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Genetic diversity and metal resistance assessment of endophytes isolated from *Oxalis corniculata*

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Abstract

In this study, we have evaluated the genetic diversity and plant growth promoting traits of endophytes isolated from Oxalis corniculata. These strains were also screened for heavy metal resistance. Twenty-one endophytic bacteria were isolated from surface sterilized roots, stem and leaves of Oxalis corniculata. These isolated strains were characterized by QTS and further identified by gene sequencing. The 16S rRNA gene sequencing and phylogenetic analysis revealed that the selected bacterial strains were closely related to Pantoea agglomerans, Agrobacterium tumefaciens, Bacillus pumilus and Kocuria rhizophila. The selected isolates were positive for phosphate solubilization, IAA, HCN production, catalase activity and ammonia production. K. rhizophila showed highest resistance against heavy metals such as Cu, Ni, Pb and Cr (0.1 mg mL^{-1}) while B. pumilus showed lowest resistance by agar well diffusion method. All the isolated strains showed great genetic diversity at each level of taxon and possessed multiple PGPR traits. These strains also showed resistance to heavy metals and could be used in bioremediation studies. These isolates from O. corniculata have been reported for the first time from Pakistan. The findings of this study established the base line data for the field of bioremediation and plant growth promotion.

Keywords: Endophytes, heavy metals, *Oxalis corniculata*, PGPR, phylogenetic analysis

Introduction

Oxalis corniculata is a medicinally important plant. It is used against many infectious diseases. It is used as antifungal, antibacterial, anthelmintic, anti-inflammatory, astringent, depurative, diuretic, emmenagogue, febrifuge, lithontriptic, stomachic and styptic (Duke and Ayensu, 1985). *Oxalis corniculata* has significant activity against fungal strains including *Fusarium solani*, *Aspergillus flexneri* and *Aspergillus flavus* (Rehman *et al.*, 2015). It also possesses antibacterial activity against *E. coli*, *S. aureus*, *P. aeruginosa*, *P. vulgaris*, and *B. subtilis* (Taley *et al*., 2012). *Oxalis* genus harbor many endophytes mainly belonging to *Firmicutes, Proteobacteria, Actinobacteria* and *Bacteroidetes* (Peng *et al.,* 2013*)*. However, little work is reported on isolation of endophytes from *O. corniculata*. Recently, *Methylobacterium oxalidis* was isolated from leaves of *O. corniculata* in Japan (Tani *et al.,* 2012*).*

Endophytic bacteria have plant beneficial properties and generally referred to as plant-growth promoting bacteria. Isolation of endophytes is of immense importance as they are involved in the production of various bioactive compounds e.g. flavonoids, tannins, indoles and phenolic compounds. They play role in plant vitality as bio-control agent and enhance tolerance against environmental stress (Hallmann *et al*., 1997). Several endophytes have been isolated from surface-sterilized roots and stem of agronomically important food crops e.g. wheat, corn, fruits and vegetables (Venierak *et al.*, 2011). These PGPR traits like HCN, nitrogen-fixation, phosphate solubilization, ammonium exudation and siderophore production enhance plant resistance by reducing disease severity hence can be employed as bio-fertilizers. These traits favor the intake of water and minerals by enhancing the root system of crop plants (Glick *et al.*, 1995).

The combination of endophytic bacteria with plants has been employed for the removal of toxic metals from contaminated soil (Rajkumar *et al.,* 2009). *O. corniculata* has capacity to remediate heavy metals as cited by Bahnika and Baruah (2014). The ongoing research has reported that endophytic and rhizospheric bacteria accumulate heavy metals as they have metal binding proteins to endure high metal concentration. They help to decrease the toxicity caused by heavy metals in plants (Rojas *et al.*, 2001).

Biological nitrogen fixation by endophytes is an important process when N is a limiting factor for the growth of organisms. Some of the endophytes are able to enhance plant growth by nitrogen fixation. Many potential endophytes *such as Azospirillum halopraeferans*, *Acetobacter diazotrophicus*, *Herbaspirillum* spp. and *Azoarcus* spp. interact with legume and non-legume plants to fix free nitrogen (Vessey, 2003).

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As regarding the scarce information related to isolation of endophytes from *O. corniculata*, the current study was designed to investigate the endophytic genetic diversity. In order to establish base line data for scientific research we aim to explore the plant growth promoting traits and resistance to selected heavy metals.

Materials and Methods

Isolation and purification of the strains

The sample of *O. corniculata* was collected from field area of Quaid-i-Azam University, Islamabad (33.7167° N, 73.0667° E). Roots, stem and leaves were cut into small pieces for surface sterilization. The plant tissues (10 g) were soaked in 3% (v/v) H_2O_2 for 4 min and washed with deionized water. The plants were surface sterilized using 0.1% $HgCl_2$ for 5 min and washed with sterile distilled water for 5-7 times. Plant parts were macerated in 1 mL sterile distilled water and transferred to the test tubes having 5 mL Dobereiner nitrogen (DN) free semisolid media. These test tubes were incubated for 7 days at 37 ^oC. These strains were further subcultured on DN agar plates until pure bacterial strains appeared (Chaudhary *et al.*, 2012).

