J. Agric. Sci. Mansoura Univ., 32 (2): 1391 - 1411, 2007

ISOLATION, CHARACTERIZATION, INTRINSIC ANTIBIOTIC RESISTANCE AND SURVIVAL OF AZOSPIRILLA INTRODUCED AS BIOFERTILIZER IN RHIZOSPHERE SOIL

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ABSTRACT

This experiment was performed to isolate and characterize *Azospirillum* present in Egyptian soils. Intrinsic antibiotic resistance against 11 antibiotic were determined. The ability of *Azospirillum* introduced as seed inoculation, to survive in soil and to colonize the rhizosphere was tested. Wheat, Faba bean and Squash grown in clay soil were inoculated with two antibiotics marked isolates. Introduced bacteria were always detected in high numbers in the rhizosphere of test plants than in soil apart but they generally represented a small fraction less than 3% of total *Azospirillum* in the rhizosphere.
Keywords: *Azospirillum* spp, isolation, characterization, antibiotic resistance

INTRODUCTION

Azospirillum is an important symbiotic N_2-fixing bacterium known to colonize the rhizosphere of some plants, where it promotes plant growth under appropriate conditions. Many investigations were carried out concerning the prevalence and distribution of these bacteria in Egyptian soils in addition to their beneficial role as a rhizosphere inhabitant (Ishac, 1989). However, a relatively limited number of research was undertaken to characterize azospirilla prevailing in Egyptian soils (Eman *et al*. 1984; Girgs, 1985, Gomaa, 1995 and Ali *et al*.,. 2002). Therefore, it was found of importance to gain further knowledge about these indigenous soil bacteria and their distribution in cultivated soils in Egypt. An antibiotic resistance marker permits the selective recovery and enumeration of a known isolate from soil containing a normal mixed microflora. *Azospirillum* spp. has been reported as streptomycin, rifampicin, tetracycline and streptophenicol-resistant (Dobereiner and Baldani, 1979; Murray *et al*., 1990; Mashhoor *et al*., 1993 and Ali *et al*., 2002).

The present research aims to get further information about intrinsic antibiotic resistance (IAR) and maximum tolerable concentration of individual antibiotic to development of antibiotic resistant strains of *Azospirillum* in Egyptian soils.

MATERIALS AND METHODS

1- Isolation and characterization of azospirilla:

Representative azospirilla prevailing in Egyptian soils were isolated from different locations of cultivated lands in many governorates. They were chosen to represent the soils fertility soils and different textural classes and those cultivated with various crops. Isolates were grown in the enrichment

semisolid malate nitrogen free medium (NFM) recommended by Dobereiner *et al*., 1976). Isolates were checked for purity by the examination of Gram stained preparations as well as by streaking on potato-dextrose agar plates. Pure cultures were also tested for nitrogenase (N_2-ase) activity, then inoculated on nutrient agar and preserved at 4°C in the refrigerator and subcultured every 2 weeks. Pure cultures being G^-short rods of spinning motility, able to form fine subsurface white pellicle, producing alkalinity in NFM medium and of different N_2-ase activity were selected.

2- Intrinsic antibiotic resistance (IAR):

Isolates were tested for their ability to resist 11 antibiotics using Oxoid and Bio-EDWIC discs of standard antibiotic concentrations. Nutrient agar was distributed in Petri dishes at the rate of 7 ml and after solidification 5 ml of seeded agar (24 old cultures) was evenly distributed over the surface. The antibiotic discs were placed side down on the seeded agar and gently pressed with the tip of a sterile forceps, then the petri dishes felt in a remain at refrigerator for one hour and incubated at 30 °C for 24 hours. Zones of inhibition developing around the discs were measured to nearest 1mm and mean of three replicates was recorded.

3- Survival of azospirilla, introduced as a biofertilizer, in soil and rhizosphere:

A pot experiment was conducted under the greenhouse conditions to investigate the survival and distribution of azospirilla introduced to soil through inoculation. The used isolates used were highly efficient N_2-fixers, actively motile and adapted to relatively high concentrations of specific antibiotics as a marking criterion.

a- Maximum tolerable concentration of individual antibiotics:

A total of 36 isolates obtained from various origins, differing in N_2-ase activity but tolerating more than four different antibiotics were chosen to adequate them for high concentrations of four antibiotic mixture. Maximum tolerate concentrations of streptomycin, rifampicin, doxycycline and tetracycline was determined by spotting on nutrient agar plates provided with appropriate concentrations of each of the four test antibiotics.

b- Development of antibiotic resistant strains:

From the results of maximum tolerable concentration of individual antibiotics, 15 isolates characterized by their tolerance towards the 4 antibiotics were selected to develop antibiotic marked strains. The gradient plate technique developed by Bryson and Szybalski (1952) was applied.

