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# ISOLATION AND IDENTIFICATION OF *AZOSPIRILLUM* AND *AZOTOBACTER* SPECIES FROM *ACACIA* SPP. AT RIYADH, SAUDI ARABIA

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*Keywords:* Isolation, Identification, *Azospirillum, Azotobacter, Acacia*

**Abstract**

Populations of *Azospirillum* and *Azotobacter* isolated and identified from the soil rhizosphere of *Acacia ehernbergiana, A. gerrardii* and *A. tortilis* from three sites varied widely irrespective of tree species, size and site. *Azospirillum* strains were purified and characterized through morphological, microscopic and biochemical tests for motility, cell shape, pigmentation, colony color, gram reaction, optimal temperatures, biotin requirement, pH growth, acidification of peptone glucose medium, ability of nitrogen fixation, growth on 3% NaCl, and D-glucose and D-ribose utilization. Twenty *Azospirillum* isolates were divided into four matching characters. They were identified as *A. lipoferum, A. brasilense, A. dobereinerae* and *A. zeae.* The same above tests with omission of temperatures, biotin requirement, and acidification of peptone and addition of starch hydrolysis, $H_2S$, urease, oxidase and sucrose, maltose and mannitol as carbon source were applied to *Azotobacter* strains. *Azotobacter* isolates were divided into three matching characters. They were identified as *A. chroococcum, A. beijerinckii* and *A. salinestris*.

## Introduction

Biological nitrogen fixation is one of the most important biological processes in nature. The utilization of biological nitrogen fixation technology can also decrease the use of urea-N, prevent depletion of soil organic matter and reduce environmental pollution to a considerable extent (Bhardwaj *et al.* 2014). Diazotrophic -associated bacteria plays a key role in plant growth, development and nutrition by numerous mechanisms (Glick 2012). Application of biofertilizers scan decrease the use of chemical fertilizers and also can increase the yield of crops (Bhardwaj *et al*. 2014, Ajmal *et al*. 2018). Among N-fixing bacteria, *Azospirillum* and *Azotobacter* spp. have high adaptation ability to colonize different host plants and have been isolated from numerous geographical regions of the world (Upadhyay *et al*. 2015). The two genera are known to be associated with roots of various wild and cultivated plant species and soils of several regions and may increase the crop yield up to 30% (Saharan and Nehra 2011). Currently the genus *Azospirillum* is comprised of 10 species, including *A. amazonense* and *A. doebereinerae* (Lavrinenko *et al*. 2010).

*Azospirillum* colonizes the root region of crop plants in large numbers and fixes substantial amount of nitrogen (Saikia *et al.* 2013). Crop production response to bacteria with *Azospirillum* inoculants was almost equivalent to that of application of 15 - 20 kg N/ha (Hossain *et al.* 2014). Inoculation with *Azospirillum brasilense* increased grain production of maize (Morais *et al*. 2016). Further, *Azospirillum* also produces growth regulating substances (Bashan and de-Bashan 2010). Moreover, inoculation with *A. brasilense* was effective in elevating endogenous nutrient content

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in rice plants and improved its growth and yield (Sahoo *et al*. 2014). *Azospirillum brasilense* and *A. lipoferum* were isolated from roots of wheat cultivars (Rojas *et al*. 2013) indicating the potential of these two isolates in fixing nitrogen in most species.

The genus *Azotobacter* is comprised of seven species, including *A. chroococcum* and *A. vinelandii* (Mazinani and Asgharzadeh 2014). Azotobacters have extremely high respiration rates and it was estimated that with its inoculation, the total benefit to crops and soil was 73.8 kg N/ha/yr after one soybean-wheat rotation (Rawat *et al*. 2012).

Both *Azospirillum lipoferum* and *Azotobacter chroococcum* can improve seed tolerance of *Dodonaea viscosa* L. to salinity and increase its germination percentage (Yousefi *et al*. 2017). In a study on N-fixing bacterial endophyte diversity associated with *A. ehrenbergiana* and *A. tortilis* in a desert region, Boukhatem *et al.* (2016) indicated that the isolated nodular endophytes revealed strong tolerance to salinity and high temperature. Similarly, Alnohait (2015) reported *Azospirillum* and *Azotobacter* genera in the rhizosphere of *A. gerrardii*. The objectives of this study were to isolate, characterize and identify *Azospirillum* sp. and *Azotobacter* sp. from three *Acacia* plant species (*Acacia tortilis, A. ehrenbergiana* and *A. gerrardii*) of different sizes in Riyadh, Saudi Arabia.