Biochemical identification of bacterial strains

The representative bacterial strains were characterized by Gram staining and biochemical tests like sodium citrate; sodium malonate (MALO), Lysine decarboxylase (LDC), Arginine dihydrolase (ADH), Orthinine decarboxylase (ODC), H_2S= H_2S production; urea hydrolysis (URE), Tryptophan deaminase (TDA), Indole; VP (vogesproskauer), Acetion; Gelatin (GELl), hydrolysis; GLU (Acidic from glucose); MAL (Acid from maltose), SuC (Acid from sucrose), MAN (Acid from mannitol), ARA (Acid from arabainose), RHA (Acid from Rhamnose), SOR (Acid from sorbitol), INO (Acid from inositol), ADO (Acid from adonitol), MEL (Acid from Melibiose), RAE (Acid from raffinose) (DESTO Laboratories Karachi, Pakistan). The overnight grown bacterial cultures were suspended in saline solution (0.85% NaCl) before inoculation to QTS kits.

SDS PAGE and 16S rRNA Gene sequencing and analysis

The grouping and identification of strains isolated from different samples were performed by analysis of whole-cell proteins using SDS-PAGE. Strains were cultured on LB agar plates at 37˚C for 24 h. The harvested strains were collected in 1.5 mL eppendorf tubes followed by the addition of sample buffer in accordance with the formula that 66.7 µL of sample buffer was added for every 0.01 g of bacterial strains. Bacterial colony was mixed well in sample buffer in order to break down proteins into monomers. SDS-PAGE was performed as described by Tan *et al.* (2011). Fragments of the 16S rRNA gene were amplified from genomic DNA of the endophytes by using forward and reverse primers sequence as explained by Hurek *et al.* (1997). The purified PCR-products were sequenced by Invitrogen Co., Malaysia by using the sequencing primers. The obtained sequences together with some associated sequences were aligned with FASTA3 program package (Jiang *et al.*, 2007). Neighbor-joining method was used to deduce the tree topology. TREECON software package was used to visualize and bootstrapped phylogenetic tree by 1,000 times of re-sampling (Van de Peer *et al.*, 1997).

In-vitro screening of isolates for plant growth promoting activities

Phosphate solubilization and quantitative estimation of phosphate

The isolates were screened for phosphate solubilization on Pikovskaya agar plates that contain insoluble tri-calcium phosphate. Each bacterial strain was streaked on agar plates and incubated at 30±1 ^oC for 24-48 h. The strains exhibiting halo zone were considered as P-solubilizers. The P-solubilizers were purified by repeated streaking and stocked for further use (Gupta *et al.*, 1994). Quantitative evaluation of inorganic phosphate solubilization was performed by using 500 µL of each bacterial culture inoculated in 50 ml of 0.5% tricalcium-phosphate Pickovskaya (PVK) broth medium in triplicates. The flasks were incubated at 30±0.1 °C and 180 rpm for 5 days in incubator shaker. The optical density (OD) of supernatant was measured at 430 nm in UV/Visible Spectrophotometer (Nautiyal, 2001).

Detection of IAA

Indole-3-acetic acid was estimated by colorimetric assay (Loper *et al.*, 1986). Nutrient broth (50 mL) amended with 0.1% DL tryptophan was inoculated with 500 mL of overnight grown bacterial cultures. The broth was incubated in refrigerated incubator shaker at 180rpm for 48 h in dark at 30±1 °C. The 90 bacterial cultures were centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatant (1 mL) was mixed with 4 mL Salkowski reagent and the appearance of pink color was the indication of IAA (Gordon and Weber, 1951). The absorbance of the resultant pink color was measured after 30 min at 535 nm in UV/Visible Spectrophotometer. The IAA production was calculated from the regression equation of standard curve and the results were expressed as mg mL^{-1} over control.

Ammonia and hydrogen cyanide (HCN) production

Endophytes were tested for ammonia production by the method of Cappuccino and Sherman (1992). Overnight

grown bacterial cultures were inoculated in 10 mL peptone broth and incubated at 30±1 °C for 48 h in incubator shaker. After incubation, 0.5 mL of Nessler's reagent was mixed. The formation of faint yellow to dark brown color indicated the production of ammonia. Similarly, overnight grown bacterial cultures were used to test HCN production (Lork, 1948). Bacterial cultures were streaked on nutrient agar medium modified with 4.4 g glycine per liter. Whatman filter paper No.1 was soaked in sodium carbonate (2%) and picric acid (0.5%) solution. Wet filter paper was placed inside the lid of each plate and they were closed with parafilm in order to protect gas from being released. Plates were kept in incubator at 30±1 °C for 4 days. Development of brown to red color pointed out the production of HCN.

Catalase activity

Bacterial colony was mixed with a drop of 3% H_2O_2 on a glass slide. Formation of bubbles of oxygen indicated catalase activity (Joseph *et al*., 2007).

Heavy metal tolerance

The activity of isolated microbes against heavy metals (Copper, Lead, Nickel and Chromium) was assessed by agar well diffusion method. The minimum inhibitory concentration (MIC) was calculated as described by Saurav and Kannabiran (2009). The MIC is the determination of lowest concentration of heavy metal that appears to inhibit the growth of bacteria. The activity of bacterial strains was tested at various concentrations i.e. 0.025 mg mL^{-1}, 0.05 mg mL^{-1}, 0.075 mg mL^{-1} and 0.1 mg mL^{-1} for the above mentioned heavy metals. After 24 h of incubation, activity was evaluated by measuring the zones around the wells. Lesser the length of the zone, more the bacteria was resistant to heavy metals.

