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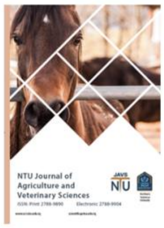

Evaluation of the effectiveness of biofertilization with native bacteria in improving the growth and yield of *Trifolium alexandrinum* under Gypsiferous soil conditions

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ABSTRACT

The excessive use of chemical fertilizers, particularly nitrogen-based ones, has significantly exacerbated environmental problems.The excessive accumulation of these fertilizers in soil and water leads to severe pollution, threatening biodiversity, disrupting ecological balance, and adversely affecting human and animal health over the long term., the world uses advanced agricultural methods, including biofertilizers, and natural sources of plant nutrients. For this reason, a field experiment was conducted at University of Tikrit, Iraq on 2022, to evaluate the effect of a bio-inoculant prepared from local bacterial isolates, namely B1 Ensifer meliloti, B2 Pseudomonas azotoformans, and B5 Sinorhizobium meliloti, , on the growth and yield of Egyptian clover (Trifolium alexandrinum) under different nitrogen levels (0, 40, and 80 kg N ha-1). The experiment demonstrated that seed inoculation with bacteria B1 and B3 significantly positively impacted plant growth compared to uninoculated seeds or those inoculated with pseudmonas azotoformans. Additionally, increasing the amount of nitrogen fertilizer improved growth, but the best results were obtained when the highest fertilizer level was combined with inoculation using bacteria B3.

Introduction

Due to the negative environmental impacts of intensive agriculture, there has been a growing interest in sustainable agriculture that relies on reducing the use of chemical inputs. Legumes are a key component of this type of agriculture, as they contribute to improving soil fertility and maintaining environmental balance through their symbiotic relationship with rhizobia bacteria, which fix atmospheric nitrogen, thus reducing the need for chemical fertilizers. [1]. Increasing the amount of biologically fixed nitrogen provided by the symbiotic relationship between legumes and Rhizobium bacteria can reduce the use of chemical nitrogen fertilizers and the undesirable environmental impacts associated with the misuse of nitrogen fertilizers, such as air and groundwater pollution [2,3]; There is a pressing need for solutions that allow for food production without the excessive use of agricultural chemicals [4].Managing the rhizosphere and introducing biofertilizers to enhance nutrient efficiency in the soil and promote plant growth and productivity is one such solution [5]. Biofertilizer refers to a solid or liquid material containing live microorganisms that is applied to the soil, seeds, or seedlings to stimulate plant growth and yield by providing essential nutrients, growth-promoting substances, and enhancing the plant's ability to absorb nutrients and resist various environmental stresses [6] Egyptian clover is one of the most prominent forage legume crops cultivated for green fodder and ranks second after alfalfa in Iraq in terms of importance, as it is one of the most productive winter forage plants in central and southern Iraq [7]. Clover produces a significant amount of green fodder that is almost a complete food for animals due to its high content of digestible crude protein with high nutritional value. It is also rich in calcium, phosphorus, vitamins, and carotene, making it easily digestible and palatable for various types of animals [8].

Materials and methods

1. Selection of bacterial isolates

A new biological inoculant was produced from indigenous bacteria isolated from different clover species in Iraq. These bacteria, registered globally under the names of Iraqi researchers Alkafaf, H.S and Alkurtany, A.E., have the ability to enhance plant growth and fertility. This inoculant has been used in field trials to demonstrate its effectiveness.

2.Steps of bacterial vaccine preparation

A pure isolate of Sinorhizobium meliloti, Pseudomonas azotoformans, Ensifer meliloti was cultivated of sterile YEMB medium [6].

Field experiment

A field study was conducted at Tikrit University, on October 2022. The experiment was designed using a Randomized Complete Block Design (R.C.B.D) with three replications.

