SURVIVAL OF *AZOSPIRILLUM BRASILENSE* INOCULATED INTO CARRIER MATERIAL USING THE IMMUNE-ENZYMATIC ELISA TECHNIQUE

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Abstract

Immune-enzymatic ELISA protocol was used to clarify the specificity of the polyclonal antiserum (anti-*Azospirillum brasilense* NO40) against *Azospirillum brasilense* strains WAS123 and WAS220.The optimal conditions for the serological characterization of *Azospirillum brasilense* strains resulted from this experiment were, bacterial count of 10^6 as it was the density that gave maximum optical density readings and the polyclonal antibodies dilution 10^{-3} as it gave full saturation for coating the cells.

Detection of the survival of *Azospirillum brasilense* strains WAS123 and WAS220 beside NO40 as a reference strain, inoculated into carrier composed of fine peat (25%), vermiculite (75%) and $CaCO_3$ (5%) using ELISA technique at five intervals (1, 7, 14, 30 and 60 days) after inoculation. All strains were capable to survive for 60 days.

In a pot experiment, the effect of *Azospirillum brasilense* strains WAS123 and WAS220 compared with *Azospirillum brasilense* Sp7 and NO40 as reference strains on rice cv: Giza 172 at different growth periods 30, 60, 90 and 120 days from transplanting was studied. The initial density of the mass cultures of all bacterial strains used was 10^8 cells/ml.

A good response of rice plant to inoculation with *Azospirillum brasilense* WAS220 was noticed. Inoculation had the highest effect on rice plant growth, followed by strain WAS123 and NO40 compared with the uninoculated control. *Azospirillum brasilense* Sp7 showed no effect on rice plants.

INTRODUCTION

Rice is considered the base of the nutrition of nearly half of the world population especially in the countries that suffered from high shortage of food. In Egypt, chemical fertilizers are heavily used to maintain the soil fertility and to ensure crop production. Biological dinitrogen fixation is the most important alternative for the ever-increasing demands of N fertilizers.

Immunological methods can be used for the identification, quantification and enrichment of specific bacteria in extracts and for the visualization of cells *in situ*. To improve the detection level, a simple time-limited, liquid-enrichment procedure was

developed, based on limited multiplication of *A. brasilense* in conventional semisolid medium and counting the bacteria in the enriched medium by ELISA or by most probable number (MPN) techniques. The method can be used as a complementary procedure to ELISA and MPN techniques when low numbers of *A. brasilense* are present in the roots (Bashan *et al.*, 1991).

The main aims of this study are: 1) determination of the sensitivity of antiserum-NO40 to antigens of the selected N_2 fixing bacteria isolated from rice plant by ELISA technique. 2) detection of some N_2 fixing bacteria inoculated in the carrier (inoculant) using ELISA technique. In addition, a pot experiment was conducted in a greenhouse to investigate the effect of inoculation by some nitrogen fixing bacteria on rice plant.

MATERIALS AND METHODS

Materials used

The soil used in this study was collected from Sakha Agricultural Research Station (Kafr El-Sheikh Governorate). The soil was clay, non saline and the pH was 8.1.

Seeds of rice cultivar Giza 172 were kindly supplied by the Crops Research Institute of the Agricultural Research Center (ARC). *Azospirillum brasilense* strains were obtained from Soils, Water and Environment Res. Inst., ARC. The reagents and materials were used in ELISA techniques are according to Levanony and Bashan (1990).

Preparation of Bacteria

The Bacterial cells were prepared and centrifuged at 10.000 rpm for 20 minutes. The obtained precipitants were washed with phosphate buffer solution (PBS) to be freezed well at – 80 °C, without any loss of their antigenic properties. The count of bacteria was carried out by direct enumeration on Thoma slide (Mavingui, 1992).

Procedures of Immune-Enzymatic Reactions

ACP-ELISA: protocol used was according to that proposed by Mavingui (1992). The adsorption of the antigens on the surface of the wells (coating) was done according to this the protocol.