Statistical analysis

The mean values were statistically analyzed using Statistix 8.1 (Steel *et al.*, 1997). ANOVA was obtained at 0.05 significance level.

Results

Isolation and SDS PAGE of bacterial isolates

A total of 21 bacterial strains were isolated from root, stem and leaves of *O. corniculata* by dilution plate technique. Protein profile of isolated bacteria was conducted by SDS-PAGE analysis. The strains with similar protein profiles were grouped into different clusters on the basis of visual comparison of band pattern formation. As a result, 11 bacterial strains were grouped into 4 clusters (I - IV) on the basis of similarities. Difference among the band pattern of each group was clearly visible.

Biochemical characterization of bacterial endophytes

The biochemical characteristics of isolated strains were tested by microbial identification kits QTS-24. The study showed utilization of different nitrogen and carbon sources by the tested strains. *K. rhizophila* and *B. pumilus* were gram positive while *A. tumefaciens* and *P. agglomerans* were gram negative. Carbon substrates were highly utilized by *P. agglomerans* while *K. rhizophila* utilized the lowest amount of carbon substrates (Table 2).

16S rRNA sequencing and phylogenetic analysis of the selected sequences

16S rRNA gene sequencing was carried out with each representative of group. Among the isolated strains, 13a showed 100% similarity with *P. agglomerans*. Phylogenetically, it was closest to *P. agglomerans* (KF 875449) among the *Pantoea* genus (Figure 1). Similarly, 14asp, 18a and 12b3 showed 100% similarity with *K. rhizophila* (KF 875448) (Figure 2), *B. pumilus* (KF 875447) (Figure 3) and *A. tumefaciens* (KF 875446) (Fig. 4) among the genus *Kocuria*, *Bacillus and Agrobacterium*, respectively (Table 1).

In vitro screening of endophytic bacteria for PGP traits

Screening of isolates for phosphate solubilization and IAA production

Isolated strains were screened for phosphate solubilization on modified pikovskaya agar medium. Out of which three isolates showed considerable phosphate solubilization zones ranging from 9 mm to 18 mm. *B. pumilus* and *K. rhizophila* showed highest solubilization zones i.e. 18 mm and 16 mm, respectively. Lowest zone of 9 mm was shown by *A. tumefaciens.* Quantitative estimation of tri-calcium phosphate solubilization was recorded between 0.01-0.07 µg ml^{-1} (Figure 5). All strains were positive for IAA, ranging between 0.05-0.45 µg mL^{-1} (Figure 6). The *P. agglomerans* was found to be the most efficient P-solubilizer and also showed the highest production rate of IAA (0.45 µg mL^{-1}).

Screening of isolates for ammonia, catalase and HCN production

All the four identified isolates have the ability to produce ammonia and showed catalase activity. The highest HCN was produced by *B. pumilus* and *P. agglomerans* while *K. rhizophila* and *A. tumefaciens* did not produce at all (Table 3).

Table 1: PCR based sequencing result of 4 representative strains from each cluster

Cluster representative	Base pairs	Closest 16S rRNA match with gene bank No.	Max. Identity %
13a	480	*Pantoea agglomerans* (AJ233423)	100 %
14asp	895	*Kocuria rhizophila* (Y16264)	100 %
18a	1496	*Bacillus pumilus* (AY456263)	100 %
12b3	1435	*Agrobacterium tumefaciens* (DQ383275)	99 %

Table 2: Morphological and biochemical characterization of isolated colonies 13a, 14asp, 18a, 12b3 obtained from plant *Oxalis corniculata*

Colonies	13a	14asp	18a	12b3
Cell morphology	Round/ off white	Round/ Yellowish	Round/ white	Round/ Creamyoff white
Gram test	Pink round	Purple round	Purple rod	Pink round
ONPG	+	-	+	-
CIT	+	-	+	-
MALO	+	-	+	-
LDC	-	-	+	+
ADH	-	-	-	+
ODC	-	-	-	-
H_2S	-	-	-	-
URE	-	-	+	+
TDA	+	+	+	+
IND	-	-	-	-
VP	-	-	-	-
GEL	-	-	-	-
GLU	+	+	+	+
MAL	+	+	+	+
SUC	+	+	+	+
MAN	+	+	+	+
ARA	+	-	+	+
RHA	+	-	-	+
SOR	+	-	-	+
INO	-	-	-	-
ADON	+	-	-	-
MEL	+	-	-	-
RAF	+	-	-	-

13a = *Pantoea agglomerans*, 14asp = *Kocuria rhizophila*, 18a = *Bacillus pumilus*,12b = *Rhizobium pusense.* Microbial identification kits (Qts-24, DEStO laboratory and Karachi) were used for biochemical tests for performing these tests 24 h old isolated cultures were used and results were noted after 18 h of incubation at 30ºC, ONPG = orhto nitro phenyl β-D-galactopyranoside; CIT = sodium citrate; MALO = sodium malonate; LDC = Lysine decarboxylase; ADH = Arginine dihydrolase; ODC = orthinine decarboxylase; H_2S = H_2S production; URE = urea hydrolysis;TDA = tryptophan deaminase; Indole; VP = (vogesproskauer) = Acetion; GELl = Gelatin hydrolysis; GLU = Acid from glucose; MAL = Acid from maltose; SuC = Acid from sucrose; MAN = Acid from mannitol; ARA = Acid from arabainose; RHA = Acid from Rhamnose; SOR = Acid from sorbitol;INO = Acid from inositol; ADO = Acid from adonitol; MEL = Acid from Melibiose; RAE = Acid from raffinose

