J.Agric.Chem. and Biotech., Mansoura Univ., Vol. 2 (10): 229 - 238, 2011

ACTIVITY OF *Azotobacter* AND *Azospirillum* IN THE RHIZOSPHERE OF ONION PLANT

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ABSTRACT

This study was carried out to evaluate the influence of inoculation with nitrogen fixing bacteria (*Azotobacte*r and *Azospirillum*) that were isolated from onion rhizosphere. The isolates were applied in field experiment to investigate the effect of inoculation with *Azotobacter* and *Azospirillum*, on Nitrogenase activity . Isolation and enumeration of *Azotobacte*r and *Azospirillum* from the soil of onion plants were studied and determination of the enzymatic activities of nitrogen fixing bacteria (nitrogenase) that were isolated from the soil of onion plant during the growing periods at 0, 30, 60, 90, 120 and 150 days after sowing and selected active isolate of *Azotobacter* and *Azospirillum*. The obtained results revealed increases in *Azotobacter* and *Azospirillum* numbers as well as nitrogenase activity in rhizosphere onion regions. Also these activities reflect on onion plant productivity. This study recommended the application of *Azotobacter* and *Azospirillum* as inoculants in onion plant productivity.

Keywords: Isolation *Azotobacter, Azospirillum* , Nitrogenase activity .

INTRODUCTION

Onion plants have gotten some interest on seed-inoculated micro-organisms and it was clear that the asymbiotic biological nitrogen fixation was highly correlated to the existence of specific micro-organisms that posses nitrogenase properties such as stutied by(Hegazi, *et al*., 1980, Shoukri, 2006 and Abd el-Halim 2009) that high nitrogenase activity was obtained by the collected samples of *Azospirillum* from plant rhizosphere (Mohy el- Deen 2002 and Narolia, *et al*., 2006) .Nitrogenase activity in onion soil as measured by acetylene reduction technique was found to be high and that indicates positive correlation between *Azotobacter* and higher plants such as onion (Dobereiner *et al*., 1973 and Chungwoo Kim *et al*., 2005) . Results showed that the isolates of nitrogen fixing bacteria (*Azotobacter* sp. and *Azospirillum* sp.) exhibit a highly activity of nitrogenase enzyme were used as a bacterial inoculants in field experiment , the isolate of *Azotobacter* sp. was 94.09 nmoles C_2H_4/g soil / hr in clayey soil and with the isolate of *Azospirillum* was 10.20 nmoles C_2H_4/g soil / hr in clayey soil . This study aims to find out and enumeration of non symbiotic nitrogen fixing bacteria (*Azotobacter* and *Azospirillum*) from onion rhizosphere, selection of most efficient biological nitrogen fixing bacteria and their influences on onion production at different types of soils.

MATERIALS AND METHODS

Materials:

Onion seeds:

Onion seeds (Giza 6) were kindly supplied by Onion Research Department, Field Crops Research Institute, ARC, and Giza, Egypt.

Experiment This experiment was to study rhizosphere and soil apart of onion plant. This study was carried out in pots (35cm) under green house conditions.

Soil used: The types of the used soils that were collected from the top layer (25 cm depth); a fertile clayey soil and a silty soil were collected from the farm of Malawi Agricultural Research Station. Soils were dried, ground to pass through 2mm sieve, their chemical and physical analyses were presented inTable (1).

Table (1): Physical and Chemical properties of the field soil experiment .

			Soil types	
			Clayey	Silty
Physical properties		pH	8.90	8.20
		Sand %	19.90	30.50
		Silt %	32.60	39.00
		Clay%	47.50	30.50
Chemical properties	Anions (-)	$CO_3^=$	Nil	Nil
		HCO_3^-	2.20	1.25
		Cl^-	2.00	0.90
		$SO_4^=$	0.94	0.76
	Cations (+)	Ca ++	1.30	0.50
		Mg++	2.10	.030
		Na+	1.60	1.80
		K+	0.14	0.31
Organic carbon %			2.4	1.2
Electrical conductivity (mhos/cm)			0.56	0.28
C / N ratio			3.9	2.06
Total N_2 %			0.19	0.11
Available P %			16.8	2.11

.A soil analysis was done according to the method described by Jackson, (1973). Analyses were carried out in Soil and Water Institute, ARC. Giza.

