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Isolation and Characterization of *Azospirillum* Isolates from Soil and Their Effect on Growth and Yield of Wheat (*Triticum aestivum* L.) under Different Levels of Nitrogen Fertilizer

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Abstract

This study involved isolation of *Azospirillum* from soils that are different in texture from Aswan governorate. Also, study the response of wheat *(Triticum aestivum* L.) to inoculation with the isolated *Azospirillum* strain (M-1, M-2 and M-3) in presence of different levels of nitrogen fertilization (40, 60 and 80 kg N/fed.) was tested in field experiments in the two seasons 2014/2015 and 2015/2016. Results of the morphological, cultural and physiological characteristics of the three *Azospirillum* strains (M-1, M-2 and M-3) indicated that they belong to *Azospirillum brasilense*. Wheat seed inoculation with any of the isolated *Azospirillum* strains (M-1, M-2 and M-3) induced highly significant increases in all measured growth parameters as well as in total and grain yields and % N in grains. Strains M-1and M-3 were more simulative than M-2 producing the highest total and grain yields. The two interacting factors (inoculation X N-fertilization level) showed most pronounced mutual effects at the lowest level of 40 kg N/feddan with any of the tested strains compared with the uninoculated treatment. *Azospirillum* strain M-1was the most reactive at all N-levels. The results also indicate that wheat inoculation with effective *Azospirillum* strain could save between 20-40 Kg N-fertilizer /feddan.

Keywords: *Isolation; Azospirillum; Inoculation; Biofertilizers; Chemical fertilizers.*

Introduction

More recently, a real challenge faces the workers in the agricultural research field to stop using the high rates of agro-chemicals which negatively affect human health and environment. It is well known that wheat crop is considered among the most important cereal crops either in Egypt or all over the world that consumes huge quantities of chemical fertilizers. Many attempts have been tried to replace a part of those harmful chemical fertilizers by biofertilizers to get yield of a good quality without loss in its quantity (El-Kholy *et al*., 2005). Mishra *et al*., (1995) have succeeded to reduce the recommended doses of chemical fertilizers needed for corn and millet by 50% using biofertilizers without loss in the yield.

Azospirillum is one of the plant growth-promoting bacteria that has been found in the rhizosphere and the intercellular of the cereals and other plant roots. Nitrogen fixation, plant growth-promoting hormones production and consequently improving the water and nutrients uptake, increasing the insoluble-phosphates solubility, siderophores and vitamins production, controlling pathogens, synergistic relationship with other useful soil bacteria, nitrite production, bioremediation of the sewage and toxic residues decomposition considered as some of the beneficial characteristics of this bacterium that ultimately causes to increase the efficiency and yield of crops (Payne *et al.,* 1981; Brock and Madigan, 1991; Stacey *et al*., 1992). Produced phytohormones by *Azospirillum* are effective on respiration rate metabolism, growth and development of root, so they increase water and nutrients uptake in inoculated plants (Holguin *et al*., 1999). Increasing the efficiency of nutrients uptake through plants was probably due to increase of root surface adsorption as the result of inoculation with growth hormones-producing bacteria such as *Azospirillum* (Bashan *et al*., 2004).

The current investigation was launched to isolate and identify *Azospirillum* strains from rhizosphere soil and assess the effect of isolated *Azospirillum* strains on growth and yield of wheat (*Triticum aestivum* L.) under different levels of nitrogen fertilizer.

Materials and Methods

Collection of samples:

Three Azospirillum strains were isolated from samples of soils that are different in texture collected from Aswan governorate. Strain (M-1) was isolated from the clayey soil and strain (M-2) and (M-3) were isolated from two samples of the sandy soil taken from two separate locations that were cultivated with wheat plants.

Isolation of Azospirillum isolates

Nfb (nitrogen free bromothymol) semi-solid medium in screw-capped tubes was inoculated with 0.1 ml of each sample suspension using a sterile pipette and was incubated at 37°C for 72 hours. After incubation *Azospirillum* appeared in the tubes forming characteristic thin dense, white pellicle few mm below the surface at the medium (Dobereiner, 1980). The pellicles were examined microscopically for the presence of gram negative, vibroid and actively motile cells. According to Krieg (1981), a loopful of the pellicle developed in tubes was transferred to fresh semi-solid Nfb-medium in screw-capped tubes and the tubes were incubated at 37°C. The white sub-surface pellicle formed after 72 hours in the fresh medium was checked by microscopic examination for the presence of gram negative, curved ‹motile cells and transferred into the fresh semi-solid Nfb-medium thrice, each transfer being made at 72 hours intervals. Then a loopful of the pellicle was streaked on the plates of Nfb-medium solidified with 1.5% agar. The plates were incubated at 37°C for one week. Pure colonies (small, dry, slightly convex and rugose) were transferred to the slants of solid Nfb-medium and incubated at 37°C for 5 days after then the slant maintained at 4 ºC in a refrigerator.

