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# RESPONSE OF THE MILLET PLANT *PENNISETUM AMERICANUM* L. TO INOCULATION WITH *AZOSPIRILLUM BRASILENSE*

**Mohammed Saeed Haran[1], Galib A.A. Alkaabi[1] and Ghofran, S. Al-Azzawi[2]**
**[1]Southern Technical University, Shatrah Technical College, Iraq**
**[2]Private Sector, Iraq**
**E-mails:** mohammed.harran@stu.edu.iq , *ghaleb.muhaibis@stu.edu.iq ,*
Ghofran.senan1203a@coagri.uobaghdad.edu.iq

**Abstract**
The study aims to isolate and characterize *Azospirillum* bacteria for the purpose of studying its effect alone or in combination with nitrogen fertilization on the growth of *Pennisetum americanum* L. millet by growing it in a greenhouse. (4) isolates of *Azospirillum* bacteria, which were isolated from the roots of millet plant, were collected from Al-Rifai city / Dhi Qar. All isolates were of A. *brasilense*, and isolate A1 of A. *brasilense* was selected for use in the greenhouse experiment, on the basis of its high activity and efficiency in secreting IAA compared to the rest of the isolates. The result of the study showed that inoculation with bio-fertilizer (*Azospirillum*) gave a significant increase in the dry matter weights of the vegetative and root groups, plant length, root length and number of lateral roots compared to the control treatment (without bio-chemical fertilization), and this indicates that the bacterial bio-fertilizer has supplied millet plants with their needs. Dietary component nitrogen during the growth phase. The results also indicate the ability of *Azospirillum* isolates to produce IAA, and the highest produced amount was 21.7 mg $L^{-1}$, which was recorded by isolate A1, which gave a significant superiority for the dry weight of the root group, root length and number of lateral roots compared to the control treatment (N%0) and the treatment of nitrogen fertilization not inoculated with fertilizers Viability (N%100), this indicates that the bacterial biofertilizer prepared millet plants with IAA, which in turn stimulated the root group to grow in addition to fixing atmospheric nitrogen.
**Keywords: millet plant, *Pennisetum americanum* L., *Azospirillum brasilense***

## Introduction

The use and spread of bio-bacterial fertilizers has expanded in the last two decades of the last century, as a result of the scarcity of energy sources and the consequent rise in the prices of chemical fertilizers, as well as modern trends in reducing the sources of environmental pollution and the adoption of bio-fertilization as one of the modern techniques to reduce the excessive use of chemical fertilizers (Haran and Thaher,2019).

Azospirillum bacteria have played a major role in biofertilization by contributing to the fixation of part of the nitrogen through symbiosis with Grass plants. It also affects plant growth as a result of its production of substances that encourage the growth and development of plant roots (Thaher,2001). Al-Shamma, (2013) He also indicated that Azospirillum is a non-specialized bacterium that fixes atmospheric nitrogen in varying quantities and improves plant growth through the secretion of some hormones, enzymes, and vitamins, which is positively reflected in plant growth and increased productivity.

Several studies have indicated that plant hormones such as (IAA) Indol-3-acetic acid, which are produced by Azospirillum bacteria, encourage plant growth by stimulating root growth (Akbari *et al*., 2007), and they also stimulate the formation of additional root hairs and side roots. The root and side roots are not only attributable to IAA but also to the production of other hormones (Thaher,2001).

Millet is one of the important crops, especially under the irrigated conditions in the central and southern regions to suit the environmental conditions for it, and some people in the world depend on it for human and animal consumption (FAO, 2013).

Therefore, the research aims to find out the production of IAA by Azospirillum bacteria in liquid cultures and the effect of inoculation with Azospirillum bacteria on millet plant growth and root group development in the presence of different combinations of nitrogenous chemical fertilizers.

## Materials and working methods

### Collecting soil and roots samples for the purpose of isolating bacteria:

Soil samples were collected from the rhizosphere area. The roots of millet plants and rhizosphere soil were carefully extracted from a field located in the Rifai / Dhi Qar area. The samples were placed in clean plastic bags sterilized with alcohol and kept in the refrigerator until used for isolation purposes. Samples were also taken from the surface layer (Zero - 30 cm). From the soil far from the root to conduct the chemical, physical and biological tests shown in Table 1.

