The Journal of Animal & Plant Sciences, 25(4): 2015, Page: 1081-1086
ISSN: 1018-7081

IDENTIFICATION OF *AZOSPIRILLUM* SPECIES FROM WHEAT RHIZOSPHERE

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ABSTRACT

Bacteria play an important role in maintaining the health status of soil ecosystem by preforming many biological processes. Among PGPR, *Azospirillum* is considered as an important genus which is closely-associated with plants and shows potential to degrade organic contaminants, improve the plant health and increase crops yield. The present study was carried out for the isolation of *Azospirillum* spp, which can be used as crop inoculants. Four bacterial strains were isolated from wheat rhizosphere. The isolates were characterized on the basis of colony, cell morphology, shape, size and motility. *Azospirillum* strain Azo LR3 has cream/ pink colony color and plump rod, 3µm in size, with typical helical motility. This isolate showed ability to produce indole-3-acetic acid (11.5 µg/ml of culture medium) and gibberellic acid (12.8 µg/ml of culture medium). 16S rRNA genes sequence analysis revealed that isolate belongs to the genus *Azospirillum* with 96% similarity and phylogenetic tree represent that it was different from the remaining other member of the genus *Azospirillum.*

Key words: *Azospirillum,* rhizosphere, IAA, GA, 16SrRNA

INTRODUCTION

Rhizo-bacteria living in association with plant roots and rhizosphere soil exert a positive effect on plant growth. They hold great potential for sustainable agriculture by increasing productivity and the growth of many commercial crops such as wheat, barley, canola, oat, peas, lentils, potatoes, soy and tomatoes, cotton, rice, cucumber, black pepper, banana, maize, chilli. The rhizospheric soil contains diverse types of plant growth promoting rhizobacteria (PGPR) communities including *Alcaligenes, Azospirillum, Burkholderia, Klebsiella, Azotobacter, Enterobacter, Arthrobacter, Bacillus, Pseudomonas,* and *Serratia* have been identified and involved in increasing the plant growth (Kloepper *et al.,* 1989; Ortíz-Castro *et al.,* 2008; Joseph *et al.,* 2012). Beneficial bacteria (PGPR) were used as inoculum in different crops for improved growth and gaining better yield. Beijerinck (1925) reported first *Azospirillum* and now azospirilla contain 15 *Azospirillum* sp. identified from rhizosphere of different plants (Massena Reis *et al.,* 2011; Shigueru *et al.,* 2013). Silva *et al.* (2004) described that bacteria belonging to the genus *Azospirillum*are are typically aerobic and Gram-negative, have spiral movements, measuring 0.8 to 1.0 µm in diameter and 2 to 4 µm in length and present in intracellular granules of polyhydroxybutyrate. *Azospirillum* sp. colonizes the plant roots and stimulates plant growth. It is free-living bacteria and widely distributed in soils of tropical and subtropical climate in the roots of grasses of great economic importance. About 30 to 90% of soil samples collected from different part of the globe had *A. brasilense* or *A. lipoferum* (Bashan *et al.,* 2004).

Auxins are the most abundant phytohormone secreted by most plant-associated bacteria. *Azospirillum* spp. are known for the production of indole-3- acetic acid, gibberellic acid and kinetin whereas *Azotobacter chroococcum*is identified to produce, gibberellic acid, indole-3- acetic acid and cytokinnin. PGPR alter root growth in grasses by producing phytohormone. Cassán *et al.* (2009) described the same effect via legume seedlings inoculation with *Azospirillum brasilense* and *Bradyrhizobium japonicum*. Bacterial differentiation has been reported by physiological, morphological and biochemical characterization (Krieg and Dobereiner, 1986), 16S ribosomalDNA sequences (Xie and Yokota, 2005). The aim of this study was to identify PGPR strains especially *Azospirillum* spp., from wheat rhizosphere soil.

