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Occurrence and Microbiological Characteristics of *Azospirillum* Strains Associated with Leguminous and Non-Leguminous Plants in Al Jabal Al Akhdar Eco-Region, Libya

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Abstract: Various strains of *Azospirillium* spp. were isolated from the bulk and the rhizospheric soils of 23 leguminous and non-leguminous plants distributed in a unique Mediterranean-type climate, Al Jabal Al Akhdr eco-region, in eastern Libya. The CFU counts were ranged from 1.1 to 130.2 × 10^3 CFU/g^{-1} soils with the highest counts were observed in the rhizosphere of legumes. Based on the morphology of the cells and cultural and biochemical characteristics, 15 strains of *A. lipoferum* strains were identified. These *Azospirillum* strains were examined for siderophore production by testing their capability to grow in iron-limiting medium (M-9). Seven strains (F18, R6, R7, R9, R17, R23 and P8) were able to grow well in the medium, whereas three other strains exhibited moderate growth and the remaining strains failed to grow. The distributions and characterization of these strains were varied based on to the plant species and soil properties.

Key words: *Azospirillum* · Leguminous and non-leguminous plants · Al Jabal Al Akhdr eco-region · Libya

INTRODUCTION

Claims of *Azospirillum* specificity for certain cereal species were documented [1]. However, data published in recent years, showed that *Azospirillum* had no preference for crop plants or weeds, or for annual or perennial plants and can be applied successfully to plants that have no previous history of *Azospirillum* in their roots. Thus, it appears that *Azospirillum* is a great root colonizer and is not a plant-specific bacterium [2].

In numerous studies, *Azospirillum* inoculation have been reported to reduce the use of chemical fertilizers in particular nitrogen by 20%-50%. The interactions showed better results especially when organic fertilizers were incorporated with the associative N_2 fixers *Azospirillum* spp. in many grasses and cereals [3]. The first species of *Azospirillum* was isolated by Beijerinck [4] from different tested soils (N-poor sandy soils) in the Netherlands and originally named *Spirillum lipoferum*. The nitrogen-fixing spirillum isolated by Becking [6] from the collected African soils was not completely identified as *S. lipoferum*, which suggested a possible relationship between *Spirillium* and *Azosprillum*.

The introduction of semisolid, N-free media [3] led to the rediscovery of this bacterium as one of the most widely-distributed, N_2-fixing bacteria, especially in warm regions. More than 60% out of one 1000 grass roots and about 200 soil samples from tropical Africa and South America contained azospirilla in numbers ranging from 10^5 to 10^7 CFU/g^{-1} of root or soil. Since then, *Azospirillum* has been isolated from the roots of numerous wild and cultivated grasses, cereals and legumes and from tropical, sub-tropical and temperate soils world-wide [6-16, 2]. Thus, it appears that *Azospirillum* is a universal bacterium found almost everywhere.

Based on physiological and morphological differences between various strains and on DNA homology experiments, Tarrand *et al.* [17] proposed the genus *Azospirillum* and distinguished two species: *Azospirillum brasilense* and *Azospirillum lipoferum*. Study of the taxonomy, biochemistry, physiology and genetics of *Azospirillum* have revealed a very interesting microorganism [18-20], which serves as a model for elucidating the mode of action of beneficial plant-rhizobacterial interaction [21, 2].

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Nitrogen fixation was the first mechanism proposed to explain the improvement of plant growth following *Azospirillum* inoculation. Moreover, careful experimentation using the ^{15}N-isotope dilution technique with some cultivars of sugarcane, *Panicum maxima* and *Paspalum notatum*. Lima *et al.* [22] have demonstrated that up to 50% of the plant N is derived from beneficial nitrogen fixation (BNF). This has been demonstrated in certain wheat and maize cultivars [23-25]. However, Bashan *et al.* [26] reported that the contribution of N_2 fixation by *Azospirillum* was smaller than 5% increase, while the mutants were capable of increasing plant growth similar to the wild type [26].

