity of soils (Tilman *et al.*, 2001). So, alternative strategies for crop fertilization and pathogen control with minimum effect on the environment are getting fame in the recent years. The ultimate benefit of the use of PGPR is not only their plant growth promoting attributes, but also their environment friendliness and their cost-effective nature (Kaymak, 2011).

The PGPR being potential tools for plant growth promotion, soil health, and ecosystem-friendly have proved their worth in agriculture with decreased reliance on synthetic chemicals for crop growth (Adesemoye *et al.*, 2009; Souza *et al.*, 2015).

Pakistan meets only 34 % of edible oil requirement with local oil production; the rest has to import causing a huge burden on economy (GOP, 2011-12). We have targeted sunflower as it is one of the most important candidates of oil seed crops that can bridge up the gap between production and consumption in Pakistan (Škorić*et al.*, 2008; Nayidu *et al.*, 2013).

Microscopic observations revealed motile and Gram negative nature of AF-163. The dominance of Gram-negative short rod PRPRs in these soil conditions is also described (Ambrosini *et al.*, 2012).

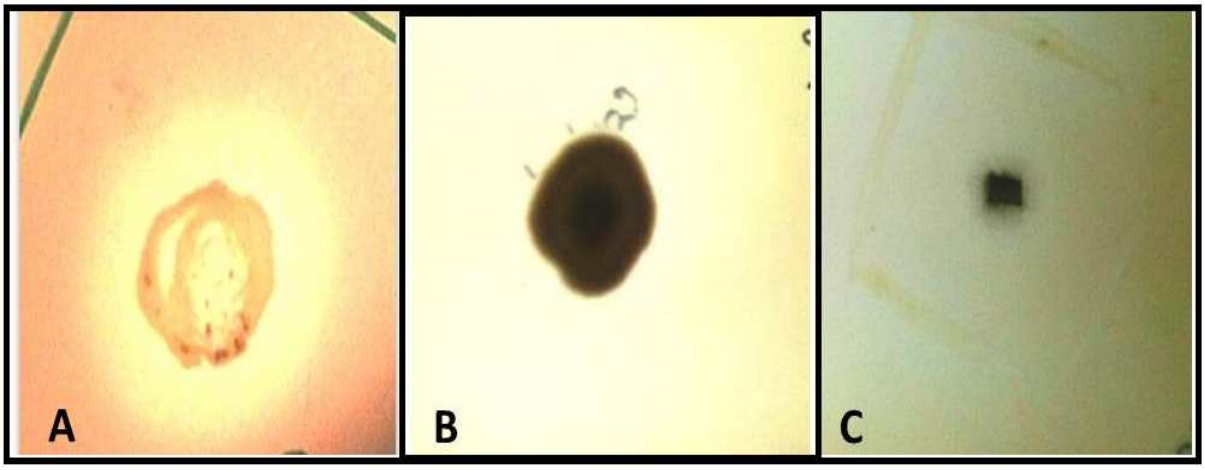

Fig. 1. Inorganic tri-calcium phosphate solubilization and bio-control activity by *Arthrobacter* sp. AF-163.
Halo zone formation as an indicator of inorganic P-solubilization on Pikovskaya's agar plate (panel A), fungal growth (Fusarium oxysporum) as a control treatment (panel B), in vitro bio-control activity on potato dextrose agar plate (panel C).

It was identified as *Arthrobacter* sp. strain through 16S rRNA gene analysis. The bacterium AF-163 was able to utilize a large number of carbon sources and substrates, confirming its metabolically diverse nature. Bacteria develop metabolic adaptations to inhabit special niches as individual plants produce specific carbon sources (Berg and Smalla, 2009); hence, metabolically versatile bacterial strains are the most successful competitors in plant microbe interaction (Wielbo *et al.*, 2007). AF-163 showed resistant to large number of antibiotics as well. These characteristics support the competency and adoptability of this bacterium in the rhizosphere of the host plant over other microbes, as reported earlier by Wielbo *et al.* (2007).