AB–ELISA: protocol used was adapted according to Levanony and Bashan (1990). Finally, the revelation of the enzymatic activity was identical to that of ACP–ELISA protocol.

Sensitivity of antiserum–NO40 to antigen of Azospirilla

Antiserum-NO40 was used in all immune-enzymatic reactions (ELISA) against antigens of strains WAS123 and WAS220. Procedures of ELISA were conducted as previously recorded by Gouzou (1992).

Survival of bacterial strains inoculated into carrier

The carrier material was composed of vermiculite (75 %), fine peat (25 %) and $CaCO_3$ (5 %). This carrier was gamma irradiated at 2.5 M Rad. The carrier was inoculated with *Azospirillum brasilense* NO40 (as a reference strain), *Azospirillum brasilense* WAS123 and WAS220 strains. These strains were grown on LB medium to obtain mass cultures (10^8 cells/ml). Inoculum size was 100 ml / 300 gm carrier material. Carrier bags were stored at room temperature for two months. Survivals of bacterial cells in the carrier bags were detected by ELISA technique at five intervals (1, 7, 14, 30 and 60 days) after inoculation. The dilution of antiserum-NO40 used was 10^{-3} (diluted in washing buffer).

Inoculation of rice by *Azospirillum brasilense*: Each pot was filled with 7 kilograms soil. Urea (46.5 % N) as a mineral nitrogen fertilizer was added as the recommended rate of 40 kg N/feddan. The experimental design was complete randomized design. The treatments used were as follows

1- Control (Uninoculated).
2- Inoculation with *Azospirillum brasilense* strain WAS123.
3- Inoculation with *Azospirillum brasilense* strain WAS220.
4- Inoculation with *Azospirillum brasilense* strain NO40.
5- Inoculation with Azospirillum *brasilense* strain 5p7.

Bacterial strains were grown in nutrient broth medium for 24 hours at 32 °C to obtain maximum density (10^8 cells /ml). These suspensions of cells were centrifuged at 4000 rpm for 30 minutes at 5 °C for three successive times. Between these centrifugations, the pellets were washed with physiological saline solution (0.08 % KCl). Finally, the washed pellet of each strain was suspended in 200 ml of the physiological saline solution to be counted on Thoma slide and inoculated into pots (10^8 cells/ml).

Samples for analysis were taken after 30, 60, 90 and 120 days after transplanting of rice plants. Dry weights of rice shoots were determined. Nitrogen uptake in shoots was determined by Kjeldahel method as described by Jackson (1967). Total bacterial count and *Azospirillum* count in the rhizosphere of rice plants were determined according to MPN method (Vincent, 1970). Statistical analysis was done according to Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

Sensitivity of antiserum-NO40 to antigen of *Azospirillum brasilense* strain WAS123

Data presented in Table 1 show that: in case of using dilution 10^{-3} of antiserum (anti-NO40), the optical density (O.D) increased with the increasing of the bacterial count. There was no significant difference between the values of optical density and the bacterial log 106, 107 and 10^8 (O.D were 1.584, 1.583 and 1.583 respectively). In case of using dilution 10^{-4} of anti-NO40, there was no reading for optical density with the bacterial count from 10^1 to 10^4. There was no significant difference between the values of optical density with the bacterial count 106, 107 and 108 (O.D were 1.637, 1.644 and 1.649 respectively).

Sensitivity of antiserum-NO40 to antigen of *Azospirillum brasilense* strain WAS220

Data presented in Table 2 show that in case of using dilution 10^{-3} of antiserum (anti-NO40), there was no significant difference between the values of optical density (O.D) and bacterial log 10^6, 10^7 and 10^8 (O.D were 1.499, 1.491 and 1.489 respectively). In case of using dilution 10^{-4} of anti-NO40, there was no reading for optical density with the bacterial count from 10^1 to 10^4. There was no significant difference between the values of optical density with the bacterial count 10^6, 10^7 and 10^8 (O.D were 1.549, 1.570 and 1.557 respectively). It could be concluded that ELISA protocol was modified to fit the serological characterization of *Azospirillum brasilense* strains. The anti-NO40 was highly specific to the antigen of *Azospirillum brasilense* strains of WAS123 and WAS220.

