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SIGNIFICANCE OF *HERB ASPIRILLUM SEROPEDICAE* INOCULATION AND (OR) MAIZE STRAW AMENDMENT ON NITROGEN FIXATION AND GROWTH OF WHEAT USING ^{15}N -DILUTION TECHNIQUE

[38]

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ABSTRACT

Pot experiments were conducted to investigate the significance of *Herbaspirillum seropedicae* (Z78) inoculation and (or) maize straw (0, 5 and 10 T ha^{-1}) amendment on growth and nitrogen fixation of wheat using ^{15}N -dilution technique. Inoculation resulted in accumulation of fixed nitrogen and %nitrogen derived from atmosphere (NdFa) recorded 24.6% and 26.5% in wheat shoots and grains, respectively. Straw amendment reduced %NdFa to 16.1% and 20.2% at high straw level (10 T ha^{-1}). Rational nitrogen fertilization at rate of 180 Kg N ha^{-1} completely inhibited nitrogen fixation by *Herbaspirillum* inoculation. Bacterial inoculation increased dry shoots and grains yields up to 23% and 31% respectively. The highest levels of shoots and grains dry mass (46.5% and 42.4%, respectively) were obtained by N-fertilization in both inoculated and uninoculated plants. Total shoots and grains N-yields were increased irrespective of organic matter amendment by *H. seropedicae* inoculation up to 9% and 25% respectively. N-fertilized plants recorded a maximum increase in N-yield (57% and 51%). *H. seropedicae* was reisolated from inoculated wheat histosphere after harvesting (90 days from sowing). Neither organic matter nor mineral nitrogen applications had marked effect on bacterial total counts colonized wheat histosphere. Moreover, no symptoms of mottled stripe disease were observed on leaves and stems of inoculated plants.

Key words: *Herbaspirillum seropedicae*, ^{15}N-dilution method, Straw amendment, Nitrogen fixation.

INTRODUCTION

Bacterial taxonomists working in Belgium found that the bacterium known as *Pseudomonas rubrisubalbicans*, a sugar cane endophyte which causes mottled stripe disease in some varieties in USA and other countries, was found to be closely related genetically to a nitrogen–fixing bacterium called *Herbaspirillum*

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(**Gills** *et al* **1991**). *Herbaspirillum seropedicae* was first isolated from the roots of maize and other cereals by **Baldani** *et al* **(1986)**, but it has also been found in stems and leaves of rice, sugarcane and maize plants (**Palus** *et al* **1996**). Most *P. rubrisubalbicans* isolates were found to be able to fix nitrogen and were identical in most other respects to *Herbaspirillum* (**Pimentel** *et al* **1991**). Moreover, results of DNA/rRNA hybridization and computer–assisted auxanographic tests have established that this plant endophyte *Pseudomonas rubrisubalbicans* must be included in the genus *Herbaspirillum* (**Gills** *et al* **1991**). *Herbaspirillum seropedicae* and *H. rubrisubalbicans* were confirmed by their DNA/DNA homologous as well as serological methods (**Olivares** *et al* **1994**). Recently, a third *Herbaspirillum* species, *H. frisingense*, isolated from C-4 fiber plants was also differentiated on physiological basis (**Kirchhof** *et al* **2001**).

Results of ^{15}N-gas incorporation technique confirmed that *H. seropedicae* and *H. rubrisubalbicans* isolates grown in semisolid JNFb medium effectively fixed nitrogen (**Boddey** *et al* **1995**). The preliminary inoculation experiments with *Herbaspirillum* in nonsterile soil under greenhouse and monoxenic N-free Hogland solution conditions, showed that about 40% increase in total plant nitrogen, dry mass and nitrogenase activity has been achieved (**Dobereiner** *et al* **1994**). Moreover, *H. seropedicae* and *Burkholderia brasilense* are among some of the endophytic bacteria,isolated from rice, and the contribution of these strains to plant N *via* BNF has been demonstrated (**Baldani** *et al* **2000**). However, the pathogenic nature of *H. seropedicae* on sorghum and pennisetum and its closely relation to the sugar cane pathogen *Pseudomonas rubrisubalbicans* preclude its use as an inoculant for agriculture (**Triplett, 1996**).