MATERIALS AND METHODS

Isolation of *Azospirillum* sp: Wheat root adhering soil, 2-3 mg, was added into 17 ml capacity vialscontaining semisolid nitrogen free malate medium (NFM). The vials were incubated at 28 °C for 48 hrs. A loop fullfrom growth streaked on LB plates to get single colonies of bacteria. Isolated bacterial colonies were characterized on the basis of cell, colony morphology and 16S rRNA gene sequence analysis.

Detection of Indole-3- Acetic Acid by spot test and quantification by HPLC: Indole-3- Acetic Acid (IAA) spot test was performed as described by Sasirekha and Shivakumar, (2012). The samples turned to pink were

subjected to ethyl acetate extraction and analyzed by HPLC. Bacterial cultures were grown in LB liquid medium containing tryptophan for 3 days. The cells were removed by centrifugation for 10 min at 8000 rpm. The supernatant was separated and its pH was adjusted to 2.8 by HCl as described by Tien *et al.,* (1979). 50 ml cell free liquid medium was mixed with equal volume of ethyl acetate in a separating funnel. Ethyl acetate fraction was collected and evaporated to dryness and residue was dissolved in 1-2 ml methanol. The 20 µl samples were analyzed on HPLC by using methanol: acetic: water (30:1:70) as mobile phase, C18 column and UV detector at wave length 260 nm. The IAA and GA were identified and quantified on the bases of retention time and peak area of standard IAA and GA.

DNA isolation and amplification of 16S rRNA gene: Bacterial cells culture was grown overnight in LB liquid medium at 28°C. 1.5 ml of culture medium centrifuged at 10,000 rpm for 2 minutes to form a pellet. Pellet was resuspend in 600µl of Lysis Solution (TE buffer 576µl, 30 µl of 10% SDS, 3 µl of 20mg /ml of proteinase K) and incubated at 37°C for 1h and then added 100 µl of 5M NaCl and 80 µl of CTAB freshly prepared was added and incubated for 10 minutes at 65 ºC. Chloroform isoamyl alcohol 780 µl were added and mixed it. Upper layer was transferred in an empty tube after centrifugation and then 20 µl of 3M sodium acetate solution was gently mixed. 1ml of absolute ethanol added and centrifuged at 13,000 rpm for 5 minutes. DNA pellet was washed with 70% ethanol and finally dissolved the pellet in TE buffer.

PCR reaction mixture was prepared with 2X PCR master mix (Fermentas) 12.5 µl, Forward primer 1µl (12-14 ng/µl), Reverse primer 1µl (12-14 ng/µl), bovine serum albumin (BSA) 0.2µl (20 mg/ml), DNA 3 µl, nuclease free water up to 25 µl. Primer sequences (F2-AGAGTTTGATCATGGCTCAG, R2-GGTTACCTTGTTACGACTT) were used for bacterial 16S rRNA gene amplication (Weisburg *et al.,* 1991). Amplification was performed in Bio-Red Thermal Cycler, programmed for an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of 94 ºC for 1 min, 54 ºC for 1 min, 72 ºC for 3 min and a final elongation step at 72 ºC for 10 min.1% agarose gel was used to separate DNA.DNA sample and PCR products 5-10 µl, mixed with 6X loading dye (Fermentas), were transferred into the wells in the agarose gel. DNA fragments were separated by electrophoresis by using 1X TAE buffer. The gel was run at 80 V for 45 minutes and then visualized under UV light and printed image through BioPrint (Syngene doc).

RESULTS

Isolation and characterization of *Azospirillm* species: Isolations of bacteria carried out from roots adhering soil of wheat. Nitrogen free malate (NFM) medium was used for isolation of *Azospirillum* species. Azospirilla made wheel like white pellicle in semisolid NFM medium. The isolates were characterized on the bases of colony and cell morphology. Colony change the color from transparent to pink was typical *Azospirillum* character (Fig 1). These isolates formed circular/ wrinkled colonies when grown on agar plates. The AzoLR3 was gram-negative medium rods 3mm in size and helically fast motility wasobserved under light microscope. The colony morphology of AzoLR3 was circular, flat, shiny and producing red pigment on maturity. Isolate B2 was circular, raised, gummyand white containing water inside. B3 and B4 were circular, yellow in color having pungent smell and nearly circular, flat, entire off-white change to brown in color having pungent smell respectively (Table 1). Morphological studies of the other three isolates isolated from wheat rhizosphere, belong to *Staphylococcus* and *Bacillus* spp.