### Isolation and purification of Azospirillum spp:

The roots of millet plants were taken after removing the rhizosphere soil from them. Some roots were cut into small pieces of 5-8 mm length and transferred by sterile forceps to test tubes containing sterile semi-solid nitrogen free malate medium (Nfb) to obtain isolates of Azospirillum bacteria according to (Thaher,2001).

With the appearance of ring growth in the nitrogen-free medium, three successive transfers were carried out on the same medium and incubated at 30 °C for 48 hours.

The dishes were incubated at 37 °C for 72 hours according to the method (FNCA, 2014), and after the appearance of the small carmine-coloured colonies on the medium, each colony was purified again by re-planning on the same medium to ensure its purity and to observe the shapes of the colonies belonging to the isolate and the shapes of its cells were examined microscopically, then the isolates were preserved on the medium of the ashes Slant feeder (Slant) for the purpose of conducting diagnostic tests on it.

Bacterial diagnostic methods:

The biochemical characteristics, as well as the phenotypic and microscopic characteristics of nitrogen-fixing Azospirillum isolates, were studied, taking into consideration the study of the characteristics related to the diagnosis of the genus Azospirillum and its dependent species (Thaher,2001).

**Table 1: Some physical, chemical and biological waste of potting soil**

| Adjective | Shatrah | Al Rifai | Unit |
|---|---|---|---|
| pH | 7.3 | 7.2 | —— |
| Ece | 3.11 | 3.4 | $ds.m^{-1}$ |
| O.M | 7.2 | 6.8 | $g.kg^{-1}$ |
| N | 37.1 | 33.9 | $mg\ Kg^{-1}$ Soil |
| P | 7.92 | 6.46 | |
| Clay | 180 | 192 | $g.kg^{-1}$ |
| Silt | 518 | 535 | |
| Sand | 302 | 273 | |
| Texture | Mixed | Mixed | |
| Total bacteria | $10^6 \times 2.9$ | $10^6 \times 2.1$ | cfu. $gm^{-1}$ dry soil |
| Azospirillum bacteria | $10^4 \times 84$ | $10^4 \times 69$ | |

***Results represent an average of three replicates.**

**Extraction of plant hormones produced by Azospirillum bacteria:**

Azospirillum strains were grown in Nfb medium supplemented with DL-Tryptophan. The medium is distributed in 200 ml bottles containing 100 ml of it, and the bottles are inoculated with 5 ml of the inoculum of different isolates of Azospirillum, which has a light density of 0.85 (this optical density is equal to the optical density of the standard turbidity that contains ($1.5\times10^8$ bacteria to $ml^{-1}$) according to the method (Baron and Finegold, 1990), then incubated in the shaking incubator (100 cycles $min^{-1}$) at a temperature of 28°C for 24 hours, and the bacterial cultures growing in 100 ml of medium are extracted by centrifugation in a centrifuge 7700 cycles for 30 minutes according to the method (Thaher,2001). Then 50 ml of the filtrate was taken and the pH was adjusted to 2.5 using 2N hydrochloric acid (HCl), and then the partitioning process was carried out with a similar volume of ethyl acetate for four times. The organic phase containing plant hormones was evaporated using a rotary evaporator at a temperature of 35 °C according to the method (Hanna and Mullinix, 2007), then 5 ml of methanol was added to it, then the samples were analyzed using a High performance liquid chromatography (HPLC). The auxins were estimated at a wavelength of 280 nm according to (Audus, 1972), the standard solutions of IAA were prepared at concentrations (0, 2, 5, 10 and 20 mg $L^{-1}$) using IAA auxin, as the standard solutions were injected and the readings were taken for them. The standard curve between the standard solutions of IAA and its readings in the device, then the readings obtained for the unknown samples were projected to obtain the concentration of IAA in units of amalgam per liter.

