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Isolation and Identification of *Azospirillium zeae* from *Acacia tortilis* at Riyadh, Saudi Arabia

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Authors' contributions

This work was carried out in collaboration among all authors. Author KHS designed the study, wrote the protocol and wrote the first draft of the manuscript. Author FNB performed the laboratory work and data collection. Author AMA performed the statistical analysis. Author AESOK managed the literature searches and amended the comments raised by the reviewers. Hereby, we read and approved the final manuscript.

Original Research Article

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ABSTRACT

The Study was conducted at Riyadh, Saudi Arabia to Isolate and Identify the Azospirillium zeae from *Acacia tortilis*. Useful bacteria associated with the plant roots have the potential to alleviate the burden of using costly and harmful agrochemicals in harsh environments. Finding of novel and appropriate bacteria for enhancing plant growth is among the main primary challenges involved in achieving the agronomic application of plant beneficial bacteria. A bacterium isolated from the rhizospheric soil of the legume *Acacia tortilis* was described based on several biochemical, morphological and molecular analyses. The bacterium H1P is motile, negative in biotin medium, no growth in 3% NaCl, negative D-Ribose, positive D- glucose. Identification of the isolate via 16s rDNA via the BLASTn revealed that the isolate is an *Azospirillum* species with a 96.9% similarity to *Azospirillum zeae* H1P. Phylogenetic analyses with MEGA6 software showed that the isolate shares an ancestor with *Azospirillum zeae*, eventually branching off into a separate taxon. *Azospirillum zeae* isolated from *Acacia tortilis* it is considered first report.

Keywords: Acacia tortilis; rhizosphere; Saudi Arabia; Azospirillum; BLASTn.

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1. INTRODUCTION

The genus *Azospirillum* is a member of the class Alphaproteobacteria and subclass of proteobacteria [1] are free-living bacteria. The genus *Azospirillum* was first described by Tarrand et al. [2] and comprised two species; *Azospirillum lipoferum* and *Azospirillum brasilense*. Currently thirteen species were added to the genera: *A. amazonense*, *A. halopraeferens*, *A. irakense*, *A. largimobile*, *A. doebereinereae*, *A. oryzae*, *A. melinis*, *A. canadiana*, *A. zeae*, *A. rugosum*, *A. palatum*, *A. picis* and *A. thiophilum* [3,4]. *Azospirillum* spp. have been isolated from various geographical regions of the world covered arid and semi arid areas include Saudi Arabia, their metabolic versatility allows them to also live in harsh environments [5] Nitrogen-fixing *Azospirillum* bacteria mainly distributed in soils and frequently associated with grasses, cereals and crops [6]. These bacteria important soil microbial community for the trees by increasing supply of the nitrogen contents. Further, *Azospirillum* also considered as colonizes the root region of crop plants in large numbers and fixes substantial amount of nitrogen [7], plant growth-promoting substances [4] when applied as inoculants, by a diverse array of mechanisms, some of which are linked to production of plant growth regulators and have beneficial effects on plant growth and crop yields. Moreover, *Azospirillum* are most extensively studied for their beneficial effects on the growth and yield of many agronomically important crops. Latterly, new *Azospirillum* sp. such as *A. zeae* have been isolated from cultivated soil [8], corn (maize) rhizosphere (Shahabi et al., 2019), rhizosphere of wheat [9] which scattered in different environmental conditions. No works have been done in arid and semi arid areas in different aspect of *Azospirillum* sp particularly in acacias. The present work was undertaken to study the taxonomic status of a newly isolated diazotrophic bacterium from acacia tortilis rhizosphere in Riyadh, Saudi Arabia.

2. MATERIALS AND METHODS

The study was conducted in Riyadh region, Saudi Arabia viz. Wadi Huraymila (25°04'N, 46°03'E) (Fig. 1). The vegetation of the study area reflects typical desert flora dominated by shrub species (Alatar et al. 2015). Soil rhizosphere samples along of Acacia tortilis were collected during January to March, 2016 from the site *viz*. W. Huraymila of Riyadh region, Saudi Arabia. Three soil samples were collected from the root rhizosphere of the acacia. Also, three soil samples away from the root rhizosphere (free soil) were collected. Samples were wrapped in polyethylene bags in ice box and brought to the laboratory. Soil physical and chemical properties were fully described by (Rowell, 2014; Suliman et al. [5].