On October 10, 2022, the land was prepared for the experiment through tillage, leveling, and smoothing. The area was divided into three main plots, and each plot was further divided into twelve experimental units, each measuring 2 × 1.5 square meters. There was a 1-meter distance between plots and a 75-centimeter distance between units. Soil samples were collected from a depth of 0-20 cm for physical, chemical, and biological analyses. Table 1 presents the initial results of these analyses.

An experiment was conducted on Egyptian clover (*Trifolium alexandrinum*) on October 20, 2022. The experiment involved applying nitrogen fertilizer (urea) at different rates (0, 40, and 80 kg N ha^{-1}), phosphorus fertilizer (triple superphosphate) at a rate of 80 kg P ha^{-1}, and potassium fertilizer (potassium sulfate) at a rate of 120 kg K ha^{-1}. All fertilizers were applied as a single dose before planting. Seeds were sown in rows with a spacing of 20 cm between rows, and the plots were irrigated using a drip irrigation system.

Table 1. shows properties of the soil

Parameter	value
pH	7.3
N	19
P	3.9
K	119
B.D $g.cm^{-3}$	1.38
Sand $g.cm^{-3}$	546
Silt $g.k^{-1}$	219
Clay $g.k^{-1}$	235
Texture	Sandy Clay Loam
Total bacteria gm^{-1} Scfu	$5.3*10^6$
Total fungi gm^{-1} Scfu	$3.9*10^3$

Results and discussion

Root nodule number per Egyptian clover plant

The results in the table 2 indicate that inoculation with *Sinorhizobium meliloti* strain B3 and *Ensifer meliloti* strain B1 significantly increased the number of root nodules in Egyptian clover plants compared to the treatment inoculated with *Pseudomonas azotoformans* strain B2 and the uninoculated treatment B0. The number of nodules for treatments B3 and B1 was 26 and 20 nodules per plant, respectively, while for treatments B2 and B0, it was only 6 and 7 nodules per plant, representing a percentage increase of 333.33% and 271.42%, respectively. This significant increase in the number of nodules can be attributed to the efficiency of these two strains in nitrogen fixation, indole-3-acetic acid production, and phosphate solubilization, as shown in Tables 17 and 18 and Figures 5 and 6. Additionally, the effectiveness of rhizobia bacteria and their increased numbers in the roots of leguminous plants may have contributed to this increase, which is consistent with the findings of [9]. These results are in line with those of [10], who found that inoculation with rhizobia led to increased plant growth, nitrogen fixation efficiency, and improved values for most of the studied traits, including the number of root nodules. This finding is also in agreement with the results of [11] and [12]. The low efficiency of the uninoculated treatment B0 can be attributed to the low abundance and effectiveness of

native bacteria, as well as the efficiency of soil sterilization [6]. On the other hand, the *Pseudomonas azotoformans* strain B2 treatment resulted in the lowest number of nodules, with only 6 nodules per plant, which may be due to its lower efficiency in promoting plant growth.

Regarding nitrogen fertilizer levels, the highest average number of root nodules was observed at the N80 kg ha^{-1} level, with 18 nodules per plant, compared to 10 nodules per plant at the N0 level, representing an 80% increase. This increase can be attributed to the fact that this nitrogen level was stimulatory for root nodule growth and development, as plants require nitrogen, especially during their early growth stages. This added nitrogen acts as an "initiator" that stimulates the formation of root nodules, indicating that this nitrogen level was stimulatory rather than inhibitory. These results are in line with those of [13] and [14] in their studies on clover.treatment B3N40 significantly outperformed the other treatments, with a root nodule number of 31 nodules per plant compared to only 5 nodules per plant in the untreated and uninoculated treatment, representing a 520% increase. Meanwhile, treatment B1N80 had 26 nodules per plant, compared to 13 nodules per plant in treatment B1N0, indicating a 100% increase. This can be attributed to the efficiency of *Rhizobium meliloti* in infecting the roots of clover plants and forming root nodules, as reported by [15].These results are in line with the findings of [16] and [17].

