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ISOLATION AND SELECTION OF EFFICIENT INDIGENOUS *RHIZOBIUM LEGUMINOSARUM*, *AZOSPIRILLUM* SPP. AND *AZOTOBACTER* SPP. FROM SHARKIA GOVERNORATE SOILS

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ABSTRACT: **This work was carried out to select efficient indigenous isolates of *Rhizobium leguminosarum bv. vicieae*, *Azospirillum* spp. and *Azotobacter* spp. to be used as biofertilizers for Broad bean (*Vicia faba* L.) and Wheat (*Triticum aestivum* L.) plants. Sixty isolates of *Rhizobium leguminosarum bv. vicieae*, sixty isolates of *Azospirillum* spp. and 120 isolates of *Azotobacter* spp. were isolated from different locations in Sharkia governorate. Thirty two *Rhizobium* isolates were selected as effective strains for nodulation on broad bean, 18 isolates of *Azospirillum*, and 14 isolates of *Azotobacter* were efficient in nitrogenase activity in liquid cultures. Five isolates showing high values in nitrogen fixation of *R. leguminosarum bv. vicieae* and the highest five effective isolates of each of *Azotobacter* spp. *and Azospirillum* spp. were used for GA_3, IAA and IBA production in liquid cultures. In conclusion, isolate RZ11 (isolated from Zagazig) proved to be the most efficient isolate of *R. leguminosarum bv. vicieae* in terms of plant growth, nodulation, nitrogenase activity and production of growth-promoting substances. Isolate ZH21 of *Azotobacter* spp. and isolate ASH21 of *Azospirillum* spp. (isolated from El-Hessenia) showed the highest nitrogenase activity and production of growth-promoting substances. These isolates will be used as biofertilizers in further studies.**

Key words: ***Rhizobium, Azotobacter, Azospirillum*, nitrogenase activity, growth-promoting substances.**

INTRODUCTION

Nitrogen fixation by the legume-*Rhizobium* symbiotic partnership represents an inexpensive alternative to the use of chemical nitrogen fertilizers in the production of food protein and oil. The process requires that the host be adequately nodulated by effective root-nodule bacteria. However, there is interest in developing co-inoculants containing other micro-organisms which are able to improve legume growth (Mishra *et al.*, 1999 and Rodelas, 1999). These include rhizobacteria which promote nodulation, nitrogen fixation, plant vigour and yield via such mechanisms as phytohormones, antibiotic or metal binding compound production, bacteria or fungi which protect against specific root pathogens and other which aid in nutrient supply via phosphate solubilization. For instance, Rodelas *et al.* (1999) pointed out that co. inoculation of broad bean with *R. leguminosarum bv. vicieae* plus plant-growth-promoting *Azotobacter* and *Azospirillum* led to changes in total content, concentration and/or distribution of the macro and micronutrients, K, P, Ca, Mg, Fe, B, Mn, Zn and Cu when compared with plants inoculated with *Rhizobium* only.

Azospirillum, an associative microaerophillic nitrogen fixer commonly found in loose association with roots of cereals and grasses which is of great interest. High nitrogen fixation capacity, low energy requirements and abundant establishment in the roots of cereals and tolerance to high soil temperature (30-40 °C) are responsible for its suitability under tropical conditions (Hedge *et al.*, 1999).

The mechanism of bacterization resulting in yield increase with decrease or no increase in N concentration may be attributed to enhanced N_2-fixation or increased N assimilation by plant (Aggarwal and Chaudhary, 1995 and Bashan and Holguin, 1997); enhanced mineral uptake in the plant (Stancheve *et al.*, 1995); improved root growth and functions (Sarig *et al.*, 1992 and Fallik *et al.*, 1994); nitrate production in nitrate respiration (Bothe *et al.*, 1992);in vitro *Azospirillum lipoferum* produces siderophores when grown in iron defined medium may improve iron-nutrition of plant (Hedge *et al.*, 1999); produces in vitro phytohormones

IAA, gibberllins, Cytokinin and ethylene. These phytohormones, especially IAA play an essential role in plant growth stimulation in general and in stimulating symbiosis between legumes and rhizobia and affect plant cell metabolism from outside the cell which suggests that bacteria are capable of excreting and transmitting a signal(s) which crossed the plant cell wall and is recognized by plant membranes and promoted N_2-fixation (Bashan and Holguim, 1997).

Inoculation with indigenous strains is an important procedure when studying their inherent capacity to benefit crops. In some cases, indigenous strains can perform better than introduced strains in promoting the growth of the plants due to their superior adaptability to the environment.

MATERIALS AND METHODS

This study was carried out in laboratory and greenhouse of Agric. Microbiology Dept. at the Faculty of Agriculture, Zagazig University, Egypt, during the period of 2002 to 2005, in order to select efficient indigenous isolates of *Rhizobium leguminosarum bv. vicieae*, *Azospirillum* spp. and *Azotobacter* spp. to be used as biofertilizers in further studies for Broad bean (*Vicia faba* L.) and Wheat (*Triticum aestivum* L.) plants.