Methods

Azotobacter isolates:

Azotobacter isolates used through out the present investigation were isolated from rhizosphere of standing onion plants of 45 days old .

Azospirillum isolates:

Azospirillum isolates were isolated from rhizosphere of the onion plants of 45 days old. One isolate of Azospirillum was isolated from the soil apart, under onion plants.

Isolation and characterization of Azotobacter and Azospirillum from rhizosphere of onion plants

Isolation and purification of the Azotobacter;

Isolation of Azotobacter from the onion rhizosphere was carried out according to the method given by Bilal *et al*, 1990. Ten grams portions from the mixed rhizosphere soil samples were transferred to a sterile 250 ml sampling bottle containing 90ml sterile tap water. The bottles were shaken on a rotary shaker for 10 minutes and further serial dilutions were done. One ml from the appropriate dilution was transferred to 100ml of Stanier basal medium free nitrogen (Stanier, *et al*.; 1963) in flasks 250ml, and incubated at 30˚C for 72hrs. from positive tubes, formed pellicle is transferred with sterile loop; streaked surface of basal medium free nitrogen. Incubated at 30˚C for 72hrs. The colonies of different morphologies developed on the N-deficient medium were picked up and streaked to obtain a single colony (Abd-El Malek and Ishac, 1968).

Isolation and purification of Azospirillum;-

From root rhizosphere the enrichment cultured technique was adapted using the nitrogen deficient semisolid malate (NFM) recommended by (Dobereiner and Day, 1976) dispensed in test 7ml/tube. Root free soil was homogenized by thoroughly mixing and shaking for 10 minutes first dilution (1:10 w/v) was prepared by transferring 5 ml of roots together with adhering soil in to sampling bottles containing a suitable volume of sterile tap water this method was carried out by Holm and Jensen (1972). Bottles were shaken vigorously for 5-10 min and further serial dilutions were prepared. Isolates of Azospirillum were inoculated in the NFM medium, for enrichment culture for further purification by striking plates to single colony isolation.

Methods of identification of the isolated Azotobacter and Azospirillum strains:-

Nine isolates of Azotobacter and Nine isolates of Azospirillum were tested for cell shape , motility , Gram reaction slime production and pigmentation. All tests were carried out on 24 hrs, old cultures using the nitrogen free medium of (Stanier,*et al*; (1963). The isolates were allowed to grow for 5 days for testing slime production and for14 days for observation of pigmentation (Bergey's Manual, 2008). The following microbiological tests were followed to identify the isolates:-

Morphology and Gram stain:, Shape, arrangement of the bacterial cells, as well as the Gram reaction were microscopically observed in strain preparations of 24-28hrs.

Motility test:, was observed in fresh preparations 24 hrs by hanging drop technique.

pigmentation:, formation of pigments was reported for 14 days old cultures.

Glucose fermentation:, sterile tubes containing 0.5 % glucose and brom-thymol blue (1.0%) as an indicator were inoculated with 0.1 of 24hrs, old cultures. After incubation at 30˚C, the change in colour to yellow and the production of gas noted in Durham s tubes.

Starch hydrolysis:, plates of starch agar were streaked with tested isolates and incubated at 30˚C for 5 days. Starch hydrolyzing microorganisms were

distinguished by the appearance of clear zones around their growth when the plates were flooded by iodine solution.