## Materials and Methods

The study was conducted in three sites in Riyadh region, Saudi Arabia *viz.* Sheibh Washlah (24º26'N, 46º38'E), Raudhat Khuraim (25º25'N, 47º15'E) and Sheib Huraymila (25º04'N, 46º03'E) (Fig. 1). These three sites were referred to as Washlah, Khuraim and Huraymila, respectively. The vegetation of the study area reflects typical desert flora dominated by shrub species (Alatar *et al.* 2015). Soil physical and chemical properties were fully described by Suliman *et al*. (2017). Generally, soil ranged from sandy loam to clay loam with slightly alkaline pH. The soils of all studied sites were calcareous in nature and poor in nutrients and organic matter content.

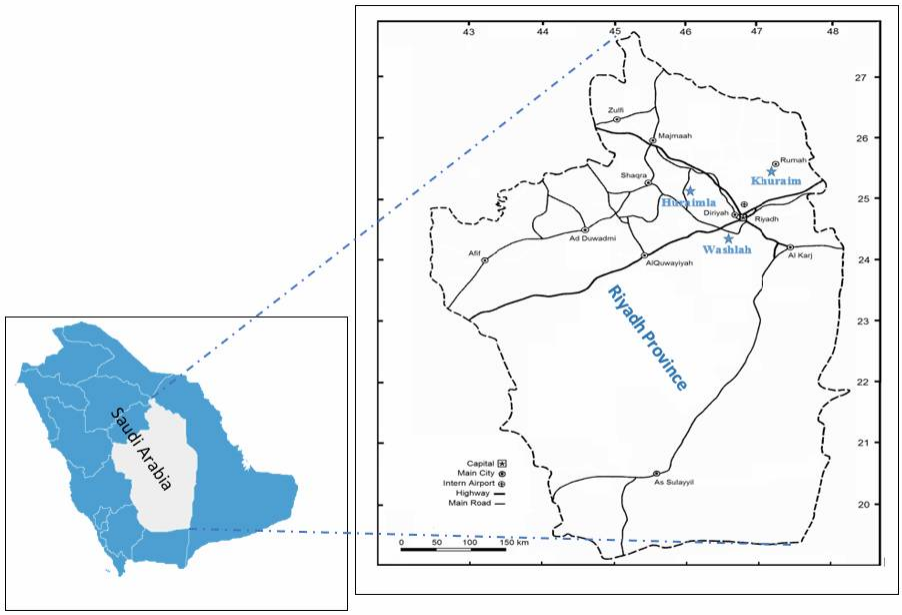


Fig 1. Map of the study site and sampling location.

Soil rhizosphere samples along with fine roots of *Acacia tortilis*, *A. ehrenbergiana* and *A. gerrardii* were collected during January to March, 2016 from the three sites *viz.* Washlah, Khuraim and Huraymila of Riyadh region, Saudi Arabia. In Khuraim site, only *A. ehrenbergiana* and *A. gerrardii* were found. In each site, *Acacias* were categorized into three size classes; small (1- 1.5 m), medium (1.5 - 3 m) and large (more than 3 m high). From each site, three soil samples were collected from the root rhizosphere of the different size classes of the three species. Also, three soil samples away from the root rhizosphere (free soil) were also collected from each site. Samples were wrapped in polyethylene bags in ice box and brought to the laboratory.

Different isolation methods were used to isolate indigenous *Azospirillum* and *Azotobacter* species from Acacia rhizosphere. A subsample of 10 g of soil was used for serial dilution method. *Azospirillum* sp. was isolated in screw cap tubes containing approximately5ml sterilized semi-solid N-free malate medium (L-malic 5 g, $K_2HPO_4$ 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) (Usha and Kanimozhi 2011) under aseptic conditions. The tubes were incubated at 28$^o$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 *A. brasilense*, *A. lipoferum* and *A. doebereinerae* and *A. zeae* were incubated on M medium without biotin (5.0 g sodium malate, 0.02 g $CaCl_2.2H_2O$, 0.2 g $MgSO_4.7H_2O$, 0.1 g $K_2HPO_4$, 0.4 g $KH_2PO_4$, 0.1 g NaCl, 10 mg $FeCl_3$, 2 mg$Na_2MoO_4.2H_2O$, 0.1 g yeast extract, 1.0 l distilled water, pH 6.8) (Xie and Yokota 2005) and basal medium with sucrose (BMS) agar per 1 lire distilled water (potatoes 200 g, L-malic acid 2.5 g, KOH 2.0 g, raw cane sugar 2.6 g, vitamin solution 1.0 ml bromthymol blue (0.5% alcoholic solution) 2 drops and agar (15 g).