Different isolation methods were used to isolate indigenous *Azospirillum* from Acacia rhizosphere. A subsample of 10 g of soil was used for serial dilution plating technique. *Azospirillum* sp. was isolated in screw cap tubes containing approximately 5ml sterilized semi solid N-free malate medium (L-malic 5 g, K_2HPO4 0.5 g, $MgSO_4.7H_20$ 0.2 g, NaCl 0.02 g, trace element solution 2 ml, bromthymol blue (0.5% dissolve in KOH) 2 ml; Fe EDTA (1.64% solution) 4 ml, KOH 4g, Agar 1.75 g, final pH 6.8 with KOH) for solid medium added 15 g/l) [10] under aseptic conditions. The tubes were incubated at 28°C for two weeks and observed for growth of *Azospirillum* as subsurface pellicle.

For enrichment, semi-solid NFb medium was used and streaked on the plates of solid NFb medium containing 0.02 g/l yeast extract of *Azospirillum* sp and H1P strain were incubated on M medium without biotin (5.0 g sodium malate, 0.02 g $CaCl_2.2H_2O$, 0.2 g $MgSO_4.7H_2O$, 0.1g K_2HPO_4, 0.4g KH_2PO_4, 0.1g NaCl, 10mg $FeCl_3$, $2MgNa_2MoO_4.2H_2O$, 0.1g yeast extract, 1.0 l distilled water, pH 6.8) [11]. Microorganisms were identified based on cultural, morphological and biochemical characteristics as per Bergey's Manual of Systematic Bacteriology and 16S rDNA region Sequencing Analysis. Motility, cell shape, color, consistency and gram stain were described for morphological characterization of bacterial isolates. Motility of *Azospirillum* isolates were tested by hanging drop method [12]. Slides were prepared with cultures and motility was observed under oil immersion. Different biochemical tests were performed, and the protocols followed are briefly outlined below.

The biotin requirements of the Bacteria isolates were tested using semi-solid nitrogen free malic acid medium prepared in two sets of tubes; one set of medium prepared with the addition of biotin (100 µg/l) and the other without biotin. The growth was observed by the change in color from yellowish green to blue [13]. For the determination of the efficiency of N_2 fixation of *Azospirillum*, isolates were grown in semi-solid Nfb medium to fix nitrogen. A loopful of the pure culture was inoculated into test tubes containing

10 ml of sterilized in semi-solid Nfb malate medium. Non-inoculated test tubes containing the same medium served as control. All the tubes (duplicate for each strain) were incubated at 30°C for 10 to15 days. Total amount of N_2 fixed by each isolate was expressed as mg N/g of malate after deducting the amount of nitrogen in control samples. The distillate was collected into 10 ml 3% boric acid solution having bromocresol green and methyl red mixed indicator. Final samples were titrated against 0.01N sulphuric acid.

Additional test of isolates were grown on semi-solid Nfb malate medium having concentration of sodium chloride 3% for growth ability test. For carbon utilization, a loopful of *Azospirillum* culture was grown at semi-solid Nfb malate medium having 1.0% replaced of the sugars D-glucose and D-ribose which were sterilized separately by filtration. The development of yellow color was observed after incubation period.

Azospirillum temperature tolerance was tested by growing on semi-solid Nfb malate medium and incubation at 30, 37 and 41°C for 24 hrs and then inoculation and incubation at 30ºC for 72 hrs on solid Nfb malate medium plates. A presumably novel strain H1P was selected for further phenotypic and phylogenetic characterization.

