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GENE EXPRESSION OF *NIFX* IN BROAD BEAN ROOT NODULES UNDER RHIZOBIAL INOCULATION AND MOLYBDENUM APPLICATION

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ABSTRACT

This study aims to investigate the expression of the *nifX* gene under the influence of bacterial inoculation and molybdenum application in selected cultivars of broad bean (*Vicia faba* L.), A field study was conducted in the winter season of 2023–2024 in the research fields of Baghdad University to evaluate the effect of Rhizobium leguminosarum inoculation and foliar application of molybdenum (Mo) on the root characteristics and expression of the nitrogen-fixing nifX gene in on broad bean (*Vicia faba* L.) cultivars. The experiment was laid out in a randomized complete block design (RCBD) in a split-split plot arrangement with three replications, that included bacterial inoculation (Bo: control, no inoculation; B_1: 2×10^8 CFU mL^{-1}), molybdenum concentrations (M_0: 0 mg L^{-1}, M_1: 5 mg L^{-1}, M_2: 10 mg L^{-1}), and three broad bean cultivars (V_1: Hannaoui, V_2: Spanish, V_3: Loz). The results revealed an inverse relationship between CT values and gene expression, where both bacterial inoculation and molybdenum application enhanced nifX gene expression. The highest nifX expression levels were observed in treatments V1B1 M2, V2B1M1, and $V_3B_1M_1$, with the Hannaoui cultivar exhibiting peak expression at 10 mg L^{-1} Mo, while Spanish and Loz cultivars performed optimally at 5 mg L^{-1} Mo. Notably, the $V_1M_1B_1$ treatment recorded the highest active nodule count (845 nodules $plant^{-1}$) and root dry weight (48.935 g), highlighting the synergistic role of Rhizobium inoculation and Mo supplementation in improving nitrogen fixation efficiency and root growth traits in broad bean.

Key words: **Biofertilizers, Cultivars, Food Security, Plant-based protein, Sustainability**
***Part of MSc thesis of 1st Author**

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INTRODUCTION

Broad bean (*Vicia faba* L.) is one of the oldest crops cultivated by humans since ancient times and is considered a winter crop with high protein content (25–30%) (Sabouh et al., 2011). It is the third most important legume crop (after soybean (*Glycine max*) and pea (*Pisum sativum*) in the world (Manolikaki and Diamadopoulos, 2019, Pandit et al., 2020). The performance of broad bean cultivars differs from environmental conditions, as environmental conditions affect the productivity and genetics pattern of the growth and yield. Specifically, soil bacteria, especially those of the rhizobia, are important for nitrogen dynamics and availability, which relate strongly with organic matter distribution in rhizosphere (Carrion et al 2019, Liu et al 2021). Rhizobia (*Rhizobium spp*.), which belong to the bacterial family Rhizobiaceae and reside symbiotically in root nodules of legumes or are free-living organisms in the soil (Brom et al 2000). Microorganisms, including bacteria, differed in their functions for plants. They had the ability to fix nitrogen, and their effect on plant growth was through