Heavy metal tolerance tests

All the four strains of bacteria showed resistance against heavy metals. The most heavy metal tolerant strain was *K. rhizophila* at concentration 0.025-0.1 mg mL^{-1}. *B. pumilus* exhibited resistance against heavy metals at lower concentration of 0.05 mg mL^{-1} and 0.025 mg mL^{-1}. *A. tumefaciens* and *P. agglomerans* were resistant to Pb and Cu at all concentrations (0.025-0.1 mg mL^{-1}) (Figure 7 and 8) but showed sensitivity against Cr and Ni at higher concentration of 0.1 mg mL^{-1} (Figure 9 and 10).

Discussion

Medicinal plants harbor a wide variety of potential endophytes. Endophytes are diverse group of organisms producing beneficial substances having symbiotic association with higher life forms (Mengoni *et al*., 2003).

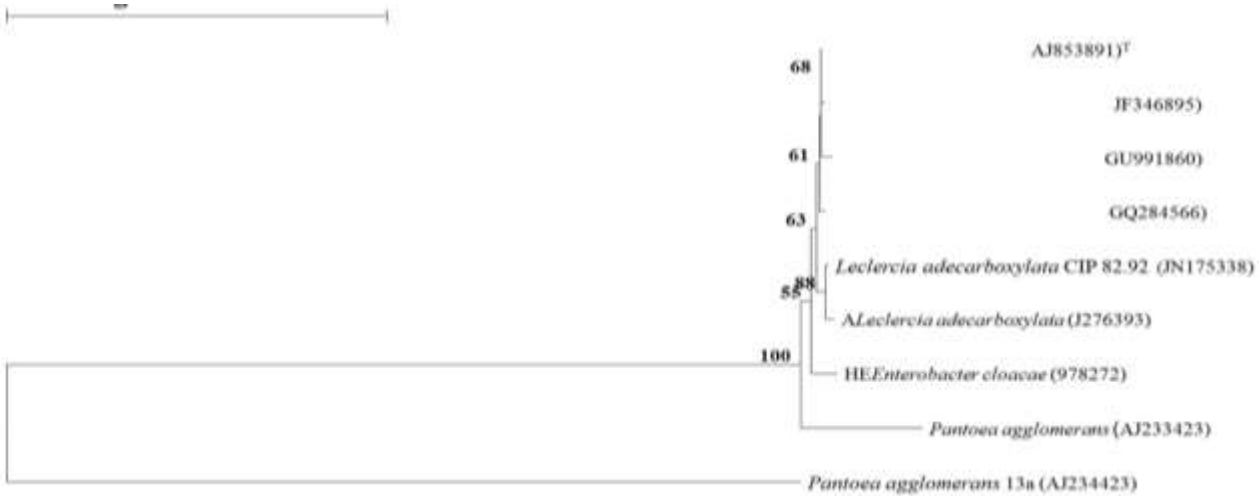

Figure 1: Phylogenetic trees based on comparative analysis of 16S rRNA gene sequences showing the relationship of the representative strain 13a among the family Enterobacteriacea. The gene bank/ accession numbers of the sequence are given in parentheses.

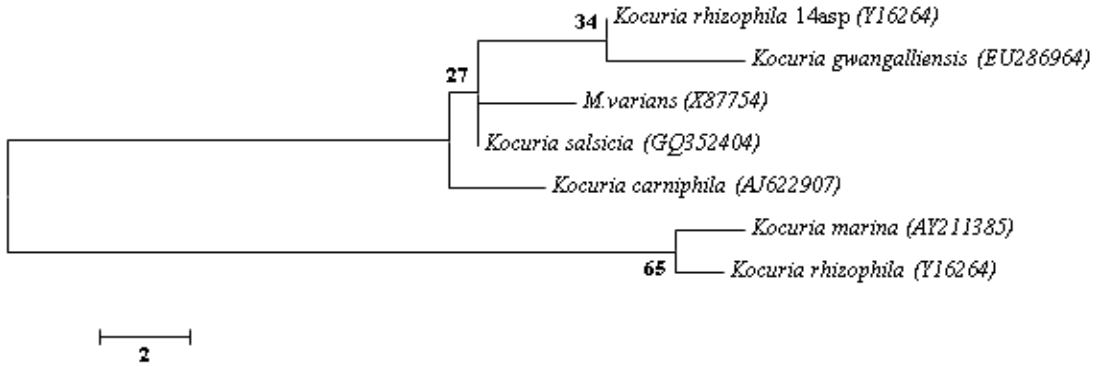

Figure 2: Phylogenetic trees bassed on comparative analysis of 16S rRNA gene sequences showing the relationship of the representative strain 14asp among the family micrococciaceae. The gene bank/ accession numbers of the sequence are given in parentheses

