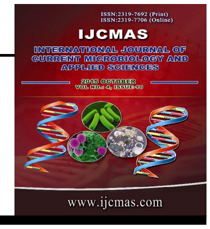

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 10 (2015) pp. 238-248**
http://www.ijcmas.com

Original Research Article

Enhancement of Plant Growth by Soil Inoculation with *Azospirillum brasilense* HM1 Isolated from Soil of Saudi Arabia

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A B S T R A C T

Several *Azospirillum* species are plant growth-promotive bacteria whose beneficial effects have been postulated to be partially due to production of phytohormones, including gibberellins (GAs). The aim of this study was to evaluate the performance of wheat inoculated with *Azospirillum brasilense*, a nitrogen-fixing bacterium under two level of nitrogen (full and half strength). In this work, *Azospirillum brasilense* strain HM1 was isolated from rhizosphere soil, characterized and identified according to morphological and physiological and biochemical factors. Culture filtrate contained IAA which increases seed germination. For greenhouse experiments, seedling of wheat plants were inoculated with *Azospirillum brasilense* in sand soil and plant irrigated with nutrient solution contained full and half nitrogen contents. After 30 days, plants were collected and analyzed. Inoculation of soil with *Azospirillum* promoted, sheath elongation, root depth, fresh weight of roots, fresh and dry weight of shoots, total nitrogen and bacterial counts in soil. The results showed that wheat inoculated with *Azospirillum* had a higher growth, mineral and chlorophyll when the plants were not supplemented with nitrogen. Data emphases the potential of *Azospirillum* as a biofertilizer, reducing costs and improving agricultural sustainability. In conclusion, soil inoculation with *Azospirillum* enhanced plant growth and protein content in addition to N and P content. Moreover, IAA content was enhanced.

Keywords

Bio-fertilizer, *Azospirillum brasilense*, Plant growth, IAA, Protein, N, P, K & Mg

Introduction

Azospirillum brasilense, a nitrogen-fixing bacterium found in the rhizosphere of various grass species, and increased the plant growth by growth substances produced by the bacteria like indole acetic acid and indole lactic acid which produced by *A. brasilense* from tryptophan (Tien et al., 1979; Okon and Vanderleyden, 1997; Bashan *et al.*, 2004). Evaluation of 20 years of data indicated that 60–70% of field experiments were successful, with yield increases ranging from 5 to 30% with *Azospirillum brasilense* inoculation (Okon and Labandera-Gonzalez, 1994). Yield increases could not be explained based on nitrogen fixation alone. The morphology of pearl millet roots changed when plants in solution culture were inoculated. The number of lateral roots was increased, and all lateral roots were densely covered with root hairs and combinations of indole acetic acid, gibberellin, and kinetin produced changes in root morphology of pearl millet similar to those produced by inoculation with *A. brasilense* (Glick, 1995; Perrig *et al.,* 2007).

Azospirillum spp. is considered to be important plant growth promotive rhizobacteria that can improve the growth and yield of at least several plant species (Okon and Labandera-González, 1994). However, the mechanism by which *Azospirillum* spp. and other promotive rhizobacteria promote plant growth has yet to be elucidated (Glick *et al.,* 1999). Phytohormone production, including gibberellins (Gas) was reported (Tien *et al.*, 1979; Okon and Kapulnik, 1986; Fulchieri *et al.*, 1993; Lucangeli and Bottini, 1997), is one mechanism that has been proposed for plant enhancement by *Azospirillum brasilense*.