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the production of plant hormone (Al-Shakarchi and Hisyan, 2021). These bacteria act as biofertilizers: they fix atmospheric nitrogen (N_2) and provide plants with key nutrients (N, P, and K) in exchange for carbohydrates, which is required for their survival and metabolic activity (Pankievicz et al., 2019, Spaepen et al., 2007). Gedamu et al. (Gedamu et al., 2021) found that significant increases in plant height (61.733 cm), root nodule count, dry nodule weight, and grain yield (3806.5 kg ha^{-1}) were due to inoculating the broad bean plants with strain EAL-1018 of the genus Rhizobium, when compared with the control. (Youseif et al., 2017) stated that inoculation with Rhizobium leguminosarum sv. The increase included; plant height (134 cm), branch number (3.7 $plant^{-1}$), pod count (24 pods $plant^{-1}$), seed yield (4.36 t ha^{-1}), dry nodule weight (814 mg) and root dry weight (2.53 g) by Viciae. Biological nitrogen fixation (BNF) by legumes, such as broad bean, requires two types of molybdenum-dependent nitrogenase enzymes. Rhizobia also have this enzyme in root nodules, which reasons the specific needs of legumes for more molybdenum than non-legume crops and also molybdenum concentration (Kliewer and Kennedy., 1978, Togay et al., 2008). Molybdenum (Mo) is an essential micronutrient involved in key nitrogen metabolism pathways, including nitrification, nitrate assimilation and denitrification, and therefore affects overall nitrogen cycling in ecosystems. It also serves as a cofactor for many key enzymes including (but not limited to) nitrate reductase and nitrogenase (responsible for biological nitrogen fixation in soil). This key member of bacterial enzymatic complexes underpins the ability of these microorganisms to transform atmospheric nitrogen (N_2) to ammonia (NH_3), Molybdenum (Mo) plays an important role in plant metabolism, but its role in enhancing nitrogen fixation through its effect on rhizobia bacteria that grow symbiotically with legumes is of particular agricultural importance (Sergey et al, 2023).the only bioavailable available that plants can absorb. Hence, the potential use of rhizobacteria and improved nitrogen fixation mechanism where the contribution of the *nif* gene is pivotal that can enhance agricultural productivity. The nitrogen fixation (nif) operon encodes ATP-dependent enzymes that perform the conversion of atmospheric nitrogen (N_2) into ammonia (NH_3). There have been several studies conducted on the symbiotic relationship between nitrogen-fixing bacteria and legumes; this specific group of bacteria is known as rhizobia (Rhizobia). So far, these bacteria have been classified taxonomically and physiologically into two major groups the alpha (α)-rhizobia and the beta (β)-rhizobia. Rizobial genera with an important presence in these groups have been *rhizobium, bradyrhizobium, mesorhizobium, sinorhizobium and azorhizobium*. Indeed, for important symbiotic bacterial species such as *Sinorhizobium meliloti, Rhizobium leguminosarum* and, particularly, *Bradyrhizobium japonicum*, genetic data have provided insights into the evolution of the genome, structural adaptations as well as the molecular conversation between plants and microbes, as well as the physiological diversity of legumes-associated microorganisms (Maclean et al., 2007). Atmospheric nitrogen fixation is an abiotic process that accomplishes the conversion of inert atmospheric nitrogen (N_2) into an organic form (NH_3) via energy intensive ATP-dependent biochemical pathways. This is mainly mediated by nitrogen-fixing microorganisms like the bacteria, Rhizobium, mediated by nitrogen-fixing (nif) genes. The nitrogenase enzyme which centralizes this involves two oxygen-labile metalloproteins, the iron (Fe) and molybdenum-iron (MoFe) proteins. Nitrogenase's FeMo-cofactor (FeMo-co) biosynthesis involves at least five *nif* genes: *nifV*, *nifB*, *nifH*, *nifN*, and *nifE*. The FeMo-co structure comprises molybdenum, iron, sulfur, and homocitrate in a 1:7:9:1 ratio. Its synthesis requires ATP, dithionite, molybdate, homocitrate, NifB-co (the metabolic product of NifB), NifNE, and NifH-mediated nitrogen reduction (Shah et al., 1999). Recent studies propose that genes such as *nifZ*, *nifW*, *nifX/NafY*, and *nifP* are primarily involved in maturing the Mo-Fe protein complex (Nonaka et al., 2019). Additionally, (Lee et al., 2000) identified *nifN*, *nifE*, and

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nifX as accessory factors critical for Fe-Mo cofactor synthesis, underscoring their role in optimizing nitrogenase activity. Plant responses to abiotic stresses such as nutrient deficiency, drought, and salinity require the production of critical metabolic proteins involved in synthesizing essential compounds, including regulatory proteins that mediate signal transduction pathways. These proteins, termed transcription factors (TFs), regulate gene expression by binding to DNA sequences of target genes involved in stress-response pathways. This coordinated regulation is governed by a proportional regulatory system, which ensures precise control of gene expression to adapt to environmental challenges (Salman and Aljuboori 2019). This study aims to investigate the expression of the *nifX* gene under the influence of bacterial inoculation and molybdenum application in selected cultivars of broad bean (*Vicia faba* L.), along with an assessment of key root system traits

MATERIALS AND METHODS

A field experiment was carried out to investigate the effect of *Rhizobium leguminosarum* inoculation and molybdenum (Mo) foliar application on root traits and nifX gene expression in some cultivars of broad bean (*Vicia faba* L.). The experiments were conducted at the research fields of the College of Agricultural Engineering Sciences, Baghdad University, in the winter season: 2023–2024. A drip system was used to irrigate crops, and standard agronomic practices were implemented.