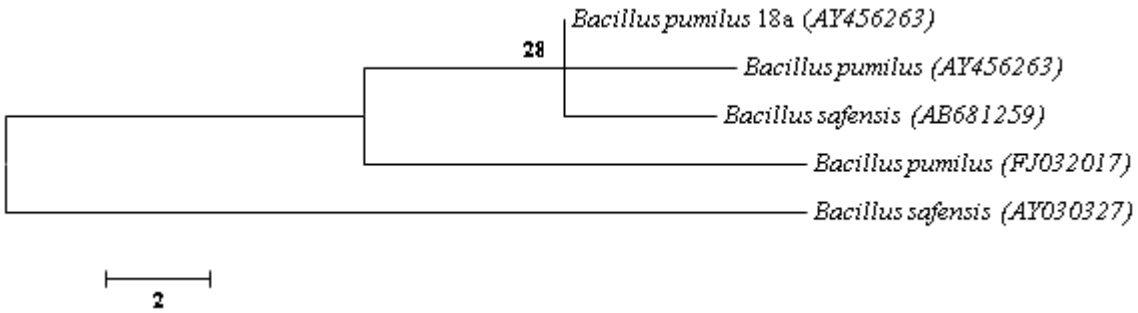

Figure 3: Phylogenetic trees bassed on comparative analysis of 16S rRNA gene sequences showing the relationship of the representative strain 18a among the family baciallaceae. The gene bank/ accession numbers of the sequence are given in parentheses.

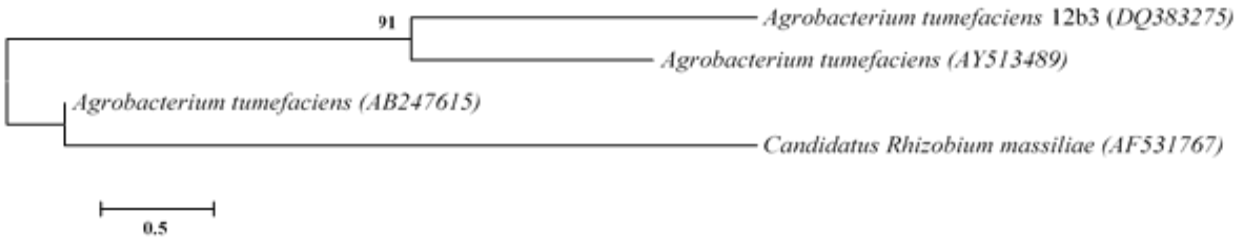

Figure 4: Phylogenetic trees bassed on comparative analysis of 16S rRNA gene sequences showing the relationship of the representative strain 12b3 among the family rhizobiaceae. The gene bank/ accession numbers of the sequence are given in parentheses

As little work is stated on isolation of endophytes from *O. corniculata*, previously Tani *et al.* (2012) isolated endophytes for the first time in Japan. We have isolated endophytes from root, stem and leaves but more number of endophytes were present in leaves. Similarly Okane *et al.* (1997) has also reported more diversity of endophytes in the leaves, one of the reasons in distribution of endophytes is the difference in substrate and structure of plant parts.

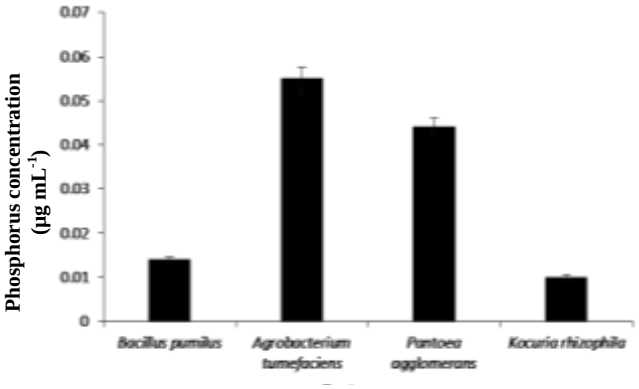

Figure 5: Phosphate solubilization shown by bacterial endophytes (*Bacillus pumilus, Pantoea agglomerans, Agrobacterium tumefaciens* and *Kocuria rhizophila*)

In cluster I, II, III and 1V bacterial strains were closely related to *P. agglomerans, B. pumilus, K. rhizophila and A. tumefaciens.* The phylogenetic tree constructed using 16S rRNA gene sequence revealed that the isolated endophytes belonging to diverse bacterial groups distributed in family Enterobacteriaceae, Baciallaceae, *Micrococciaceae* and *rhizobiaceae*. Previous studies established the endophytic nature of these bacterial isolates. *P. agglomerans* has been reported as endophytic bacteria from sugarcane (Quecine *et al.,* 2012). Its colonization has also been reported from Eucalyptus seeds and sweet potato stem in Japan (Ferreira *et al.,* 2008). *A. tumefaciens* has an endophytic association with root nodules of *Melilotus dentatus* as studied by Wang *et al.* (2006). It was also identified as a diazotrophic endophytic strain isolated from sugarcane (Xing *et al.,* 2006). *B. pumilus* was previously isolated as the dominant endophyte from maize, lemon and roots of cotton stem (Mcinroy *et al.,* 1995). However, previously very limited data was available on endophytic nature of *K. rhizophila*.