Table 1. Sensitivity of antiserum – NO40 to antigen of *Azospirillum brasilense* strain WAS123 using ELISA technique

Log number of bacteria/well	1	2	3	4	5	6	7	8
Dilution of Anti- NO40	10^{-3}							
Absorbance at 405 nm	0.229	0.200	0.109	0.157	0.615	1.642	1.638	1.638
	0.131	0.126	0.100	0.099	0.512	1.608	1.608	1.604
	0.156	0.186	0.130	0.083	0.585	1.502	1.502	1.506
Mean (O.D)	0.172	0.171	0.113	0.113	0.571	1.584	1.583	1.583
Standard Error	0.084	0.065	0.025	0.06	0.088	0.12	0.119	0.113
Dilution of Anti- NO40	10^{-4}							
Absorbance at 405 nm	0	0	0	0	0.269	1.566	1.577	1.560
	0	0	0	0	0.216	1.630	1.659	1.658
	0	0	0	0	0.302	1.716	1.697	1.728
Mean (O.D)	0	0	0	0	0.262	1.637	1.644	1.649
Standard Error	0	0	0	0	0.072	0.125	0.102	0.140

This occurred because both *Azospirillum brasilense* NO40 and *Azospirillum brasilense* WAS220 are taxonomically related. They were isolated from rice plant cultivated in Sakha soil and the similarity of NO40, WAS123 and WAS220 to the original profile of *Azospirillum brasilense* is within the ratio of 82.29 % and 64.58 % respectively in the BIOLOG bacteria identification technique. The optimal conditions for the serological characterization of *Azospirillum brasilense* strains were, (a) bacterial count of 10^6 as it was the density that gave maximum optical density (O.D) reading.

Table 2. Sensitivity of antiserum – NO40 to antigen of *Azospirillum brasilense* strain WAS220 using ELISA technique

Log number of bacteria/well	1	2	3	4	5	6	7	8
Dilution of Anti- NO40	10^{-3}							
Absorbance at 405 nm	0.268	0.227	0.123	0.189	0.708	1.540	1.597	1.572
	0.147	0.149	0.120	0.114	0.593	1.504	1.464	1.490
	0.173	0.207	0.147	0.094	0.653	1.453	1.411	1.404
Mean (O.D)	0.196	0.194	0.130	0.132	0.651	1.499	1.491	1.489
Standard Error	0.106	0.067	0.024	0.083	0.095	0.071	0.158	0.140
Dilution of Anti- NO40	10^{-4}							
Absorbance at 405 nm	0	0	0	0	0.321	1.517	1.509	1.476
	0	0	0	0	0.265	1.519	1.572	1.589
	0	0	0	0	0.358	1.612	1.628	1.606
Mean (O.D)	0	0	0	0	0.315	1.549	1.570	1.557
Standard Error	0	0	0	0	0.078	0.090	0.099	0.116

(b) The polyclonal antibodies (PcAbs) dilution of 10^{-3} instead of 10^{-4} as it gave full saturation for coating the cells. These optimal conditions for best results of ELISA technique are in accordance with those of El-Haddad *et al.* (2000).

Survival of azospirilla inoculated into carrier by ELISA technique

Data recorded in Figure 1 present the survival of bacterial strains in the carrier after one day of inoculation and showed that, in case of strain NO40, there was no significant difference between the values of optical density and the bacterial counts, while in case of both *Azospirillum brasilense* strains WAS123 and WAS220, the optical density increased with the increase of the bacterial count. Results indicated that, after one day of carrier inoculation the bacterial strains were still alive and adapted to the carrier that composed of 75 % vermiculite and 25 % fine peat (Omar and Hassan, 1994).