The procedure was repeated till the strain reached a maximal tolerable concentration. Resistant strain was resuspended in nutrient broth either with or without antibiotics provided maximum concentration. Antibiotic resistant strains were tested again for motility and N_2-ase activity to ensure that they still possess the same characters of the corresponding parents.

c- Experimental design:

A top of 10 cm fertile clay soil obtained from the Experimental Farm, Agric. Res. Center at Giza was air dried, crushed to pass through a 2 mm sieve was distributed in plastic pots at the rate of 2 kg/pot. Soils prepared for cultivating wheat, faba bean and squash were respectively, supplemented with 100, 20, and 150 ppm P as superphosphate (15.5% P_2O_5) and 50, 100 and 100 ppm K as potassium sulphate (48% K_2O). Uncultivated control was provided with 100 ppm P plus 100 ppm K. The fertilizer were throughly mixed with soil before planting and in no case N did. Tap water was added to adjust soil moisture to 70% FC surface, disinfected seeds were sown at the rate of 7 seeds /pot distributed at equal distance from each other and gently pressed to a depth of 0.5 cm. Two antibiotic resistant strains of *Azospirillum* were used for pot inoculation. One day old culture in nutrient broth containing Ca 10^7 CFU/ml was prepared and over head inoculation was carried out. Faba bean seeds were additionally inoculated with 1 ml of 2-days old culture of *Rhizobium leguminosarum* biovar viceae added simultaneously with the *Azospirillum* inoculum. Moreover, unplanted control pots were similarly inoculated with both antibiotic-resistant *Azospirillum* strains added separately in 7 marked locations in the pot. Sampling was carried out at seven-day intervals to determine the number of indigenous and introduced azospirilla in both rhizosphere and soil apart using MPN technique. Enumeration was carried out for each sample using NFM medium of Dobereiner *et al*., (1976) to determine total count of Azospirilla as well as with the same medium provided with known concentration of the four antibiotic mixture tolerated by the introduced *Azospirillum* strains to determine the antibiotic-marked bacterial count. The antibiotic mixture was prepared in distilled water at known concentration, sterilized by filtration and added aseptically before inoculation in amounts sufficient to give to final desired level of antibiotics. Five tubes of each liquid medium were inoculated for each dilution with 1 ml. After 5 days incubation, the positive tubes were recognized by the presence of the subsurface with pellicle and alkalinity production. MPN figures were obtained from Cochran's table (1950).

4- Statistical analysis:

Results were statically analysed according to the procedures outlined by Little and Hills (1977) and Snedecor and Cochran (1980). Treatment means were compared using the least significant Difference (LSD) test (Waller and Duncan (1969) at 5% level of probability.

5- Media:

Nutrient broth (Difco, 1985), Potato agar medium (Dobereiner *et al*., 1976), Nitrogen deficient malate medium (Dobereiner et al., 1976), Enumeration of antibiotic marked Azospirilla carried out in the "persistence of introduced Azospirilla in soils" experiment, was performed by using the same medium but provided with streptomycin, rifampicin, doxycycline and tetracycline in concentration (μg/ml): 1280, 20, 40 and 600 for isolate No.I and 760, 120, 20 and 600 for isolate No. II. Just before inoculation, the filter sterilized antibiotics solution was aseptically added to the sterilized medium at known volume to obtain the final antibiotic required. Yeast malate broth (Day and Dobereiner, 1976).

RESULTS AND DISCUSSION

1- Isolation and Characterization:

All of the 110 isolates formed a fine subsurface pellicle when grown in the nitrogen-deficient malate medium, turning to thick white as growth continues, which is a growth behavior well defined for *Azospirillum*. Microscopic examination of stained preparation revealed that cells were Gram-negative straight or slightly curved short rods. Cultural characteristics almost resembled those mentioned by Tarrand *et al*., (1978) and Dobereiner (1991). Colonies on potato agar became visible after 48 hours being smell whitish, flat with elevated borders and smooth. After one week, colonies turned light pink and became dry wrinkled. Mobility in wet mounts revealed that 18-24 hour old isolates were slightly to actively motile (Table, 1). Tested isolates could be divided in to three groups:
(1) Slow movement; worn-like motility (+), (2) active spiral motility (++) and (3) very active spinning motility (+++). The same groups were previously mentioned in literature to be among the characteristics of *Azospirillum* spp. However, pattern of motility could change as the culture became old (36-48 hrs). As shown in (Table, 1), all of 110 isolates possessed nitrogenase enzyme as they could reduce C_2H_2 to C_2H_4. Nitrogenase activity expressed as n moles C_2H_4/hour/culture widely varied among the isolates. Three levels of the enzymatic activity could be recognized, namely low, moderate and high. From the physiological point of view, several biochemical tests have been carried out by previous investigators to characterize members belonging to genus *Azospirillum*. However, there are three important tests which are frequently applied and were recommended by Tarrand *et al*. (1978); Reinhold *et al*. (1987), Bilal *et al*. (1990) and Dobereiner (1991) as decisive parameters for differentiation between azospirilla. All isolates were therefore, tested for biotin requirement, ability to use glucose as a sole of carbon source applied in nitrogen free semi solid medium of Day and Dobereiner (1976) with some modification as described by Tarrand *et al*. (1978) and acidification of glucose in culture broth under aerobic and anaerobic conditions. Results of the biochemical tests for individual isolates in defined groups according to their characteristics as shown in Table (2).

2- Intrinsic antibiotic resistance (IAR):

Intrinsic antibiotic resistance is one of the important characters found in many groups of microorganisms, which play a role in various kinds of environmental activities. Response towards eleven antibiotics was carried out using the antibiotic disc technique. Detailed information about the extent of sensitivity of isolates towards the different antibiotics is expressed as mean values of inhibition zones in Table (3). Total number of antibiotics resisted by each *Azospirillum* isolate is summarized in column No. 7 in Table 1. All isolates examined were resistant to penicillin G (10 μg) but their response towards the other 10 antibiotics widely varied.

T1

T1

t1

T1

T1

The majority of isolates i.e., 90, 68, 67 and 78% could respectively resist ampicillin (10 μg), chlormphenicol (30 μg), amoxycillin (25 μg) and erythromycin (15 μg). From 45 to 75% of isolates were slightly sensitive (inhibition zone from 3.0 to 7.8 mm) towards streptomycin (10 μg), kanamycin (30 μg), refampicin (30 μg), gentamycin (10 μg), doxycycline (30 μg), and tetracycline (30 μg), while from 7 to 37% of isolates were moderately sensitive towards these antibiotics having a range of inhibition zone of (7.9-11.0 mm). Relatively few number of azospirilla were highly sensitive to the different antibiotics tested where 1-23% of the isolates gave an inhibition zone of more than 11 mm (Fig.1). It is interesting to note that, out of the 105 tested *Azospirillum*, 4 isolates could resisted 2 and 3 antibiotics, respectively. The majority of azospirilla being 24, 35 and 19 isolates representing 23, 33 and 18% of the total number under investigation could resist 4, 5 and 6 antibiotics, respectively, while only 6 isolates (5.7%) resist 7 antibiotics. This results suggests that most of isolates belonging to the genus *Azospirillum* are characterized by their efficient IAR and they can resist a wide spectrum of antibiotics. To find out whether there is a distinct relationship between the property of IAR and the origin of the microorganism, number of sensitive and resistant isolates and their percentage in total isolates was calculated and arranged in four groups namely, root-free soil, rhizosphere soil, washed roots and root interiors (Table 4). All of the 13 isolates obtained from the root free soil were sensitive to streptomycin (10 μg), Kanamycin (30 μg) and rifampicin (30 μg). On the other hand, 5-8, 5-18 and 0-13% of Azospirilla, respectively, isolated from the rhizosphere, washed roots and root interiors were resistant to the aforementioned three antibiotics.