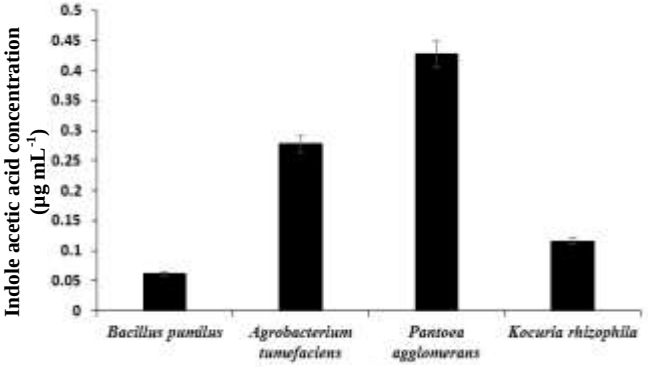

Figure 6: Indole acetic acid production shown by bacterial endophytes (*Agrobacterium tumefaciens, Pantoea agglomerans, Kocuria rhizophila* and *Bacillus pumilus*)

These endophytic bacteria were predicted to have diazotrophic activity as explained earlier by Feng *et al.* (2006). They are stress tolerant, bioremediator, resistant to antibiotics, involved in production of biofuel and antifungal agents. They have significant role in agriculture as biocontrol agent, clinical and industrial microbiology and in environmental bioremediation (Ding *et al.,* 2005, Radwan *et al*., 2010).

The current study proposes that endophytic bacteria have a significant role in the promotion of plant growth and

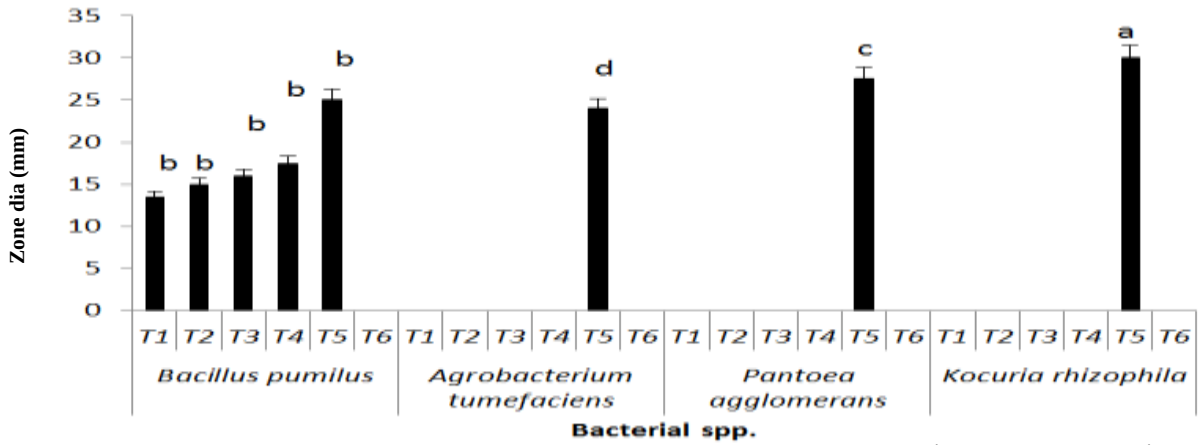

Figure 7: Heavy metal resistance of bacterial strains against lead. T_1 (0.025 mg mL^{-1}), T_2 (0.05 mg mL^{-1}), T_3 (0.075 mg mL^{-1}), T_4 (0.1 mg mL^{-1}), T_5 (PC1) and T_6 (DMSO). *Bacillus pumilus, Agrobacterium tumefaciens, Pantoea agglomerans* and *Kocuria rhizophila*, positive control (DMSO) and negative control (PC1)

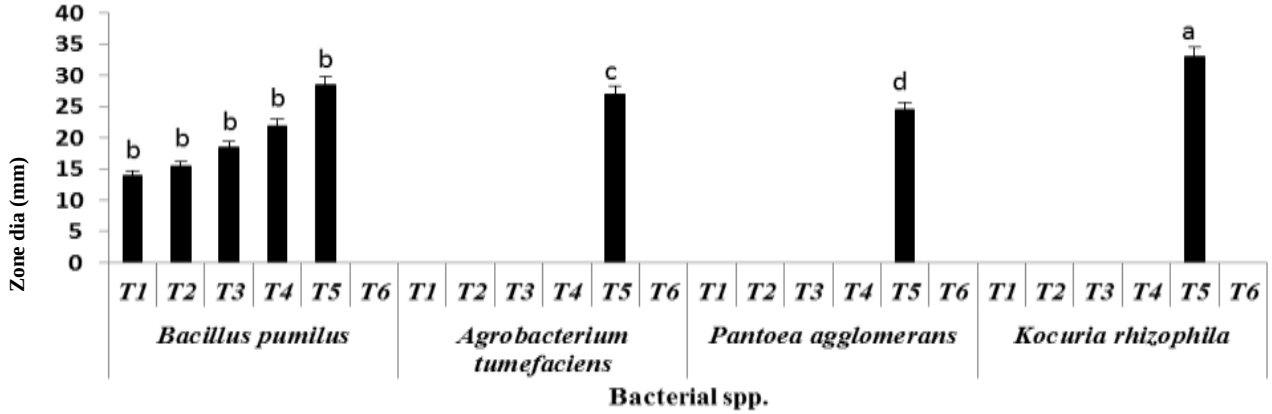

Figure 8: Heavy metal resistance of bacterial strains against copper. T_1 (0.025 mg mL^{-1} T_2 (0.05 mg mL^{-1}), T_3 (0.075 mg mL^{-1}), T_4 (0.1 mg mL^{-1}), T_5 (PC1) and T_6 (DMSO). *Bacillus pumilus, Agrobacterium tumefaciens, Pantoea agglomerans* and *Kocuria rhizophila*, Positive control (DMSO) and negative control (PC1)