Moreover, most researchers agree that the number of *Azospirillum* cells in roots, though considerable, is too small to enhance crop yield significantly by means of N_2 fixation [27-30]. *Azospirillum* also produces phytohormones and the production of phytohormones may enable *Azospirillum* to enhance plant growth and even when occurring in small quantities in roots [31, 32]. In this respect special attention was given to the production and release of indole acetic acid (IAA) and gibberellins by the bacterial cells [33-36]. It has been shown that in wheat both inoculation with *Azospirillum brasilense* Cd and application of pure IAA to the roots increased root length, numbers of lateral roots and the number of root hairs [37]. Information regarding *Azospirillum* inoculation under different levels of N-fertilizer is still controversial [1].

The objectives of the present study were to enumerate and isolate strains of *Azospirillum* species of the bulk and the rhizosphere soils of legume and non-leguminous plants in the Mediterranean-type climate, Al Jabal Al Akhdar eco-region, in Libya. The study included isolation of the N_2-fixing *Azospirillum* spp. from 23 soil and root samples collected from the region and to monitoring the effect of inoculation with *Azospirillum* spp. at different levels of organic-matter (wheat straw) on growth and N_2-fixation as well as some physiological activities of wheat and maize plants. This is the first report demonstrated the occurrence of *Azospirillum* spp in Al Jabal Al Akhdar eco-region.

MATERIALS AND METHODS

Determination of Soil Type: The soil type was determined by the hydrometer method [38]. The probe of the hydrometer was calibrated in order to read directly the percentage of soil remaining in a suspension. A weighed quantity of air-dry soil, equivalent to 50 gm of oven-dry soil was transferred to a 800 ml beaker and approximately 50-60 ml of H_2O_2 were added.

The mixture was then allowed to settle in the cold overnight. Then the beaker was immersed in the boiling water bath for 5 minutes. When the contents of the beaker were cool down, 2N hydrochloric acid were added. The acid and soil were allowed to react for 1 h, then the mixture was filtered through Buchner funnel and the soil was washed with four successive portions of 50 ml 0-2 N hydrochloric acid. After the soil has been wash, it was transferred to a beaker and 200 ml of distilled water and 15 ml of 0-5 N sodium oxalate were added. The soil was then diluted to 1 litter solution.

Soil Chemical Analysis:

Total Soluble Salts: For the determination of total soluble salts, 100 g of each soil sample was shaken in a 500 ml of distilled water for about 30 min and the mixture was left overnight to settle. The soil extract was then filtered evaporated in an oven at 105°C. The dry residue was then weighted and the amount of total soluble per gm oven-dry soil was calculated.

Organic Matter Content: The organic content of the soil sample was determined according to Walkly and Black method [39, 40], minor modification by Sahrawat was adopted [41]. The 0.5 g of sieved soil sample was digested by chromic acid (for oxidation of organic matter to carbon dioxide) and the excess chromic acid was back titrated against standard ferrous sulphate solution using diphenylamine as an indicator.

pH Value: A Beckman pH meter was used for the determination of soil pH. The electrodes were immerse in the soil paste with water to a ratio of 1:1 to avoid the error arising through higher dilutions [40].

Isolation of *Azospirillum* spp. From Rhizosphere and Free-Soil Samples: For isolation of N_2-fixing *Azospirillum* spp., the free soil was removed from the plant roots by shaking the roots and the soils were subjected to soil-chemical analysis. Roots were cut into approximately 0.5-1 cm long segments. One hundred mg of the root pieces were collected from different localities and both the soil and roots samples were introduced into a sterile test-tubes containing 4 ml of semisolid NFb-medium [42].