Streaking of serial soil dilution was performed on plates containing Ashby Mannitol Agar (per 1l): 20 g mannitol, 0.2 g $K_2HPO_4$, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.1 g $K_2SO_4$, 5 g $CaCO_3$, 15 g agar (Hi-Media Laboratories Pvt. Ltd. Mumbai, India) (Mazinani *et al*. 2012, Ahmad *et al*. 2016). Enrichment on Burk's Medium has the following composition: (g/l) $K_2HPO_4$ 0.64, $KH_2PO_4$ 0.2, $MgSO_4.7H_2O$ 0.2, $NaCl_2$ 0.2, $CaSO_4.2H_2O$ 0.05, $Na_2MoO_4$ 0.001, $FeSO_4$ 0.003, sucrose 20 sterilized separately, agar 1.5% (W/V), followed by streaking on to Ashby Mannitol Agar. The plates were incubated at 28$^0$C for 3-7 days presenting growth of *Azotobacter* were revealed by the appearance of smooth, opaque, low convex, mucoid slimy and glistening colonies.

Enumeration of microorganisms in soil samples were carried out by most probable number (MPN). One milliliter successive dilutions of $10^3$,$10^4$,$10^5$ soil samples were transferred to the test tube containing semi-solid NFb medium and plates of Ashby medium for *Azospirillum* and *Azotobacter* isolates, respectively. Tubes and plates were then incubated under suitable temperature.

$$\text{Bacterial population} = \frac{\text{MPN value} \times \text{middle dilution} \times \text{middle dilution used}}{\text{Dry weight of the soil sample}}$$

Microorganisms were identified based on cultural, morphological and biochemical characteristics as per Bergey's Manual of Systematic Bacteriology. Motility, cell shape, color, consistency and gram stain were described for morphological characterization of bacterial isolates. Motility of *Azospirillum* and *Azotobacter* isolates were tested by hanging drop method (Narendran *et al*. 2016). 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 *Azospirillum* 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 (Chari *et al*. 2015)

*Azospirillum* isolates were tested by the method of (Garrity *et al*. 2005). The medium composition was as follows (g/l): peptone 2.0, $(NH_4)_2SO_4$ 1.0, $MgSO_4.7H_2O$ 1.0, $FeCl_3.6H_2O$ 0.002, $MnSO_4.H_2O$ 0.002, bromothymol blue 0.025, (Dissolved in dilute KOH). The medium was made up to the volume of 950 ml, the pH was adjusted to 7.0 and sterilized by autoclaving at 15 lbs for 20 minutes. After cooling, 50 ml of 20% (W/V) solution of glucose (sterilized by filtration) was added aseptically. The medium was inoculated with a loopful of 24 hrs culture from semi-solid Nfb malate medium. The tubes were incubated at $30^0$C for 96 hrs. The development of yellow color was observed after incubation period.

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 $30^0$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.

*Azotobacter* isolates were grown in sterilized 10 ml Ashby medium (without agar) in 20 ml test tube on a rotary shaker (125 rpm) under continuous airflow at $30^o$C for 72. Cell concentrations were determined as $10^6$ CFU/ml of each isolate. The non-inoculated media served as control. Nitrogen was determined by micro-Kjeldahl analysis (Kanimozhi and Panneerselvam 2010).

The amount of total nitrogen in the sample was calculated by using the formula:

$$\text{Total nitrogen} = \frac{(\text{ml standard acid} - \text{ml blank}) \times \text{N of acid} \times 1.4007}{\text{Weight of sample in grams}}$$

Only *Azospirillum* 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 D-glucose and D-ribose which were sterilized separately by filtration. The development of yellow color was observed after incubation period. For the *Azotobacter* bacteria isolates, sucrose, maltose and mannitol were used. The test was done following the method described by Garrity *et al*. (2005). Sterilized screw tube with Durham tube contained 5 ml of the phenol red carbohydrate broth medium composed (g/l) : protase peptone 10, NaCl 5, beef extract 1.0, phenol red 0.018 and carbohydrate 10.0 used single carbohydrate for each test. Tubes were inoculated with bacteria growth and incubated under 28ºC for 18 - 48 hrs. Gas production indicates positive results and reddish or pink color indicates negative results.