**The potting experience in the greenhouse:**

In order to obtain information about the ability of Azospirillum bacteria to affect millet plants in soil, the pot experiment was carried out in the shade and using a completely randomized design (CRD) with three replications and the characteristics were analyzed using the analysis of variance. The averages of the transactions were compared using the least significant difference (RLSD) test using the calculator at a probability level of 0.05, depending on the SPSS statistical program (Al-Rawi *et al*., 2000). So, soil was taken from a field located in the Shatrah / Dhi Qar area, whose chemical, physical and biological characteristics are shown in Table 1, air-dried, crushed and sieved of 4 mm, then placed in a pot with an amount of 5 kg $Pot^{-1}$ on the basis of dry weight, fertilized with nitrogen fertilizers (according to The fertilizer recommendation is 250 kg N $ha^{-1}$) in the form of urea fertilizer (46% N) and in two batches, one with planting and the second one month after germination, then the superficially sterilized Pennisetum americanum L. millet seeds were inoculated with the liquid inoculum for isolate (A1) and three replications with Some pots were left without inoculation as a comparison treatment, then planted directly at a rate of 20 seeds per pot and irrigated with tap water to the extent of the field capacity and compensated for the lost moisture on the basis of weight. The height of the plants and the vegetative and root group of the plants were obtained and the dry weight of the vegetative and root group was obtained. The roots and vegetative parts were dried in an oven at 65° m for 48 hours and their dry weight was taken, then the length of the root and the number of lateral roots were measured.

**Results and Discussion**

**Isolation and identification of Azospirillum bacteria:**

Four isolates of Azospirillum bacteria were taken from an area planted with millet in the city of Al-Rifai, as they were isolated from the roots of the millet plant.

By studying the cultivar, microscopic and biochemical characteristics of the studied isolates shown in Table 2, and depending on the special criteria for differentiation between species according to researchers (Akbari *et al*., 2007; Al-Shamma, 2013), it appeared that all isolates belong to the species (A.brasilense) as shown in Table 3.

Table 2: microscopic and biochemical characteristics for the diagnosis of Azospirillum isolates.

| **Isolation number** | **Phenotypic characteristics of colonies** | | | **Microscopic characteristics** | | | **Biochemical characteristics** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Density** | **Appearance** | **Color** | **Shape** | **Movement** | **Pigment gram** | **Oxidase** | **Catalase** | NaCl%3 | pH (6) | pH (7.5) |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | +++ | **Dry** | **Red** | **Stick** | **spiral** | $G^{-ve}$ | ++ | + | + | ++ | ++ |
| A2 | + | Dry | **R**ed | **S**tick | spiral | $G^{-ve}$ | ++ | ++ | + | + | + |
| A3 | + | Dry | **R**ed | **S**tick | spiral | $G^{-ve}$ | ++ | ++ | + | + | ++ |
| A4 | ++ | **Glitter** | **Pink** | **S**tick | spiral | $G^{-ve}$ | + | + | + | + | + |

**(-) No growth (+) Weak growth (++) Medium growth (+++) Heavy growth**

Table 3: differential characteristics to distinguish the dependent species of Azospirillum isolates.

| Isolation / Exams | | A1 | A2 | A3 | A4 |
|---|---|---|---|---|---|
| **Use of multiple carbon sources** | **Glucose** | – | – | – | – |
| | **Maltose** | – | – | – | – |
| | **Lactose** | – | – | – | – |
| | **Sucrose** | – | – | – | – |
| | **Trehalose** | – | – | – | – |
| | **Mannitol** | – | – | – | – |
| | **Pectin** | – | – | – | – |
| **In the midst of the need for biotin** | | – | – | – | – |
| **Growth at pH 7.5** | | + | + | + | + |
| **Growth at pH 6.0** | | + | + | + | + |
| **Growth with 3% NaCl** | | + | + | + | + |
| **Type of isolation** | | A.brasilense | A.brasilense | A.brasilense | A.brasilense |

**(-) No growth (+) Weak growth (++) Medium growth (+++) Heavy growth**

Amount of IAA secreted by A.brasilense:

Table 4 indicates the difference of isolates in their production of IAA, and the highest amount of IAA was 21.7 mg $L^{-1}$ recorded by isolate A1, as some isolates have high production of IAA and some are low, and this difference may be due to the nature and ability of Azospirillum bacteria to produce these hormones (Akbari *et al*., 2007).