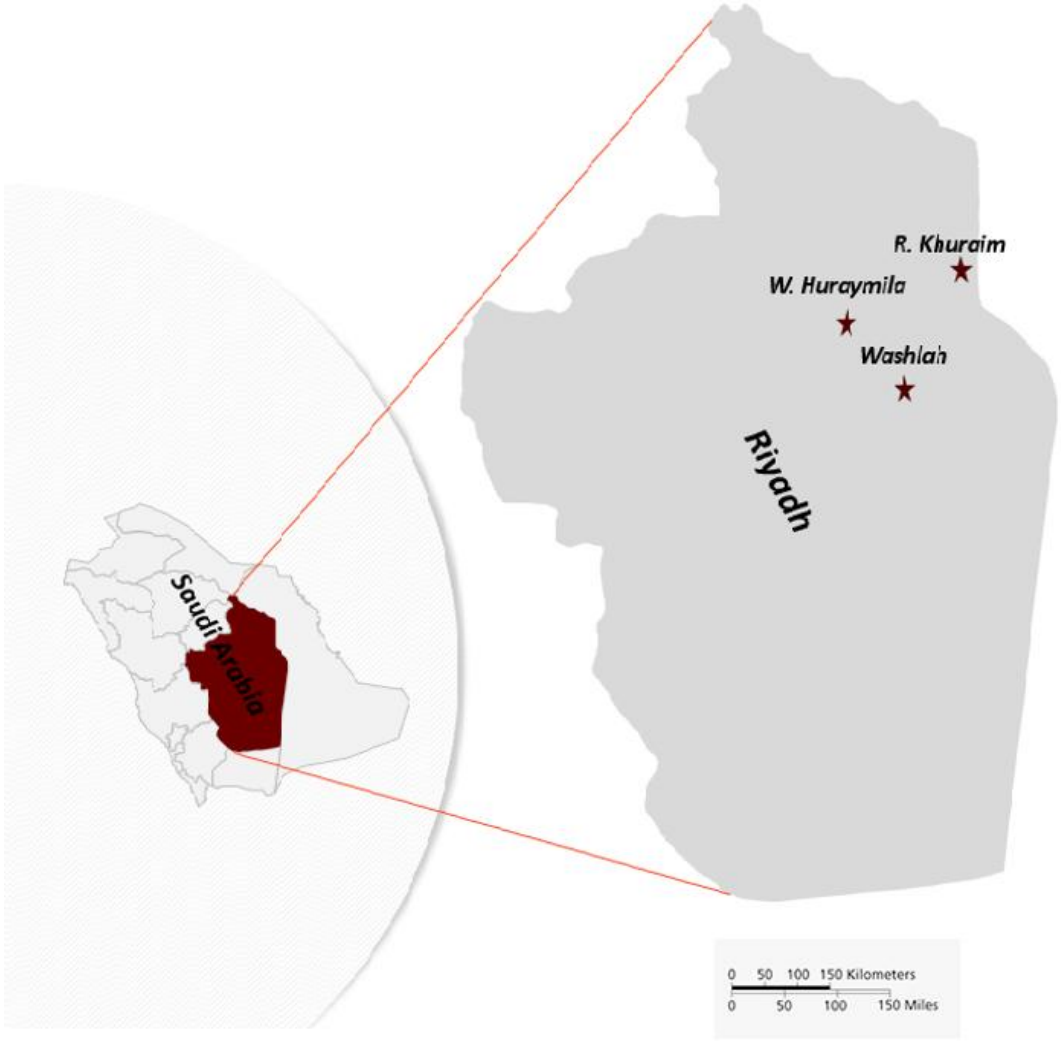

Fig. 1. Map of the study site and sampling location

3. PARTIAL 16S rRNA GENE SEQUENCING

16S rDNA Sequence was applied for H1P strain (suggested *Azospirillum zeae*) used PCR (BIO-RAD) Primer Sequences 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' (Haiyambo and Chimwamurombe 2018) The PCR reaction was performed with 20 ng of genomic DNA, Macrogen, South Korea. Activation of Taq template in a 30 $\mu\ell$ reaction mixture by using a EF-Taq (Sol Gent polymerase at 95°C for 2minutes, 35 cycles of 95°C for 1minute, 55°C and 72°C for 1minute each were performed, finishing with a 10-minute step at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit [1]. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems Foster City, CA). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The evolutionary history was inferred using the Neighbor-Joining method [14]. The evolutionary distances were computed using the Maximum Composite Likelihood [15] and are in the units of the number of base substitutions per site. The analysis involved 38 nucleotide sequences. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [16]. Nucleotide sequence accession numbers were obtained from National Center for Biotechnology Information (NCBI) database.