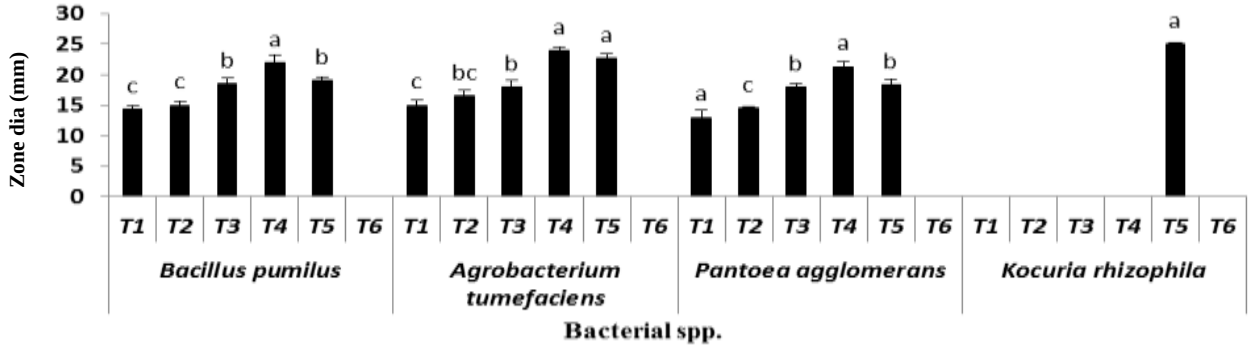

Figure 9: Resistance of bacterial strains against chromium. T_1 (0.025 mg mL^{-1}), T_2 (0.05 mg mL^{-1}), T_3 (0.075 mg mL^{-1}), T_4 (0.1 mg mL^{-1}), T_5 (PC1) and T_6 (DMSO). *Bacillus pumilus, Agrobacterium tumefaciens, Pantoea agglomerans* and *Kocuria rhizophila*, positive control (DMSO) and negative control (PC1). All the means sharing common letter(s) are insignificantly different at p≤0.05 level and vertical bars represents means ± standard error of replicates

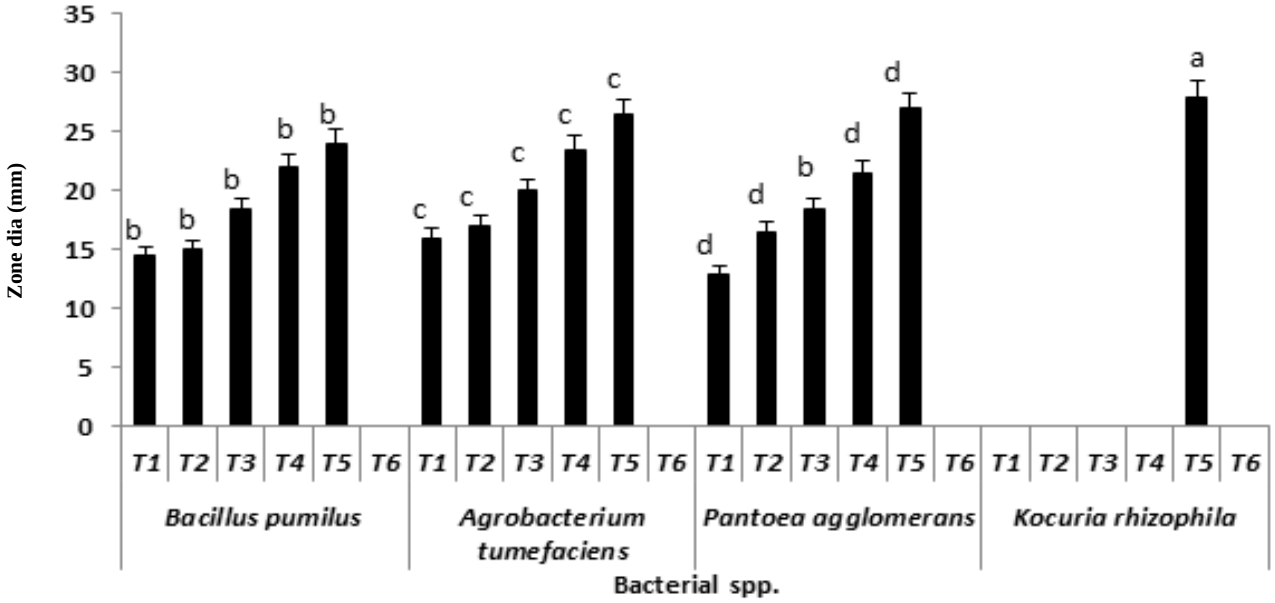

Figure 10: Heavy metal resistance of bacterial strains against nickel. T_1 (0.025 mg mL^{-1}), T_2 (0.05 mg mL^{-1}), T_3 (0.075 mg mL^{-1}), T_4 (0.1 mg mL^{-1}), T_5 (PC1) and T_6 (DMSO). *Bacillus pumilus, Agrobacterium tumefaciens, Pantoea agglomerans* and *Kocuria rhizophila*, positive control (DMSO) and negative control (PC1)