Table 2. Number of Root Nodules per Clover Plant

$N.ha^{-1}$	isolates				Average
	B0	B1	B2	B3	
N0	5.00 ff	13.00 e	6.00 ff	17.00 d	10.00 b
N40	7.00 ff	22.00 c	5.00 ff	31.00 a	16.00 aa
N80	8.00 ff	26.00 bb	7.00 f	30.00 ab	18.00 a
Average	7.00 c	20.00 b	6.00 cc	26.00 a	

Plant height of Egyptian clover (cm $plant^{-1}$)

Table 3 revealed that the application of bacterial inoculants and nitrogen fertilizers significantly affected the growth of Egyptian clover plants, particularly in terms of plant height. The results indicated that the use of inoculants alone led to a noticeable increase in plant height. Treatments inoculated with *Sinorhizobium meliloti* strain B3 and *Ensifer meliloti* strain B1 performed significantly better, with average plant heights of 99.88 cm and 91.55 cm, respectively, compared to treatments inoculated with *Pseudomonas azotoformans* strain B2 (74.11 cm) and the uninoculated control B0 (76.66 cm). This represents a percentage increase of 34.77% and 23.53%, respectively. This increase can be attributed to the efficiency of these two strains in promoting plant growth through nitrogen fixation, indole production, phosphate solubilization, and chelation, These results align with those of [18], who found that bacterial inoculation led to an increase in both the number and weight of root nodules, enhancing biological nitrogen fixation and consequently promoting plant growth and height.

Regarding nitrogen levels, the N80 kg ha^{-1} level outperformed the N0 level, with an average plant height of 88.91 cm compared to 81.66 cm for the N0 level, representing an 8.87% increase. This increase can be attributed to the role of nitrogen in vital biological processes such as protein synthesis and cell division, which enhance vegetative growth and subsequently accelerate plant growth. These findings are supported by [19].

The results showed that the interaction between nitrogen fertilizer levels and bacterial treatments had a significant impact on the height of clover plants. In particular, the treatment using bacteria B3N80 with 40 kg/ha of nitrogen performed significantly better, with an average plant height of 103 cm. Treatment B3N0 followed with a height of 100.66 cm, and then treatment B1N40 with a height of 96 cm. Compared to the control treatment (uninoculated and unfertilized), these three treatments achieved increases in height of 45.07%, 41.77%, and 35.21%, respectively. This increase can be attributed to the significant interaction between bacterial inoculation and nitrogen fertilization, which enhanced plant height. Additionally, the role of microorganisms as essential components for plant growth cannot be overlooked. These microorganisms, such as nitrogen-fixing bacteria, increase nutrient availability and promote plant growth [20]. These findings align with those of [21]. Conversely, treatment B2N0 exhibited the lowest plant height (68.00 cm), which can be attributed to the lower efficiency of *Pseudomonas azotoformans* in forming root nodules, fixing nitrogen, and solubilizing phosphate, as well as producing chelates,. This negatively impacted plant growth.

Table 3. height of Egyptian clover plants (cm $plant^{-1}$)

$N.ha^{-1}$	Isolates				Average
	B0	B1	B2	B3	
N0	fg71.00	87.00 dc	68.00 GG	100.66 ab	81.66 b
N40	78.33 def	96.00 abc	74.00 efg	96.00 abc	86.08 aa
N80	80.66 de	91.66 bc	80.33 de	103.00 a	88.91 a
Average	76.66 c	91.55 b	74.11 c	99.88 a	