Table 1. Characterization of colony morphology (size, shape and color).

Source of isolations	Isolates	Shape	Size (mm)	Elevation	Surface	Margin	Colour	Odour /pigment/ misllininess
Agricultural Wheat rhizosphere soil	Azo 3	Circular	variation present	flat	Shiny	entire	creamy turn to pink	mature colonies produced red pigment
	B2	Circular	Medium	raised	Outer dry inter water	wrinkled	White	Odourless
	B3	Circular	Small	flat	Shiny, Gummy	entire	Yellow	Pungent
	B4	Nearly Circular	Medium	flat	Gummy	entire	Off-white change Brown	Odourless

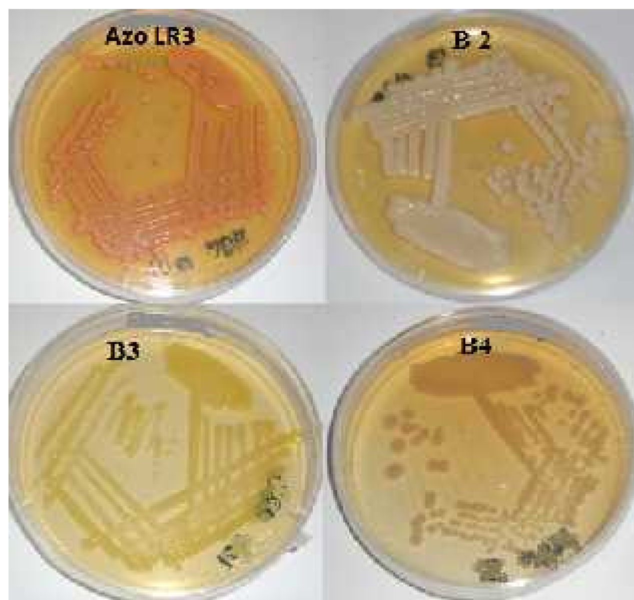

Figure 1: Bacterial isolates isolated from wheat rhizosphere soil.

Indole-3- Acetic Acid (IAA) and gibberellic acid (GA) quantification by HPLC

All four isolates were grown in LB broth containing tryptophan (100 mg/liter) for 3 days on shaker at 120 rpm at 30 ºC. 20 µl samples of culture broth and 20 µl Salkowski reagent was applied on a white Perspex sheet and observed after 20 min for development of pink color. The 100 ppm IAA standard 20 µl, was used as positive control. One of them was IAA positive. IAA production was significant for plant beneficial microbes. AzoLR3 analyzed for IAA and GA HPLC, showed 11.5µg/ml IAA and 12.8µg/ml GA in culture medium (Table 2) IAA and GA production by other isolates is given in table 2.

Table 2. Identification and quantification of IAA and GA production by isolates from wheat rhizosphereby HPLC.

Isolates codes	indole-3-acetic acid µg/ ml	GA µg/ ml
Azo 1	11.5	12.8
B 2	3.7	7.7
B 3	5.2	8.0
B 4	6.1	6.5

Identification of bacterial by 16SrRNA: Genomic DNA was used for amplification of 16S rRNA gene. (Fig 2).