4. RESULTS

4.1 Physico-chemical Properties of Soil

Physico-chemical properties of soil are given in (Table 1). The results showed that texture class of soil samples was sandy loam with slightly alkaline. The pH value of soil indicates to the limited leaching and slow rates of weathering and soil development in of the arid region accompanied by high soil content of calcium carbonate. The EC value indicates that the soil is suitable for normal plant growth. Beside that soil was calcareous in nature. On the other hand, the soil was poor in their organic matter content. These findings agree with that of Yasir et al. [17]. However, soils organic matter content of tree rhizosphere was relatively higher (two times compared to bare soil) possibly resulting from root exudates and microbial activities. Soil Na+, Ca^{2+}, Mg^{2+} and K^{1+} ions were the most dominant cations, while Cl^{-1} and SO_4^{-2} ions were the most dominant inions. This means that dry conditions and limited rainfall has led to an increase in the concentration of salts and carbonate in the surfaces layers. Moreover, low amount of rainfall also led to reduced vegetation cover in the bare soils reflecting to the low of soil organic matter. On other hand, despite of arid regional located of the soil it was characterized by high level of available P (4.95 mg/kg) and K (174.3 mg/kg) and it is vice versa a poor in total nitrogen percentage. Generally, the arid conditions and the low vegetation cover affected soil conditions and properties.

4.2 Description of Suggested *Azospirillum zeae* sp

Azospirillum zeae was isolated from A. tortilis and identified based on growth cultural, morphological and biochemical characteristics and 16S rDNA sequence (Fig. 2). New *Azospirillum* sp isolated from Acacia tree morphologically, cells are rods, motile with a single polar flagellum, gram negative, pink colonies form after 48–72 h, which become wrinkled and dried with time. These bacteria was optimum growth on M medium occurs at 30°C and pH 5–7 and failure to grow in 3% NaCl. Expected *Azospirillm* sp negative for D-Ribose and positive D- glucose, N-fixing bacteria and negative growth in the medium contained biotin as reported of Peng et al. [18]. In this study, 24 *Azospirillum* isolates were investigated for their efficiency using various biochemical tests. These isolates of *Azospirillum* was observed as the appearance of a thin white colored pellicle below the surface of the semi-solid Nfb malate medium, and then, transferred into NFb agar plates. 16 isolates were identified *Azospirillum* sp., and the remaining 8 isolates were *Enterobacter* and *Benibacillus* they were not processed further (data not shown). The 16 *Azospirillum* sp. isolates divided in to 4 categories depending on the possible morphological and biochemical characters of these isolates as A S1, A S2, A S3 and H1P (suggested *A. zeae*) (Table 2 and Fig. 2). Isolates S1, AS2 and AS3 were not studded more (data not shown).

Table 1. Physico- chemical properties of the soil in the study

Properties		*A. tortils* rhizosphere	Bare soil
pH		7.9	7.8
EC (dS/m) (1:1)		0.74	0.24
	Ca^{++}	5.46	1.43
Dissolved Cations Meq/l	Mg^{++}	1.14	0.45
	Na^{+}	0.45	0.03
	K^{+}	0.47	0.39
	Cl^{-1}	1.96	0.49
Dissolved anions Meq/l	CO^{-3}	0.0	0.0
	HCO^{-3}	1.29	1.30
	SO^{-4}	4.12	0.54
OM %		1.13	0.60
Available	K^{1+}mgKg^{-1}	174.27	138.4
	P mg Kg^{-1}	2.50	7.4
Total N %		0.14	0.04
$CaCO_3$ %		35.37	25.58
	Clay %	8.68	6.68
Particle size	Silt %	15.00	30.60
	Sand %	76.32	62.72
Texture class		Sandy loam	Sandy loam

Table 2. Physiological differences among identified Azospirillum isolates. (+): positive, (-): negative, nd: not determined

Characteristics	AS1	AS2	AS3	H1P
Motility	+	+	+	+
Water soluble pigment	nd	nd	nd	nd
Temperature (°C)	37	37	30	30
Acidification of peptone glucose medium	+	-	nd	nd
Biotin	+	-	+	-
3% NaCl	-	-	-	-
pH range for growth	7.5 - 6.5	6 -7.8	6 - 7	5 -7
D – glucose	+	-	+	+
D – ribose	+	-	-	-
Maltose	-	-	-	-
Mannitol	+	-	+	-