Bacterial Inoculum Preparation

Rhizobium leguminosarum inoculum was prepared according to Black (Black, 1965a), with a bacterial density of 2.3×10^8 CFU mL^{-1}**.** Broad bean seeds were directly sown in the field on October 18, 2023, at a spacing of 30 cm between plants and 75 cm between rows. To prevent cross-contamination, plots treated with bacteria were isolated using nylon sheets buried 30 cm deep.

Experimental Design

The experiment followed a Randomized Complete Block Design (RCBD) with split-split plot arrangement with three replications. Treatments were structured as follows:

1. Main plots: Bacterial inoculation (B):
B0: Control (no inoculation).
B1: Inoculation with *Rhizobium leguminosarum* (2×10^8 CFU mL^{-1}).
2. Subplots: Molybdenum concentrations (M):
M0: 0 mg L^{-1} (control).
M1: 5 mg L^{-1}.
M2: 10 mg L^{-1} (applied as sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$).
Foliar applications were performed in two doses: November 23, 2023**,** and December 23, 2023**.**
3. Sub-subplots: Broad bean cultivars (V):
V1: Hannaoui (local).
V2: Spanish (local).
V3: Loz.

Root System Traits

1. Number of Active and Dead Root Nodules: Plants were uprooted at the flowering stage. Roots were washed gently under tap water to remove soil, and the total number of active (pink-colored) and dead (brown/black) nodules per plant was counted using a hand lens (Beck et al., 1993).

2. Dry Weight of Root Nodules

After uprooting, roots were thoroughly cleaned and nodules were separated. Nodules were placed in labeled paper bags, oven-dried at 65°C until constant weight, and weighed using a precision balance (Beck et al., 1993).

3. Root Dry Weight

The entire root system was excised from the plant, cleaned, and placed in labeled paper bags. Samples were oven-dried at 65°C until constant weight and weighed using a precision balance to determine root dry weight.

Statistical Analysis

Data were analyzed using GenStat 12th Edition software. Treatment means were compared using the Least Significant Difference (L.S.D.) test at a 5% probability level (Al-Rawi and Khalf Allah, 2000).

Gene Expression Analysis of *nifX*

Gene expression analysis of the *nifX* gene was performed using a commercial kit according to the protocol provided by the manufacturer.

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Table 1. Kits used for gene expression analysis and tehir origin

Kits	Company/ Country
TransZol Up Plus RNA Kit	Transgen Biotech Co./ China
GoScript™ Reverse Transcription System	Promega/ USA
Perfect start green qpcr supermix	Transgen Biotech Co./ China

Primers

Table 2.1. Nucleotide sequences of primers used in this study

Primer		Sequence (5'----------3')	Company/Origin
NifX	F	AAAAATCGCGGTTCTGTCCAC	Macrogen / South Korea
	R	AGCAACGCCCCTATTCCTTC	
18SrRNA	F	GGGCATTCGTATTTCATAGTCAGAG	
	R	CGGTTCTTGATTAATGAAAACATCCT	

Table 2.2. Thermal cycling conditions for Reverse Transcription

Stage	Temperature (°C)	Time (min)	Number of Cycle
Annealing	25	5	1
Extension	42	60	1
Deactivation	70	15	1

Table 2.3. Real-time-PCR Program

Steps	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	94	2	-
Denaturation	94	0.5	
Annealing	60	0.5	40
Extension	72	0.5	
Melting curve	65-95	0.5	-

Analysis of Gene Expression Data

The ΔΔCt for all samples was calculated the difference between the ΔCt of the treated samples after normalized the gene expression to housekeeping gene (18srRNA).

ΔΔCt = ΔCt (treatment) - ΔCt (control)

Fold Change Calculation: The fold change in gene expression between the treated and control samples was calculated using the formula:

Fold Change = $2^{(-\Delta\Delta Ct)}$

This formula represents the normalized target amount in the treated sample relative to the control sample. The fold change value indicates how many times the gene expression level has changed in the treated sample compared to the control sample. A fold change greater than 1 indicates upregulation, while a fold change.