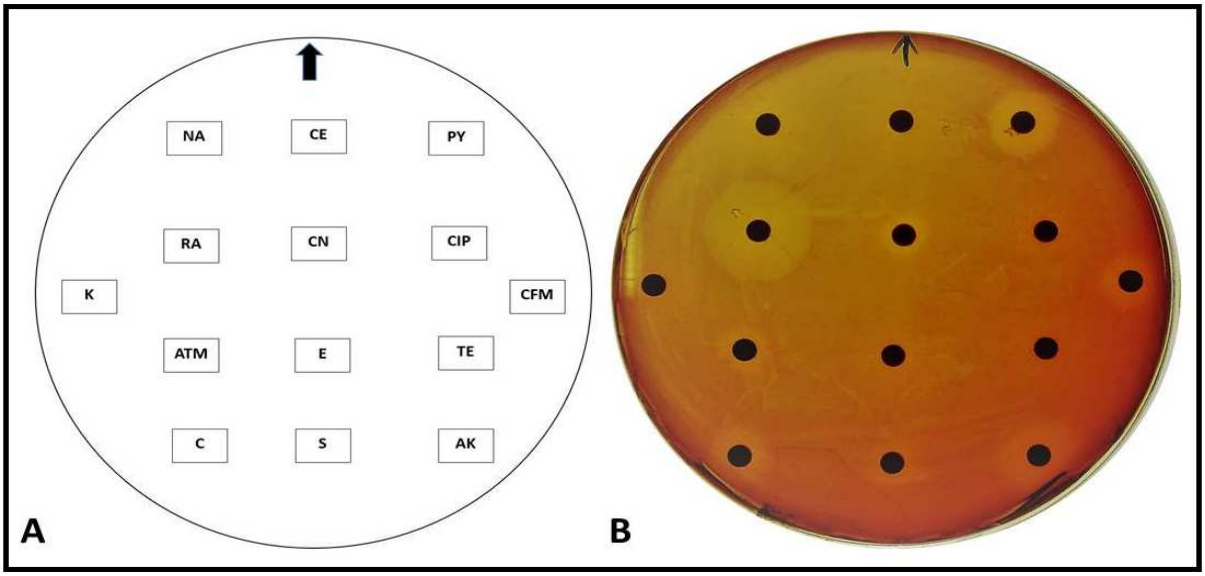

Fig. 2. Intrinsic antibiotic resistance pattern of *Arthrobacter* sp. AF-163.
Antibiosis disc pattern (panel A), antibiogram of AF-54 on antibiotic sensitivity sulphonamide agar (panel B).
AK: Amikacin (10 µg), PY: Carbenicillin (100 µg), CN: Gentamicin (10 µg) CIP: Ciprofloxacin (5 µg), CE: Cephradine (30 µg), ATM: Aztreonam (30 µg), CFM: Cefixime (5 µg), TE: Tetracycline (30 µg), NA: Nalidixic acid (30 µg), K: Kanamycin (30 µg), RA: Rifampicin (5 µg), S: Streptomycin (10 µg), E: Erthromycin (15 µg), C: Chloramphenicol (30 µg.)

Arthrobacter sp. AF-163 has shown nitrogenase activity up to 107.24 nmoles mg^{-1} protein h^{-1}. Adequate N supply is essential for plant metabolic processes involved in vegetative and reproductive plant growth enhancement (Lawlor, 2002). Plant growth promoting rhizobacteria are very well reported agents of biotic conversion of inert N through biological nitrogen fixation (Dobbelaere *et al.,* 2003; Bashan and de-Bashan, 2010; Naqqash *et al.*, 2016) as N availability to the plant is pointedly dependent on the microbial activity, even if applied as chemical fertilizer as it is not only fixed by microbes but its subsequent fate i.e., plant availability is highly dependent on microbial activity (Khan, 2005).The vast range of nitrogenase activity by nitrogen fixing bacterial isolates are documented in many studies (Islam *et al.*, 2016; Shen *et al.*, 2016).