Considering that cereals account for the production of most part of the food consumed by humans, clearly even if only part of nitrogen was provided by the association with fixing bacteria, nitrogen fertilizer would still be considerably economic. Therefore, biological nitrogen fixation is an alternative to supply part of the nitrogen needed for many plant and specially cereals. Inoculation of*Azospirillum* spp. bacteria in grasses has caused significant increases in root growth, resulting in better benefit and use of fertilizer and water and, consequently, better plant growth (Lana *et al.,* 2012). According to Okon and Vanderleyden (1997) at least part or, perhaps many of these effects caused by *Azospirillum* spp. on plants may be associated with the bacteria production of growth enhancing substances, including auxin, cytokinins and gibberellins, and not only the fixation of nitrogen. Several authors have reported the positive effects of the interaction between *Azospirillum* spp. and maize, such as increases in dry matter production, grain yield and nitrogen accumulation in inoculated plants, mainly when unimproved genotypes were involved in the presence of low nitrogen availability (Okon and Vanderleyden, 1997; Hungria*et al.,* 2010). There is also a wide variety of results in the literature regarding the relationship between inoculation and nitrogen fertilization. Barros-Neto (2008), when working with maize subjected to nitrogen fertilization levels (100 and 150 kg ha-1 of N) and inoculation of product based on *A. brasilense*, have found that inoculation provided an increase of 793 kg /ha in grain yield compared to control. The aim of the present study was isolation and identification of a potent bacterial isolate, has the ability to improve plant growth

Material and Methods

Bacterial isolation: *Azospirillum brasilense* recommended for maize and wheat was isolated from the rhizosphere of wheat plant grown in Huaalsham, western region of Saudi Arabia on semisolid medium (Döbereiner *et al.,* 1976). The bacterial isolates were purified using agar plates of the same medium. To study the colony and cell-morphology, pure bacterial colonies were grown in LB broth medium overnight at 30°C. 20 µl of bacterial culture was examined under light microscope.

Identification of *Azospirillum brasilense:* Identification was carried out according to morphological, physiological and biochemical characters (cell morphology, the color, motility, acid production and carbon sources utilization) as Döbereiner *et al.* (1976).

Bacterial growth: Bacterial strains were grown in liquid nitrogen-free biotin-based (NFb) medium with malic acid (5 g L^{-1}) and NH_4Cl (1.25 g L^{-1}) on an orbital shaker at 30°C and 80 rpm, for five days, each 24 hr., growth and IAA in the supernatant were determined. Moreover, % of wheat germination was determined each 24 hr. after seed soaking in culture filtrate and seed soaked in dist. water were used as control Mahmoud *et al.* (2004). Similarly, the isolated bacterium was grown in the previous medium until an optical density at 540 nm of 1.0 was reached. This corresponds with a concentration of approximately 10^8 CFU mL^{-1}. Bacteria were harvested by centrifugation at 8,000 rpm and 4°C for 15 min. The cellular pellet was washed twice with 0.85% (w/v) NaCl solution and re-suspended in 0.05 Mphosphate buffer (pH 7) to obtain a cell suspension of 3×10^6 CFU/mL for later inoculation of the soil. Growth of the bacterial isolate was determined using spectrophotometer by measuring optical density at 550 nm.

Extraction and detection of IAA: The isolate HM1 was grown in liquid broth medium (Döbereiner *et al.*, 1976) containing 2mg/ml L-tryptophan for 4 days at 30C and 80 rpm. The cells were collected and the supernatant was used to determine the quantity of IAA as described by Ahmad *et al.* (2005); Bano and Musarrat (2003).

Identification of the bacterial isolate: The isolate HM1 was characterized through a number of microbiological, physiological and biochemical tests as described in Döbereiner and Pedroza (1987).

The effect culture filtrate on seed germination of wheat: The supernatant of HM1 was filtered (Milipore filter, 0.45 mm) and the cell free filtrate was prepared. The Egyptian 'Giza-155' cultivar, *Triticum vulgare* L. cv. Giza-155 seeds were surface sterilized by soaking in a 10% sodium hypochlorite (NaOCl) for 5 min, followed by rinsing in sterile distilled-water. The surface-sterilized seeds were separately soaked in the culture filtrate or sterile distilled water incubated in the dark until the seedlings emerged (10 days) and germination percentage (%) was determined as described by Dhamangaonkar and Pragati (2009).