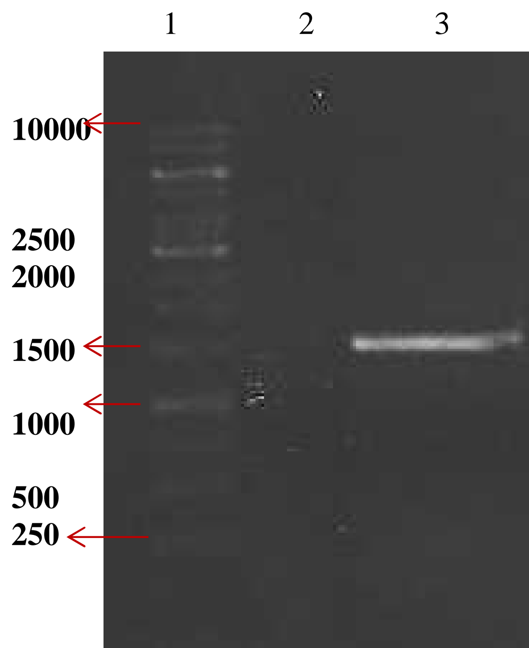

Figure 2: Agarose gel electrophoresis (1%) Lane 1 molecular size marker is a 1-kb ladder (Fermentas.) and the sizes were indicated in base pairs, Lane 2 control and Lane 3 amplified product of AzoLR3 isolate.

The present isolate have been identified by 16S rRNA gene sequence analysis. The 16S rRNA results showed that the isolate belonging to Phylum "Proteobacteria". Fasta formatted sequences of different *Azospirillum* spp. were aligned in CLUSTALX2 and MEGA5 was used to construct phylogenetic tree. A tree structure represents the evolutionary relationships among a group of organisms. AzoLR3 16S rRNA gene sequence was deposited in Genbank (EMBL) with the accession number HG931087. It was observed that AzoLR3 have shown 96% similarity with *Azospirillum brasilense*. Therefore, they may be different isolate from the other *Azospsirillum* strains of *Azospirillm brasilense* (Fig 3).

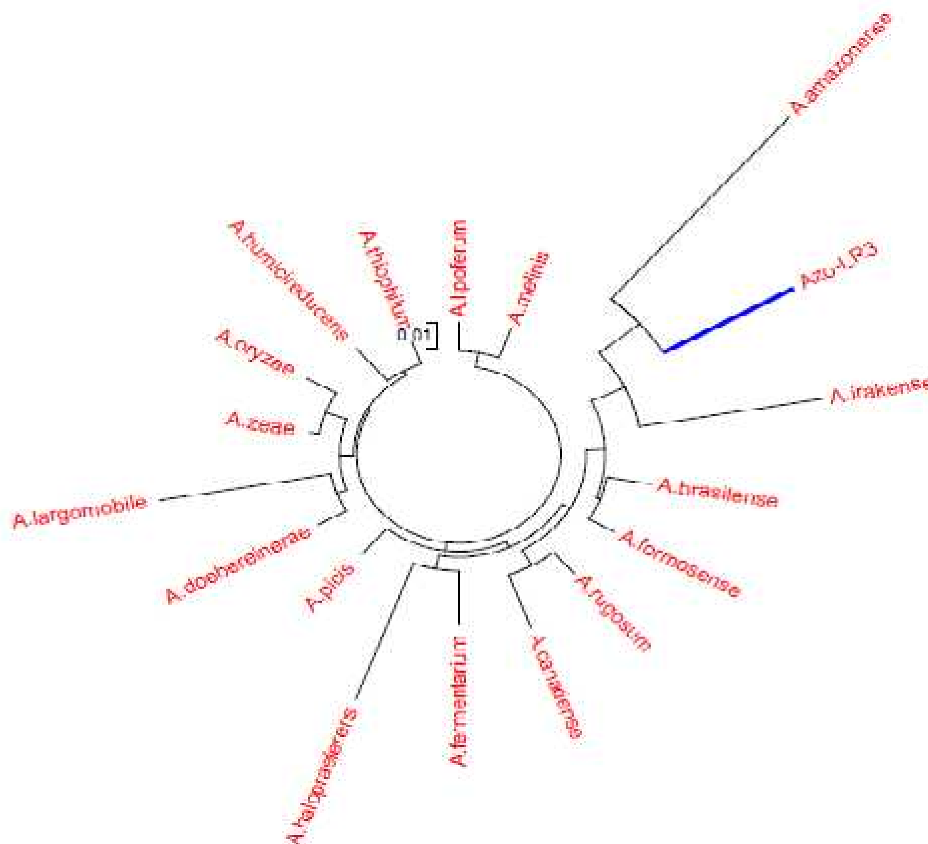

Figure 3: Phylogenetic relationships of Azo LR3 isolate with the related *Azospirillum*spp.