Percentage of wet leaves to stems in Egyptian clover %

The results in table 4 indicated significant differences among the means of bacterial isolates, nitrogen fertilizer levels, and the interaction between fertilizer levels and bacterial isolates. The treatment inoculated with *Sinorhizobium meliloti* strain B3 significantly outperformed other treatments, with a leaf-to-stem ratio of 46.44%. This was followed by *Ensifer meliloti* strain B1 with a ratio of 44.66%, compared to the uninoculated control B0 with the lowest ratio of 37.60%. This represents a percentage increase of 23.51% and 18.77%, respectively. This increase can be attributed to the fact that the addition of biofertilizers containing nitrogen-fixing bacteria promotes plant growth by influencing the uptake of minerals and water and increasing root growth, as confirmed by [22]. Additionally, the role of microorganisms in nitrogen fixation is crucial for increasing both the quantity and quality of plant production, as reported by [23]. In contrast, *Pseudomonas azotoformans strain* B2 yielded the lowest mean leaf-to-stem ratio of 38.53%.

Regarding nitrogen fertilizer levels, the N80 kg ha^{-1} level outperformed the unfertilized N0 level, with an average leaf-to-stem ratio of 44.70% compared to 38.26% for the N0 level, representing a percentage increase of 16.83%. This increase can be attributed to the fact that the addition of nutrients is a crucial stimulant for biological processes. Nutrients are essential for the growth and synthesis of enzymes. Nitrogen and phosphorus, in addition to carbon, are among the most important nutrients required by microorganisms in high concentrations [24].

B3N80 significantly outperformed other treatments, with a leaf-to-stem ratio of 50.48%.This was followed by treatment B1N40 with a ratio of 47.16%, compared to the uninoculated and unfertilized treatment, which had the lowest ratio of 33.78%. This represents a percentage increase of 49.43% and 39.60%, respectively. The addition of nitrogen and phosphate biofertilizers significantly increases the yield of inoculated plants, enhancing plant growth parameters and resulting in a yield increase of more than 40% [25,26]. In contrast, treatment B2N0 yielded the lowest mean leaf-to-stem ratio of 34.

Table 4. wet leaves to stems of Egyptian clover%

N.ha^{-1}	Isolates				Average
	B0	B1	B2	B3	
N0	33.78 gg	43.07 bcde	34.40 gg	41.80 cde	38.26 c
N40	39.97 def	47.16 ab	35.69 fg	47.05 ab	42.47 b
N80	39.06 ef	43.74 bcd	45.50 bc	50.48 a	44.70 a
Average	37.60 bb	44.66 aa	38.53 b	46.44 a	

Dry weight of Egyptian clover per (tonnes/hectare)

Table 5 shows the treatment inoculated with *Sinorhizobium meliloti* strain B3 significantly outperformed other treatments, with an average dry matter yield of 3.71 t/ha. This was followed by the treatment inoculated with *Ensifer meliloti* strain B1 with a yield of 3.52 t/ha, compared to the uninoculated control B0 with a yield of 2.56 t/ha. This represents a percentage increase of 44.92% and 37.5%, respectively, Studies have shown that the use of these bacteria in sustainable agriculture can improve plant growth, increase biomass, and enhance soil quality [27]. These results are consistent with those of [28], who found that inoculation with Rhizobium bacteria increased plant dry matter by up to 89.7% and 49.1%. In contrast, strain B2 yielded the lowest dry matter, and the lower values of growth-promoting parameters.

Regarding nitrogen fertilizer levels, the N80 level significantly outperformed the N0 level, with an average dry matter yield of 3.12 t/ha compared to 2.75 t/ha for the N0 level, representing a percentage increase of 13.45%. There were no significant differences among the lower levels. This increase can be attributed to the role of nitrogen as a key component of chlorophyll, as shown in Table 63. Chlorophyll enables plants to absorb light energy and convert it into chemical energy through photosynthesis. The increased photosynthetic efficiency leads to higher carbohydrate production, which contributes to increased plant dry matter.