Mannitol test was done following the methods of Hi-Media Laboratories Pvt. Ltd. Technical data (Atlas 2010), phenol red mannitol broth medium was composed (g/l) of Protase peptones 10, According to beef extract 1.0, mannitol 5.0, NaCl5, phenol red 0.018, pH 7.4. 5 ml of the medium in sterilized screw tube with Durham tube. Tubes were inoculated and incubated under $28^0$C at 18 - 48 hrs. Gas production indicates positive.

Tolerance to high temperature was tested by growing *Azospirillum* isolates 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.

The production of diffusible pigments in the presence or absence of the *Azotobacter* bacteria isolates was done following the method of Jiménez *et al.* (2011) the basal medium composed (g/l): $K_2HPO_4$ 1.0, $CaCl_2.{}_2H_2O$ 0.1, $MgSO_4.{}_7H_2O$ 0.2, and $NaMoO_4.2H_2O$ 0.005, Ion agar 10.0, glucose 10.0 and pH 7.3.

Starch hydrolysis test of *Azotobacter* sp. was done following the methods of Akhter *et al*. (2012) on starch agar plates. Plates were inoculated by streak method and incubated at 30ºC for 24 hrs. After growth, iodine solution was added. Blue color with clear halos surroundings bacteria growth indicates positive results and blue color without clear halos designates negative results.

The test of hydrogen sulfide for *Azotobacter* isolates was done according to Cappuccino and Welsh (2017). Isolates were cultured on slant of triple sugar iron (TSI) agar medium. Streaked TSI slant with growth culture was incubated at 28°C for 24 hrs. Slant has black color in butt indicates that positive results (hydrogen sulfide) red or orange slant color negative results.

Oxidase test for isolated *Azotobacter* sp. was measured by the method of Naz *et al.* (2012). Small piece of sterilized filter paper was moistened with 1 - 2 drops of oxidase reagent. Sterilized loopful of colony was touched with filter paper under aseptically condition. Blue color after 10 - 30 sec indicates positive results and no change designates negative results.

*Azotobacter* isolates urease activity test was measured by the method of Rasool *et al*. (2015). Urea broth medium (yeast extract 0.1 g, $KH_2PO_4$9.1 g, $K_2HPO_4$9.5 g, phenol red 0.01 g) was inoculated with test organism and incubated at 30ºC for 24 - 72 hrs.

For temperature tolerance, fresh culture colony of *Azotobacter* sp. was taken by sterilized loop and inoculated to sterilized test tubes which were filled with *Azotobacter* broth medium composed (g/l): $K_2HPO_4$ 0.8, $KH_2PO_4$ 0.2, $MgSo_4.{}_7H_2O$ 0.5, $FeSo_4.{}_6H_2O$ 0.1, $CaCl_2.{}_2H_2O$ 0.05, $NaMoO_4.{}_2H_2O$ 0.05, pH 7.5 and incubated at 32 and 37ºC for 24 hrs. Then inoculating and incubated at 30ºC for 3 - 5 days on Ashby's mannitol agar medium plates.

**Results and Discussion**

Several types of microorganisms were isolated from soil rhizosphere samples of three size classes of three different acacia species. Identification of *Azospirillum* and *Azotobacter* were based on growth cultural, morphological and biochemical characteristics of isolates (Table 1 and Fig. 2A, B-F). These bacteria were transferred successively to solid (N-free) media and purified.

In these experiments, 27 *Azospirillum* isolates were screened for their efficiency using various biochemical tests. Growth of *Azospirillum* was observed as the appearance of a thin white colored pellicle below the surface of the semi-solid Nfb malate medium, then transferred into NFb agar plates and M medium. Colonies formed in this medium were initially yellowish white, and eventually became pinkish, grey and pink colonies form after 48 - 72 hrs, become wrinkled and dried with time on M medium.

For enumeration of population density, the number of colonies was counted in range of ($0.11 \times 10^2$ - $33 \times 10^2$ CFU/ g soil) (Fig. 3 A, B and C). The highest population density was shown in small-size *A. ehernbergiana* and the lowest population density was observed in free soil at Khuraim site.