Root-Soil Core Samples

Root-soil core samples were collected following the root-soil core procedure with roots of broad bean and wheat plants grown in different locations in Sharkia governorate. The collected samples represented eight locations namely: Zagazig (Z), Fakous (F), Abo-Kibeer (K), El-Hessenia (H), Kafer-Saker (R), Abo-Hammad (A), Belbase (B) and Salhia (S). Root nodules of broad bean plants found in each soil sample were used for *Rhizobium* isolation and soil is used for *Azotobacter* isolation. Soil and roots of wheat grown in each soil sample were used for *Azospirillum* and *Azotobacter* isolation.

Isolation and Purification of *Rhizobium* Isolates

Pure cultures of *Rhizobium leguminosarum bv. vicieae* were isolated according to the methods described by Vincent (1970) and Somasegaran and Hoben (1985). Single colonies were inoculated

onto slopes of YEMA medium after microscopic testing of gram stained slides of the colonies. A total of 60 isolates of *Rhizobium leguminosarum bv. vicieae* were isolated from all the collected soil samples. Each soil sample was represented by one rhizobial isolate (Table, 1). These isolates were tested for their nodulation as described by Vincent (1970) and Somasegaran and Hoben (1985). Stock cultures of rhizobial isolates were maintained on slants of YEM agar at 4 °C and recultivated monthly.

Isolation and Purification of *Azospirillum* Isolates

Isolates of *Azospirillum* spp. were isolated from soil and roots of wheat plants, grown in different locations in Sharkia governorate (Table, 1) according to the method described by Gunarto *et al.* (1999). Pellicle formation in semi solid nitrogen free broth (NFb) medium indicated successful isolation. For final purification, these cultures were streaked out on Potato infusion agar (BMS) medium (Baldani and Döbereiner, 1980), and the typical pink, often wrinkled colonies were transferred

Table 1: The locations of soil samples selected for isolation of *Rhizobium*, *Azotobacter* and *Azospirillum*

Locations	*Rhizobium*			*Azospirillum*			*Azotobacter*		
	Total No. of isolates	No. of chosen isolates	Nomenation	Total No. of isolates	No. of chosen isolates	Nomenation	Total No. of isolates	No. of chosen isolates	Nomenation
Zagazig (Z)	9	6	RZ11, 21, 22, 23, 32, 33	9	2	ASZ11, 33	18	3	ZZ11, 13, 23
Fakous (F)	9	6	RF12, 13, 21, 23, 31, 32	9	3	ASF11, 12, 32	18	2	ZF22, 31
Abo-Kibeer (K)	9	7	RK11, 12, 13, 21, 23, 31, 32	9	3	ASK21, 23, 33	18	2	ZK12, 31
El-Hessenia (H)	9	3	RH13, 23, 31	9	2	ASH21, 31	18	3	ZH11, 21, 31
Kafer-Saker (R)	9	4	RR11, 13, 23, 31	9	1	ASR31	18	1	ZR22
Abo-Hammad (A)	9	2	RA21, 33	9	3	ASA12, 23, 33	18	2	ZA11, 31
Belbase (B)	3	3	RB11, 12, 13	3	1	ASP11	6	-	-
Salhia (S)	3	1	RS13	3	3	ASS11, 12, 13	6	1	ZS13

to an NFb agar slant containing 0.5 g yeast extract L^{-1} without vitamin solutions for storage and use for further experiments. Sixty isolates were obtained from which only 18 representative isolates were selected for their valuable nitrogenase activities.

Isolation and Purification of *Azotobacter*

Pure cultures of *Azotobacter* spp. isolated from soil samples collected from different locations in Sharkia governorate (Table, 1) were obtained according to (Abd El-Malek and Ishac, 1968). Single colonies produced were microscopically examined for pure films of gram negative, large, oval or cocci, single or in pairs. The method of identification was carried out according to Breed *et al.* (1994). One hundred and twenty isolates were obtained, from which only 14 isolates were selected for their high nitrogenase activities.

Nitrogenase Activity in Liquid Cultures

The ability of *Azotobacter* and *Azospirillum* isolates to fix atmospheric nitrogen (N_2-ase activity), was assayed using acetylene reduction technique (ARA) according to Somasegaran and Hoben (1985). The results were recorded as μ mole C_2H_4/ml/h using gas chromatography system (Hewlett Packard -HP-6890-series, USA).

Testing of Different Isolates of *Rhizobium leguminosarum bv. vicieae* for Nodulation and Plant Growth of *Vicia faba* L. grown in greenhouse

Broad bean seeds (*Vicia faba* L. cv. Giza 843) were surface sterilized by immersion for three minutes in sodium hypochlorite and rinsed five times with sterile, distilled water. Six seeds were sown in sandy soil in autoclaved 20 cm pots. Each pot was inoculated with either of *R. leguminosarum bv. vicieae* isolates at 1×10^8 cfu / ml of broth culture. The pots were placed in a greenhouse and after germination seedlings were thinned to three plants per pot, and watered daily with sterile nitrogen free nutrient solution (Somasegaran and Hoben, 1985). Treatments were replicated three times and uninoculated controls were included. Plants were uprooted after 7 weeks from planting. Shoots were cut at the crown, dried at 70 °C, weighed

and ground. The nitrogen content of the shoot was then determined colormetrically using Nessler solution (Naguib, 1969). Shoot dry weight and nitrogen content were used to compute above-ground nitrogen accumulation.