RESULTS AND DISCUSSION

Gene Expression Analysis of the *nifX* Gene in Broad Bean Root Nodules Under Field Conditions: Results revealed an inverse correlation between cycle threshold (CT) values and *nifX* gene expression levels. Bacterial inoculation (B1) and molybdenum (Mo) application (M1, M2) significantly enhanced *nifX* expression across all treatments compared to the control (B0M0). Notably, the lowest CT values—indicating the highest gene expression—were observed in V1B1M2, V2B1M1, and V3B1M1. Under the same treatment conditions, the highest increases in *nifX* expression were recorded for all studied cultivars, with the Hannaoui cultivar (V1) exhibiting peak expression at 10 mg L^{-1} Mo, while Spanish (V2) and Loz (V3) performed optimally at 5 mg L^{-1} Mo.

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Table 3. *nifX* gene expression values in broad bean root nodules under the influence of rhizobial inoculation and molybdenum foliar application

Genotypes	Treatments	18S rRNA	nifX	ΔCt	ΔΔCt	Fold
V1	B0M0	20.78	31.16	10.38	0.00	1.00
	B0M1	13.92	24.23	10.31	-0.07	1.05
	B0M2	12.46	22.23	9.77	-0.61	1.52
	B1M0	14.65	23.88	9.23	-1.16	2.23
	B1M1	15.75	26.21	10.46	0.08	0.95
	B1M2	14.21	23.93	9.72	-0.66	1.58
V2	B0M0	14.42	19.25	4.83	0.00	1.00
	B0M1	15.76	20.02	4.26	-0.57	1.48
	B0M2	16.44	21.40	4.96	0.14	0.91
	B1M0	18.38	22.72	4.34	-0.49	1.40
	B1M1	17.10	20.12	3.02	-1.81	3.50
	B1M2	18.55	22.34	3.79	-1.04	2.06
V3	B0M0	18.51	28.38	9.86	0.00	1.00
	B0M1	15.30	25.34	10.04	0.18	0.89
	B0M2	15.46	25.02	9.56	-0.30	1.23
	B1M0	15.27	24.82	9.54	-0.32	1.25
	B1M1	16.12	25.20	9.08	-0.79	1.73
	B1M2	17.26	26.99	9.73	-0.13	1.10