In some cases the semisolid medium was inoculated with soil suspensions of 10^{-5} to 10^{-7} dilutions. After 72 h of incubation period, veil-like pellicles were observed below the medium surface which indicated presence of *Azospirillum*. It is easily recognized with the blue medium (use of malic acid lead to a pH decrease and a change in medium color). *Azospirillum* were transferred to new test tubes containing the same medium. As a new pellicle was

visible, the cultures were streaked out on agar plates containing the same medium with yeast extract (20 mg l^{-1}). The small amount of yeast extract permits the growth of small colonies on the surface of the plates. Individual colonies were then maintained on NA slants for further studies.

Identification of the Bacterial Strains: Bacterial strains were streaked four times for purification on malate agar medium supplemented with (20 mg l^{-1}) yeast extract [42]. Purification and microscopic examination (using Olympus C×21 microscope) were carried out to obtain pure cultures. Fifteen strains were recognized as belonging to the genus *Azospirillum* based on the morphological, cultural and some biochemical characteristics by [17] and the schemes as described in the 9th edition of Bergey's Manual of Systematic Bacteriology [43].

The International type species, the Brazilian strain *A. brasilense* Cd. Sp7 (ATCC, 29145) and *A. lipoferum* strain 137, which was kindly supplied by Prof. Vassuyk, L.F., Academy of Science, Petersburg, Russia were used as reference for comparison with the locally isolated strains.

Cultural and Morphological Description: Putative *Azospirillum* colonies were selected on the basis of the culture plate morphology characteristics namely: opacity, pale to deep pink pigmentation, no slimy and wrinkled. Selected colonies were picked up and cultured on NA slants.

Morphological Description of the Vegetative Cells: *Azospirillum* was examined for cell-shape, Gram reaction, inclusions and motility in the semisolid malate medium after 1-3 days. Polymorphism was recorded after 2, 7 and 15 days of incubation.

Physiological and Biochemical Tests: For species determination, utilization of different carbon-sources was performed either in aerobic or anaerobic conditions. The bacterial strains were grown in semisolid malate medium containing carbohydrate together with a pH indicator (bromothymol blue) [44].

The development of a yellow color during 96 hour incubation at 30°C indicated acidification. The tests involved catalase activity, denitrification test, growth in the presence of 3% NaCl and growth on the amino acid L-Histidine as a sole C and N source [45]. Production of siderophore (growth on M-9 medium) [46] as well as starch and gelatin hydrolysis were also tested [47].

Microcosms Experiments: In order to select the more effective A*zospirillum* strain on growth of wheat, a microcosms experiment was conducted in laboratory conditions. Pots were maintained in a laboratory with a 12-h photoperiod (light source, Osram HPTI/HQI 400W; intensity and irradiance at plant-top level, 7–10 kLux and 30-35 Wm^{-2}; day/night temperatures 25±2°C/22°C, RH 30-40%). Wheat (*T. aestivum*, Giza 167) was grown in small pots (500 g capacity) filled with sterilized mixture of sand and clay soil in a ratio of (3:1, wt:wt) and inoculated with different locally isolated bacterial strain (10^6 CFU/seed).

Statistical Analysis: The data were subjected to one way-analysis of variance (ANOVA) and the means were separated by the least significant difference, LSD [48].

RESULTS AND DISCUSSION

General Description of Al Jabal Al Akhdar Soils: Table 1. shows some properties of the soil samples and the plant used for isolation. The percentages of sand, silt and clay ranged from 60.68 to 90.68, 5.45 to 30.45 and 3.87 to 15.59, respectively. The texture of all soil samples was sandy loam except for El-Haneia soil, which was sandy. The pH values were higher than 7 which means that the soils were alkaline and the highest were recorded in Kaser Libya and Zawiat Elarkob soils (with pH 8.4, see Table 1). Such alkaline reaction of the Jabal Al Akhdar eco-region can be attributed to the bed rocks, which contain high concentrations of calcium carbonates (limestone) [49].

The values of EC ranged from 0.31 to 0.92 dS/m which means that the soils were not saline. However, during drought periods of this area, which is located in Mediterraean-type climate, the soil biota may influenced by the direct and indirect effects of the salts. With respect to the soil organic matter, the values ranged from 0.8 to 4.3 % which can be attributed to the quantity and quality of the crop residues [50].