Morphological and Biochemical study of the Azospirillum isolates:

Microscopic examination of the isolates grown on Nfb semi-solid and agar media (24 hours and 7-day old cultures) were made for determinations of cell morphology, dimensions, Gram reaction, cell motility and colony characteristics. Besides, the isolates were tested for catalase test, nitrate reduction test, starch hydrolysis test, carbohydrate fermentation test, denitrification test, ammonification test and gelatin-liquification test were made on the physiological activities of the organisms. In each case growth of the isolates was recorded by visual observation. All selected isolates were identified according to Bergey's Manual of Determinative Bacteriology (1994).

Efficiency of the isolated strains in fixing of the atmospheric nitrogen:

The nitrogen fixation by each *Azospirillum* isolates was estimated according to the method described by Humphries (1956). The N free semi solid malate medium supplied with L-glutamic acid was used in this study. A quantity of 100 ml of the above medium was dispensed to a 250 ml capacity conical flask and autoclaved. The *Azospirillum* isolates grown for 24 hrs separately in Nfb broth were used to inoculate 2 ml/100 ml of the medium. Duplicate samples were maintained for each isolate. After seven days of incubation at 37°C the culture was homogenized. Five ml of the homogenized culture was collected and digested with 5 ml concentration H_2SO_4 and 200 mg catalytic mixture (K_2SO_4 : $CuSO_4$: selenium) (100:10:1 ratio) till the contents become clear. After cooling, the volume was made up to 25 ml with distilled water. Then aliquot 5 ml was transferred to microkjeldhal distill unit. An aliquot of 10 ml of 40 percent sodium hydroxide was added and steam distilled. Ammonia evolved was collected over 2 percent boric acid (20 ml) containing 2 drops of double indicator (83.3 mg bromocresol green + 16.6 mg methyl red indicator dissolved in 10 ml of 95% ethanol) and back titrated against 0.005 N H_2SO4. The nitrogen fixed in vitro was calculated and expressed in mg N fixed / g of malate supplied.

Experiments on wheat:

Response of wheat *(Triticum aestivum* L.) to inoculation with the isolated *Azospirillum* strain (M-1, M-2 and M-3) in presence of different levels of N-fertilization (40, 60 and 80 kg N/fed.) was tested in field experiments at the "Experimental farm of center for Bio-organic Agricultural Researches in Aswan" in the two seasons 2014/2015 and 2015/2016. The local cultivar Seds 1 was used in all experiments. Some physical and chemical properties of soil are presented in Table 1.

The experimental design split plot with 6 replicates was employed in the field experiments. The main plots were devoted to the different levels of N-fertilization 40, 60 and 80 kg N/feddan. The subplots were assigned for the treatments of *Azospirillum* strains. The area of the experimental unit was 1/400 feddan. Grains of each separate plot, uninoculated or inoculated were broadcasted at the rate of 150 g/plot (60 kg/fed.), then slightly covered by surface scratching the seedbed before irrigation. Peat inoculants of the tasted *Azospirillum strains* were used in these experiments.

Table 1. Some physical and chemical characteristics of a representative composite soil sample from the experimental site.

Property	Values
Clay	9.3
Silt	30.5
Sand	60.2
Texture grade	Sandy loam
Total $CaCO_3$ %	16.18
EC dS/cm^{-1} (1:1)	1.22
pH (1:1 suspension)	7.82
Total nitrogen %	0.04
Organic matter %	0.30
Available P mg g^{-1} soil	6.67

Inoculant preparation and grains treatment:

Sterile peat moss was used as a carrier for inoculant preparations. Pulverized dry peat moss, was neutralized to pH 7 with $CaC0_3$ and Ca $(OH)_2$. The treated peat was distributed in batches of 50 g each in polyethylene bags and autoclaved for 30 minutes at 121°C on three successive days. Aliquots of 50 ml of the *Azospirillum* broth culture (incubated for 6 days at 28-30°C) were added per 50 g of sterilized carrier material. The grains of each separate plot in polyethylene bag were inoculated by adding 10 ml of 40% arabic gum solution and 50 g peat inoculum, then thoroughly mixed until surface coated. Peat inocula contained 10^8 viable cells of *Azospirillum*/g, determined on plates of Nfb agar medium. Ammonium nitrate (33.5% N) was used as N-fertilizer and the tested level was add in two doses, the first after 30 days from sowing and the second 30 days later, before spike formation. The plants were irrigated once every 20-30 days until harvest after 5 months from sowing.