4.3 Identification of Isolates by 16S rRNA Sequence Analysis

Next, a PCR assay for the *Azospirillum* strain H1P was developed. Presence of the nifH Gene and phylogenetic relationships with 38 documented *Azospirillum* species; the attempt to amplify the nifH gene fragment from the genomics DNA of isolate H1P a PCR product of multiscreen filter plate. BLASTn search of the 16S rRNA gene sequence indicated that isolate H1P most similar to *Azospirillum zeae* (GenBank accession number FN813472.1 (96.9%) (Fig. 3). Based on the 16S rRNA gene sequences, the phylogenetic tree (Fig. 4) shows that the isolated bacteria species is closely related to the *A. zeae* with accession number FN813472.1. None the less, isolate H1P is attached to a branch on a different class than that of its most similar strains.

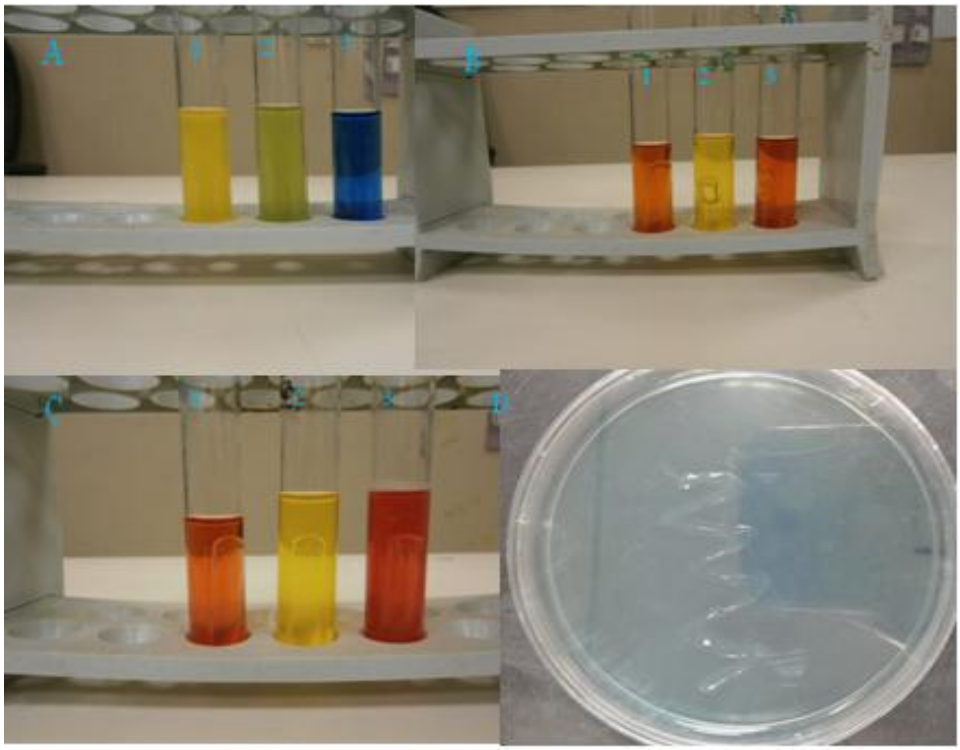

Fig. 2 (A) Acidification peptone glucose: 1, 2 positive, 3 negative, (B) mannitol: 1 negative, 2 positive, 3 control (C) D-glucose: 1 control, 2 positive, 3 negative (D) isolated H1P in solid NFb medium: 1 control, 2 positive, 3 negative (D): H1P growth in solid NFb medium

Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Azospirillum zeae	1489	1489	76%	0.0	96.87%	1448	FN813472.1
Azospirillum zeae	1485	1485	78%	0.0	96.37%	1447	FR667897.1
uncultured bacterium	1483	1483	76%	0.0	96.86%	1450	MN737242.1
uncultured Azospirillum sp.	1483	1483	76%	0.0	96.86%	1444	JQ278811.1
uncultured Azospirillum sp.	1483	1483	76%	0.0	96.86%	1445	JQ278782.1
Azospirillum zeae	1480	1480	76%	0.0	96.75%	1449	FR667885.1