In order to gain information about the latter aspect, and in previous work we investigated the survival, pathogenicity and the possible effects of *Herbaspirillum* inoculation on growth as well as nitrogen fixation (using the acetylene reduction assay) of wheat and maize plants grown in monoxenic hydroponic and in soil microcosms experiments (**El-Komy, 1999; 2001 and Abdel Wahab** *et al* **2000**). Results of our experiments indicated that *Herbaspirillum* inoculation exhibited significant increases in plant mass, total nitrogen content and nitrogenase activity, while no symptoms of mottled stripe disease were observed.

Therefore, this paper reports on the significance of *H. seropedicae* inoculation and (or) straw amendment as well as inorganic nitrogen fertilization on nitrogen fixation, and growth of wheat plants using ^{15}N – dilution method in pot experiments.

MATERIAL AND METHODS

Bacterial strain and growth conditions

H. seropedicae CD strain Z78 (ATCC 35893) isolated from sorghum roots kindly supplied by J. Dobereiner, EMBRAPA-SPI, Rio de Janeiro (Brazil) was used in this study. The medium JNFb containing 20-m mol NH4Cl was used to grow the bacterial strain at 30°C for 24 h under shaking (110 rpm) according to **Baldani** *et al* **(1986)**. Cells were harvested by centrifugation (7000 g, 15 min), washed twice in sterile phosphate buffer pH 7.0 and used as inoculum in concentration of 10^7 CFU/ml.

Pot-experiments (under greenhouse conditions)

Homogenized mixtures of newly reclaimed nonsterile sandy soil (the chemical analysis of the soil is listed in Table 1) and nonsterile maize straw (the physical and chemical compositions of maize straw are listed in Table 2) in rates of 0, 5 and 10 T ha^{-1} equal to 0, 7.5 and 15 gram straw pot^{-1} . were prepared to fill pots of 3 Kg capacity. Second pots group was prepared for fertilization by rational mineral nitrogen as ammonium sulfate at rate of 180 Kg N ha^{-1} (270 mg N pot^{-1}) . Half-N dose was applied at sowing and the remaining half dose 25 days after sowing. Recommended rates of phosphorus (50 Kg P_2O_5 ha^{-1} as superphosphate) which equal to 0.015 g P_2O_5 pot^{-1} and potassium (100 Kg K_2O ha^{-1} as potassium sulfate) which equal to 0.03 g K^2O pot^{-1} were applied before sowing. Pots were arranged in a completely randomized design with five replicates.

Grains of wheat (*Triticum aestivum* L.) var. Seds-6 provided by Agricultural Research Center, Ministry of Agriculture, Egypt were surface sterilized and germinated for two days as described by **El-Komy *et al* (1998)**. Five germinated seeds were transplanted into each pot, and each seed was inoculated with 1ml bacterial inoculum contained 10^7 CFU of *H. seropedicae* strain. After reaching 10 cm in length, the plants were thinned down to 3 per pot. Pots were equally fertilized by $(^{15}NH_4)_2SO_4$ (1% atom excess) at the rate of 100 Kg N ha^{-1} dissolved in water. ^{15}N–labeled fertilizer was divided into 3 doses as follows: 1- One third of the total ^{15}N amount was applied 2 weeks after sowing. 2- One third of the total ^{15}N amount was applied 4 weeks after sowing. 3- One third of the total ^{15}N amount was applied 6 weeks after sowing. At harvesting (90 days after sowing), densities of *Herbaspirillum* were determined in root histosphere (crushed roots washed and shaken in 2 min in 95% ethanol) using the dilution plate technique on JNFb agar containing 20 mg/l yeast extract (**Baldani *et al* 1986**). Shoots and grains were harvested, dried at 70°C, weighed, grounded and total nitrogen was determined by Kjeldahl method. Percentage of ^{15}N atom excess (^{15}N:^{14}N ratio) was determined using mass spectrometry at IAEA Seibersdorf Laboratory, Vienna, Austria.