Data presented in Figures. 2, 3, 4 and 5 clarify the survival of bacterial strains in the carrier after 7, 14, 30 and 60 days of inoculation with all inoculated strains (NO40, WAS123 and WAS220). There was a gradual increase in the optical density (O.D) with increase in the bacterial count. This means that after 7, 14, 30 and 60 days of carrier inoculation the bacterial strains were still viable in the carrier. This means that *Azospirillum brasilense* strains were capable to adapt themselves to live in the carrier for 60 days. The values of optical density indicated that no significant decrease in the five intervals (1, 7, 14, 30 and 60 days) after inoculation of strains. These results are in line with that of Omar and Hassan (1994).

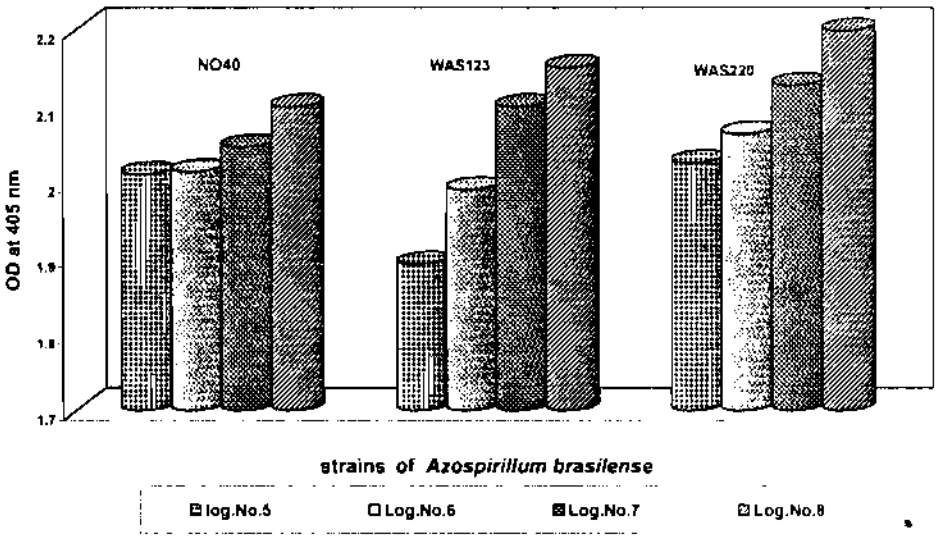

Figure 1. Identify of azospirilla inoculated in the the vermiculite carrier bags under laboratory conditions by using ELISA technique after 1 day from inoculation of bags.

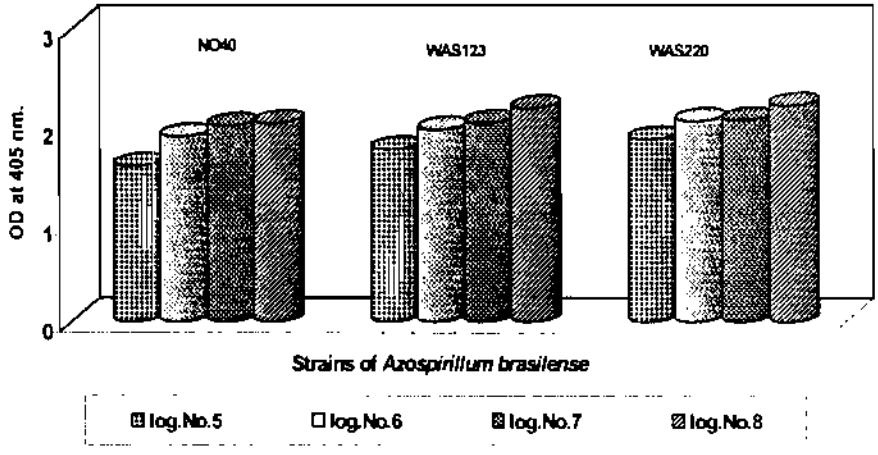

Figure 2. Identify of azospirilla inoculated in the vermiculite carrier bags under laboratory conditions by using ELISA technique after 7 days from inoculation of the bags.