Fig. 3. Highest six BLASTn search results based on the sequence of a 16s rRNA fragment amplified from genomic DNA of isolate H1P

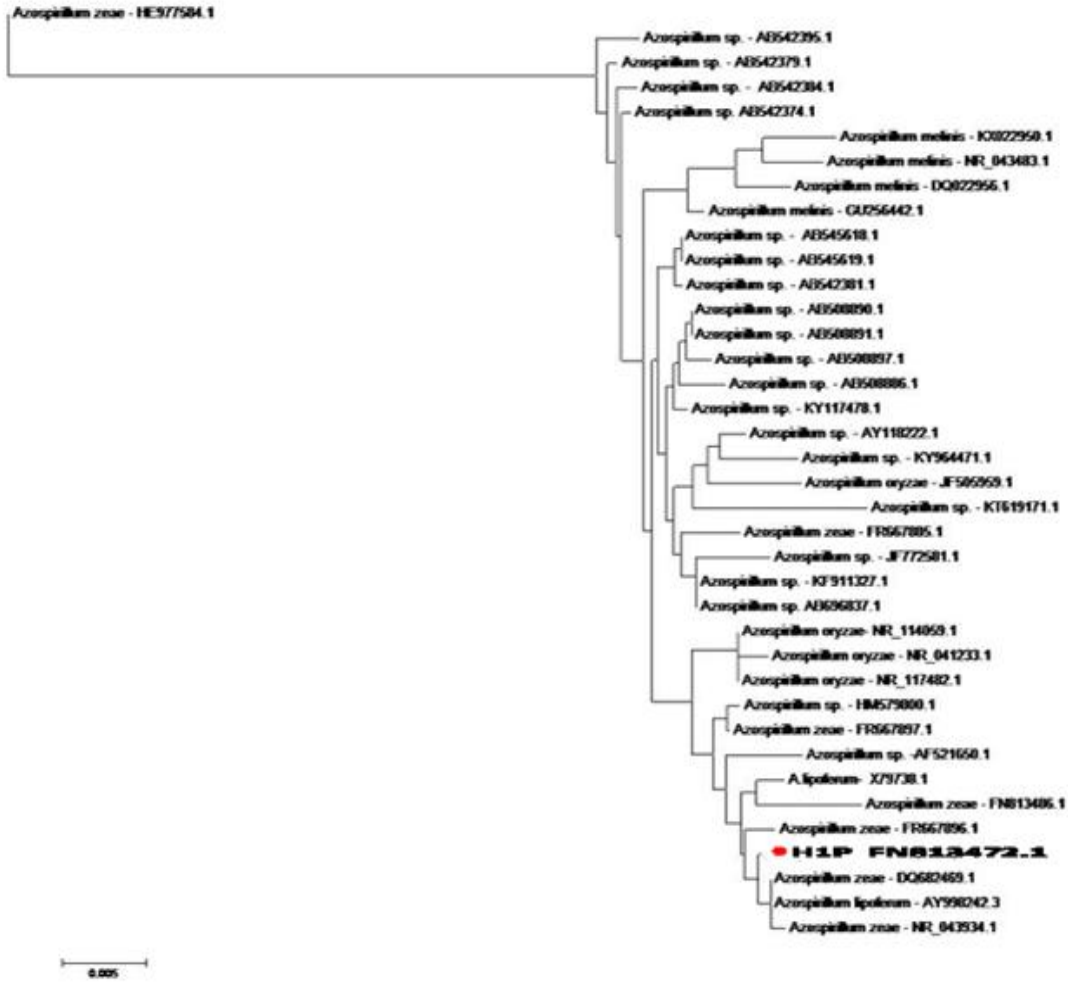

Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences. The tree was constructed by maximum likelihood method. Bootstrap values over 50% (based on 100 replications) are shown at each node Accession numbers are given after the scientific names of bacteria species. The isolates obtained in the present study are given in clear font with red bullet.