Plant sampling:

Plant samples of 10 plants were taken from each plot, 70 days after sowing. The plants of each sample were taken from different parts of the plot. Immediately after sampling, the plants were transferred to the laboratory, and roots were washed with top water to remove superfluous soil. Plant height was determined, and the sampled plants were separated to shoots and roots for fresh and dry weight determinations. Plant samples were dried at 70°C in an air forced-draft oven and their weights were determined. At harvest, total yield was determined for each plot, and plant samples of about 2.3 kg from each plot were taken for thrashing and grain weight determination, then total grain and straw yields per plot were calculated.

Statistical analysis

Data obtained in the two seasons were subjected to analysis of variance and the combined analysis of both seasons was used for comparison of means of the different treatments using the least significant difference L.S.D. (Statsoft 1995).

Results and Discussion

Characteristics of the isolated *Azospirillum* strains

In this study, 10 different colonies were isolated from rhizosphere soil samples. From these 10 colonies of primary selection 3 isolates were selected finally for further study on the basis of their ability to grow better and faster in Nfb semi-solid medium in screw capped test tubes. The selected isolates were M-1, M-2 and M-3. Results of the morphological, cultural and physiological characteristics of the three *Azospirillum* strains are presented in Table 2. According to Bergey's Manual of Determinative Bacteriology (1994), considering all the identifying characteristics selected isolates were identified as *Azospirillum brasilense*. Variations among the isolates in some characteristics such as colony morphology, starch hydrolysis and fermentation test indicated the different identity of the three isolates, although the observed variations are within the limits of species.

Table 2. Morphological, cultural and physiological characteristics of the isolated *Azospirillum* strains.

Test	M-1	M-2	M-3
Gram reaction	-	-	-
Motility	+	+	+
Pellicle formation	+	+	+
Colony morphology on Nfb medium			
Catalase test	+	+	+
Nitrate reduction	+	+	+
Starch hydrolysis	-	+	+
Gelatin hydrolysis	-	+	+
Fermentation test			
Glucose	+	+	+
Sucrose	-	-	-
Mannitol	+	-	-
Lactose	+	-	+
Fructose	+	+	-
Maltose	+	-	+
Ammonification test	-	-	+
Denitrification test	+	-	+

Efficiency of Azospirillum to fix atmospheric nitrogen

The amount of nitrogen fixed by Azospirillum strains determined by the microkjeldahl method, were 23.65, 15.32 and 19.75 mg N per gram of malate utilized, respectively. These results indicate that *Azospirillum* strain M-1 had higher potential to fix atmospheric nitrogen compared with the other strains (M-2 and M-3). These results are in accordance with the findings of Savalgi *et al*., (2009) who examined the in vitro N fixation efficiency of *Azospirillum* isolates on Nfb and reported that nitrogen fixed ranged from 1.4 to 20.96 mg /g of malate. Purushothaman *et al*., (1988) reported that the nitrogen fixation potential of *Azospirillum* sp. varied between 1.6 to 23.96 mg per gram of carbon by *Azospirillum* strains from cotton genotypes. Azospirillum lipoferum and *Azospirillum brasilense* showed nitrogen fixation in the range of 7.54 to 24.53 mg of nitrogen per gram of malic acid after seven days at 28°C. In vitro nitrogen fixation of *Azospirillum* strains isolated from sorghum under satic conditions. Of the 88 *Azospirillum* isolates, 55 per cent were identified as A. lipoferum and 41.57 per cent as *A. brasilense*. These represented isolates from the rhizosphere/ endorhizosphere of different ornamental plants. The nitrogen fixing capacity of these isolates ranged from 1.4 to 20.54 mg N per g of malic acid in the experiment conducted by Tamilvendan and Purushothaman (1996).