Preparation of inocula:

Efficient local strains of Azotobacter sp No (5)and Azospirillum sp No (1)which had been isolated from the rhizosphere of some onion plants, were used. Heavy cell suspensions of each strain were obtained by growing for 5 days at 29˚C, on Ashby and Dobereiner media for Azotobacter and Azospirillum respectively. Ten ml of Azotobacter inoculum-suspension (1.5×105 cells/ml of medium) or Ten ml. wer added of Azospirillum inoculum-suspension (1.4×105cells/ml of medium) for each plant, as sub-soil biofertilizers, in the rhizosphere area.

Enzymatic activity of nitrogen fixing bacteria:

Nitrogenase activity:

The nitrogenase activity was measured according to **(**Turner and Gibson, 1980**)** as follow:

1-Sample from the soil of onion plant was collected (25 g) in a 100 ml bottle.

2- ml sugar solution were added and well stirred, the solution is composed of 0.5 gram mannitol, 0.5 gram malice acid, 0.5 grams glucose and 0.5 gram sucrose milted in 100 ml distilled water .

3-The bottle was closed tightly and removed the air inside it then inject acetylene gas (10 % V) and put a sticker on the injection position.

4-The bottle was incubate on 30° C for 24 hours .The acetylene and ethylene were measured in the sample using gas liquid chromatography model HP6890 ,

5-The concentration of ethylene in the samples (nmols / C_2H_4 / hr) was then converted to moles by dividing these values by the volume of the molecular weight of gas (22.4 L). The results were presented as n mol / C_2H_4 / ml culture / hr.

RESULTS AND DISCUSSION

Characteristics of the Azotobacter and Azospirillum isolates:-

The isolation and purification of nine isolates of Azotobacter and nine isolates of Azospirillum were performed on their specific medium. According to their morphological and physiological characteristics as proposed by Tarrand *et al* (1978) and Bergey's manual (2008**).** Results recorded in Table (2) illustrated that isolates that developed on Abdel-Malek and Ishac media (1968) have large ovoid to rods shaped, Gram negative, encysted, capsule formation . According to Bergey's manual (2008**).**The isolates were identified as *Azotobacter spp.* Isolates that grown on NFM medium are vibriod and spiral shape, Grame negative , starch hydrolysis is negative ,motility test is positive , acid from glucose not formed . The data in table (2) showed that the organisms could be indented as Azospirillum According to Bergey's manual (2008**)** and Chahal, *et al*. (1982**)** . The most active isolates of *Azotobacter* and *Azospirillum* were used as bacteria inoculum in the field experiment under nitrogen fertilizer levels (0.175 and 350 Kg / fed.)

Table (2); Some morphological and physiological characterstics of Azotobacter and Azospirillum isolates from onion plants .

Serial number of isolates		Morphological and physiological characterstics					
		Motility	Gram stain	Cell form	Production of acid from glucose	Production of yellow green fluorescent pigment	Starch hydrolysis
Picked organisms isolated from N-deficient medium	1	-	-	Oval	+	-	-
	2	-	-	'	+	-	-
	3	+	-	'	+	-	++
	4	+	-	'	+	+	+
	5	-	-	'	+	-	-
	6	-	-	'	+	-	+
	7	+	-	'	+	+	-
	8	-	-	'	+	+	-
	9	-	-	'	+	+	+
Picked organisms isolated from NFM medium	1	+	-	Spiral	+	-	-
	2	+	-	'	-	-	-
	3	+	-	'	+	-	-
	4	+	-	'	-	-	-
	5	+	-	'	+	-	-
	6	+	-	'	+	-	-
	7	+	-	'	+	-	-
	8	+	-	'	-	-	-
	9	+	-	'	-	-	-

Data in Table (3) showed that the isolates of nitrogen fixing bacteria (*Azotobacter* and *Azospirillum*) in two soil types exhibit a highly activity of nitrogenase. The activity of the isolate *Azotobacter sp* was 94.09 nmoles C_2H_4 / 1ml culture / hr No 5 in clayey soil , while the isolate *Azotobacter* in *Azospirillum* was 10.20 nmoles C_2H_4 / 1 ml culture /hr No 1 in clayey soil. Such findings confirm those obtained by (Chungwoo Kim *et al*. ;2005 and Abd El-Halim. 2009).