Immediately after the shoots were separated from the roots, the roots were gently separated from the soil, washed and placed into reaction jars fitted with a septum. Acetylene was injected into each of the jars to achieve a final concentration of 10 % (vol./vol.) samples were incubated at 30 °C for 120 min before gas samples were withdrawn for the analysis of ethylene concentration (μ mole C_2H_4/h/g dry weight of nodules (Hardy *et al.*, 1973) using gas chromatography system (Hewlett Packard- HP- 6890- Series, USA), after the withdrawal of gas samples, the nodules were separated from the roots, counted, dried at 70 °C and weighed.

Isolation of Rhizobiophages from Soil Samples

Rhizobiophages in soil which can infect *Rhizobium leguminosarum bv. vicieae* were isolated by enrichment of soil samples from the rhizosphere of broad bean plants in Sharkia governorate using the method described by Patel and Graig (1984). Each of the phage isolates was purified by five successive single plaque isolations (Adams, 1959). All phages were stored at 4 °C in YEM broth containing 0.5 % chloroform.

Extraction and Determination of Phytohormones (IAA and GA_3) from Bacterial Isolates

Bacterial isolates were grown in 250 ml conical flasks, shaken at 150 rpm for 48 hours at 30 °C. Bacterial cultures (100 ml) were centrifuged at 5000 rpm for 20 min., then extraction was carried out according to method of Shindy and Smith (1975). The supernatants (30 ml) of the stationary phase culture were adjusted to pH 8.6 with 1 % NaOH.

Standard and extracts of hormonal compounds were fractionated with ISCO model 2360 gradient liquid chromotograph equipped with α V-UTS-250 UV according to the method described by Frankenberger and Brunner (1983) and Grolamys and Servando (1997).

RESULTS AND DISCUSSION

Testing the Efficiency of Different Isolates of *Rhizobium*, *Azotobacter* and *Azospirillum*:

A. Testing the efficiency of *Rhizobium* isolates

Thirty two isolates of *R. leguminosarum bv. vicieae* were tested in the greenhouse in order to study their efficiency on plant growth, nodulation and N_2-fixation of broad bean (*Vicia faba*) plants.

Data in Table 2 show the effect of inoculation with 32 isolates of *R. leguminosarum bv. vicieae* on plant dry weight, nodulation, nitrogenase activity, nitrogen content and phage interaction in broad bean plants.

1. Nodulation

Data in Table 2 indicated that inoculation of broad bean seedlings with different 32 isolates of *R. leguminosarum bv. vicieae* resulted in formation of root-nodules with different numbers depending on the efficiency of rhizobial-isolates. The mean number of nodules per plant after 7 weeks from cultivation ranged between 10.3 (in case of inoculation with isolate RH21) and 126.7 (in case of isolate RZ33), while the uninoculated control treatment did not form nodules because of the use of autoclaved soil. In addition, isolates RK13, RZ23 and RZ22 of *R. leguminosarum bv. vicieae* induced the highest nodules dry weight per plant, being 150.0, 143.3 and 140.0 mg / plant, respectively. These results are in agreement with Salem *et al.* (1981).

2. Nitrogenase activity

The specific nitrogenase activity of broad bean root nodules, measured as μ mole C_2H_4/h/g nodules dry weight, as affected by inoculation with different isolates of *R. leguminosarum bv. vicieae* is also shown in the same Table 2. Nitrogenase activity ranged between 7.205 (for isolate RZ11) and 0.564 (for isolate RK11) μ mole C_2H_4/h/g nodules dry weight. The results show that shoot nitrogen content of broad bean plants was highly correlated with nitrogenase activity and nodule mass in most of inoculated treatments. These results are in agreement with those reported by Narendra *et al.* (1996) and Hussein *et al.* (1997) who reported

Table 2: Shoot and root dry weight (g/plant), total nitrogen content, nitrogenase activity and phage interaction in nodulation test of bread bean plants