Response of wheat to Azospirillum inoculation and levels of N- fertilization:

The combined analysis for the data obtained on wheat growth and yield in two successive seasons is presented in Table (3) showing the main effects of N-fertilization levels and inoculation with the isolated *Azospirillum* strains. The results showing that wheat seed inoculation with any of the isolated *Azospirillum* strains (M-1, M-2 and M-3) induced highly significant increases in all measured growth parameters as well as in total and grain yields and % N in grains. Strains M-1and M-3 were more simulative than M-2 producing the highest total and grain yields (Table 3).

Under field conditions the rate of N-fertilization had significant influence on plant growth and yield, showing significant additive increases with the increase in N-level from 40 up to 80kg N/feddan. The maximum values recorded at the 80 kg-level were significantly higher than those recorded at the 60 kg-level for fresh weight of shoots and roots, total and grain yields and total N in grains.

Table (4) show the interaction effects of N-fertilization levels and Inoculated *Azospirillum* strains on plant growth and yield of wheat. The two interacting factors (inoculation X N-fertilization level) showed most pronounced mutual effects at the lowest level of 40 kg N/fed. with any of the tested strains compared with the uninoculated treatment. At the higher N-fertilization levels (60 and 80 kg N/Fed.) the increases in plant growth and yield induced by the tested strains tended to decrease, yet the differences were still significant compared with the uninoculated treatment.

Azospirillum strain M-1was the most reactive at all N-levels. At the 40 Kg N-level, this strain scored the highest grain yields of 8.35 Kg/plot compared with 4.94 Kg, 7.69 Kg and 9.22 Kg grain yield/plot produced in the uninoculated treatments with, 40, 60 and 80 Kg N/fed., respectively. (Table 4). This confirms the superiority of the isolated Azospirillum strain M-1in comparison with M-2 and M-3. The results also indicate that wheat inoculation with effective Azospirillum strain could save between 20-40 Kg N-fertilizer/feddan. The significant increase in total and grain yields recorded in the inoculated treatments are probably due to N2-fixation and the hormonal effects by the inoculated Azospirillum strains. Positive impacts on plant growth by Azospirillum through several mechanisms include enhancement of root development, production of growth regulators and nitrogen fixation (Okon *et al*., 1994; Garcı´a de Salamone *et al.,* 1996). The content of nitrogen, phosphorus, potassium and various micronutrients is higher in plants inoculated with Azospirillum (Caballero-Mellado *et al*., 1992).

Table 3. The main effects of N- fertilization levels and inoculation with Azospirillum strains on wheat, cv.Seds1. grown on a sandy loam soil Combined analysis of the two seasons of 2013/2014 and 2014 / 2015

Treatments	Average plant height cm	Shoot wt. g/plant		Root wt. g/plant		Yield kg/plot*			N-in Grains %
		Fresh	Dry	Fresh	Dry	Straw	Grains	Total	
N-fertilization (kg N/feddan)									
40	101.35	44.80	7.50	2.73	1.11	13.87	5.93	23.80	2.33
60	104.72	45.39	10.36	3.26	1.45	17.24	9.02	29.95	2.43
80	106.53	52.64	10.78	3.36	1.78	18.73	9..86	32.53	2.22
L.S.D 0.05	1.43	3.32	0.63	0.26	0.14	0.90	0.53	1.01	0.06
Inoculated strains									
Uninoc.	101.67	45.28	6.89	2.56	1.12	14.55	6.13	24.55	2.32
strain M-1	104.49	52.11	9.7	3.67	1.56	17.39	9.15	30.46	2.41
strain M-2	103.23	46.56	9.90	3.45	1.53	18.11	8.65	29.43	2.44
strain M-3	107.32	47.13	11.05	3.78	1.45	16.65	9.11	30.70	2.42
L.S.D 0.05	1.64	4.72	0.83	0.40	0.28	0.93	0.52	1.14	0.07
Season:									
1	102.19	45.71	8.35	2.87	1.25	15.53	7.63	27.26	2.42
2	106.32	49.50	10.30	3.57	2.03	16.61	8.89	29.35	2.45
L.S.D 0.05	1.15	3.35	0.62	0.31	0.20	0.65	0.36	0.80	0.05

*The area of each plot =1/400 feddan

Table 4. The interaction effects of N-fertilization levels and inoculation with Azospirillum strains on wheat, cv. Seds 1. grown on a sandy loam soil Combined analysis of the two seasons of 2013/2014 and 2014/ 2015 **.