Data presented in Table (4):Showed that the mean counts of *Azotobacter sp.* cells gradually increased in clayey and silty soils with 175 Kg N_2/fed.than soils with 350/kgN_2 /fed In clayey soil ; the highest level of mean count was 8.8 x 10^4 cells / 1 g dry soil after 60 days from sowing amended with 175 kg N_2/fed compared to control without N_2 fertilizer was 5.1 x 10^4 cells / 1 g dry soil and to 350 kg N_2/fed was 6.4 x 10^4 cells / 1 g dry soil after 120 days from sowing compared to control without N_2 fertilizer was 3.8 x 10^4 cells / 1 g dry soil . In silty soil; the highest level of mean counts with 175 Kg N_2/ fed. was 8.1 x 10^4 cells / 1 g dry soil after 90 days from sowing compared to control without N_2 fertilizer was 3.4 x 10^4 cells / 1 g dry soil after 90 days from sowing .The results obtained were in agreement with Kole *et al.* ;1988, Shoukri ; 2006 ,Abd el-Halim. 2009 and Ki-Yoon Kim *et al*., 2010. *Azospirillum sp.*

Table (3): Nitrogenase activity of the different isolates of Azotobacter and Azospirillumin clayey and silty soil.

Serial number of isolates		Origin of bacterial isolates		Nitrogenase activity nmoles C_2H_4 / 1 ml /culture/hr ml
		Dilution	Soil type	
Azotobacter *sp.*	1	10^6	Clayey	4.75
	2	10^4	Clayey	18.06
	3	10^5	Clayey	14.79
	4	10^5	Clayey	67.51
	5	10^5	Clayey	94.09
	6	10^5	Silty	10.21
	7	10^4	Silty	1.89
	8	10^4	Silty	14.42
	9	10^3	Silty	17.65
Azospirillum sp.	1	10^6	Clayey	10.20
	2	10^7	Clayey	0.58
	3	10^6	Clayey	1.85
	4	10^7	Clayey	1.27
	5	10^5	Clayey	0.34
	6	10^5	Clayey	2.26
	7	10^6	Silty	4.46
	8	10^5	Silty	3.63
	9	10^6	Silty	2.79

Table (4): Mean counts of *Azotobacter* and *Azospirillum* (10^4 cells / 1 g dry soil) in clayey and silty soil of onion plants at two nitrogen fertilizer levels.

Soil type	Bio and Inorganic fertilizer / Days after sowing	*Azotobacter sp.*			*Azospirillum sp.*		
		N fertilizers (Kg / fed.)			N fertilizers (Kg / fed.)		
		0	175	350	0	175	350
Clayey	30	4.8	7.1	5.2	3.0	4.2	3.5
	60	5.1	8.8	5.4	3.2	5.1	4.1
	90	4.2	8.4	6.0	3.0	5.6	3.5
	120	3.8	7.4	6.4	2.4	4.4	3.0
	150	3.2	7.0	4.7	2.1	4.1	3.3
Silty	30	3.8	6.8	5.2	2.2	5.1	4.1
	60	4.2	7.4	6.1	2.4	4.8	4.7
	90	3.4	8.1	6.4	2.0	5.3	4.1
	120	3.6	7.2	5.2	2.4	4.3	3.6
	150	3.1	6.4	4.8	2.1	4.0	3.7

Data presented in Table (4) indicated that the mean counts of *Azospirillum sp.* in cells gradually increased in clayey and silty soils with 175 Kg N_2/fed. . In clayey soil ; the highest level of the mean counts in clayey soil was 5.6 x 10^4 cells/1 g dry soil at 90 days after sowing compared to control without N_2 fertilizer was counts was 4.1 x 10^4 cells / 1 g dry soil after 60 days after sowing compared to control without N_2 fertilizer was 3.2 x 10^4 cells / 1 g dry soil .In silty soil ; the highest level of the mean counts was 5.3 x 10^4 cells / 1 g dry soil after 90 days after sowing compared to control without N_2 fertilizer was 2.0 x 10^4 cells / 1 g dry soil while with 350 Kg