Table 1. Soil physical and chemical characteristics

pH	8.03	K (ppm)	88.3	Sand%	87.61
Organic C %	0.15	NH_4^+ (ppm)	3.0	Silt %	6.59
Total N %	0.014	NO_3^- (ppm)	0.3	Clay %	5.82
C/N ratio	11:1	P(ppm)	4.0	Texture	Sandy

Table 2. Chemical analysis of maize straw

Organic C %	51.28	K %	2.5
Total N %	0.79	Ca %	0.6
C/N ratio	65:1	Fe (ppm)	140
P %	0.22	Mn (ppm)	198

Calculations

The following calculations were carried out according to **Fried and Middelboe (1977); Rennie *et al* (1983) and El-Komy and Abdel Wahab (1998):**

$$\text{Total N-yield (mgN pot}^{-1}) = \frac{\text{Dry matter (mg pot}^{-1}) \times \text{Total N}}{100}$$

$$\%\text{ N derived from atmosphere (\% NdFa)} = \frac{1 - \%^{15}\text{N.a.e. (Fx)}}{\%\ 15\text{N.a.e. (nFx)}} \times 100$$

Where

(Fx) and (nFx) = inoculated (fixing) and uninoculated (nonfixing) plants respectively.

$$\%\text{ N derived from fertilizer (\% NdFf)} = \frac{1 - \%^{15}\text{N.a.e. (Fx)} \times 100}{\%\ ^{15}\text{N.a.e. (nFx) fertilizer}}$$

$$\%\text{ N derived from soil (\%NdFs)} = 100 - (\%\text{NdFa} + \%\text{NdFf})$$

$$\%\text{N derived from organic matter(\%NDFo)} = 1 - \frac{\%\text{NdFf in plant with organic matter} \times 100}{\%\text{NdFf in plant without organic matter}}$$

$$N_2\text{-fixed (mg pot}^{-1}) = \frac{\%\text{NdFa} \times \text{total N yield (mg pot}^{-1})}{100}$$

Statistical analysis

Data were statistically analyzed according to **Steele and Torrie (1960).**

RESULTS

Isotope determination without straw amendment

Considering the initial enrichment of 1% atom excess, the atomic percentage of ^{15}N excess in dry shoots and grains was relatively low (Table 3). These low values could be due to the residual ^{15}N associated with soil and plant roots. *H. seropedicae* inoculation resulted in different ^{15}N atom excess, the highest values were recorded with uninoculated (control) treatments. Less significant values were obtained with *H. seropedicae* inoculation in both shoot and grain mass. This decrease in the ^{15}N enrichment of inoculated compared to uninoculated treatments indicates that the plants were receiving N_2 from the atmosphere. *H. seropedicae* inoculation resulted in the accumulation of fixed N_2 and %NdFa recorded 24.6% and 26.5% (equals to 23.7 and 32.2 mg fixed nitrogen pot^{-1}) in wheat shoot and grain respectively (Figs. 1 and 2). Data also show that when less N_2 derived from fixation was available to the plant, more fertilizer N (%NdFf) or soil N (%NdFs) or both were assimilated by the plant (Fig. 1).

Isotope determinations with straw amendment or inorganic N ferilization.

High ^{15}N abundance in control treatments as compared to the inoculated ones was recorded in straw amended plants up to 10 T ha^{-1} both in shoots and grain yield

(Table 3). This is indicative of isotopic dilution as a result of uptake of fixed nitrogen. In straw amended plants, %NdFa decreased as compared with those without straw amendment. %NdFa were 16.1% and 20.2% at high straw level (10T ha^{-1}) in shoot and grain tissues respectively, however, higher N uptake from soil and fertilizer were recorded. Data also showed that %NdFa was completely inhibited by *Herbaspirillum* inoculation at rational mineral nitrogen fertilization at rate of 180 Kg N ha^{-1} (Fig. 2).

Table 3. Effect of *Herbaspirillum* inoculation and/or straw amendment on % ^{15}N*% a.e. of wheat and bacterial counts (log CFU g^{-1} fresh root).