Isolation, Identification and Distribution of *Azospirilum* spp.: Twenty-eight spirillum strains were recovered from both free-soil (bulk soil), or from rhizosphere and rhizoplane of the collected plants. After several transfers for purification, 15 strains were identified as bacteria belonging to the genus *Azospirillum* based to the following common cultural and cell-morphological characteristics (Table 2).

Table 1: Characteristics of the soil samples and plant used for isolation and total Azospirillum counts

		Partical size distribution								
Soil No.	Place	Sand %	Silt %	Clay %	Texture	pH	E. C. ds/m	Organic matter %	Plant under cultivation	No. of Azospirillum × 103
1	Kernada	64.70	26.8	8.5	Sandy Loamy	7.7	0.27	3.3	Pimpinella anisum	2.5
2	Albyda	66.68	20.72	12.59	Sandy Loamy	8.2	0.38	4.3	Triticum vulgaris	1.1
3	ElKharika	70.68	26.72	2.59	Sandy Loamy	7.9	0.41	4.1	Phagnallon rupestre	15.0
4	Omar Al-Mukhtar	64.69	20.35	14.95	Sandy Loamy	8.2	0.47	2.4	Thapsia garganica	2.1
5	El-Faidia	60.68	23.72	15.59	Sandy Loamy	7.9	0.33	2.2	Marrubium vulgare	26.1
6	El-Mansora	63.04	30.45	6.51	Sandy Loamy	7.7	0.43	1.9	Portulaca oleracea	9.2
7	Shahat	70.68	20.72	8.59	Sandy Loamy	7.8	0.41	3.1	Thymus serpyllum	8.5
8	Wardama	70.68	24.72	4.59	Sandy Loamy	7.6	0.30	3.0	Marrubium vulgare	130.2
9	Massa	64.68	24.72	10.59	Sandy Loamy	7.7	0.38	2.7	Glycin max	25.1
10	Belhaded	66.68	24.72	8.59	Sandy Loamy	7.8	0.27	2.0	Ceratonia siligua	50.5
11	El-Waseta	68.68	18.72	12.59	Sandy Loamy	8.1	0.22	3.2	Zea mays	2.1
12	Eslenta	68.68	19.72	11.59	Sandy Loamy	7.8	0.62	3.8	Cucumis sativus	14.5
13	Kandola	68.4	19.0	12.59	Sandy Loamy	7.7	0.51	1.3	Brassica oleracea	42.5
14	Gardas	68.68	16.72	14.59	Sandy Loamy	7.8	0.32	1.0	Hordium vulgaris	46.4
15	El-Khwimat	84.68	10.08	5.23	Sandy Loamy	8.3	0.26	0.9	Artemesia herba-alba	17.5
16	Marawa	66.68	26.72	6.59	Sandy Loamy	8.3	0.13	1.4	Thapsia garganica	16.5
17	Eljehad	72.68	18.72	8.59	Sandy Loamy	7.9	0.34	3.6	Thapsia garganica	18.4
18	Kaser Libya	73.04	26.36	0.59	Sandy Loamy	8.4	0.25	1.4	Hordium vulgaris	3.5
19	Zawiat Elarkob	78.68	8.72	12.59	Sandy Loamy	8.4	0.27	1.6	Triticum vulgaris	7.5
20	Ekfenta	56.68	34.0	9.31	Sandy Loamy	8.0	0.18	1.5	Thapsia garganica	8.5
21	El-Hamama	69.68	21.72	8.59	Sandy Loamy	8.0	0.26	1.2	Artemesia sp.	56.1
22	El-Koof	73.04	18.36	8.59	Sandy Loamy	8.3	0.31	1.8	Paronychia argentina	33.2
23	El-Haneia	90.68	5.44	3.87	Sandy	7.7	0.92	0.8	Lycopersicum sp.	6.5