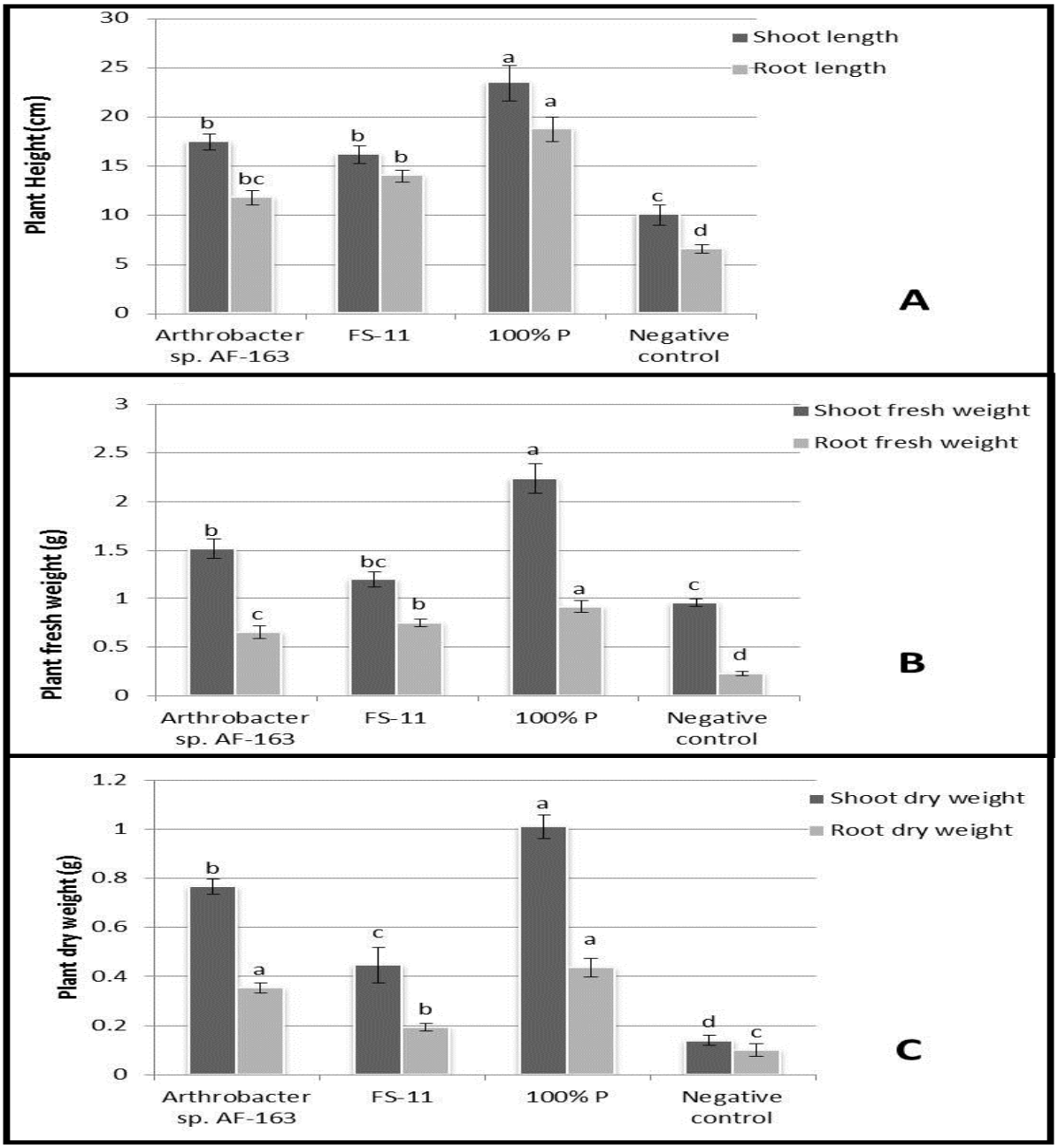

Fig. 3. Effect of *Arthrobacter* sp. AF-163 inoculation on different growth parameters of sunflower plant.
Values are the mean of three replicates. The standard errors of the means are represented as bars. Values sharing same letter do not differ significantly (P≤ 0.05) according to Fisher's LSD. FS-11= reference bacterial strains (Enterobacter sp.) used as positive control.