Straw level*	$\%^{15}N$ a.e in shoots			$\%^{15}N$ a.e. in grains			Log CFU
	0	5	10	0	5	10	
Control	0.191	0.179	0.162	0.230	0.198	0.194	0.0
Inoculation	0.144	0.141	0.136	0.169	0.160	0.158	5.8
Inorganic N	0.210			0.250			6.4
Inoculation–Inorganic N	0.220			0.278			6.6
L.S.D. 5%	0.032	0.031	0.023	0.033	0.022	0.034	

- Straw level in ton $hectare^{-1}$
- $\%^{15}N$ atom excess = $\%^{15}N$ a. e.

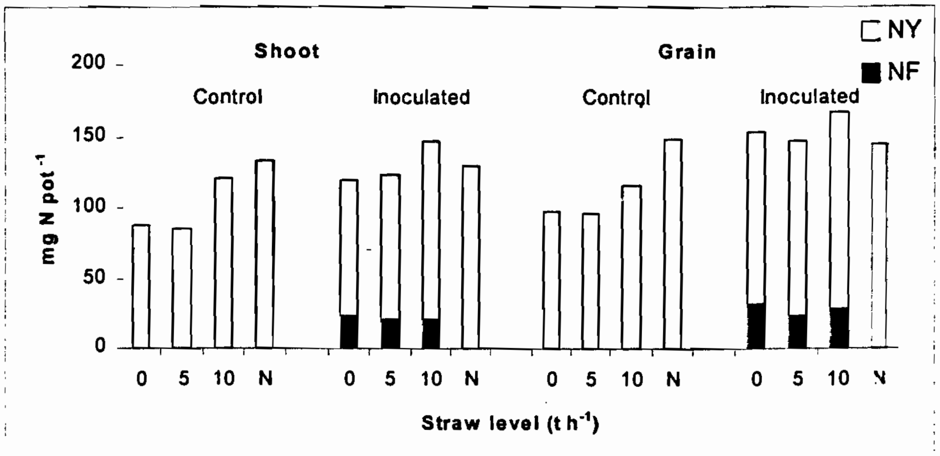

Fig. 1: Effect of *Herbaspirillum* inoculation and/or straw amendment on total N-yield (NY) and fixed nitrogen (NF) in shoots and grains yields of wheat.

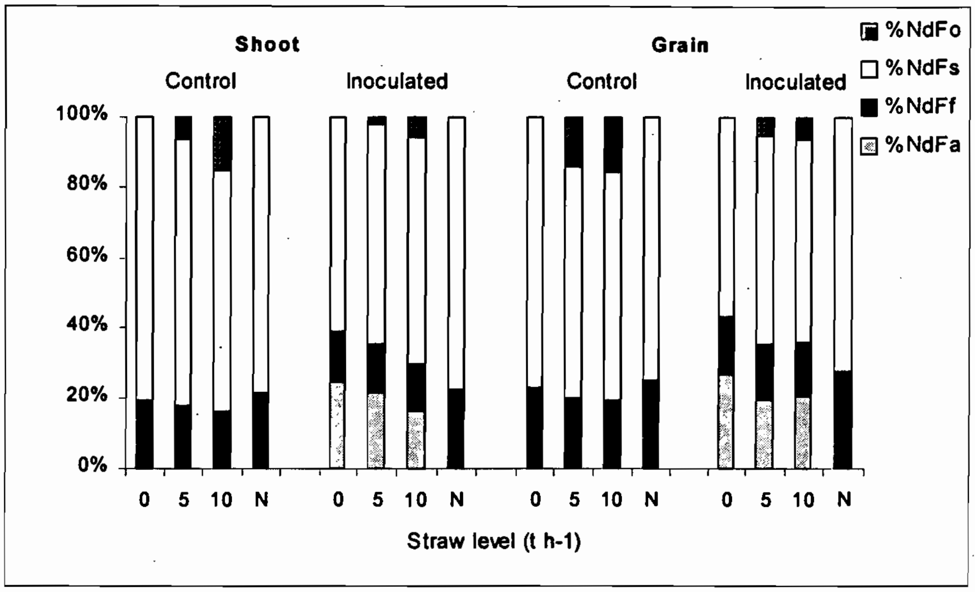

Fig 2: Effect of *Herbaspirillum* inoculation and/or straw amendment on % NdFa, %NdFf, %NdFs and %NdFo in wheat shoot and grains.