Regarding the interaction between bacterial isolates and nitrogen fertilizer levels, treatment B3N40 significantly outperformed other treatments, with an average dry matter yield of 4.07 t/ha. This was followed by treatment B1N80 with a yield of 3.59 t/ha, compared to the uninoculated and unfertilized treatment B0N0 with a yield of 2.18 t/ha. This represents a percentage increase of 86.69% and 64.67%, respectively. This increase can be attributed to the beneficial interaction between bacteria and nitrogen fertilizer, especially at the N40 level. Experimental evidence suggests that applying nitrogen at rates ranging from 30 to 70 kg/ha can significantly enhance fresh and dry matter yield of clover. The results demonstrate that nitrogen fertilization can significantly enhance the growth and yield of legume crops like clover, despite the conventional belief that legumes are self-sufficient in terms of nitrogen due to their symbiotic relationship with nitrogen-fixing bacteria [13,29].

Table 5. Dry weight (tonnes/hectare) of Egyptian clover

N.ha^{-1}	isolates				Average
	B0	B1	B2	B3	
N0	2.18 ef	3.43 bb	1.96 ff	3.45 bb	2.75 b
N40	2.63 cd	3.53 bb	2.10 ef	4.07 a	3.08 aa
N80	2.86 c	3.59 bb	2.40 de	3.62 b	3.12 a
Average	2.56 b	3.52 aa	2.15 c	3.71 a	

Amount of nitrogen absorbed by of Egyptian clover (kg N/ha)

bacterial isolates, nitrogen fertilizer levels, and their interaction on the nitrogen uptake of the first cut of Egyptian clover. The results indicate a significant effect of both bacterial isolates and nitrogen levels, as well as a significant interaction between nitrogen levels and bacterial isolates. The treatment inoculated with *Sinorhizobium meliloti* strain B3 significantly outperformed other treatments, with a nitrogen content of 95.93 kg N/ha. The treatment inoculated with bacteria B1 resulted in a plant nitrogen content of 87.50 kg_N/ha, representing a significant increase compared to the control treatment (B0) which had a nitrogen content of 40.02 kg_N/ha, This represents a percentage increase of 139.70% and 118.64%, respectively. This can be attributed to the efficient use of *Ensifer meliloti* and *Sinorhizobium meliloti* inoculants and the interaction between the bacteria and the legume plant, which enhances nutrient availability in the soil and thus prepares it for plant uptake. Rhizobia bacteria contribute to improving the nutritional status of the plant and thus provide the plant with nitrogen[30]. Additionally, the symbiotic relationship between Egyptian clover and Rhizobia bacteria, leading to the formation of root nodules as shown in Table (2), results in the fixation of atmospheric nitrogen and consequently increased plant uptake [31]. In contrast, strain B2 showed the least significant difference with a value of 20.24 g/plant.

Regarding nitrogen fertilizer levels, the N80 kg/ha level significantly outperformed the unfertilized N0 level. The nitrogen content in the N80 treatment was 65.28 kg/ha

compared to 52.99 kg/ha in the N0 treatment, an increase of 23.19%. However, there was no significant difference between the N80 and lower nitrogen levels. This increase can be attributed to the role of the applied mineral fertilizer in increasing the availability of nitrogen in the soil solution, thereby increasing its uptake by roots and its concentration in the plant's vegetative parts. These results are consistent with those of [32], who found an increase in the percentage of nitrogen uptake by plants when mineral fertilizer was applied.

Regarding the interaction between nitrogen fertilizer levels and bacterial isolates, treatment B3N40 significantly outperformed other treatments, with a nitrogen content of 103.60 kg/ha. This was followed by treatment B1N80 with a content of 90.58 kg/ha, compared to the uninoculated and unfertilized treatment B0N0 with a content of 18.62 kg/ha. This represents a percentage increase of 456.39% and 386.46%, respectively. These results are in agreement with those of [14] who studied the effect of co-inoculation and nitrogen levels on Egyptian clover. Treatment B2N80 showed the lowest nitrogen content of 18.08 kg/ha.