Preparation of inoculums: *Azospirillum brasilense* HM1 was grown on agar Medium or/and the bacterial cells were scraped from seven-day-old culture into sterile saline solution to give a bacterial suspension containing $2x10^6$ cells/ml (Döbereiner *et al.*, 1976).

Plant growth studies and analysis: A pot experiments were conducted under greenhouse conditions for a 30 day period with wheat plant Giza-155grown in sandy soil. This experiment was carried out during winter 2014. The sterile seeds were germinated in the dark and one week-old seedlings were transferred to each pot containing 2 kg of steam sterilized sandy soil. The pots were kept in a glasshouse with a temperature range of 20–22°C. Two groups of pots were established: the first one remained without any inoculation (control), the second was inoculated with *Azospirillum brasilense* HM1. When plants were grown for certain length, 15 ml of the bacterial suspension ($2x10^6$ CFU/ml) was used to inoculate each pot. For control, only water was added and all plants were irrigated by Hoagland nutrient solution (Hoagland and Arnon, 1950), with two concentrations of nitrogen, solution with full strength of nitrogen [KNO_3 and $Ca(NO_3)_2$] and solution with half nitrogen strength. The nutrient solution had the following composition, in mM: KH_2PO_4, 1.0; KNO_3, 5; $Ca(NO_3)_2$, 5; $MgSO_4$, 2; Fe- EDTA, 0.1; H_3BO_3, 0.005; $MnCl_2$, 0.010; $ZnSO_4$, 0.008; $CuSO_4$,0.004; $(NH_4)MO_7O_{24}$, 0.0002.Each pot received only 200 ml two times/week and the plants were irrigated with distilled water when needed. After 30 days, the plants were harvested and analyzed. The root depth, shoot length and no of leaves were determined. The shoot and root systems were separated and oven-dried for 10 days at 60°C and dry weights for each sample were obtained.

Photosynthetic pigments: Chlorophyll a, chlorophyll b and carotenoids of *Zea mays*, L. leaves were determined *spectrophotometrically* as the method described by Arnon (1949) and Metzner *et al.* (1965). An 85% aqueous acetone extract of a known F.W. of leaf was assayed *Spectrometrically* (*LKB NOVASPEC*) at 664, 645, 420 nm. The following equations were used to determine the concentration of the pigment fractions as γ/ml.

Chlorophyll a = 10.3 E664 – 0.918 E645 …. µg / ml. (1)
Chlorophyll b = 19.7 E645 – 3.870 E664……………….. µg / ml (2)
Carotenoids = 403 E452- (0.0264 Chl. a + 0.426 Chl.b)..……. µg / ml (3)
The pigment fractions were calculated as µg Chl./mg D.W.

Soluble sugars contents: Soluble sugars were determined using the antherone-sulphuric acid method described by Fales, (1951), Schlegel (1956) and adapted by Badour (1959).

Proteins contents: Dry samples collected during the growth study were analyzed for protein content, after precipitating the protein with 15% TCA at 4^{o}C according to Lowry *et al.* (1951).

Phosphorus, N, K and Mg concentration were estimated after acid digestion according to methods described by Allen *et al.* (1974) using Shimadzu Atomic Absorption Flame Spectrophotometer (Model AA-640-12).

Statistical analysis: Data of the shoot and root recorded was statistically analyzed by *F* test *ANOVA* and the means were compared using Duncan's multiple range (P<0.05). Where relevant, the experimental data was subjected to analysis of variance. Percentage values were transformed into arcsines according to Bliss (1973) and analysis of variance was carried out according to Snedecor and Cochran (1967).t - Test to determine whether the differences between control and treated samples were significant or not at P< 0.05.