Phosphorus is found to be the major limiting factors for crop productivity as its major fraction is present in fixed form of Ca-phosphates, Fe and Al-phosphates is soil (Ahemad and Kibret, 2014) resulting in its low bioavailability (Jorquera *et al.*, 2011). PGPR called phosphor-bacteria are well documented microorganisms that can significantly change the soil P dynamics. *Arthrobacter* sp. AF-163 was able to solubilize 40.5 μgmL^{-1} tri-calcium phosphate with a drop in pH (2.3). In soil ecosystem, mineral P solubilization is greatly accredited to the production of low molecular weight organic acids (Bianco and Defez, 2010; Lavania and Nautiyal, 2013). Thus, we also measured the nature and amount of organic acids produced by AF-163 and it was able to produced malic, ascorbic, oxalic and gluconic acid. These organic acids are known to have variable influence on P-solubilization mechanism (Patel *et al.*, 2008) and the most prevalent one is gluconic acid which plays a prime role in inorganic P-solubilization (de Werra *et al.*, 2009). A large body of scientists reported that inorganic forms of P solubilization is the result of pH decrease in combination with organic acids production (Sahin *et al.*, 2004; Richardson *et al.*, 2009; Hanif *et al.*, 2015).

Moreover, phytohormone production is considered to be one of the most important mechanisms of plant growth promotion by rhizobacteria (Spaepen *et al.,* 2007; Islam *et al.*, 2016). *Arthrobacter* sp. AF-163 produced 12.7 μgmL^{-1} indole-3-acetic acid by the induction of tryptophan which acts as a precursor of IAA. Phytohormones are the key players in plant growth and yield promotion as these are the organic compounds which effect physiological, biochemical and morphological plant processes in extremely low concentrations and serve as chemical messengers (Fuentes-Ramírez and Caballero-Mellado, 2006). Most of the PGPR are well reported IAA producers (Ahmad *et al.,* 2008; Shoebitz *et al.,* 2009; Saharan and Nehra, 2011, Naqqash *et al.*, 2016; Hariprasad and Niranjana, 2009).

In addition, phytopathogens being the cause of significant reduction of in crop yield, and usually, chemical pesticides are used for their control. Unfortunately, this approach has led to serious environmental as well as human health concerns besides developing resistance against most of these chemical remedies over time (Fernando *et al.,* 2006). This bacterium also possesses biocontrol activity against fungal phytopathogens (*Fusarium* sp.) which is a serious threat for crop production. Ali *et al.* (2014) also reported broad spectrum antifungal activity by *Bacillus* sp. RMB7 due to the production of antifungal metabolites. Antifungal metabolite production by PGPR is well reported phenomenon of biocontrol activity against phytopathogens (Haas and Defago, 2005; Medeiros *et al.*, 2011).

Plant inoculation studies

Most of the bacterial strains benefit plant growth as they exhibit multiple growth promoting properties but PGPR potential of the strains may cause differential growth responses in plants (Ghyselinck *et al.*, 2013; Naqqash *et al.*, 2016). The effect of AF-163 inoculation on host plant was evaluated in soil-less culture. The results of plant inoculation experiment showed that *Arthrobacter* sp., having multiple plant growth promoting traits, produced significant ($P \leq 0.05$) positive effects on plant growth parameters like root/shoot length, root/shoot fresh weights, root/shoot dry weights over non-inoculated treatments. Differential specificity of a particular bacterial strain might be articulated by several growth promoting traits like plant growth hormone production, nitrogen fixation, phosphate solubilization, disease suppression and biocontrol activity etc (Van Loon, 2007; Hussain *et al.,* 2015; Imran *et al.,* 2015; Hanif *et al.*, 2015; Naqqash *et al.,* 2016).

Conclusion

The current study, characterized a promising PGPR strain *Arthrobacter* sp. AF-163, from sunflower rhizosphere from an unexplored area of Azad Jammu and Kashmir, Pakistan. This PGPR strains augmented the growth of sunflower plants considerably after inoculation. Considering the harmful effects of synthetic fertilizers, their non-availability to farmers

in hilly areas like Chamyati, Dhirkot in addition to the environmental pollution, *Arthrobacter* sp. AF-163can be used as bio-inoculants to supplement chemical fertilizers after confirming its potential under field condition.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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