Data also show that the percentage of nitrogen derived from organic matter (%NdFo) in control uninoculated plants recorded 6.3% and 13.9% in shoots and grains, respectively when applied at 5 T ha^{-1} level. However, %NdFo increased up to 15.2% and 15.7% in shoot and grain respectively at high straw level (10Tha^{-1}). *H. seropedicae* inoculation reduced the percentage of nitrogen derived from organic matter in both shoot and grain, and recorded 2.1% and 5.3%, respectively at low organic matter level, and 5.6% and 6.5% at higher level (10 T ha^{-1}).

Effect of *H. seropedicae* inoculation on wheat growth and total N-yield.

Data of Table (4) show that the growth of wheat plants positively affected by *H. seropedicae* inoculation as well as by the amendment with different levels of straw. Moreover, no symptoms of mottled stripe disease (as red stripes) were observed on leaves and stems of inoculated and control plants. Without organic matter amendment, *H. seropedicae* inoculation increased the shoots and grains yields up to 23% and 31%, respectively as compared with control uninoculated plants. *H. seropedicae* inoculation at different organic matter levels recorded 9% and 12% increasing in shoot and grain yields at high organic matter level (10 T ha^{-1}). Maximum shoot and grain yields were obtained by inorganic N fertilization both with and without bacterial inoculation (Table 4).

Data of Fig. (1) show that total shoot and grain N-yields increased significantly by *H. seropedicae* inoculation without organic matter application, recording 9%

Table 4. Effect of *Herbaspirillum* inoculation and/or straw amendment on dry shoots and grains weights and %N of wheat.

Straw level	Dry shoot ($g\ pot^{-1}$)			Shoot % N			Dry grain ($g\ pot^{-1}$)			Grain %N		
	0	5	10	0	5	10	0	5	10	0	5	10
Control	1.57	1.54	1.76	0.65	0.55	0.69	4.20	4.8	4.9	2.31	2.00	2.37
Inoculation	1.93	1.57	1.92	0.50	0.65	0.66	5.5	5.8	5.5	2.21	2.12	2.51
Inorganic N	2.20			0.55			5.9			2.5		
Inoculation + Inorganic N	2.10			0.53			6.0			2.3		
L.S.D. 5%	0.31	0.21	0.19	0.11	0.11	0.11	0.52	0.41	0.38	0.40	0.40	0.40

and 25.3% increases in shoots and grains respectively as compared to uninoculated plants. Total shoots and grains N-yields increased up to 20% and 28% respectively by the amendment of organic matter at 5 T ha^{-1} level, and 5% and 19% at 10 T ha^{-1} levels. However, maximum total N-yield was obtained by N-fertilization with and without inoculation up to 57% and 51% in shoots and grains respectively as compared with control plants.

The survival of H. seropedicae in wheat histosphere

To ensure the establishment of *H. seropedicae* inoculum within wheat histosphere, crushed root which had been washed after surface sterilization, the total number of *H. seropedicae* within the root tissues was estimated by the dilution plate technique using JNFb medium. Data in Table (3) showed that *H. seropedicae* could be reisolated from wheat histosphere as small whitish smooth colonies with dark centers on JNFb medium. *Herbaspirillum* inoculation recorded 6.5 (log CFU g^{-1} fresh root), log CFU 5.8 and 6.4 in inoculated plants amended with straw at 5 and 10 T ha^{-1} respectively, and log CFU 6.6 in case of inoculation under N-fertilization. Attempts to isolate *H. seropedicae* from control uninoculated plants failed. Organic matter amendment had no marked effect on bacterial total counts in wheat histosphere.