Table 2: Morphological and Biochemical activities of the isolated Azospirillum spp. strains

	Isolates																
Tests	A. brasilense (Sp7)	A. lipoferum (137)	F8	F9	F14	F18	R6	R7	R8	R9	R10	R17	R23	P6	P8	P9	P10
Cell-shape	curved	ovoid	ovoid	ovoid	rod	rod	curved	ovoid	curved	spiral	rod	rod	ovoid	rod	ovoid	curved	ovoid
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gram-stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Polymorphism	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in L-Histidine	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 3% NaCl	+/-	+	+/-	+	+/-	+	+/-	+	+	+	+	+	+	+	+	+/-	+/-
Growth in M9 medium	+	+	+/-	-	-	+	+	+	+/-	+	-	+	+	+/-	+	-	-
Growth in JNFb medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase activity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sole carbon Source:																	
Succinate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2H2-reduction (nmole C2H4/h)	126	96.5	110	86.6	54.3	36.6	77.3	86.5	109.5	34.5	50.0	66.0	119.2	35.5	44.0	76.5	87.9

F= Isolated from free-soil. R= Isolated from rhizoshere. P= Isolated from rhizoplane

Results presented in this study indicated that *Azospirillum* was found abundantly and dominantly in all of the tested soil-samples (23-samples) collected from different localities in Al Jabal Al Akhdar eco-region, which were from cultivated areas and areas with weed plants. *Azospirillum* densities were as high as 1.1×10^3 - 13.2×10^4 CFU/g dry soil. The size of *Azospirillum* population has been estimated at 1-10% of the total soil population [51]. Until recently, procedures used for isolation N_2 fixing bacteria were not basically different from those proposed by Beijerinck [52] and Winogradsky [53]. The procedure based on the enumeration and purification of bacteria growing in N-free media inoculated with dilutions or aggregates of soil.

The addition of certain carbon source encourage the growth of certain group of bacteria. For instance, addition of manitol or glucose in N_2-free medium leads to the frequent isolation of Azotobacteraceae [54], whereas malate leads to the isolation of *Azospirillum* [55]. In the present study, *Azospirillum* was isolated and enriched from the rhizosphere, rhizoplane or bulk soil using the nitrogen-free biotin medium (NFb) in which L-malic was the sole C-source. Although reports on the isolation of *Azospirillum* from graminaceous plants are common, other reports showed that the bacterium is a natural inhabitant of many nongraminaceous plants. *Azospirillum* was isolated from roots of coconut palms grown under diverse agronomic practices [56] and within the stem nodules, root nodules and stem of *Aeschynamene indica* [57].

In the present study *Azospirillum* was isolated from cultivated plants (crops) (*Hordium vulgaris*, *Zea mays*, *Triticum vulgaris*) as well as non-cultivated weed plants (*Thapsia garganica*, *Marrubium vulgare*, *Paronychia argentina*). Moreover, *Azospirillum* was also isolated from leguminous plants such as *Ceratonia siliqua* (Crob), *Glycin max* (Soya bean)) and non-leguminous plants such as *Hordium vulgaris* (Barley), *Zea mays* (Corn).

Thus, *Azospirillum* is a general root colonizer and is not a plant-specific bacterium [2, 58]. In the present investigation, 28 spirilla were isolated from the rhizosphere, rhizoplane and free-soil from different plants grown in Al Jabal Al Akhdar region. After several transfers, 15 strains were identified as bacteria belonging to the genus *Azospirillum* based on to their common cultural and cell morphological characteristics. The characteristics were the formation of a veil like pellicle or ballon often 10 mm below the surface of semisolid N-free media. The formation of this pellicle is due to an aerotactic response of the motile bacteria towards low levels of PO_2 that permit N_2 fixation [51]. The dissolved O_2 concentration in the media was just enough for optimal respiration rates without inhibiting N_2 fixation [3], as a result of nitrogenase inhibition.