Isolates	Mean no. of nodules/plant	Mean dry weight of nodule (mg/plant)	Nitrogenase activity µ mol/h/g dry nodule	Mean shoot dry weight (g/plant)	Mean root dry weight (g/plant)	Mean shoot N content (mg/plant)	Mean root N content (mg/plant)	Phage inter-action*
control	0.00	0.00	0.000	0.58	0.19	12.41	2.28	0
RR11	43.30	86.66	2.945	1.43	0.60	55.63	12.30	VI
RR13	36.70	103.33	2.917	1.04	0.28	34.32	5.12	VI
RR23	116.70	110.00	2.590	1.49	0.34	53.49	6.22	V
RR31	47.00	116.66	1.892	0.84	0.33	31.50	6.37	II
RF12	45.00	123.33	1.509	1.32	0.33	45.14	5.78	VI
RF13	36.00	123.33	3.955	1.36	0.45	55.62	10.44	VI
RF21	57.70	73.33	6.015	1.55	0.34	64.64	9.28	V
RF23	64.00	100.00	3.605	1.19	0.34	44.15	7.31	III
RF31	66.00	26.67	3.846	1.24	0.33	47.00	6.93	IV
RF32	83.30	16.66	3.225	1.04	0.34	40.77	6.63	VI
RH13	87.70	123.33	2.410	1.21	0.44	41.38	8.58	V
RH21	10.30	50.00	2.465	0.97	0.41	35.60	7.18	IV
RH31	92.00	93.33	3.833	0.95	0.27	36.67	5.94	V
RA21	25.70	116.66	2.494	0.93	0.28	31.81	5.40	V
RA33	35.00	130.00	1.219	1.28	0.53	43.26	8.90	VI
RZ11	46.70	90.00	7.206	1.65	0.46	69.96	12.56	IV
RZ21	48.70	126.67	1.707	1.25	0.55	41.75	9.63	VI
RZ22	56.70	140.00	5.008	1.47	0.49	61.30	11.27	IV
RZ23	69.30	143.33	1.273	1.37	0.56	44.25	9.46	IV
RZ32	43.00	163.33	0.766	0.68	0.21	19.86	3.34	V
RZ33	126.70	126.66	3.923	0.87	0.38	35.58	8.47	IV
RK11	71.70	73.33	0.564	0.86	0.20	23.74	3.06	VI
RK12	49.00	70.00	4.532	1.31	0.43	53.58	9.59	VI
RK13	42.30	150.00	1.563	0.96	0.32	35.23	5.60	IV
RK21	89.70	123.33	1.355	1.35	0.34	44.55	5.88	V
RK23	101.70	116.66	1.273	1.02	0.33	32.33	5.31	IV
RK31	41.70	30.00	1.760	0.98	0.24	33.52	4.39	IV
RK32	56.70	83.33	2.365	1.10	0.37	40.37	6.22	V
RS3	61.00	46.66	2.779	1.12	0.30	43.90	5.58	IV
RB1	66.00	113.33	1.043	1.19	0.42	44.74	8.06	IV
RB2	50.00	70.00	4.331	1.37	0.34	57.13	7.89	III
RB3	43.30	76.66	3.978	1.29	0.33	51.21	6.77	0

* Group number of isolates infected with the same number of bacteriophages.

that *Rhizobium*–inoculation significantly increased the number and dry weight of nodules of broad bean cultivated in newly reclaimed soil of Egypt. They showed that *Rhizobium*-inoculation, combined with the highest rate of P and K, produced the highest number and dry weight of nodules, dry weight of shoot and N-content as well as protein yield.

3. Plant dry weight

Inoculation of broad bean seedlings with either of 32 isolates of *R. leguminosarum bv. vicieae* increased both shoots and roots dry weight as compared with the uninoculated control (Table, 2). Inoculation with isolates RZ11 and RF21 gave the highest shoots dry weight, i.e., 1.65 and 1.55 g/plant, respectively. On the other hand, isolates RR11and RZ23 showed the highest roots dry weight, being 0.60and 0.56 g/ plant, respectively. However the uninoculated control treatment yielded only 0.58 g shoots/plant and 0.19 g roots/plant. These results demonstrated that broad bean plants when inoculated with isolate RZ11 showed the highest plant dry weight as compared to the other isolates, irrespective with the number and weight of nodules which didn't show the highest values. It was observed that nodules produced by RZ11 were large enough to fix more nitrogen which was reflected on the plant dry weights. Previous investigations showed that the number of nodules is not necessarily be correlated with their efficiencies for symbiotic N_2-fixed (Salem, 1962, 1969 and Mishra *et al.*, 1999).

4. Nitrogen content

The effect of inoculation of broad bean seedlings with different isolates of *R. leguminosarum bv. vicieae* on nitrogen content of shoots and roots showed variation in their response (Table, 2). Isolates RZ11 and RF21 showed the highest shoots nitrogen contents being 69.96 and 64.64 mg N / plant, respectively. In addition, isolates RZ11 and RR11 showed also the highest roots nitrogen contents being 12.56 and 12.30 mg N / plant, respectively. The same general trends like those of plant dry weight.

From these results it can be concluded that inoculation with isolate RZ11 gave the highest nitrogen contents in shoots and roots of broad bean plants as compared to plants inoculated with other strains. This could also

emphasize that legumes responded differently when inoculated with different rhizobial-strains isolated from different localities showing varying efficiencies towards the symbiotic N_2-fixation (Salem, 1962 and 1969; Hussein *et al.*, 1997 and Brock, 2003).