It was also shown (Thaher,2001) that the nitrogen-fixing A.brasilense bacteria isolated from the rhizosphere of different weeds differ in their production of IAA, and this depends on the concentration of Tryptophen added to the medium and the age of the bacterial culture until reaching the stationary phase.

Table 4: Quantity of IAA from different isolates of A.brasilense.

| **Isolation** | **Amount of IAA mg $L^{-1}$** |
|---|---|
| A1 | 21.7 a |
| A2 | 9.6 d |
| A3 | 17.1 b |
| A4 | 13.8 c |

**± 0.28 deviation of values from their mean.**

**Plant height:**

Table 5 indicates the effect of inoculation with Azospirillum bacteria on the rate of growth of millet plants, as inoculation led to a significant ($p<0.05$) increase in the rate of plant height, (33.08%) compared to the control treatment. higher than the control treatment, which did not differ significantly from the treatments of nitrogen fertilization and combined fertilization (biological and nitrogen).

The increase in the height of plants may be due to the fixation of atmospheric nitrogen by Azospirillum bacteria and then its absorption by the plant, which leads to an increase in the height of the plant, in addition to the fact that these bacteria secrete natural growth hormones such as IAA, which works to develop the root group of the plant (Hanoon *et al*., 2020; Tahir and Sarwar, 2013).

Table 5: The effect of adding biological and nitrogen fertilizers on the rate of plant height (cm).

| **Control treatment** | **Nitrogen fertilizer 100%N** | **Bio fertilizer bacterial** | **Bacterial fertilizer and 50% N** | **Bacterial fertilizer and 100% N** |
|---|---|---|---|---|
| 26.9 b | 37.3 a | 35.8 a | 36.7 a | 38.7 a |

Dry weight of the vegetative part of millet:

It is noted from Table 6 that inoculation with Azospirillum bacteria had a significant effect on the average dry weight of the vegetative part of the plant, as inoculation with Azospirillum bacteria without adding nitrogen fertilizers increased the dry weight of the vegetative part by 33.91% higher than the comparison treatment which gave the lowest average dry weight of the vegetative part. (5.13 g Pots$^{-1}$).

The increase in the dry weight rate of the vegetative part of the plant upon inoculation with Azospirillum bacteria may be due to these bacteria fixing nitrogen biologically, which leads to an increase in plant growth and consequently an increase in the dry weight of the vegetative part of the plant (Tahir and Sarwar, 2013), and many researchers have obtained an increase in the dry weight of the plant as a result of to inoculate plants with some isolates of Azospirillum bacteria (Haran, 2021), in addition to the fact that these bacteria produce growth-regulating substances such as IAA, which leads to increased growth of the root system, which increases the absorption of water and nutrients from the soil (Haran *et al.*, 2021) and encourages the absorption of nitrates and phosphates. and potassium (Hanoon *et al.*, 2020).

Table 6: Effect of adding biological and nitrogen fertilizers on the dry weight of the vegetative part of the plant (g pots$^{-1}$).

| **Control treatment** | **Nitrogen fertilizer 100%N** | **Bio fertilizer bacterial** | **Bacterial fertilizer and 50% N** | **Bacterial fertilizer and 100% N** |
|---|---|---|---|---|
| 5.13 b | 7.11 a | 6.87 a | 6.94 a | 7.33 a |

**Plant root lengths:**

Table 7 indicates a significant increase in the average root lengths of plants when inoculated with Azospirillum bacteria, as the percentage of increase when inoculated with Azospirillum bacteria without adding nitrogen fertilizers was (51.77%), which was significantly superior to the treatment of nitrogen fertilizer 100% N% without bacterial inoculation, while The lowest average was (14.1 cm) when the comparison treatment. These results are consistent with what was indicated by (Akbari *et al.*, 2007) that inoculation of plants with Azospirillum species led to an increase in root length.