Results and Discussion

In this experiment, *Azospirillum* was isolated from rhizospehere soil sample on semisolid medium recommended by Döbereiner *et al.* (1976). Bacterial cells were having a diameter of 1.0 µm when grown in broth medium and they had a single polar flagellum, but when grown on agar at 30C lateral flagella of shorter wavelength were also formed (Table 1). It was identified as *Azospirillum brasilense* HM1 according to Tarrand *et al.* (1978). Within the phylum of Proteobacteria there are multiple subgroups; *A. brasilense* belongs to the alpha subclass of Proteobacteria, Alphaproteobacteria (Okon, 1994). The bacterium belongs to group IV of Alphaproteobacteria, Rhodospirillales and the phenotypic differentiation of *A.brasilense* from other diazotrophic members of group IV is based upon the size and shape of the bacterial cell, and the mode of nitrogen fixation among other things (Okon, 1994). Within its family, *Azospirillum* can be distinguished from other members by lack of phototropy, the inability to form root and stem hypertrophies, and G+C content (Okon, 1994). *A. brasilense* can be distinguished from other Azosprillum species based upon the ability of utilization of ribose and mannose Extensive genetic, biochemical and ecological studies have ranked *Azospirillum* as one of the best characterized genera among associative plant growth promoting rhizobacteria (Steenhoudt and Vanderlayden, 2004).

Azospirillum brasilense are generally regarded as rhizosphere bacteria, but display strain-specific differences in the way they colonize roots (Döbereiner and Pedroza, 1987, Hungria *et al.*, 2010). They predominantly colonize the root surface. *Azospirillum* species can promote plant growth and increase crop productivity (Okon and Vanderleyden, 1997). However, such effects are variable probably due to interaction between the plant genotype and the bacterial strain, soil properties and climatic factors.

An *Azospirillum*–plant root association can only be successful if the bacterium is able to survive in the soil and attain significant populations on the host root system. In the rhizosphere, decreasing nutrient gradients from the root to the surrounding soil are generated by plant root exudates. Motility and chemotaxis enable the bacteria to move towards plant roots where they can benefit from root exudates as carbon and energy source, and may therefore contribute to survival and rhizosphere colonization. *Azospirillum* can convert atmospheric nitrogen into ammonium under microaerobic conditions at low nitrogen levels (Hartmann, 1988; Huergo *et al.*, 2008).

In liquid broth medium, *Azospirillum brasilense* growth and IAA production were increased with time up to 96 hr (Table 2). Indole acetic acid (IAA) production increased with increasing tryptophan concentration from 1 to 100 µg/ml increased with the age of the culture until bacteria reached the stationary phase (Tien *et al.*, 1979). Shaking favored the production of indole acetic acid, especially in a medium containing nitrogen. A small but biologically significant amount of gibberellin was detected in the culture medium. Also at least three cytokinin-like substances, equivalent to about 0.001 µg of kinetin per ml, were present.

Moreover, soaking seed in culture supernatant enhanced significantly % of seed germination compared to control. Wheat plats were grown in serial soil and inoculated with *Azospirillum brasilense* HM1 under two nitrogen levels (full and half dose) and growth was measured and compared to control pants (non inoculated plants). It was found that under 100% nitrogen level, root depth, root fresh and dry weights, shoot length, and shoot fresh and dry weights were maximum. Furthermore, at half nitrogen level, all the previous parameters significantly decreased (Table 3).

Positive effect of inoculation was obtained in the absence of nitrogen, with a higher production of shoot dry biomass and growth in relation to treatment without inoculation. As it is well known, nitrogen is very important for plant growth and metabolism and is considered the mineral element that plants need in the greatest amount (Taiz and Zeiger, 2004) for grain production and to raise protein content. Zheng (2009) reported that cellular carbon and nitrogen metabolism must be tightly coordinated to sustain optimal growth and development for plants and other cellular organisms.