Screening of different strains for carbohydrate utilization differed markedly with respect to the *Azospirillum* species and to the carbon source (Table 3). However, all the 15 strains effectively oxidized the tested organic acids (Succinate, Malate and Pyruvate) when used as a sole carbon source auxanotrophically [in presence of (NH_2SO_4)]. The preference of the organic acids by different *Azospirillum* species was reported earlier by Reinhold *et al.* [59]. This can be explained on the basis that organic acids were the major source of nutrients for the microflora in the rhizosphere [60].

The results of the present study also indicated that *A. lipoferum* related strains were able to utilize large group of carbohydrate, while *A. brasilense* strain (SP7) was more restricted in its use of carbon sources including glucose which was not used by *A. brasilense* (SP7). Glucose catabolized by *Azospirillum* spp. by the action of NAD (P)-glucose-6-p-dehydrogenase, is required for 6-phosphoglucanate dehydrogenase synthesis which is a key enzyme of the ED pathway for glucose catabolism. It was the first enzyme produced in high level by *A. lipoferum* but was undetectable in *A. brasilense* [61]. However, *A. amazonense* has remarkable ability to grow and fix N_2 in media containing disaccharides [62], which is a characteristic of this species. The isolated *Azospirillum* spp. in the present study were not able to utilize sucrose (Table 3).

Siderphores are low-molecular weight compounds produced by the microorganisms which are able to bind iron from the environment. The binding to the siderphore allow transfer of iron to the cell, enabling bacteria to compete for this otherwise unavailable element [63]. *Azospirillum* spp. produces siderophores that represent an important factor for their competition and survival in the rhizosphere [64]. The siderophore spirilobactin produced by *A. brasilense* strain RG, was also reported by Bachhawat and Gosh [46]. The siderophore iron uptake of *A. basilense* SP6 was studied by using molecular genetic approach and the *Lon* gene was found to be involved in the iron uptake of *A. brasilense* [65]. Our *Azospirillum* strains were examined for siderophore production by testing their capability to grow in iron-limiting (M-9) medium (44). Table (2) indicated that seven strains (F_{18}, R_6, R_7, R_9, R_{17}, R_{23}, P_8) were able to grow well in the later medium, whereas three other strains exhibited moderate growth and the remaining strains cannot grow in that special medium.

Table 3: Oxidation and Fermentation of different carbon compounds by the isolated Azospirillum spp. strains in comparison with type species

	Carbon Source															
	Glucose		Fructose		Sucrose		Xylose		Maltose		Dextrose		Lactose		Manitol	
Isolates	Ox.	Fer.	Ox.	Fer.	Ox.	Fer.	Ox.	Fer.	Ox.	Fer.	Ox.	Fer.	Ox.	Fer.	Ox.	Fer.
A. brasilense (Sp7)	-	-	-	-	-	-	±	±	+	+	-	-	-	-	-	-
A. lipoferum (137)	+	+	±	±	-	-	-	-	+	+	±	±	±	±	+	+
F8	+	+	+	+	-	-	-	-	±	±	+	+	+	+	+	+
F9	+	+	±	±	-	-	-	-	+	+	+	+	+	+	±	±
F14	+	+	±	±	-	-	-	-	+	+	+	±	+	+	+	+
F18	+	+	+	+	-	-	-	-	+	+	+	±	+	+	+	+
R6	+	+	+	+	-	-	-	-	±	±	+	+	+	+	+	+
R7	+	+	±	±	-	-	-	-	+	+	+	+	+	+	±	±
R8	+	+	+	+	-	-	-	-	±	±	±	±	+	+	+	±
R9	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+
R10	+	+	±	±	-	-	-	-	+	+	±	±	+	+	+	+
R17	+	+	±	±	-	-	-	-	+	+	+	+	+	+	+	+
R23	+	+	±	±	-	-	-	-	+	+	±	±	+	+	+	+
P6	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+
P8	+	+	+	+	-	-	-	-	+	+	+	±	+	+	+	+
P9	+	+	±	±	-	-	-	-	+	+	+	+	+	+	+	+
P10	+	+	±	±	-	-	-	+	+	+	+	+	+	+	+	+