The inoculation with Azospirillum bacteria led to a significant increase in the average length of plant roots, due to its secretion of an appropriate amount of IAA (Table 4), as it leads to an increase in the absorption capacity of nutrients from the roots as a result of the development of the root system (Thaher,2001).

Table 7: Effect of adding biological and nitrogen fertilizers on plant root lengths (cm).

| **Control treatment** | **Nitrogen fertilizer 100%N** | **Bio fertilizer bacterial** | **Bacterial fertilizer and 50% N** | **Bacterial fertilizer and 100% N** |
|---|---|---|---|---|
| 14.1 c | 17.9 b | 21.4 a | 22.8 a | 19.5 ab |

**Dry weight of the root part of the plant:**

The results showed that the inoculation with Azospirillum bacteria led to a significant ($p<0.05$) increase in the dry weight of the root as shown in Table 8. The percentage of increase in dry weight of the root part of the plant when inoculated with Azospirillum bacteria without adding nitrogen fertilizers (42.75%) was higher than the control treatment, which did not differ significantly from the combined fertilization treatments (biological and nitrogen) and differed significantly from the nitrogen fertilization treatment only.

The increase in the rate of dry weight of the root part of the plant when inoculated with Azospirillum bacteria may be due to the bacteria producing growth-regulating substances such as IAA, which leads to an increase in the growth of the root system by increasing root length, number of lateral roots and the density of root hairs, which increases the absorption of water and nutrients from Soil (Thaher,2001). Many researchers have obtained an increase in the dry plant weight as a result of inoculation of plants with some isolates of Azospirillum bacteria (Haran, 2021).

Table 8: Effect of adding biological and nitrogen fertilizers on the dry weight of the root part of the plant (g. pots$^{-1}$).

| **Control treatment** | **Nitrogen fertilizer 100%N** | **Bio fertilizer bacterial** | **Bacterial fertilizer and 50% N** | **Bacterial fertilizer and 100% N** |
|---|---|---|---|---|
| 1.31 c | 1.58 b | 1.87 a | 1.91 a | 1.67 ab |

**Average number of lateral roots of plants:**

Table 9 indicates a significant increase in the average number of lateral roots of millet when inoculated with Azospirillum bacteria, as the highest rate of the number was (74) when inoculated with Azospirillum bacteria, which significantly outperformed most of the treatments, but did not differ significantly from the treatment of bacterial and nitrogen fertilizers 50% N. While the lowest rate was (40) for each pot when the control treatment, and inoculation with Azospirillum bacteria increased the number of lateral roots by 85% higher than the control treatment.

Vaccination with Azospirillum bacteria led to a significant increase in the number of lateral roots of the plant. This may be due to the bacteria's ability to produce IAA in appropriate quantities (Table 4), which in turn works to develop the root group, increase the number of lateral roots and increase its absorption of nutrients (N, P, K) and thus increase Meristem tissue division and elongation (Wasim, 2006).

Table 9: Effect of adding biological and nitrogen fertilizers on the number of lateral roots for each pot.

| Control treatment | Nitrogen fertilizer 100%N | Bio fertilizer bacterial | Bacterial fertilizer and 50% N | Bacterial fertilizer and 100% N |
|---|---|---|---|---|
| 40 d | 52 c | 74 a | 68 ab | 60 bc |

**Conclusions and Recommendations**

1- The isolates of A.brasilense varied in their production of IAA, Isolate A1 outperformed the rest of the isolates in its production of IAA.

2- Fertilization with A1 isolate of A.brasilense led to a significant increase in the studied growth parameters of potted millet plants compared to the control treatment, as it did not differ significantly from the treatment of nitrogen fertilization 100%N with respect to plant height and dry weight of the vegetative part of millet, while it was significantly superior About the treatment of nitrogen fertilizer 100%N in relation to the dry weight of the root, root length and number of lateral roots, and thus this reduces production costs and reduces environmental pollution when using nitrogen fertilizer.

3− We recommend inoculating millet plants with A.brasilense.

4 - Conducting research on the presence of natural growth hormones secreted by Azospirillum bacteria in general, determining their quantities and studying their effect on plants.

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