Table.1 Morphological character of the selected isolate HM1

Tested character	Results
Gram stain	Gram negative
Source of isolation	Soil
Motility of spore	Present
Shape of cell	Spiral shape
Colony color	Whitish to light pink
Size	Short plump rods
Aeration	Microaerophylic
Isolation	On semisolid medium

Table.2 Growth and IAA production by *Azospirillum* isolate HM1 and *wheat* germination after different growth periods

Time (Hours)	Growth (540nm)	IAA (mg/ml)	% Seed germination	
			Control	Bacterial supernatant
24	0.276	4.0±1.50	70.0	79.7*
48	0.733	6.0±0.85	80.0	90.4*
72	1.533	6.8±0.44	81.8	90.5*
96	1.941	7.8±0.15	94.5	97.5*
120	1.999	5.8±2.50	97.0	97.9

**: Significant results at $p<0.5$*

Table.3 Effect soil inoculation with *Azospirillum* on root depth (cm), shoot length (cm) and root and shoot dry weight (g/plant) of wheat plants grown in sterile soil and inoculation or uninoculated with *Azospirillum* HM1

Treatments	Nitrogen level	CFU/g $X10^8$	Root			Shoot			
			Depth cm	**Fresh weight g/plant**	**Dry weight g/plant**	**Length cm**	**Fresh weight g/plant**	**Dry weight g/plant**	**No. leaves No./plant**
Without inoculation (Control)	**100 %N**	**ND**	**11.0**	**1.9**	**0.99**	**20.9**	**3.92**	**1.9**	**7**
	50% N	**ND**	**8. 8***	**1.3***	**0.77***	**20.1**	**3.42**	**1.4***	**6**
Inoculation with *Azospirillum brasilense*	**100 %N**	**2.11**	**8.3***	**3.2***	**1.54***	**24.1***	**5.31***	**2.6***	**8**
	50% N	**6.88**	**13.4 ***	**4.9***	**2.44**	**29.1**	**6.81***	**3.2***	**8**

* Significant results at $p<0.5$

Table.4 Effect of soil inoculation with *Azospirillum* on mineral content (mg/g) of wheat roots and shoots

Treatments	Nitrogen level	Root				Shoot			
		N	**P**	**K+**	**Mg++**	**N**	**P**	**K**	**Mg**
Without inoculation (Control)	**100 %N**	**10.55**	**7,88**	**7.99**	**3.33**	**15.62**	**10.2**	**11.19**	**4.18**
	50% N	**8.88***	**4.65***	**7.67**	**1.99***	**11.29***	**14**	**10.41***	**4.33***
Inoculation with *Azospirillum brasilense*	**100 %N**	**12.69***	**8.88***	**10.1***	**4.83***	**18.44***	**14.3**	**14.34***	**6.31**
	50% N	**9.99**	**9.67***	**8.96***	**3.54**	**14.66**	**12.1**	**14.8***	**5.8**

* Significant Results at p <0.5

Table.5 Effect of soil inoculation with *Azospirillum*HM1 on chlorophyll content (mg/g) of leaves and shoot protein (mg/g), sugars (mg/g) and IAA (mg/g) of wheat plants grown in sterile soil

Treatments	Nitrogen level	Leave pigment		Shoot		
		Chlorophyll a+b (mg/g)	**Carotenoids**	**Protein (mg/g)**	**Soluble sugar**	**IAA (mg/g)**
Without inoculation (Control)	**100 %N**	**3.99**	**0.88**	**0.99**	**77.3**	**20.11**
	50% N	**3.14***	**0.76***	**0.60***	**60.9***	**23.08***
Inoculation with *Azospirillum brasilense*	**100 %N**	**4.11**	**1.07**	**0.99**	**91.0***	**27.19***
	50% N	**6.70***	**0.90**	**1.70***	**83.0***	**31.91***

* Significant Results at p <0.5

All full nitrogen level used in nutrient solution, inoculation of soil with cells of *Azospirillum brasilense* enhanced all measured parameters except root depth compared to un-inoculated plants at the same nitrogen level. Al lower nitrogen level and soil inoculation with *Azospirillum brasilense*, root depth, root fresh and dry weights, shoot length, and shoot fresh and dry weights were increased significantly compared to control at the same nitrogen level.