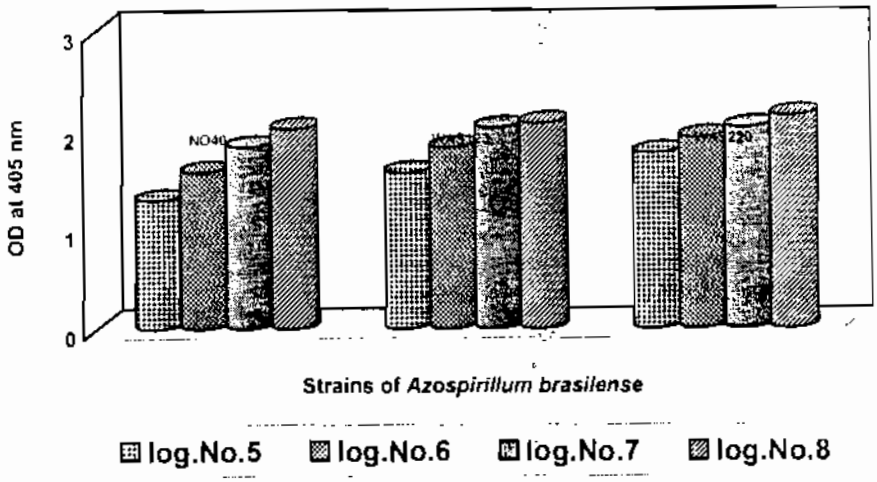

Figure 3. Identify of azospirilla inoculated in the vermiculite carrier bags under laboratory conditions by using ELISA technique after 14 days from inoculation of the bags .

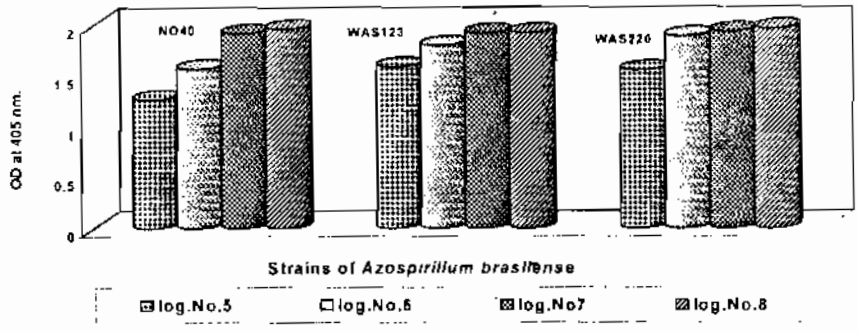

Figure 5. Identify of azospirilla inoculated in the vermiculite carrier bags under laboratory conditions by using ELISA technique after 60 days from inoculation of the bags.

Data presented in Table 3 show the dry weight of shoots of rice plants in four growth periods (30, 60, 90 and 120 days) after transplanting of rice plants. It was clear that in all plant growth periods, the highest dry weight of shoots was recorded in the treatment inoculated with WAS220 (5.97, 9.14, 9.70 and 10.63 g, respectively), then in the treatment inoculated with WAS123 (4.47, 8.46, 9.13 and 9.91 g, respectively) compared with the uninoculated control (4.54, 5.48, 5.41 and 6.53 g) respectively and the reference strain NO40 (4.36, 5.6S, 5.77 and 6.14 g, respectively). The treatment inoculated with the reference strain Sp7 did not show any positive effect on the dry weight of shoots (4.65, 5.22, 4.88 and 5.05 g, respectively). Results