DISCUSSION

The discovery of endophytic diazotrophs (such as *Herbaspirillum* spp. and *Acetobacter diazotrophicus*) colonizing roots, stems and leaves of many graminaceous plants may finally explain the high contributions of biological nitrogen fixation to these crops (**Cavalcante & Dobereiner, 1988; Gillis *et al* 1989 and 1991**). Results of this study indicated that *Herbaspirillum seropedicae* inoculation of wheat plants resulted in N_2-fixation by this organism and %NdFa recorded 24.6% and 26.5% in shoots and grains respectively. Elegant acetylene reduction assay (ARA) and ^{15}N isotope dilution experiments have shown that *Herbaspirillum seropedicae* inoculation of rice in nonsterile soil and monoxenic N-free Hoagland solution, plants obtained from 54%-31% of their N from nitrogen fixation (**Baldani *et al* 2000**). Recently, **James *et al* (2002)** showed that two rice varieties exhibited nitrogenase activity when inoculated with a beta–glucoronidase (GUS)–marked strain of *Herbaspirillum seropedicae* (Z67) and recorded 30% increase in plant N content above that of the uninoculated controls.

Application of organic matter to the soil has beneficial effects in improving its productivity by several mechanisms, such as improving the soil biological activity (**Roper and Ladha, 1995**). However, in this study, *Herbaspirillum* populations in wheat histosphere remained more or less unchanged in the presence of maize straw. This could be explained on the basis that the stimulatory effects of straw are very likely related to the enhancement of other groups of soil diazotrophs such as *Azotobacter* spp., *Clostridium* spp. and *Bacillus* spp. rather than *Herbaspirillum* (**Hegazi *et al* 1983; Shaban *et al* 1997**).

Results of this study also showed that, maize straw amendment enhanced plant shoot and grain yields as well as their total N-yield as compared with control untreated plants (Table 4 and Fig.1).

Results also showed that in uninoculated plants amended with straw, the percentage of nitrogen derived from organic matter (%NdFo) raised from 6.3 % and 13.9 % at 5 T ha^{-1} to 15.2% and 15.7% at 10 T ha^{-1} in shoots and grains, respectively. **Roper and Ladha (1995)** reported that N gain in straw amended soil depends on different environmental factors such as temperature, soil moisture, pH, oxygen, soil texture as well as straw management and its C:N ratio. In *Herbaspirillum seropedicae* inoculated plants, %NdFo recorded the lowest levels of 2.1%-5.3% at 5 T ha^{-1} in wheat shoots and grains yields. The reduction in %NdFo in inoculated plants compared to control treatments could be explained on the basis that, inoculated plants received nitrogen from other sources, for example *via* nitrogen fixation. Such results were obtained previously in *Azospirillum* – maize associations at different levels of farmyard manure amendment **(El Komy *et al* 1998)**.

It has been observed that nitrogen fertilization gave the maximum effect in all growth parameters studied in both inoculated and uninoculated plants. The present results together with previous reports clearly indicate that wheat crop responded positively to high N fertilization up to 200 Kg N ha^{-1} **(Zambre *et al* 1984)**. Nevertheless an increased growth and total N-yield by *Herbaspirillum* inoculation over uninoculated control at high N level is not necessarily due to the nitrogen fixation since %NdFa was completely inhibited at such N level, but might be due to the growth hormones and better utilization of added fertilizer.

In straw amended plants, the percentage of nitrogen derived from nitrogen fixation (%NdFa) was reduced and was completely inhibited by N fertilization (Fig. 2). This could be explained by organic matter mineralization which resulted in higher inorganic nitrogen accumulation that inhibited nitrogenase activity, as well as the reported inhibitory effect of higher N-levels on *Herbaspirillum* nitrogenase activity **(Fu & Burris, 1989 and Kassen *et al* 1997)**.