Table 2: Grouping of *Azospirillum* isolates according to certain differentiating characteristics

		biochemical reaction for			
Proposed species	**Isolate number**	**Biotin require-ment**	**Glucose used as sole carbon source**	**Acidification of glucose**	
				aerobic	**anaerobic**
A. Lipoferum					
23 isolates:	28, 29, 34, 49, 52, 54, 55, 66, 68, 69, 70, 73,74, 80, 81, 85, 86, 89, 101, 102, 103, 105,106	+	+	+	+
A. brasilense					
52 isolates :					
(a) 25 isolates	13,14, 15,16, 27, 32, 40, 50, 51, 61, 67, 76, 77, 82, 84, 91, 92, 93, 94, 95, 96, 97, 98, 100, 104	-	-	+	+
(b) 16 isolates	2, 3, 5, 6, 8, 10, 11, 24, 30, 38, 39, 41, 48, 107, 108, 109	-	-	-	-
(c) 6 isolates	12, 31, 33, 83, 59, 44	-	-	+	-
(d) 5 isolates	20, 23, 35, 36, 110	-	-	-	+
Unidentified (I)					
34 isolates:					
(a) 25 isolates	9, 17, 18, 19, 21, 22, 25, 26, 37, 53, 56, 57, 58, 60, 62, 63, 64, 71, 72, 75, 78, 79, 87, 88, 90	-	+	+	+
(b) 6 isolates	1, 4, 7, 43, 45, 47	-	+	+	-
(c) 2 isolates	42, 46	-	+	-	+
Unidentified (II)					
1 isolates:	99	+	-	+	+

Regarding the other tested antibiotics, it could be stated that the percentages of isolates obtained from rhizosphere, washed roots and surface sterilized roots which could resist chloramphenicol (30 μg), amoxycillin (25 μg) or erythromycin (15 μg) tended to be higher than the corresponding on as of the root-free soil isolates. For the remaining four antibiotics, no clear differences could be observed in the percentages of resistant isolates in relation to the source of isolation.Moreover, the magnitude of sensitivity of the non resistant isolates towards ampicillin, gentamycin, doxycycline or tetracycline as indicated by the width of inhibition zones and the calculated standard deviation was, in general, not related to the origin of *Azospirillum* isolation. Comparison between the 11 test antibiotics in their lethal or suppresive effect on *Azospirillum* isolates under investigation, revealed that most inhibitory compounds were tetracycline, rifampicin, streptomycin and doxycycline where about 96, 95, 91% of total isolates showed various degrees of sensitivity towards these compounds, respectively.

From the aforementioned results it could be stated that there is a better chance for the prevalence of antibiotic resistant *Azospirillum* in the vicinity of plant roots than in the soil apart.

Results obtained are supported by the findings of previous investigators who pointed out to the more incidence of antibiotic resistant bacteria in the rhizosphere of some plants that in root – free soil (Baldani and Dobereiner, 1980). The early work of Krasil'nikov (1958) and Katznelson (1965) showed that plant roots can stimulate antibiotic-producing actinomycetes and certain antibiotics could be detected in different concentrations in plant roots and adhering soils. Recently, almost similar openions were given by Dobereiner and Boddey (1981) and Dakora (1985). Incidence, in higher percentages, of antibiotic-resistant *Azospirillum* in rhizosphere and histoplane found in the present study as well as in previous work could be explained by the role of the antibiotic-producing microorganisms in soil. The activity of these microbes in root region can affect the equilibrium between the indigenous sensitive and resistant *Azospirillum* strains. Antibiotic–resistant strains having a better chance to exist in the root region.

3- Survival and distribution of azospirilla, introduced as a biofertilizer in soil and rhizosphere:

Data illustrated by Figs.(2) a and b represent the MPN of total azospirilla determined on the nitrogen deficient malate medium as well as the MPN of each of the introduced isolates determined on the same medium but provided with the appropriate tolerable concentration of antibiotics mixture. Initial count of indigenous azospirilla in the clay soil under investigation was high being 10^5 MPN/g. Counts in the unplanted control decreased with time to attain their lowest number of 2×10^3 /g at the end of the experiment. It is clear shown that rhizosphere soils always harbourd high numbers of these bacteria generally exceeding 10^6 /g during the first 21 days then count tended to decrease to reach their minimum at the end of the experiment.

T3

T3

t3

f1

T4

F2

F2b

Total azospirilla in the rhizosphere soils of the three plants inoculated with either *Azospirillum* isolates were generally higher than the corresponding ones of the uninoculated treatments. These results agreed with Falik and Okon (1996), Solaiman *et al*., (2003) and Hana *et al*., (2005).

Regarding the presence of the introduced antibiotic resistant *Azospirillum* isolates in the rhizosphere soils, showed or indicated that both isolates were invariably encountered throughout the successive periods. Population densities between 10^4 and > 10^5 /g were found to be much higher than in the root-free soils. However, these high numbers represented only a small fraction in total azospirilla in the rhizosphere.