revealed that the dry weight of both shoots and roots tended to increase with inoculation by *A. brasilense* WAS220 and WAS123 over those of control and reference strain NO40 specially at the flowering stage (90 days of transplanting) and heading stage (120 days after transplanting). These results are almost similar to those obtained by Farag (1998). The increase in dry weight of shoots and roots due to inoculation with *Azospirillum brasilense* could be explained by the role of the microorganisms which are supplying the rice roots with their fixed nitrogen and consequently improving the vegetative growth. On the other hand, the inoculation of rice plants by *Azospirillum brasilense* Sp7, which are showed that no response was observed on plant growth.

Table 3. Dry weight of shoots (g/plant) of rice plants, inoculated with azospirilla, with 40 kg of mineral nitrogen fertilizer / feddan.

Periods days after transplanting Treatments	30	60	90	120
Control (uninoculated)	4.54	5.48	5.41	6.53
Inoculated by WAS 123	4.47	8.46	9.13	9.91
Inoculated by WAS 220	5.97	9.14	9.70	10.63
Inoculated by NO 40	4.36	5.65	5.77	6.14
Inoculated by Sp7	4.65	5.22	4.88	5.05
LSD 0.05	1.229	3.709	1.881	2.514

Data presented in Figure 6 show N-uptake in shoots of rice plants in the four plant growth stages (30, 60, 90 and 120 days) after transplanting of rice plants. It is noticed that the N-uptake in shoots was increased significantly by the inoculation with *Azospirillum brasilense* where it was higher than that of the uninoculated control. The highest N-uptake of shoots was recorded in the treatment inoculated by WAS220 (96.1, 237.55, 310.11 and 370.45 mg N/plant respectively), then in the treatment inoculated by WAS123 (62.17, 185.18, 288.41 and 314.84 mg N/ plant respectively) and in the treatment inoculated with NO40 (53.71, 75.65, 101.61 and 11.979 mg N/plant respectively) as compared with the uninoculated control (20.56, 28.3, 29.26 and 41.46 mg N/g shoot respectively). The treatment inoculated with the reference strain Sp7 did not show any increase in the N-uptake of the shoots (29.95, 22.39, 23.95 and 15.09 mg N/plant respectively) as compared with the uninoculated control.

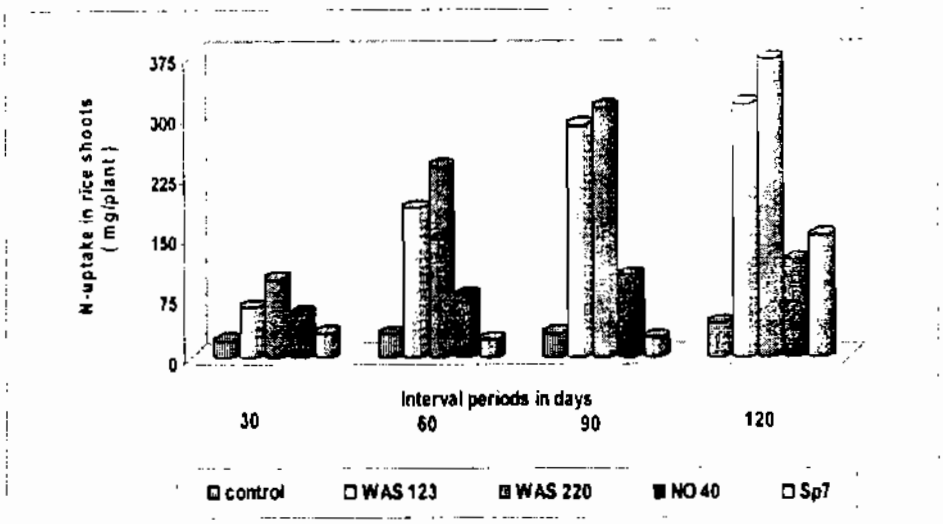

Figure 6. N- uptake in shoots of rice plants,inoculated with azospirilla with 40 kg of mineral nitrogen fertilizer/fed.