phosphate solubilization. Phosphate solubilizing bacteria are involved in the conversion of insoluble phosphorous to simpler organic and inorganic compounds. Currently, all four bacterial strains had shown phosphate solubilization although maximum phosphate solubilization was shown by *P. agglomerans*. It has been reported by Walpola and Yoon *et al.* (2013) that *P. agglomerans* acted as an efficient phosphate solubilizer. The mechanism behind P-solubilization is the production of acid phosphatase and organic acids involved in mineralization of organic phosphorous (Chen and Rekha *et al.,* 2006). IAA is the major signal molecule, regulating many physiological processes taking part in plant development as reported by Zhao (2010). In the present study, all the bacterial strains were positive for IAA production but highest production has been shown by *P. agglomerans* as similar to Walpola and Yoon *et al.* (2013).

Another important trait of PGPR is the production of ammonia that directly influences the plant growth. Every fertilizer contains nitrogen either in the form of ammonia or compounds derived from ammonia. Currently, all the isolates have the ability to produce ammonia. Among all the strains, *P. agglomerans* was the most efficient PGPR which produced ammonia, IAA and solubilized insoluble phosphorous. This study will promote the use of presently isolated endophytic PGPRs as inoculants. Hydrogen cyanide is a secondary metabolite produced by bacteria that plays a vital role in plant defense system. Currently, *B. pumilus* and *P. agglomerans* produced HCN, while *K. rhizophila* and *A. tumefaciens* did not produce it at all. The present results showed maximum production of HCN by *B. pumilus* that are in accordance with *Godinho et al*. (2010) and Dastager *et al.* (2009). All the bacterial isolates in the present study are able to produce catalase. It has been studied that bacterial strains showing catalase activity must be highly resistant to environmental stress (Dey *et al*., 2004).

Microorganisms have adopted mechanisms to survive in an environment contaminated with heavy metals. All these endophytes isolated from *O. corniculata* showed resistance against heavy metals. In the present study, *K. rhizophila* showed maximum resistance to heavy metals such as Ni, Cu, Pb and Cr at higher concentrations (0.1 mg mL^{-1}). It was reported by El-Sharouny *et al.* (2013) in which two strains of *Koccuria* sp. showed positive results for biosorption of Zn, Cu, Co, Cd and Pb. *B. pumilus* showed resistance to heavy metals at lower concentrations (0.025 mg ml^{-1}) and at higher concentration it was sensitive. The present findings are contrary to the findings by Andreazza *et al.* (2011) where maximum Cu was incorporated by *B. pumilus* (121.82 mg L^{-1} OD unit in 24 h). *A. tumefaciens* has ability to resist against higher concentration of heavy metal. It had shown higher resistance against Cu and Pb as compared to Cr and Ni. However, limited work has been reported on the effect of heavy metals on *A. tumefaciens.* To the best of our knowledge, this report is the first ever report on *A. tumefaciens* showing resistance against toxic heavy metal.

Previously, effect of heavy metals on *A. tumefaciens* had been examined by Hao *et al*. (2012) in which it showed Zn-Pb resistance but unable to remove Cu. *P. agglomerans* showed resistance against Pb and Cu in higher concentration (0.1 mg mL^{-1}) results are similar to findings of Al-Mathkhury *et al.* (2011). It showed resistance against Cr and Ni at lower concentration (0.025 mg mL^{-1}) which is in accordance with the study conducted by Kaewchai and Prasertsan (2002). Evolution of heavy metal resistance evolved through the modification of metal uptake genes found on chromosomes. Their function is to compartmentalize heavy metal away from sensitive cellular functions (Doty, 2008). The reason behind that is some bacteria showed resistance against heavy metals while others did not. This is the presence of the metal resistance efflux system using a chemiosmotic transporter. Genes involved in the efflux system, assist in the conversion of toxic form of metal to less toxic form (Aydinalp, 2009). However, further studies are therefore needed to explore its potential and to study the mechanism of resistance against toxic heavy metals. Furthermore, our data also signifies the potential of isolated strains in the removal of toxic metals from the contaminated soil to protect the environment.

Endophytic bacteria isolated from *O. corniculata* showed prominent genetic diversity, different from each other even at the phylum level. Moreover, isolation of these strains is the first report from Pakistan. Assessment of bacterial strains against heavy metal stress showed their potential for bioremediation. In future, we can evaluate the diazotrophic nature of these isolated bacterial strains. Diazotrophic bacteria play an important role in agriculture. The bacterial strains having potential to tolerate heavy metals that might be used in future to reclaim heavy metal contaminated soils. Beneficial microorganisms can be a significant component of management practices to achieve the desired yield. Plant growth promoting traits of these isolated strains can be used to improve the crop yield along with potential of tolerance to heavy metal stress.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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