Microscopic examination of *Azospirillum* isolates revealed that they were pleomorphic forms, vibrioid, slightly curved rods in shape and motility levels of all isolates showed spiral, single polar flagellum and winding or snake-like movement. Based on the staining, all isolates were Gram-

**Table 1. Physiological differences between identified *Azospirillum* and *Azotobacter* isolates.**

| Characteristics | *Azospirillum* | | | | *Azotobacter* | | |
|---|---|---|---|---|---|---|---|
| | *A. lipoferum* | *A. brasilense* | *A. dobereinerae* | *A. zeae* | *A. chroococcum* | *A. beijerinckia* | *A. salinestris* |
| Motility | + | + | + | + | + | - | + |
| Water soluble pigment | nd | nd | nd | nd | - | - | b |
| Optimum temperature (°C) | 37 | 37 | 30 | 30 | nd | nd | nd |
| Acidification of peptone glucose medium | + | - | nd | nd | | | |
| Biotin | + | - | + | - | | | |
| 3% NaCl | - | - | - | - | | | |
| pH range for growth | 5.7-6.8 | 6- 7.8 | 6-7 | 5-7 | | | |
| D-glucose | + | - | + | v | | | |
| D-ribose | + | - | - | - | + | d | nd |
| Starch hydrolysis | | | | | + | d | nd |
| $H_2S$ production (thiosulfate) | | | | | d | d | nd |
| Urease | | | | | + | + | + |
| Oxidase | | | | | + | + | + |
| Sucrose | | | | | + | + | + |
| Maltose | - | - | - | - | + | d | nd |
| D-Rhibose | + | - | - | - | | | |
| Mannitol | + | - | + | - | + | d | nd |

**(+) : Positive, (-): Negative, D : Doubtful, nd : Not determined, b : Brown black color, rv : Red-violet color.**

negatives which are characteristics of genus *Azospirillum* (Cassán *et al.* 2015). The efficiency strains of *Azospirillum* sp. to utilize carbon was tested as per the procedures described earlier. Isolates grew in D-glucose and D-ribose (1%) utilization test using semi-solid Nfb malate medium (without malate), turned to yellow color after incubation period. Some strains showed no change in the medium color and grew well in the medium containing biotin (Pandiarajan *et al.* 2012). Strain showed positive growth in the peptone-based glucose which turned the medium to yellow color due to acidification after 24-96 hrs of incubation time. In BMS, the colonies formed became pinkish as they grew larger. Some isolates were salt tolerant up to 3% NaCl at the same way of Akhter *et al.* (2012) who reported that N-fixing bacteria tolerated 6% NaCl. Having optimal temperature of 37ºC, pleomorphic cells (S-shape or helical), non-motile with 48 hrs and vibrioid,