Treatments N-fert. x inocu-lated (kg N/fed.)	strain	Average plant height cm	Shoot wt. g/plant		Root wt. g/plant		Yield kg/plot*			N-in grains %
			Fresh	Dry	Fresh	Dry	Straw	Grains	Total	
40	Uninoc.	99.76	38.30	5.14	2.11	0.59	10.10	4.94	14.45	2.23
	strain M-1	103.10	61.26	8.65	2.76	1.10	16.23	8.35	24.58	2.32
	strain M-2	101.45	44.82	8.12	2.64	1.07	18.29	7.27	25.53	2.24
	strain M-3	102.93	44.91	8.36	2.29	1.33	15.13	7.57	22.70	2.36
60	Uninoc.	101.76	43.11	9.22	2.55	0.95	17.18	7.69	24.88	2.38
	strain M-1	103.80	50.80	11.27	4.39	1.25	18.55	10.92	29.50	2.46
	strain M-2	104.33	43.54	10.12	3.44	1.45	18.53	10.63	29.23	2.46
	strain M-3	111.91	53.17	12.66	3.28	1.96	18.57	10.77	28.36	2..50
80	Uninoc.	103.33	55.46	8.49	2.61	1.00	19.12	9.22	28.26	2.38
	strain M-1	106.71	55.90	8.36	4.11	1.81	20.05	11.22	31.28	2.42
	strain M-2	106.00	56.09	11.53	4.07	1.540	20.18	11.26	31.51	2..46
	strain M-3	110.10	54.95	12.16	3.90	1.60	19.27	11.18	31.28	2.45
L.S.D 0.05		3.81	8.80	1.73	0.75	0.40	2.43	1.50	2.65	0.16

*The area of each plot =1/400 feddan .

** Each value is a mean of 6 replicates.

Most of the studies of the *Azospirillum* plant association have been conducted on cereals and grasses (Tyler *et al.,* 1979) and only a few other plant families have been investigated (Bashan *et al.,* 1989). Plant response to inoculation with *Azospirillum* in cereals and non cereals are often reported in terms of increased grain yield, plant biomass, nutrient uptake, grain and tissue N contents, nitrogenase activity, early flowering, tiller numbers, greater plant height and leaf size, increased number of spikes, grains per spike, test weight, increased root length and volume. The responses varied with crops, cultivators, locations, seasons, agronomic practices, bacterial strains, soil fertility and interaction with native soil microflora (Wani, 1990). The increase in the stem diameter of maize and increase in the number of ear and tillers in wheat was attributed to the production of plant growth promoting substances by *Azospirillum* (Kapulnik *et al.,* 1981). Among the three plant growth promoting substances produced by the *Azospirillum*, auxins were quantitatively more. The bacterial IAA biosynthesis has been thoroughly studied in *A. brasilense*. *Azospirillum* possess at least 3 IAA biosynthesis pathways of which, two are tryptophan dependent and the other is tryptophan independent. Piccinin *et al.* (2011) evaluated the agronomic efficiency of *Azospirillum brasilense* in physiological parameters and yield components of wheat under reduced N_2 fertilization. Their work showed that the use of half level of N_2 associated with the inoculation of seeds with *A. brasilense* promoted results on the agronomic performance and productivity of wheat.

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عزل وتوصيف الأزوسبيريلا المعزولة من التربة وتأثيرها على نمو ومحصول القمح تحت مستويات مختلفة من التسميد النيتروجيني

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الملخص

تم في هذا البحث عزل ثلاث سلالات من بكتريا الأزوسبيريلا من أراضي مختلفة القوام بمحافظة أسوان.

وقد أوضحت دراسة الصفات المورفولوجية والمزرعية والفسيولوجية للسلالات الثلاث المعزولة (M-1,M-2,M-3) علي أنها تتبع نوع بكتريا *Azospirillum brasilense* .

كذلك تم دراسة تأثير التلقيح بالسلالات المعزولة علي نمو ومحصول نبات القمح تحت مستويات نيتروجين مختلفة (٤٠ ، ٦٠ ، ٨٠ كجم نيتروجين/فدان) ، وذلك في تجربة حقلية خلال الموسمين ٢٠١٤/٢٠١٣ و ٢٠١٥/٢٠١٤ .

وقد أوضحت نتائج هذه التجربة أن التلقيح بأي من سلالات الأزوسبيريلا الثلاث أدي إلي زيادة معنوية في كل قياسات النمو ومحصول الحبوب. كم أوضحت النتائج أن السلالة M-1 كانت أفضل السلالات تحت كل مستويات النيتروجين وأن التلقيح بالأزوسبيريلا يمكن أن يوفر من ٢٠ – ٤٠ كجم نيتروجين/فدان.