Significant increases in the total N-yield over the control treatments by *H. seropedicae* inoculation at different straw levels might be attributed to greater values recorded for shoot and grain weights over the control plants rather than increasing in the percentage of nitrogen content (measured by Kjeldahl method). Even if such increase in the total N-yield was attributed to the percentage of total N content, they were mainly derived from soil or fertilizer N (%NdFs and %NdFf), or from both. These results are in agreement with those of **Caballero–Mellado *et al* (1992) and Hamdia & El Komy (1998)**. These authors concluded that the significant response of rhizobacteria inoculation was mainly due to increased uptake of soil and fertilizer N as a response of the bacterial hormonal activity.

This paper confirms that the nitrogen fixing capacity of *H. seropedicae* may have great economic importance with wheat despite of its reported pathogenic characters to cause mottled stripe disease in some sugar cane cultivars **(Palleroni, 1984)**. However, in Egypt, this disease is hardly ever observed **(Abo Arkob, 2000)**. Moreover, results of this investigation and previous studies showed that no symptoms of mottled stripe disease were observed in wheat inoculated plants **(El-Komy, 2001)**, rice **(Dobereiner *et al* 1994 and Boddey *et al* 1995)**, and maize **(El Komy, 1999)**. Even though the extent of pathogenicity of this organism must be confirmed with different plant genotypes. Moreover, like the phytopathogenic bacterium *Clavibacter michiganensis*, *H. seropedicae* must be applied after genetic manipulation to eliminate its pathogenic characteristics (**Meletzus** ***et al*** **1993**) opening new approaches for investigation of plant-pathogen-diazotroph interactions.

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دراسة أهمية التلقيح ببكتيريا الهرباسبيرلام سيروبدكا فى وجود أو غياب قش الذرة على تثبيت النيتروجين ونمو القمح باستخدام تقنية تخفيف النيتروجين المرقم-١٥

[٣٨]

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أجريت تجارب أصص لدراسة أهمية التلقيح ببكتريا هيرباسبيرلام سيروبدكا (سلالة Z78) فىغياب أو وجود قش الذرة بمعدلات ٥ ، ١٠ طن للهكتار باستخدام تقنية النيتروجين المرقم- ١٥ على تثبيت النيتروجين و نمو نباتات القمح.

أثبتت النتائج أنه عند التلقيح بالبكتريا محل الدراسة قد أدت إلى تثبيت نيتروجين بنسبة ٢٤،٦ % و ٢٦،٥ % فى كل من المجموع الخضرى والحبوب على التوالى ، كما أدت إضافة القش إلى تناقص فى النسبة المئوية للنيتروجين المثبت إلى ١ ، ١٦%- ٢٠،٢ % عند أعلى معدل إضافة قش (١٠ طن للهكتار).

كما أدى تسميد النباتات بالسماد المعدنى بمعدل (١٨٠ كجم للهكتار) إلى تثبيط كامل لعملية التثبيت الحيوى بواسطة بكتريا هيرباسبيرلام سيروبدكا وأدى التلقيح إلى زيادة فى محصول القش والحبوب بنسبة ٢٣% و ٣١ % على الترتيب. وكانت أعلى زيادة فى محصول القش والحبوب فى معاملات التسميد المعدنى سواء مع التلقيح أو غير الملقح و التى سجلت ٤٦,٥% و ٤ ، ٤٢% بالمقارنة بالنباتات غير المعاملة. أدى إضافة القش والتلقيح إلى زيادة النيتروجين الكلى بغض النظر عن مستوى لتسميد المعدنى المضاف وكانت الزيادة ٩ %و ٢٥% فى القش والحبوب على التوالى. وسجلت معاملات التسميد بالنيتروجين المعدنى أعلى زيادة فى محتوى النيتروجين الكلى (٥٧%, ٥١%) فى القش والحبوب على التوالى. أمكن عزل بكتيريا الهيرباسبيرلام من منطقة الهستوسفير وذلك عند الحصاد (٩٠ يوما من الزراعة).

وسجلت النتائج أن إضافة القش أو التسميد المعدنى ليس له أى تأثير ملحوظ على أعداد البكتيريا. كما أظهرت النتائج أيضا عدم تسجيل أى إصابة للنباتات بأعراض مرض تخطيط الأوراق أو سيقان نباتات القمح الملقح.

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