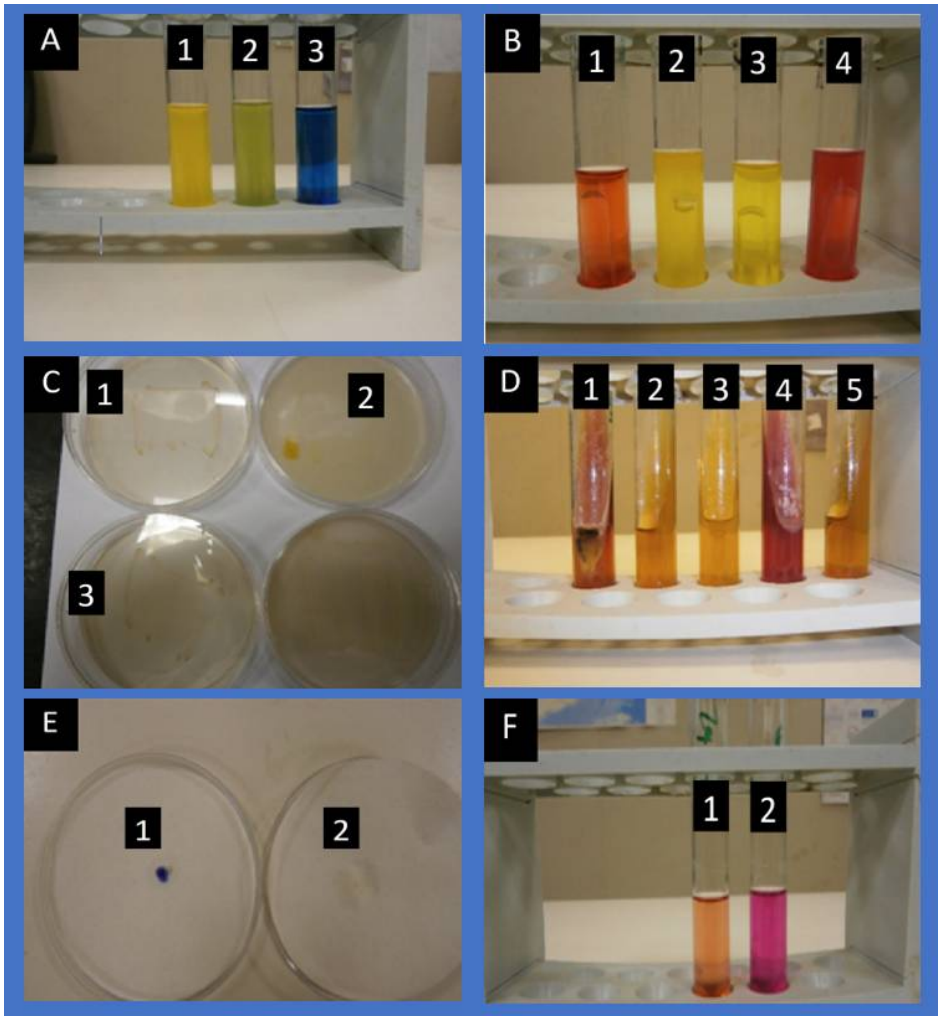


Fig. 2 (A) Acidification peptone glucose: 1, 2 positive, 3 negative, (B) sucrose utilization: 1 control, 2 positive with gas, 3 positive without gas, 4 negative, (C) pigments: 1,3 brown black, 2 yellowish green, (D) $H_2S$ produced 1, red slant re red butt $H_2S$ formed, 2, 3 yellow slant, 4 red butt red slant, 5 control (E) oxidase test 1 positive, 2 negative and (F) urease 1, negative 2, positive.

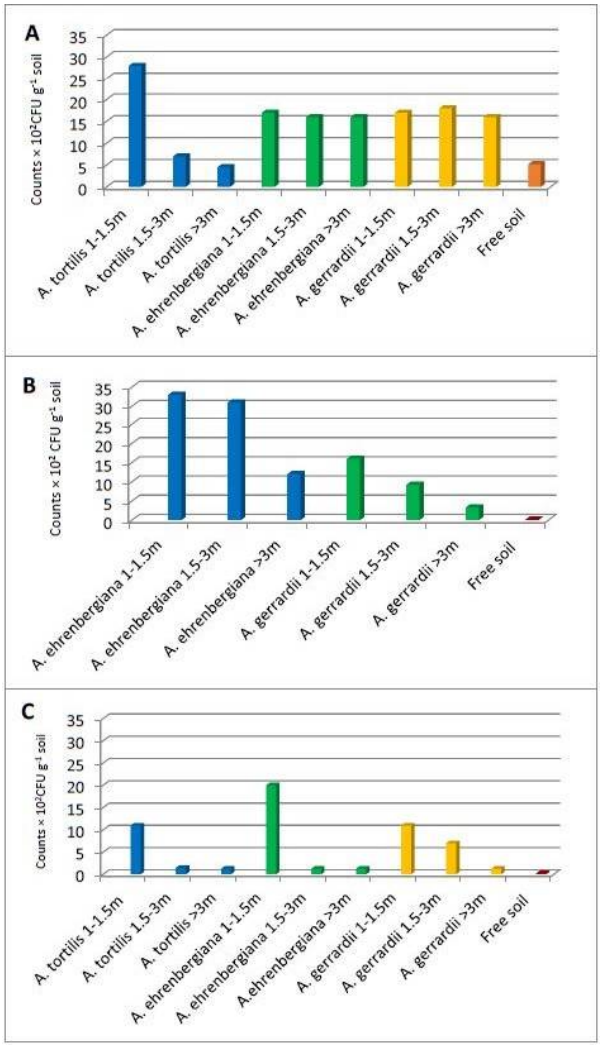


Fig. 3. A, B and C. *Azospirillum* density counts (CFU/g soil) in Acacia rhizosphere at A. Washlah, B. Khuraim and C. Huraymila sites.