N_2/fed. the highest level of the mean counts was 4.7 x 10^4 cells / 1 g dry soil after 60 days after sowing compared to control without N_2 fertilizer was 2.4 x 10^4 cells / 1 g dry soil. The results obtained were in agreement with those of Omer and Abdel-Satar; 2001, Jayathilake *et al;.* 2003, Shoukri,A A.; 2006,Abd el-Halim,M.S.; 2009 and Ki-Yoon Kim *et al*., 2010

Nitrogenase activity:

Data in Table (5) showed the highest increases of nitrogenase activity of *Azotobacter sp* and *Azospirillum sp* under microbial inoculation and N-fertilization .

In clayey soil the activity was 82.89 nmoles C_2H_4 / g soil / hr at 175 kg N_2/fed. after 90 days from sowing compared to 350 Kg N_2/ fed. was 61.0 nmoles C_2H_4 / g soil / hr at 90 days after sowing compared to control was 47.12 nmoles C_2H_4 / g soil / hr.

In silty soil ;the highest nitroganase activity of *Azotobacter chroococcum* was 78.65 nmoles C_2H_4 / g soil / hr with 175kg N_2/fed while with 350 Kg N_2/fed. Was 39.18 nmoles C_2H_4 / g soil / hr compared to control 11.79 nmoles C_2H_4 / g soil / hr.

The obtained results in Table (5) showed the values nitroganase activity of *Azospirillum brasilense*

In clayey soil were 62.39 and 46.21 nmoles C_2H_4 / g soil / hr with 175 and 350 Kg N_2/fed. respectively at 90 days after sowing compared to control 16.33 nmoles C_2H_4 / g soil / hr .

In silty soil; the highest values were 58.95 nmoles C_2H_4 / g soil / hr with 175 Kg N_2/fed. and 21.96 nmoles C_2H_4 / g soil / hr with 350 Kg N_2/fed. after 90 days from sowing respectively compared to control 4.56 nmoles C_2H_4 / g soil / hr .The results obtained were in agreement with those of Hegazi *et al.*, 1983**,**. Chezhiyan,*et al*.,2003; shalan,2005;lakshmanan,*et al*.,2005 and Abd El-Halim.M.S.; 2009.

Table (5): Nitrogenase activity of Azotobacter and *Azospirillum* nmoles C_2H_4 / 1m1culture/h at interval growth periods at two nitrogen fertilizer levels in the used different soil types.

Soil type	Bio and inorganic fertilizer / Days after sowing	*Azotobacter No (5)* N_2 fertilizers (Kg / fed.)			*Azospirillum No (1)* N_2 fertilizers (Kg / fed.)		
		0	175	350	0	175	350
Clayey	30	3.23	5.64	2.75	4.02	12.1	18.62
	60	18.46	15.2	16.27	4.76	3.47	25.85
	90	47.12	82.89	61	16.33	62.39	46.21
	120	23.42	65.96	42.67	1.62	3.31	23.72
	150	21.82	64.34	36.34	0.4	4.72	4.66
Silty	30	2.92	1.79	1.91	2.81	2.68	3.46
	60	5.47	60.64	13.69	2.09	2.12	17.13
	90	11.79	78.65	39.18	4.56	58.95	21.96
	120	2.86	58.51	30.91	6.53	4.16	6.14
	150	5.43	38.34	14.53	2.25	2.46	4.29

Recommendation

This study recommended that application of non symbiotic nitrogen fixing bacteria (*Azotobacter* and *Azospirillum*) isolated from onion rhizosphere resulted a highly active of nitrogenase and so the organisms should be used as active biofertilizer in onion production.