In general conclusion, inoculation of broad bean (*Vicia faba*) seedlings with *R. leguminosarum bv. vicieae* isolates increased nodulation, plant dry weight, nitrogen content and nitrogenase activity as compared with uninoculated control treatment. The percent of increases differed from rhizobial-isolate to another depending on the eco-systems of the isolates and the physicochemical properties of the soil. (Alexander, 1977; Russel, 1975; Salem *et al.*, 1981 and Pathak *et al.*, 1997). The highest five isolates of *R. leguminosarum bv. vicieae* in nitrogen fixation were RZ11 and RZ22 isolated from Zagazig and RF21, RB2, and RK12 from Fakous, Belbase and Abo-Kibeer, respectively. From data in Tables 2 and 3, it could observed that the more efficient rhizobial isolates were isolated from nodules of plants grown in soils characterized by being fertile clay soil with normal salinity having EC ranging between 0.93 - 5.60 dsm^{-1} (Table, 3). In this respect Hassan *et al.* (1990) estimated the N_2-fixed by broad bean plants grown in three soil types. They found that the amount of N_2-fixed differed markedly depending upon inoculation treatment, dose of N-fertilizers applied, and soil type. Also, in this respect, inoculation of broad bean plants with isolate no. RH21 (isolated from El-Hessenia) resulted in the lowest no. of nodules (10.3 nod./plant) with relatively low values of dry weight (0.97 mg/plant), nitrogenase activity (2.465 μ mole C_2H_4/h/g nodule dry weight) and nitrogen content (35.6 mg/plant). The latter rhizobial-isolate had been isolated from plants grown in El-Hessenia soil of high salinity (EC 9.4 dsm^{-1}). However, broad bean plants inoculated with rhizobial-isolate no. RH31 showed higher values of nodules number and dry weight, nitrogenase activity and total nitrogen content of the plant, although the inoculated *Rhizobium* was isolated from soil of relatively higher salinity (EC 19.7 dsm^{-1}) than RH21. This suggests that isolate RH31 is relatively resistant to soil salinity and could be used as inoculants in such saline soil.

Table 3: Physical and chemical analysis of soil in different locations of Sharkia governorate

Locations		Physical analysis			Mechanical analysis				Chemical analysis						
									Cations				Anions		
Locations	dem	PH	E.C dsm^{-1}	O.M. %	Sand %	Silt %	Clay %	Type of soil	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	Hco_3^{-}	Cl^{-}	So_4^{-}
Zagazig (Z)	1	7.86	2.67	2.43	33.22	13.91	52.89	Clay	0.55	0.60	1.65	0.07	0.28	0.81	1.70
	2	7.45	0.93	2.79	43.55	13.10	43.31	Clay	0.33	0.31	0.45	0.03	0.41	0.32	0.07
	3	8.09	1.45	2.50	35.0	30.2	43.7	Clay	0.19	0.45	1.31	0.3	0.54	0.42	0.68
Fakous (F)	1	7.51	4.3	1.68	25.5	40.1	34.2	C. loam	5.7	6.9	15.9	0.31	3.6	13.4	11.7
	2	7.50	5.6	0.87	55.1	25.5	19.6	S. loam	10.3	12.8	31.7	0.32	3.0	29.8	22.8
	3	8.00	6.4	1.82	38.8	5.6	55.6	Clay	10.7	11.5	43.5	0.54	3.0	32.6	30.6
Abo-Kibeer (K)	1	7.75	4.8	1.22	58.0	8.1	33.8	S.C. loam	6.8	6.8	34.8	0.4	3.6	8.6	3.9
	2	7.95	1.2	1.59	35.0	30.2	34.7	C. loam	7.1	5.5	3.5	0.5	4.1	41.3	51.7
	3	8.01	8.8	1.52	70.8	7.6	21.6	S.C. loam	11.4	13.8	71.7	0.36	3.1	8.0	3.2
El-Hessenia (H)	1	7.85	1.5	2.08	18.22	7.8	74.00	Clay	5.7	2.7	4.4	0.36	3.1	8.0	3.2
	2	7.85	9.4	1.89	9.58	10.33	80.09	Clay	27.81	26.2	60.90	0.08	2.5	43.2	65.0
	3	7.95	19.7	1.98	34.32	12.97	52.82	Clay	40.0	27.8	119.6	0.5	2.7	132.4	52.8
Kafer-Saker (R)	1	8.20	9.08	1.95	36.0	20.1	43.8	Clay	26.2	23.2	60.9	0.08	2.5	4.32	12.1
	2	7.55	7.20	2.31	40.1	35.2	24.7	Loam	51.5	24.0	7.5	3.2	3.6	9.6	75.7
	3	7.60	32.8	2.13	38.8	7.7	53.5	Clay	29.9	37.8	163.0	1.3	3.5	148.8	79.6
Abo-Hammad (A)	1	7.75	4.8	1.8	32.2	27.6	39.6	Clay loam	6.8	6.3	34.8	0.41	3.4	28.4	16.2
	2	7.5	2.8	1.33	35.0	31.6	33.3	Clay loam	14.8	3.3	3.01	1.9	2.2	8.1	4.2
	3	8.1	8.8	1.96	15.4	24.2	60.1	Clay	11.4	13.8	71.7	0.50	4.1	41.3	51.7
Belbase (B)	1	7.1	7.0	1.22	87.2	1.6	11.2	S. loam	26.2	22.1	34.8	0.46	2.4	38.4	42.7
Salhia (S)	1	7.75	4.2	1.33	89.0	5.7	5.1	Sandy	27.1	15.3	20.1	0.31	2.3	43.3	18.1