Ox.= Oxidation (aerobic) Fer.= Fermintation (anaerobic condition)

Table 4: Effect of inoculation with different Azospirillum lipoforum strains on growth of wheat in microcosms experiment

	Shoot-system					Root-system			
Treatment	Length /cm	Leaf area /Cm2	**Fresh weight	**Dry Weight	(%) increase	Root length/cm	**Fresh weight	**Dry weight	(%) increase
Control	28.0	9.8	0.54	0.08	-	20.5	0.09	0.03	-
F8	30.5*	11.3*	0.56	0.08	-	20.8	0.10	0.04	-
F9	29.5*	9.6	0.79*	0.09	12.5	22.0*	0.12*	0.05*	33.0
F14	29.5*	12.6*	1.04*	0.10*	25	20.0	0.13*	0.04	66.6
F18	32.5*	12.1*	0.57	0.07	-	22.0*	0.15*	0.04	33.0
R6	29.0	12.0*	0.63	0.07	-	20.0	0.13*	0.04	33.0
R7	30.0*	12.0*	0.64	0.08	-	21.5*	0.10	0.04	33.0
R8	31.0*	14.5*	0.89*	0.09*	12.5	21.5*	0.20*	0.04	33.0
R9	30.5*	9.6*	0.75*	0.08	-	21.0	0.15*	0.04	33.0
R10	30.0*	11.7*	0.49	0.08	-	21.0	0.06	0.03	-
R17	28.5	12.2*	0.56	0.07	-	21.0	0.09	0.03	-
R23	32.0*	14.4*	0.86*	0.19*	137.5	22.5*	0.19*	0.06*	100.0
P6	29.0	10.3	0.84*	0.07	-	21.0	0.12*	0.04	33.0
P8	31.0*	12.6*	0.67*	0.08	-	20.0	0.10	0.03	-
P9	28.5	14.0*	0.76*	0.08	-	22.0*	0.12*	0.04	33.0
P10	28.0	11.9*	0.54	0.07	-	20.0	0.11	0.03	-
L.S.D 5%	1.3	0.9	0.22	0.01	-	0.9	0.02	0.01	-

* Significant Effect

More studies are needed to examine the efficiency of such indigenous *Azospirillum* strains for the nitrogen fixation. Hence, the most efficient strain could be used for increasing the soil fertility and plant production. Likewise, the counts of such strains in the rhizosphere of legumes should also be further investigated.

Microcosms Experiment of Wheat to Select the Most Effective *Azospirillum lipoferum* Strain: Table (4) showed that wheat plants generally responded positively to inoculation with most of the studied *Azospirillum* strains. This is indicated by the significant increases in the growth parameters of inoculated plants compared to

the control. *A. lipoferum* strain R_{23} recorded the highest effect on wheat growth, since it recorded 137.5% increase in dry soot-mass and 100% increase in dry root-mass if compared with control of non-inoculated plants. Results presented in the microcosms experiment with wheat as well as results of the nitrogenase activity of the pure cultures of *Azospirillum* using the acetylene reduction assay (ARA) (Table 2) showed that *A. lipoferum* strain (R_{23}) was the most potent isolate, therefore it has been selected for further investigations in the pot-experiments.

CONCLUSIONS

In conclusion, the data presented in this study indicate that the mode of action of *Azospirillum* is most probably composed of multiple mechanisms. Further studies are needed to clarify possible interaction in *Azospirillum*-host plant association. Therefore, the increased use of the various biological processes in soil, of which some examples have been given in the present study will decisively contribute to make agriculture more productive with less harm to the environment. This fact may be of importance for developing countries where the use of fertilizers is costly. It is hoped for substantial increase in food production in order to eliminate under-nourishment and poverty, which is the main goal to be achieved by using biofertilizers.

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