Table 6. nitrogen amount (kg N/ha) of Egyptian clover

N.ha^{-1}	Isolates				Average
	B0	B1	B2	B3	
N0	468.4 dd	1044.0 a	493.8 dd	808.0 abc	703.58 aa
N40	543.9 cd	935.1 aa	550.6 cd	869.9 ab	724.88 aa
N80	671.3 bcd	8447.2 ab	688.7 bcd	876.2 ab	770.8 c
Average	561.21 bb	942.10 a	577.71 b	851.37 aa	

Seed yield of Egyptian clover (kg/ha)

The results presented in Table **7** clearly demonstrate the superiority of the treatments inoculated with bacteria compared to the control (uninoculated) treatment. The treatment inoculated with bacteria B1 achieved the highest average seed weight (942.10 kg/ha), followed by the treatment inoculated with bacteria B3 (851.37 kg/ha). In contrast, the control treatment recorded the lowest average (561.21 kg/ha). This increase in seed yield can be attributed to the efficiency of the inoculant in forming root nodules, which play a significant role in fixing atmospheric nitrogen.This increased nitrogen content in the vegetative parts of the plant enhances protein synthesis, improving plant growth and yield. Additionally, the improvement in carbohydrate reserves during growth led to better seed formation and increased root activity for nitrogen uptake. These findings are in agreement with those of [33,34], and [35] in their studies on Egyptian clover.

Regarding nitrogen fertilizer levels, the N80 kg/ha level resulted in an average seed yield of 770.83 kg/ha compared to the unfertilized N0 level, which yielded 703.58 kg/ha, representing a percentage increase of 9.55%. This can be attributed to the role of nitrogen, which is an essential macronutrient required by plants to complete their life cycle. Nitrogen is involved in chlorophyll synthesis, protein synthesis, cell division, and the formation of new cells [36]. Regarding the interaction between nitrogen fertilizer levels and bacterial isolates, treatment B1N0 significantly outperformed other treatments with a seed yield of 1044.0 kg/ha, followed by treatments B1N40 and B3N2 with yields of 935.1 kg/ha and 876.2 kg/ha, respectively. Compared to the control treatment B0N0 with a yield of 468.4 kg/ha, this represents a percentage increase of 122.88%, 99.62%, and 87.13%, respectively. This can be attributed to the efficiency of co-inoculation with nitrogen fertilizer, which increases nitrogen fixation, improves the availability of macronutrients, and promotes the production of chelating agents and plant growth hormones, thereby enhancing the growth and yield of legumes and maintaining soil fertility.Treatment B2N0 yielded a lower seed weight of 493.8 kg/ha, while treatment B2N80 increased the yield to 688.7 kg/ha .This can be attributed to the role of nitrogen in stimulating the synthesis of proteins, nucleic acids, protoplasm, and chlorophyll [37].

Table 7. the seed yield (kg/ha) of Egyptian clover.

$N.ha^{-1}$	Isolates				Average
	B0	B1	B2	B3	
N0	18.62 dd	87.31 ab	18.08	87.96 ab	52.99 b
N40	49.94 cc	84.63 b	19.83 dd	103.60 a	64.50 aa
N80	51.49 c	90.58 ab	22.82 d	96.22 ab	65.28 a
Average isolates	40.02 b	87.50 aa	20.24 c	95.93 a	

Conclusions

We can conclude from the results of the current study that the root nodules of *Trifolium alexandrinum* plants may contain other bacterial genera that do not belong to the Rhizobiaceae family, such as, and *Pseudomonas azotoformans*, in addition to containing species belonging to the Rhizobiaceae family that can be used in the production of biofertilizer in environmental cleanliness and sustainable agriculture. It was found that inoculation with the vaccine prepared from the two isolates B1 and B3 *Ensifer meliloti* and *Sinorhizobum meliloti* was superior in most of the studied traits, while inoculation with the isolate B2 *Pseudomonas azotoformans* led to a reduction in all plant growth traits. The fertilized treatment at the level of 80 kg N ha^{-1} was significantly superior to the unfertilized treatment in most of the studied traits.

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