5. The phage reaction of rhizobial isolates

Phage typing is a common technique used to discriminate between various strains of *Rhizobium* (Kowalski *et al.*, 1974). Phage typing potentially is more discriminatory than the use of serology and as such can be used to type rhizobia beyond the level of antigenic reaction. The 32 isolates of *Rhizobium leguminosarum bv. vicieae* were shown to fall into six categories when typed with phage isolated from the rhizosphere of broad bean plants in Sharkia governorate. The six categories were: no. susceptibility (RB3 isolate), susceptible to two (RR31), three (RF23, RB2), four (RF31, RH21, RZ33, RZ11, RZ23, RK13, RK31, RK23, RS3 and RB1), five (RR23, RF21, RH31, RH13, RA21, RZ32, RK21, RK32) and the rest with all six phages.

No trend was observed between phage infection and ability of the isolates to nodulate broad bean and fix atmospheric nitrogen.

Rhizobiophages have been detected in soils of many countries

and are usually associated with legumes (Vincent, 1977). Some reports suggested that rhizobial phages are present in the rhizosphere of legumes and are absent in non-rhizosphere soils, (Golepiowska *et al.*, 1971 and 1976). Also, in Egypt rhizobiophage were found to be common in the Nile Valley soils cultivated with leguminous plants (Emam *et al.*, 1983; Othman, 1986; El-Didamony, 1990 and Salama, 1992) The presence of rhizobiophage in fields, therefore, suggest that they could play an important role in selection, propagation or elimination of *Rhizobium* genotypes in nature (Vincent, 1977).

B. Testing the Efficiency of *Azotobacter* and *Azospirillum* Isolates in Liquid Culture

Acetylene reduction assay was set up in liquid culture to measure the efficiency of nitrogenase activities of 14 different isolates of *Azotobacter* and 18 isolates of *Azospirillum* (Table, 4). Nitrogenase activity of *Azotobacter* isolates ranged between 0.69 (in case of isolate ZR22) and 52.44 μ mole C_2H_4/ml/h (in case of isolate ZH21). Isolates ZH21, ZZ23, ZZ13 and ZH31 showed the highest nitrogenase activity being 52.44, 40.27, 33.83 and 29.32 μ mole C_2H_4/ml/h, respectively. On the other hand, isolates ZR22, ZZ11 and ZH11 resulted in the lowest nitrogenase activity being 0.69, 12.04, and 13.64 μ mole C_2H_4/ml/h, respectively. The highest five isolates of *Azotobacter* spp. in nitrogen fixation were ZZ23, ZS3, ZH31, ZZ13 and ZH21 (Table, 4). It seams that the isolated *Azotobacter* efficiencies differed from one isolate to another depending on the environmental conditions prevailing the location from which they were isolated. For example *Azotobacter* isolates ZZ23 and ZZ13 which showed high efficiency for N_2-fixation where isolated from fertile clay soil from Zagazig region having relatively high organic matter percentages (2.79 % and 2.43 % respectively). This could be the reason for isolation of *Azotobacter* of high N_2-fixation efficiency from such soil because of the impact of eco-system and synergistic effect of other microbes execrating growth-promoting substances that proliferate high densities of *Azotobacter*. Alexander (1977) stated that a number of environmental factors govern the rate and magnitude of

Table 4: Nitrogenase activity in *Azotobacter* and *Azospirillum* isolates.

Azotobacter isolates	Nitrogenase activity μ mole C_2H_4/h/ml	*Azospirillum* isolates	Nitrogenase activity μ mole C_2H_4/h/ml
ZH21	52.44	ASH21	0.76
ZZ23	40.27	ASF32	0.62
ZZ13	33.83	ASF11	0.51
ZK31	22.83	ASA12	0.47
ZR22	0.69	ASZ11	0.41
ZS3	28.03	ASA23	0.39
ZH31	29.32	ASK23	0.31
ZA11	16.41	ASK33	0.08
ZK12	15.26	ASB1	0.06
ZF22	25.64	ASZ33	0.06
ZF31	27.94	ASH32	0.16
ZA31	26.98	ASA33	0.09
ZH11	13.64	ASF12	0.10
ZZ11	12.04	ASR31	0.06
		ASS2	0.02
		ASS3	0.01
		ASK21	0.04
		ASS1	0.03

non-symbiotic N_2-fixation, and transformation is markedly affected by the physical and chemical characteristics of the habitat.

Another eco-factor could affect the proliferation of *Azotobacter* and their efficiencies for N_2-fixation is that of the Ca-content of the soil used for isolation. For example isolate ZH21 of *Azotobacter* which showed the highest nitrogenase activity (52.44 μ mole c_2H_4/h /ml) was isolated from El-Hessenia soil having high Ca content (27.81 %). In this regard Alexander, (1977) reported that a requirement for calcium has been demonstrated during N_2-assimilation by the blue green alge and some species of *Azotobacter*, but the calcium can sometimes be replaced by strantium.