The results in this experiment could conclude the effect of *Azospirillum brasilense* inoculation on the N-uptake in shoots of rice at the four plant growth stages. The results revealed that N-uptake in shoots tended to increase with inoculation by *A. brasilense* WAS220, WAS123 and NO40 respectively over those of uninoculated control at the four plant growth stages (30, 60, 90 and 120 days) after transplanting of rice plants. The positive effect of *Azospirillum brasilense* may be due to the nitrogen fixed by it and in turn its beneficial effect on the shoots nitrogen content. These results are in line with Omar (1995).

Data presented in Table 4 show that in all plant growth stages (30, 60, 90 and 120 days) after transplanting of rice plants, the total bacterial count and *Azospirillum* count in the rhizosphere were increased by inoculation with *Azospirillum brasilense*, where they were higher than those of uninoculated control. The percentage of azospirilla to the total number of bacterial cells showed an increase in the inoculated treatments than that of the uninoculated control indicating the good colonization of *Azospirillum* spp. in the rhizosphere of rice. The highest percentage (%) of *Azospirillum* cells was recorded in the treatment inoculated with WAS220 in the four plant growth stages (4.44 %, 22.36 %, 31.67 % and 36.21 % respectively) and in treatment inoculated with WAS123 (4.17 %, 10.00 %, 25.00 % and 30.30 % respectively), then in treatment inoculated with NO40 (3.00 %, 5.00 %, 16.36 % and 18.00 % respectively). The lowest percentage of *Azospirillum brasilense* cells was recorded in the treatment inoculated with Sp7 in the four plant growth stages (2.67 %, 3.00 %, 5.00 % and 4.63 % respectively) as compared with the uninoculated

control (0.50 %, 1.67 %, 2.17 % and 3.33 % respectively). These results are in complete harmony with those obtained by Hammouda and Afify (2000).

The changes in population of azospirilla were clearly noticed and the results revealed that the population was increased due to the inoculation by *Azospirillum brasilense*. Hence, the results of the present study suggest that root exudation; generally play an important role in the colonization of the rhizosphere by *Azospirillum* spp.

Table 4. Log number of total bacteria ,log number of azospirilla and % of azospirilla in total bacterial count in inoculated rice plants with 40 kg N/fed at different interval periods in days .

Treatments	Log No.Of total bacteria				Log No. of azospirilla				% of azospirilla / total bacteria			
periods in days	30	60	90	120	30	60	90	120	30	60	90	120
Control uninoc	9.69	9.95	10.3	10.2	7.39	8.17	8.7	8.7	0.50	1.67	2.1	3.3
Inoc byWAS123	10.77	10.98	11.3	11.2	9.39	9.97	107	10.7	4.17	10.0	25.0	30.3
Inoc.by WAS220	10.95	11.24	11.5	11.5	9.60	10.60	10.9	11.0	4.44	22.86	31.7	36.2
Inoc.by NO40	10.74	10.90	11.0	11.0	9.22	9.60	10.2	10.3	3.00	5.0	16.4	18.0
Inoc.by Sp7	9.88	10.0	10.6	10.7	8.30	8.47	9.3	9.3	2.67	3.0	5.0	4.6

This is supported by the observations of Martin *et al.* (1989). They reported that, the extra nitrogenase activity and more establishment of inoculated *Azospirillum* spp. in the rhizosphere soil and roots of several grasses were roughly proportional to the concentration of the exuded photosynthates and presumably to the microbial population density and nitrogen-fixing microorganisms in particular.

The results of the pot experiment exhibited a good response of rice plants to inoculation by *Azospirillum brasilense*. However, these results are supposed to be attributed to the synergetic effects of the association of plant with *A. brasilense* that play an important role in the superiority of N uptake by *Azospirillum* inoculated plants.