5. DISCUSSION

In the present experiment, the rhizosphere soil of a wild *Acacia tortilis* was collected and used for isolation of *Azospirilla bacterium* like many strains, which are known to improve the growth and productivity of many agricultural crop species [19]. The genus Azospirilla have been isolated from roots of various wild and cultivated grasses, cereals, and legumes and from tropical, subtropical,and temperate soils [9]. *Azospirillum* sp. was isolated and indentified initially based on cultural, morphological and biochemical characters compared to Bergey's Manual of Systematic Bacteriology [20]. Isolated bacterium growth excellent in the semi solid N-free malate [11], rod shape, motile with a single polar flagellum. On the M medium they formed pink colonies form after 48–72 h, which become wrinkled and dried with time and the optimum growth occurs at 37°C. These organisms that they are potential nitrogen fixers, similar to azospirilla [21]. The obtainment of numerous carbon sources is a good indicator of a high level of adaptability and distinguishes genus members polyphasic a taxonomic approach. Multiple carbon sources and other retaliated characters description of novel suggested isolate and development of improved culturing and storage media [22]. *Azospirillum* a diazotrophic bacterium isolated from roots of numerous plants [8] such as *Azospirillum zeae* isolated from *Zea mays*

rhizosphere (Mehnaz, 2015), *Azospirillum brasilense* and *Azospirillum zeae* from the wheat rhizosphere [9]. Most of the *Azospirillum* sp. have ability to fix nitrogen when examined by means of the acetylene reduction assay (Mehnaz, 2015). On the basis of the sequence analysis of the 16S rRNA gene, isolate H1P was affiliated to the genus *Azospirillum* with high similarity to *A. zeae* (96.9%). Similarly of that percentage closest relative to *A. zeae* isolated from cultivated soil [1]. H1P shared a cluster with the *A. zeae* type strain, and the other four isolates formed a cluster with *A. melinis* and *A. oryzae* strains. Our findings revealed that the Acacia rhizosphere is colonized by *Azospirillum* as well as *A. zeae* - related isolates. This is the first report indicating the presence of an *A. zeae* - like isolate in the acacia rhizosphere in Saudi Arabia. The *A. zeae* type strain was basically isolated from roots of maize in Canada [23]. Initially, this isolate was identified as *Azospirillum lipoferum* [24] but was later re-identified on the basis of a polyphasic taxonomic approach, including morphological characterization, Biological analysis, DNA–DNA hybridization, and phylogenetic analysis based on 16S rRNA gene sequences [25]. Roots exudates are useful for nutrients resources and organic compounds from the plant rhizosphere in order to establish a relationship with the plant in the root zone. *Azospirillum* sp.association with host plants by possess several chemotaxis signal transduction pathways to granting it an advantage by making the due adjustments for survival [26]. The high adaptability and association between plant and *Azospirillum* sp. in wide range of different conditions encourage to use *Azospirillum* sp. as biofertilizer and plant growth promoting (Mehnaz, 2015).

6. CONCLUSION

The Current paper is concluded that *Azospirillum zeae* was isolated from *A. tortilis* and identified based on growth cultural, morphological and biochemical characteristics and 16S rDNA sequence. New *Azospirillum* sp isolated from Acacia tree morphologically, cells are rods, motile with a single polar flagellum, gram negative, pink colonies form after 48–72 h, which become wrinkled and dried with time. These bacteria was optimum growth on M medium occurs at 30°C and pH 5–7 and failure to grow in 3% NaCl. Expected *Azospirillm* sp negative for D-Ribose and positive D- glucose, N-fixing bacteria and negative growth in the medium contained biotin. Moreover, in this study, 24 *Azospirillum* isolates were investigated for their efficiency using various biochemical tests. These isolates of *Azospirillum* was observed as the appearance of a thin white colored pellicle below the surface of the semi-solid Nfb malate medium, and then, transferred into NFb agar plates. 16 isolates were identified *Azospirillum* sp., and the remaining 8 isolates were *Enterobacter* and *Benibacillus* they were not processed further (data not shown). The 16 *Azospirillum* sp. isolates divided in to 4 categories depending on the possible morphological and biochemical characters of these isolates as A S1, A S2, A S3 and H1P (suggested *A. zeae*).

COMPETING INTERESTS

Hereby, the authors declared that no competing interests exist.

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