On the other hand, the eco-factor could also deleteriously affect the efficiency of isolated *Azotobacter* if salinity is prevailing the soil used for its isolation (Russell 1975; Bashan and Holguin 1997 and Subba Rao, 1999). In this respect, isolate ZR22 of *Azotobacter* which showed lower nitrogenase activity was isolated from Kafr Saker soil which is characterized by being saline soil (EC 7.2 dsm^{-1}) with relatively low organic matter percentage (2.13 %).

Concerning *Azospirillum* isolates nitrogenase activity ranged between 0.01 (isolate ASS3) and 0.76 μ mole C_2H_4/h/ml (isolate ASH21). Isolates ASH21, ASF32 and ASF11 showed the highest nitrogenase activity values being 0.76, 0.62 and 0.51 μ mole C_2H_4/h/ml, respectively. However, isolates ASS3, ASS2 and ASS1 reflected the lowest values of nitrogenase activity being 0.01, 0.02 and 0.03 μ mole C_2H_4/ml/h, respectively. The highest five isolates of *Azospirillum* spp. in nitrogen fixation were ASH21, ASF32, ASF11, ASA12 and ASZ11 (Table, 4).

Azospirillum isolates showed also some correlations between location of their isolation and their efficiencies in fixing nitrogen in liquid culture. It was also observed that the efficient isolates were isolated from fertile clay soil of either Zagazig region (ASZ11) or Fakous (ASF11 and ASF32) or El-Hessenia (ASH21).

Here, also an additional proof that efficiency for N_2-fixation of *Azospirillum* isolates is correlated to some extent with the ecosystem and habitat of these organisms, in addition to the physico-chemical properties of the soil they survived, (Döbreiner, 1974; Okon, 1984 and Okon *et al.*, 1995).

C. Determination of Growth-promoting Substances in Liquid Cultures of *Rhizobium*, *Azotobacter* and *Azospirillum* Isolates

The highest five N_2-fixing isolates of each of *R. leguminosarum bv. vicieae*, *Azotobacter* spp. *and Azospirillum* spp were used for determination of GA_3, IAA and IBA in liquid culture.

1. GA_3, IAA and IBA in *Rhizobium* isolates

Data in Table 5 indicate that the highest GA_3 content in culture filtrate was obtained by rhizobial-isolate RZ11 (48.398 μg/100 ml), followed by RK12 isolate (25.825 μg/100 ml). The lowest content of GA_3 in culture filtrate was obtained by RF21 isolate (5.865 μg/ 100 ml). In addition, the highest content of IAA in culture filtrate was obtained by RK12 isolate (0.22 μg/100 ml) followed by RZ22 isolate (0.17 μg /100 ml). While, the lowest content of IAA in culture filtrate was obtained by RF21 isolates (0.00 μg/ 100 ml). However, isolates RF21 and RZ22 produced small

Table 5: GA_3, IAA and IBA in liquid culture of *Rhizobium leguminosarum bv. vicieae*, *Azotobacter spp.* and *Azospirillum spp.* isolates

Isolates	GA_3 (µg/100ml)	IAA (µg/100ml)	IBA (µg/100ml)
	***Rhizobium* isolates**		
RF21	5.865	0.00	29.96
RZ22	10.614	0.17	18.93
RK12	25.825	0.22	0.00
RB2	9.507	0.12	0.00
RZ11	48.398	0.10	0.00
	***Azotobacter* isolates**		
ZZ23	2.164	0.04	0.00
ZS3	0.933	0.06	0.00
ZH31	1.071	0.04	0.00
ZZ13	10.712	0.04	0.00
ZH21	17.735	0.15	0.00
	***Azospirillum* isolates**		
ASF32	118.416	1.07	362.48
ASA12	17.973	0.93	22.20
ASZ11	17.943	1.05	111.42
ASF11	16.208	0.15	1480.28
ASH21	48.518	1.91	15174.85

amounts of IBA being 29.96 and 8.93 µ g/100 ml, respectively. However, IBA was not detected in culture filtrate of RK12, RB2 and RZ11.

2. GA_3, IAA and IBA in *Azotobacter* isolates

Data in Table 5 indicated that production of GA_3 in *Azotobacter* spp. isolates generally, ranged between 0.933 and 17.735 µg/100 ml. The highest content of GA_3 in culture filtrate was obtained by ZH21 (17.735 µg/100 ml) followed by ZZ13 isolate (10.712 µg/100 ml). The lowest content was obtained by ZS3 isolate (0.933 µg/ 100 ml).

The culture filtrate content of IAA produced by *Azotobacter* isolates generally, ranged between 0.04 and 0.15 µg/100 ml. The highest content of IAA in culture filtrate was obtained by isolate ZH21 (0.15 µg/ 100 ml). The lowest content was obtained by isolates ZZ23, ZZ13 and ZH31 (0.04 µg/100 mlof each). However, the culture filtrates of all examined *Azotobacter* isolates were IBA free.