DISCUSSION

Identification of *Azospirillum*by morphological: *Azospirilla* are free-living bacteria and which are widely distributed in soils of tropical and subtropical climate in the roots of grasses of great economic importance. About 30 to 90% of soil samples collected from different part of the globe had *A. brasilense* and/ or *A. lipoferum* (Bashan *et al.,* 2004). The physiology and genetics of *Azospirillum lipoferum* and *Azospirillum brasilense* were well studied (Tarrand *et al.,* 1978)**.** It is gram-negative and aerobic bacteria, with spiral movement, measuring 0.8 to 1.0 µm in diameter and 2 to 4 µm in length. Eckert *et al.,*2001 described that *Azospirillum* species were curved rods or S-shaped, 1.0–1.5 µm in width and 2.0–30 µm in length, and a wide variation was found in size and pellicle thickness (1-4 mm) by (Murumkar *et al.,* 2013). Optimum growth observed at 30 ºC and at 6- 7 pH. Cells were about 1.0 um x 3.5 mm in size with single flagellum. In the present study, wheat isolates formed a wheel like white pellicle in bluish back ground of semisolid NFM medium and circular/ wrinkled colonies changed the color from transparent to creamy and then dark pink on agar plates, clearly indicated that they were *Azospirillum* sp. as they showed typical characteristics of *Azospirillum* sp. Under light microscope, they were gram negative, plump rods ranged from 3- 5 µm size and showed fast helically motility which confirmed their identification as *Azospirillum* sp.

Azospirillum spp. are reported to stimulates plant growth by production of phytohormones. In present studies, IAA and GA production was 11.5 µg/ml and 12.8 µg/ml respectively observed in *Azospirillum* sp. *Pseudomonas* sp. and *Azospirillum* ER-2 and ER-20 produced higher amounts of IAA (upto 35 ug/ml) in the liquid medium containing tryptophan and NH4Cl (Rasul, 1999). In the present study, production of IAA and GA by AzoLR3 and other isolates confirmed the presence of plant beneficial traits in these strains like *Azospirillum* sp. The exact IAA role in bacterial -legume symbiosis is not clearly known yet. Various auxins like indole-3-pyruvic acid, indole-3- acetic acid, indole lactic acid and indole-3-butyric acid gibberellins were found in liquid medium (Bottini *et al.,* 1989; Costacurta *et al.,* 1994; Kang *et al.,* 2012; Bruijn, 2013). However, we only identified IAA and GA by HPLC in the liquid medium of these wheat isolates.

Identification of *Azospirillum*by 16S rRNA gene sequence analysis: 16S rRNA gene sequence analysis has been widely used for identification of bacteria

(Saxena *et al.,* 2014). In present studies, PCR amplifications of 16S rDNA and sequencing of the amplified products has been carried out. AzoLR3 and *Azospirillum* reference sequences which were obtained from Gene Bank were aligned using CLUSTALX and a phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987). The phylogenetic analysis and comparison of isolates with other members of genus *Azospirillum* showed evolutionary relationship among them. AzoLR3 isolate showed 96% similarity with *Azospirillum* genus. Therefore, the bacterial strain AzoLR3 was identified as *Azospirillum* sp. On the basis of 3 % difference in 16S rDNA sequences, novel bacterial species has been proposed (Stackebrandt and Goebel, 1994; Xieand Yokota, 2005). .

Acknowledgement: This work was funded by the Higher Education Commission, Islamabad, Government of Pakistan.

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