Conclusion

There is a need to reassess the technique applied for introducing selected or manipulated *Azospirillum* strains in soil as biofertilizers.

Distribution, survival and persistence of introduced bacterial strain will be affected by the technique of inoculation besides the pronounced effect of the environmental conditions prevailing, including the population density of the indigenous similar bacteria, which will be competed with the introduced strain. Antibiotic marked strain of *Azospirillum* is recommended as a practical technique in that respect.

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عـزل وتوصـيف الآزوسـبيريللا المقاومـة للمضـادات الحيويـة والمسـتخدمة كلقـاح حيوى فى منطقة الريزوسفير

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قسم بحوث الميكروبيولوجيا الزراعية– معهد بحوث الأراضى والمياه والبيئة– مركز البحوث الزراعية

أجريت هذه الدراسة لعزل وتوصيف بكتريا الأزوسبيريللا فى الأراضى المصرية. تناول البحث أيضاً دراسة مدى قدرة الآزوسبيريللم المميزة بقدرتها على مقاومة المضادات الحيوية على البقاء حية وكذلك توزيع إنتشارها فى التربة وذلك عند إضافتها التربة فى صورة سماد حيوى.

وشملت هذه الدراسة ما يلى:

1- **تم الحصول على عزلات الآزوسبيريللا المتوطنة** فى عديد من الأراضى المصرية بمختلف المحافظات حيث عزلت من التربة الخالية من الجذور والريزوسفير والجذور المغسولة ومن داخل انسجة الجذور وكانت جميع العزلات ذات صفات مورفولوجية ومزرعية متطابقة مع ما هو معروف عن بكتريا الآزوسبيريللم، كما تميزت تلك العزلات بإحتوائها على إنزيم النيتروجينيز.

2- **الخاصية الذاتية لمقاومة المضادات الحيوية:**

- أختبرت عزلات الآزوسبيريللا للخاصية الذاتية لمقاومة المضادات الحيوية بإستخدام طريقة أقراص المضادات الحيوية.
- كانت جميع المزارع المختبرة (105) عزلة مقاومة للبنسلين بتركيز (10 مكيروجرام) فى حين تباينت درجة إستجابتها تجاه عشرة مضادات حيوية أخرى.
- أمكن للغالبية العظمى من العزلات بنسبة 91، 68 ،67، 78% من مقاومة الإمبيسيلين (10ميكروجرام) ، الكلورامفينيكول (30ميكروجرام) ، الأموكسى سيلين (25ميكروجرام)، الإريثروميسين (15ميكروجرام) على التوالى.
- أظهرت نسبة تتراوح من 45 إلى 75% من العزلات حساسية ضعيفة لكل من الإستربتومايسين (10ميكروجرام) ، الكاناميسين (30ميكروجرام) ، فى حين كانت النسبة (7-37%) من العزلات ذات حساسية متوسطة تجاه هذه المضادات الحيوية.
- أظهر عدد من العزلات (1-23%) حساسية شديدة لمختلف المضادات الحيوية المختبرة ويعتبر كل من التتراسيكلين، الريفمبيسين ، الإستربتومايسين والدوكسى سيكلين اكثر المضادات الحيوية تثبيطاً للعزلات المختبرة.
- اظهرت العلاقة ما بين الخاصية الذاتية لمقاومة المضادات الحيوية وبين مواقع عزل تلك العزلات ان هناك فرصة اكبر لتواجد الآزوسبيريللا المقاومة للمضادات الحيوية فى محيط الجذور عنه فى التربة البعيدة عن الجذور.

3- **تجربة الزراعة:**

تم إختبار قدرة الآزوسبيريللم المضافة إلى التربة عن طريق التلقيح الحيوى على البقاء حية والتوطن فى محيط جذور النباتات فقد زرعت بذور القمح والفول والكوسة فى تربة طينية ثم التلقيح بعزلتين من الآزوسبيريللم المقاومة للمضادات الحيوية. أمكن التعرف على تواجد هذه البكتريا فى ريزوسفير النبات وبأعداد عالية، غير أنها لم تكن تمثل إلا نسبة ضئيلة لا تزيد عن 3% من أعداد الآزوسبيريللم الكلية فى التربة.

1392 1393 1394 1395 1396 1397 1398 1399 1400 1401

1402 1403 1404 1405 1406 1407 1408 1409 1410 1411

1392 1393 1394 1395 1396 1397 1398 1399 1400 1401

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