In this respect Taler and Wong (1989) reported that *Azotobacter*

vinelandii can produce cytokinine like substance in culture medium.

3. GA_3, IAA and IBA in *Azospirillum* isolates

Data in Table 5 indicate that production of GA_3 in *Azospirillum* spp. isolates generally, ranged between 16.208 and 118.416 µg/ 100 ml. The highest value was obtained by ASF 32 isolate (118.416 µg/ 100 ml), followed by isolate ASH21 (48.518 mg/100 ml), while, the lowest content was obtained by isolate ASF11 (16.208 µg/ 100 ml).

IAA of culture filtrate in *Azospirillum* isolates generally, ranged between 0.15 and 1.91 mg/ 100 ml. The highest content was obtained by isolates ASH 21 (1.91 mg/ 100 ml), followed by ASF32 (1.07 mg/ 100 ml), while the lowest content was obtained by isolate ASF 11 (0.15 mg/ 100 ml).

However, the culture filtrate content of IBA generally, ranged between 22.20 and 15174.85 µ g/100 ml. The highest content was obtained by isolate ASH21 (15174.85 µ g/100 ml) followed by ASF11 (14802.28 µ g/ 100 ml). However, the lowest content of IBA was obtained by isolate ASA12 (22.20 µ g/ 100 ml).

In this respect Tien *et al.* (1979) reported that *Azospirillum* in culture produces plant growth-promoting substances. Hartmann *et al.* (1983) obtained IAA over producing mutants of *Azospirillum* in culture media. Baca *et al.* (1994) stated that indole-3-acetic acid (IAA) is excreted by different wild strains of *Azospirillum* spp. They added that microorganisms can produce IAA during the late stationary growth phase and significant increase in IAA production occurred when tryptophan is added. Also, Dobbelaera *et al.* (1999) reported that auxine produced by *Azospirillum* is believed to play a major role in the plant growth-promoting effect.

These results suggest that isolate RZ11 of *R. leguminosarum bv. vicieae* was the highest efficient isolate in terms of plant growth, nodulation, nitrogenase activity and production of growth-promoting substances. In addition, isolate ZH21 of *Azotobacter* spp. reflected the highest nitrogenase activity and production of growth-promoting substances. Moreover, isolate ASH21 of *Azospirillum* spp. showed also the highest nitrogenase activity and production of growth-promoting substances.

The three isolates RZ11, ASH21 and ZH21 of *R. leguminosarum bv. vicieae*, *Azospirillum* spp. and *Azotobacter* spp., respectively were chosen as the best indigenous isolates to be used as inoculants for seedlings of broad been and wheat plants in further studies .

Utilization of plant-growth-promoting bacteria in order to minimize fertilizer application should be recommended both economically and also to avoid environmental pollution by excessive applications of industrially produced fertilizers to cultivated fields.

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عزل وانتخاب سلالات محلية عالية الكفاءة من بكتريا الريزوبيوم و الأزوسبيريللم والأزوتوباكتر من أراض بمحافظة الشرقية

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أجريت هذه الدراسه لانتخاب أكفأ العزلات المحلية من بكتريا الريزوبيوم (*Rhizobium leguminosarum bv. vicieae*) وبكتريا الأزوسبيريللم (*Azospirillum* spp.) و بكتريا الأزوتوباكتر(*Azotobacter* spp.) لاستخدامهم كأسمدة حيوية لنباتات الفول البلدي والقمح. وقد تم عزل٦٠ عزلة من بكتريا الريزوبيوم و٦٠عزلة أخرى من بكتريا الأزوسبيريللم و ١٢٠ عزلة من بكتريا الأزوتوباكتر من مواقع مختلفة من محافظة الشرقية. تم انتخاب ٣٢عزلة من بكتريا الريزوبيوم لإجراء اختبار التعقد على الفول البلدي كما تم انتخاب ١٨عزلة من بكتريا الأزوسبيريللم و١٤عزلة من بكتريا الأزوتوباكتر وذلك لتقدير نشاط إنزيم النيتروجينيز في البيئات السائلة. تم استخدام أعلى خمس عزلات من كل من الريزوبيوم و الأزوسبيريللم و الأزوتوباكتر التي أوضحت أعلى قيم في تثبيت النيتروجين في إنتاج حمض الجبريليك (GA3) و إندول حمض الخليك (IAA) و إندول حمض البيوتيريك (IBA) في البيئات السائلة. و أثبتت الدراسة أن العزلة RZ11 كانت أعلى عزلات الريزوبيوم كفاءة في التأثير على نمو النبات والتعقد ونشاط إنزيم النيتروجينيز و إنتاج الهرمونات النباتية، بالإضافة إلى ذلك فأن العزلة ASH21 من بكتريا الأزوسبيريللم و العزلة ZH21 من بكتريا الأزوتوباكتر كانت أعلى العزلات في نشاط إنزيم النيتروجينيز و إنتاج منظمات النمو. ولذلك سوف ستخدم هذه العزلات كأسمدة حيوية في دراسات لاحقة.