Similarly, Heulin *et al.* (1987) observed that, the *A. brasilense* colonized in the rhizosphere of rice caused an increase in exudations, (17 %), compared with control.

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تقييم لقاح الازوسبيريللم مع نباتات الأرز باستخدام طريقه الأليزا المناعية

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استخدم تكنيك الاختبار المناعي (الاليزا) في هذه الدراسة لكشف و تحديد تخصص الانتيسيرم المتعدد لسلالة ازوسبيريللم برازيلينس NO40 للتوافق مع السلالات WAS123 و WAS220. اظهر الانتيسيرم الخاص بسلالة ازوسبيريللم برازيلينس NO40 تخصصا مرتفعا جدا للانتيجين من كلا السلالتين WAS123 و WAS220 حيث أن كليهما مع السلالة المرجعية NO40 ينتمون تقسيميا إلى نفس الأصل الوراثي و كذلك تم عزلهم من نفس العائل النباتي و هو نبات الأرز النامي على تربة من ارض سخا بمحافظة كفر الشيخ.كانت الظروف المثلى للصفات السيرولوجية لسلالة ازوسبيريللم برازيلينس و التي نتجت من هذه التجارب هي: كثافة العدد البكتيري 10^{7} هو العدد الأمثل للقراءة على جهاز الاليزا و كذلك تخفيف الانتيسيرم المتعدد حيث أعطى التخفيف 10^{-3} الحد التشبعي لتغطية الخلايا. حيوية السلالتين ازوسبيريللم برازيلينس WAS123 و WAS220 و كذلك السلالة المرجعية NO40 و التي تم استخدامها في تلقيح الحامل البكتيري المكون من البيت الناعم ٢٥% و الفرميكولايت ٧٥% و كربونات الكالسيوم ٥%. حيث استخدم تكنيك الاليزا فى معرفة حيوية هذه السلالات في الحامل البكتيري على فترات مختلفة من التخزين و هي ١ ، ٧ ، ١٤ ، ٣٠ و ٦٠ يوما بعد التلقيح.و يمكن تلخيص النتائج التي تم التوصل إليها بأن كل السلالات البكتيرية التي استخدمت في التلقيح لها القدرة على التأقلم و الحيوية في الحامل البكتيري المستخدم لمدة ٦٠ يوما حيث ظلت الخلايا البكتيرية حية و لم تتأثر بطول فترة التخزين في الحامل البكتيري و هي فترة مناسبة لتطبيق اللقاح بالحقل أثناء الموسم الزراعي.

أقيمت تجربة أصص لدراسة تأثير التلقيح البكتيري بالسلالات البكتيرية ازوسبيريللم برازيلينس WAS123 و WAS220 مقارنة بالسلالات المرجعية ازوسبيريللم برازيلينس Sp7 و NO40 على نبات الأرز صنف جيزة ١٧٢ على فترات نمو مختلفة (٣٠ ، ٦٠ ، ٩٠ و ١٢٠ يوما من شتل الأرز) و كانت كثافة العدد البكتيري للخلايا المستخدمة 10^{8} خلية/سم^{3} و استخدمت اليوريا كسماد ازوتى معدني بمعدل ٤٠ كيلوجرام نيتروجين/فدان.

أظهرت النتائج أن هناك استجابة عالية للتلقيح البكتيري باستخدام سلالة ازوسبيريللم برازيلينس لنباتات الأرز.

وسجلت السلالة WAS220 أعلى تأثير على صفات النمو في نباتات الأرز التي اختبرت في هذه التجربة.

وتبعتها السلالة WAS123 و أخيرا السلالة المرجعية NO40 مقارنة بالمعاملات غير الملقحة. بينما أظهرت السلالة المرجعية ازوسبيريللم برازيلينس Sp7 تأثيرا سلبيا على نباتات الأرز حيث أنها غير متلائمة مع نباتات الأرز و غير متلائمة مع الأراضي المصرية.