and curved rods in alkaline medium (pH for growth 5.7 -7.8), the strains were identified as *A. lipoferum* and *A. brasilense* as reported by (Raffi and Charyulu 2012). Some strains were characterized by 30°C optimal temperature, failed to grow in 3% NaCl, not requiring biotin for growth, pH growth 6.0 -7.0, no acidification of peptone-based glucose and D-glucose or Dribose, curved rods or S-shaped and motile with a winding or snake-like movement. These strains were identified as *A. dobereinerae* and were similar to the findings of Eckert *et al.* (2001). Those strains having rod cells, motile with a single polar flagellum, formed pink pigment colonies after 48-72 hrs, became wrinkled and dried with time. Their optimum growth on M medium occurred at 30°C and pH 5-7 but failed to grow in 3% NaCl. They were negative for D-ribose and D-glucose and had negative growth in medium containing biotin. They were identified as *A. zeae* according to the criteria of Reis *et al.* (2015). Among 27 tested isolates, 7 were identified as *Enterobacter* sp. and

*Benibacillus* sp. They were not processed further. The remaining 20 isolates were identified as *Azospirillum* and were divided into 4 categories depending on the characterizations and were identified as *A. lipoferum, A. brasilense, A. dobereinerae* and *A. zeae* (Table 1).

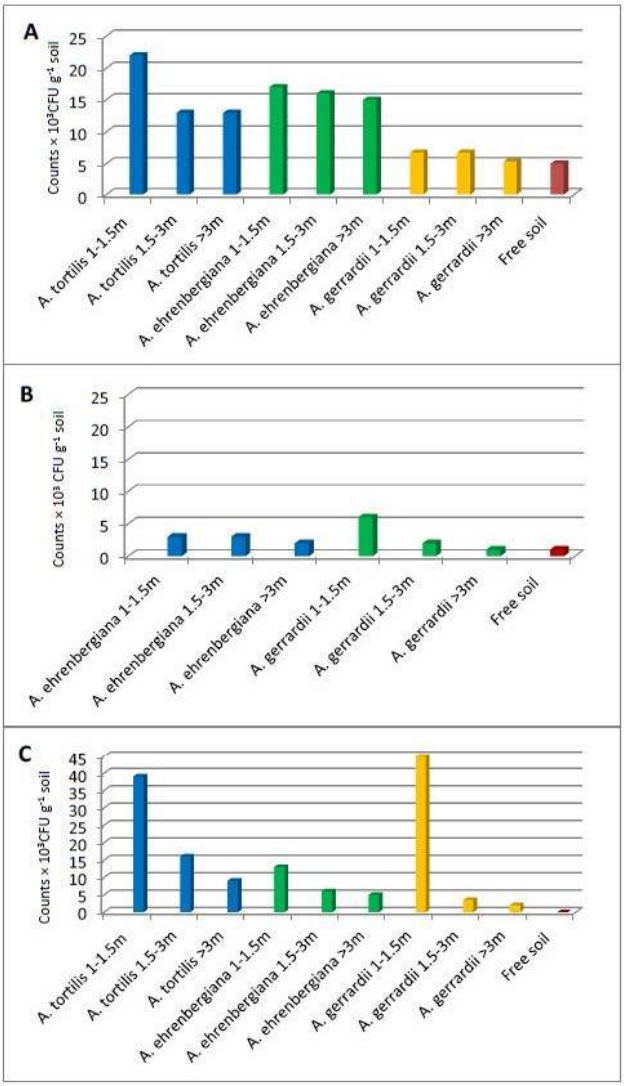


Fig. 4. A, B and C. *Azotobacter* density counts (CFU/g soil) in Acacia rhizosphere at A. Washlah, B. Khuraim and C. Huraymila.

For *Azotobacter* isolates, from the total of 27 soil samples were tested using various characterizations and biochemical tests. Growth of *Azotobacter* sp. was observed as an appearance of slimy, glistening, opaque, convex, smooth, mucoid and oval in N-free Ashby mannitol agar. From the census of population density, the number of colonies was counted in the range of ($1 \times 10^3$ - $45 \times 10^3$ CFU/g soil) (Fig. 4 A, B and C). Abundance was very low possibly due to high

pH and low organic matter content in soil. The highest population density was observed in soil rhizosphere of small-size *A. gerrardii* at Huraymila and the lowest in large sized *A. gerrardii* at Khuraim. Earlier, Alnohait (2015) recorded *Azotobacter* sp. in the rhizosphere of *A. gerrardii*.