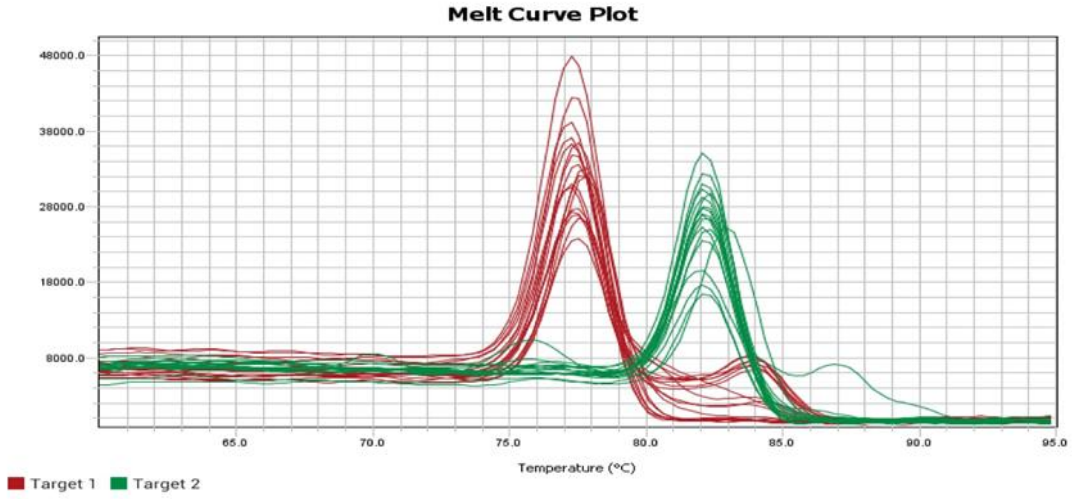

Figure 1. Melt curve plot

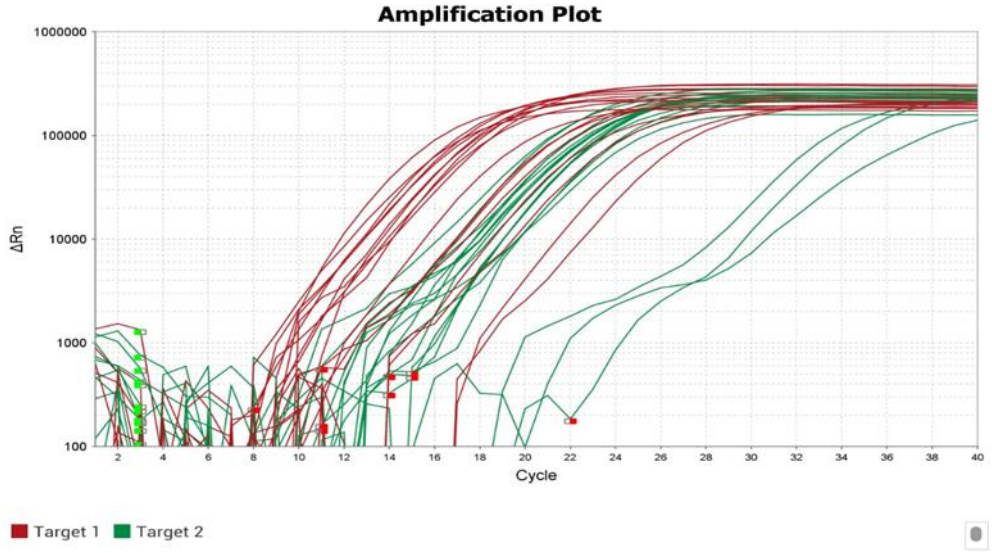

Figure 2. Amplification curve plot

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Effect of cultivars, molybdenum foliar application, and bacterial biofertilizer on some root traits: The three-way interaction of genotype, molybdenum, and bacterial inoculation significantly influenced root traits: Treatment V1M1B1 recorded the highest active nodule count (845 nodules plant^{-1}) and root dry weight (48.935 g), while $V_1M_1B_0$ exhibited the highest dry nodule weight (3.035 g). In contrast, $V_2M_1B_0$ and V3M2B0 showed the lowest values for nodule count (133 nodules plant^{-1}) and root dry weight (10.9 g), respectively, with V2M0B0 yielding the lowest dry nodule weight (0.5950 g). The study revealed varying degrees of nifX gene expression in broad bean root nodules, with molybdenum (Mo) playing a critical role in stimulating transcriptional activity. Mo functions as a cofactor, playing an important role in the biosynthesis of the Fe-Mo cofactor needed for the activity of nitrogenase, the enzyme that catalyzes atmospheric nitrogen fixation (Shah et al., 1999). The nif gene family is responsible for the synthesis of the Mo-Fe nitrogenase complex, and truly symbiotic organisms contain the nifBHDKENX operon and lack in non-nitrogen-fixing bacteria. But these findings are somewhat discordant with those reported by (Black et al., 2012) which found that nifX and nifZ were absent from Rhizobium leguminosarum. This discrepancy highlights the coordination complexity of rhizobial symbiosis based on an intricate network of molecular signals, biochemical interactions and transcriptional control between bacteria and their host plants. This study uncovers new information regarding the role of the nifX gene in rhizobia in the context of its interaction with molybdenum, shedding light on its role in optimizing biological nitrogen fixation.

Table 4. Effect of genetic composition, molybdenum foliar application, bacterial biofertilizer, and their interaction on root growth indicators

Cultivars	Treatments	Root dry weight (g)	Nodule dry weight (g)	Number of active root nodules (nodules per plant)
	B0M0	22.415	1.4450	313.5
	B0M1	33.175	3.035	758.5
	B0M2	18.150	1.0067	267.5
V1	B1M0	20.250	0.7033	633.0
	B1M1	48.935	2.3867	845.0
	B1M2	30.130	1.2700	300.0
	B0M0	11.395	0.5950	286.0
	B0M1	26.550	0.8400	133.0
	B0M2	26.785	2.0633	404.0
V2	B1M0	34.135	0.8200	656.0
	B1M1	29.495	1.5233	577.0
	B1M2	22.785	2.8800	526.0
	B0M0	15.145	1.4250	304.5
	B0M1	10.900	1.0000	386.0
	B0M2	18.030	0.9333	306.5
V3	B1M0	32.930	1.5333	617.5
	B1M1	15.045	0.8667	258.5
	B1M2	39.150	2.1700	628.0
LSD	0.05	1.4332	0.10467	28.92