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نشاط الازوتوباكتر والازوسبيريللم فى ريزوسفير البصل

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تهدفُ هذه الدراسةِ إلي الحصول علي أكثر العزلات كفاءة لُتثبيت النتروجين والتى تم عزلها مِنْ منطقة ريزوسفير البصل وتأثيرها على إنتاجية البصلِ تحت ظروف نوعينِ مِنْ التُرَبة مِع أقل تُكلّفُة وخفض تلوث البيئـةَ النـاتجَ مِـنْ الإسـتعمالِ المفـرطِ للأسـمدة المعدنيةِ.وشـملت الدراسـة عمليـة عـزل وعـد بكتريـا الأزوتوباكتروالأزوسبيريللم مِنْ منطقـة ريـزو سفير البصـل مـع تقـدير نشـاط إنـزيم تثبيـت النتـروجين الجـوي (إنزيم النيتروجينيز) بواسطة بكتيريا الأزوتوباكتر والأزوسبيريللم من ريزوسفيرنبات البصل مع مستويين 175 و350 كيلو جرام / فدان من تسميد النتروجينِ المعدني خلال فترات النمو (0 ,30 ، 60 ، 90 ، 120 ، 150 يومِ من الزراعة) أوضحت النتائج زياده فى عددَ خلايا بكتريا الأزوتوباكتر بشكل تدريجي ووَصلَ الى الحدَ الأعلى بعد 60 يومِ من الزراعة مع معدل تسميد نتروجين معدني 175 كجم /فدان حيث وصل العدد 8.8 **x** 10^4 خليةَ / جم تربة جافة لريزوسفيرالبصل في التربة الصفراء. فى حين وصل عددَ خلايا بكتريا الأزوتوباكتر الى 8.1 **x** 10^4 خليةَ / جم تربة جافة لريزوسفير البصل بعد 90 يومِ من الزراعة بمعدل تسميد 175 كجم نتروجين / فدان, أيضا وصل عدد بكتريا الأزوسبيريللم في التربة الطينيَة و الصفراء الى 5.6 ، 5.3 **x** 10^4 خليةَ / جم تربة جافة لريزوسفير البصل علي التوالي . كما أوضحت نتائج العزلات التي تم عزلها من تربة نبات البصل من نوعي التربة (الطينية و الصفراء) أن أعلي عزلة رقم(5) حققت أعلي نشاط لإنزيم النيتروجينيز كانت لبكتريا الأزوتوباكتر التي أعطت 94.09 نانومول ك$_2$يد$_4$/ جم تربة/ ساعة في التربة الطينية بينما كانت بكتريا الأزوسبيريللم رقم (1) اعطت 10.20 نانومول ك$_2$يد$_4$/ جم تربة/ ساعة في التربة الطينية والتي تم إستخدامهما كلقاح في تجربة الحقل في التربة الطينية بعد ذلك . وكان نشاطُ إنزيم النيتروجينيز مع بكتريا الأزوتوباكتر في التربة الطينية و الصفراء بعد 90 يومِ من الزراعة ومعدل تسميد 175 كجم نتروجين / فدان هو 82.89 ، 78.75 نانومول ك$_2$يد$_4$/ جم تربة/ ساعة على التوالي. فى حين كان مع بكتريا الأزوسبيريللم في التربة الطينية و الصفراء كان أعلى نشاطُ لإنزيم النيتروجينيز بعد 90 يومِ من الزراعة بمعدل تسميد 175 كجم نتروجين / فدان هو 62.39 ، 58.95 نانومول ك$_2$يد$_4$/ جم تربة / ساعة على التوالي .ويوصى البحث تطبيق اللقاح الحيوى لبكتيريا الأزوتوباكتر والأزوسبيريللم فى انتاج البصل وخفض مستوى التسميد المعدنى و التلوث البيئى.

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