Lana *et al.* (2012) found that the inoculation with *Azospirillum* spp. enhanced both shoot dry biomass and yield and provided similar means for the treatments with and without nitrogen topdressing fertilization, which suggesting the possibility of replacement of nitrogen topdressing fertilization by the use of inoculation. They added that nitrogen topdressing fertilization, associated with the use of inoculation, has reduced yield and the shoot dry biomass production. Considering that nitrogen fertilizers represent about 40% of total production costs of maize (Machado *et al.,* 1998), such replacement would allow considerable savings in production costs. Piccinin *et al.* (2013) evaluate the efficiency of seed inoculation with *Azospirillum brasilense* on the agronomic performance and yield of wheat in randomized blocks design using three doses of nitrogen (zero, half and full dose). They found that at the point of harvest, the number of seeds per year, seed mass and weight were increased by either inoculation due to nitrogen fixation or nitrogen fertilization. Inoculation of soil with *Azospirillum brasilense* increased all root and root mineral on tents specially N, P, K and Mg compared to control (un-inoculated plates) at the two tested nitrogen level (Table 4).

Moreover, plant chlorophyll, proteins, soluble sugar and IAA were enhanced by *Azospirillum brasilense* inoculation (Table 5). Increases in the production of shoot dry biomass and yield provided by inoculation in this study could be attributed to the stimulus that *A. brasilense* provides to the development of the root system, with increase in density and root hair length and on the volume and number of lateral roots, resulting in greater uptake ability and utilization of water and nutrients like reported by Huergo *et al.* (2008).

In the early studies on the associations between plants and *Azospirillum* spp., researchers believed that the benefits were derived only from biological nitrogen fixation (Dobbelaere *et al.,* 2001). However, later studies demonstrated that the positive effects provided by these nitrogen-fixing micro-organisms are due primarily to morphological and physiological changes in the roots of inoculated plants (Okon and Vanderleyden, 1997). Such changes in the root system are related to the production of growth enhancing substances by bacteria, since plant growth regulators (phytohormones), mainly the indole acetic acid (IAA), excreted by *Azospirillum* spp. play an important role in fostering the growth of plants in general (Bashan and Holguin, 1997).

The incorporation of higher amount of nitrogen fertilizer to soil, due to topdressing fertilization, has undermined the effect of inoculation (Lana *et al.*, 2012). According to Hartmann (1988), the efficiency of biological fixation in *Azospirillum* spp. is rapidly reduced or even inhibited in the presence of higher concentrations of nitrogen compounds in the soil, especially ammonium, which causes the rapid inhibition of nitrogenase activity in bacteria, responsible for the conversion of the atmosphere nitrogen (N_2) to a form that is acceptable by the plants (Lopes, 2007). This process of control and regulation of nitrogenase complex activity by modulator environmental factors (concentration of assimilated nitrogen and oxygen) becomes necessary due to the high demand for energy involved in the process of reducing atmospheric nitrogen (N_2) by diazotrophic organisms, some 28 moles of ATP per mole of ammonia produced (Baldani*et al.*, 2009)

In conclusion, *Azospirillum brasilense* is one of the most well-studied plant growth promoting bacteria. It is considered a free-living soil bacterium that has the ability to affect the growth of numerous agricultural crops worldwide through the excretion of various hormones and the bacteria's ability of nitrogen fixation.

Acknowledgements

The authors are thankful to *Prof. Dr. Ali, Magda M.* Biology Department, (Microbiology), Faculty of Science, King Abdul-Aziz University for her great help in planning and carrying out the field work and also for offering all facilities at preparation of the manuscript.

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