This study demonstrates that *Rhizobium leguminosarum* inoculation (B1) combined with molybdenum (Mo) foliar application (M1/M2) significantly enhances *nifX* gene expression and root traits in broad bean cultivars. The Hannaoui genotype exhibited peak *nifX* expression and root biomass under Mo supplementation, while Spanish and Loz cultivars responded optimally to lower Mo levels. The negative correlation between the CT values and the expression of the nitrogenase genes indicates that Mo acts as a regulator in the biosynthesis of nitrogenase and therefore plays a crucial role in the

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symbiotic fixation of nitrogen. Our findings illustrate genotype specific responses to biofertilizers and Mo, which provide a sustainable approach to promote legume productivity and reduce dependency on synthetic N inputs.

CONCLUSION

This study demonstrates that *Rhizobium leguminosarum* inoculation (B1) combined with molybdenum (Mo) foliar application (M1/M2) significantly enhances *nifX* gene expression and root traits in broad bean cultivars. The Hannaoui genotype exhibited peak *nifX* expression and root biomass under Mo supplementation, while Spanish and Loz cultivars responded optimally to lower Mo levels. The negative correlation between the CT values and the expression of the nitrogenase genes indicates that Mo acts as a regulator in the biosynthesis of nitrogenase and therefore plays a crucial role in the symbiotic fixation of nitrogen. Our findings illustrate genotype specific responses to biofertilizers and Mo, which provide a sustainable approach to promote legume productivity and reduce dependency on synthetic N inputs.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR/S DECLARATION

We confirm that all Figures and Tables in the manuscript are original to us. Additionally, any Figures and The authors declare that this manuscript is original, has not been published previously, and is not currently under consideration by any other journal. All figures and tables are original and prepared by the authors. Any material obtained from third parties has been included with the required permissions. All authors have read and approved the final manuscript.

AUTHOR'S CONTRIBUTION STATEMENT

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تعبير الجين *nifX* في العقد الجذرية لنبات الباقلاء تحت تأثير التلقيح بالرايزوبيا والرش بالموليبدنوم

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المستخلص

يهدف البحث لدراسة التعبير الجيني للجين nifX بتاثير التلقيح البكتيري والمولبدنوم لبعض التراكيب الوراثية من الباقلاء ودراسة بعض صفات المجموع الجذري، نفذت تجربة حقلية لدراسة تأثير التلقيح ببكتريا *Rhizobium Leguminosarun* الرش الموليبدنوم في صفات الجذور والتعبير الجيني للجين nifX المسؤول عن تثبيت النيتروجين لبعض التراكيب الوراثية لنبات الباقلاء. أجريت التجربة في الحقول البحثية بجامعة بغداد للموسم الشتوي 2023–2024 باستخدام تصميم القطاعات العشوائية الكاملة وبترتيب الالواح المنشقة المنشقة وبثلاث مكررات. تضمنت المعاملات التلقيح بالبكتيريا B0 بدون إضافة، B1 بتركيز 2×10^8 الرش بالموليبدنوم بتراكيز (M0: 0 ، M1: 5، M2: 10 ملغم لتر$^{-1}$) وثلاثة أصناف من الباقلاء (V1: حناوي، V2: الاسباني، V3: لوز. أظهرت النتائج وجود علاقة عكسية بين قيم CT والتعبير الجيني؛ إذ أدت معاملات اللقاح والموليبدنوم إلى زيادة التعبير الجيني لـ *nifX* سجلت أعلى زيادة في التعبير الجيني عند معاملة V1B1M2 و V2B1M1 و V3B1M1 أعطى الصنف "حناوي" أعلى تعبير جيني عند التركيز 10 ملغم لتر$^{-1}$ من الموليبدنوم، بينما تفوق الصنفان "الاسباني" و"لوز" عند التركيز 5 ملغم لتر$^{-1}$. كما تفوقت المعاملة V1M1B1 معنوياً في عدد العقد النشطة (845 عقدة) والوزن الجاف للجذور (48.935 غم)، مما يشير إلى دور التلقيح والرش بالموليبدنوم في تعزيز كفاءة تثبيت النيتروجين وتحسين صفات النمو.

الكلمات المفتاحية: اسمدة احيائية، أصناف، امن غذائي، بروتين غذائي، استدامة.

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