Cell morphology appears as rods to ellipsoid and ended-rods. All grown isolates were Gram-negatives. These results are similar to the findings of Akhter *et al.* (2012). Some isolates were motile, produced $H_2S$ on phenol red carbohydrate broth medium containing 1% sucrose or maltose which agrees with the results obtained by Upadhyay *et al*. (2015) and Yorukce *et al*. (2017) who utilized mannitol, starch, urease and oxidase.

Some isolates were non-motile, oval rod shape, smooth colonies, positive reaction of sucrose and negative reaction of maltose and hydrolysis of starch. These results are in alignment with those of Upadhyay *et al*. (2015). Strains having urease, oxidase positive results, motile by means of peritrichous flagella and oval with pointed ends, utilizing of urease, hydrolysis of starch and sucrose and negative $H_2S$ are characteristic features of *A. beijerinckii*. The characters of isolates were also similar to those of *A. salinestris* (Patel *et al.* 2013). Depending on characterization, isolates were identified as *A. chroococcum, A. beijerinckii* and *A. Salinestris* (Table 1). All isolated strains had nitrogen fixing efficiency. Stella and Suhaimi (2010) also reported that among the *Azotobacter, A. chroococcum, A. vinelandii* and *A. beijerinckii* exhibited high growth, nitrogen fixation and *in vitro* production of phytohormone.

Among the 27 soil sample isolates, 19 were belonged to *Azotobacter* and the remaining 8 were *Sinorhizobium* sp., *Mesorhizobium* sp. and *Bacillus* sp. They were omitted and were not processed further. *Azotobacter* species which has the same characters were placed in one category and were identified as *A. chroococcum, A. beijerinckii* and *A. salinestris.*

**Table 2. Nitrogen fixation by the isolates of *Azospirillum* sp. and *Azotobacter* sp.**

| Identified isolates | Mg N fixed/g |
|---|---|
| *Azospirillum lipoferum* | 6.9 |
| *A. brasilense* | 9.3 |
| *A. dobereinerae* | 5.4 |
| *A. zeae* | 3.3 |
| *Azotobater chroococcum* | 6.3 |
| *A. beijerinckia* | 7.6 |
| *A. salinestris* | 2.7 |

Nitrogen fixing capacity was estimated by Kjeldhal method. Results showed both *Azosirillum* sp. and *Azotobacter* sp. have the efficiency to fix nitrogen. The range of nitrogen fixing ability with *Azospirillum* sp. was between 3.3 to 9.3 mg N/g. Among them, the maximum nitrogen fixing ability (9.3 mg N/g) was recorded from *A. brasilense* and minimum (3.3 m N/g) was recorded in *A. zeae*. (Table 2). Among other factors, the nitrogen fixing abilities of *Azospirillum* isolates depend on nitrogenase enzyme activity (Sant'Anna *et al.* 2011). Hossain *et al.* (2014) reported that nitrogen fixing efficiency of *Azospirillum* sp. ranged from 10.03 to 13.11 mg N/g. Further, *A. brasilense* had high fixed N values compared to other species as a result of NifA which is inactive in conditions of excess nitrogen (Lin *et al.* 2011).

Results showed that pellicles were greater in roots than in the soil samples which agrees with the findings of da Silva *et al.* (2014). In general, *Azospirillum* isolates showed higher $N_2$ fixing abilities than *Azotobacter*. Similar results were obtained by Tanvir *et al*. (2017). Among

*Azotobacter* sp., *A.beijerinckii* N-fixation was high (7.6 N mg/g) which is comparable to results of Upadhyay *et al*. (2015) who reported that *A. chroococcum* $N_2$ fixation was high compared to *A. beijerinckii.*

Based on the performed study of isolation and identification of free N-fixing bacteria *Azospirillum* and *Azotobacter* species were identified as *Azospirillum lipoferum, A. brasilense, A. dobereinerae, A. zeae* and *Azotobacter chroococcum, A. beijerinckii* and *A. salinestris.* The microbial activities were naturally distributed in the soil rhizosphere of *Acacia* species in the Riyadh region. *Azospirillum dobereinerae* and *Azospirillum zeae* were identified for the first time in Saudi Arabia. Isolates showed variation among species and their characters. The two genera, *Azospirillum* and *Azotobacter* are active in the maintenance of soil fertility and productivity of vegetation. Isolation, purification and screening for highly efficient N-fixers from natural vegetation preparation need further research to find better inoculants. Further, identification of *Azospirillum dobereinerae* and *Azospirillum zeae* need to be confirmed by means of molecular technique**.**

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