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The effect of various nitrogen compounds on the growth of bacteria belonging to the genus *Rhizobium*

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ABSTRACT

Nitrogen compounds can influence the early stages of legume–rhizobium symbiosis. This study examined their effects on the growth of six *Rhizobium* isolates obtained from legume rhizospheres in the Ganja–Tovuz region of Azerbaijan and on the attachment of *Rhizobium leguminosarum* to pea (*Pisum sativum* L.) roots. Bacterial growth was estimated by measuring optical density at 590 nm, and root attachment was assessed by fluorescence microscopy over 24 h. Compared with the water-treated control, 60 mM potassium nitrate and ammonium sulfate had little or no effect on *R. leguminosarum* growth. Potassium nitrite reduced growth by 19.6% at 3.5 mM, while sodium nitroprusside, a nitric oxide donor, reduced growth by 31.2% at 0.02 mM. Growth inhibition increased with sodium nitroprusside concentration. In the pea seedling assay, ammonium sulfate and potassium nitrite increased root attachment by up to 18% and 10%, respectively. Potassium nitrate had little effect, while sodium nitroprusside reduced attachment by up to 65%. These findings show that the response of rhizobia depends on the compound, its concentration, and the process measured. Potassium nitrite inhibited growth in culture but increased attachment to pea roots, whereas sodium nitroprusside inhibited both processes.

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INTRODUCTION

In the presented work was studied the influence of nitrogenous compounds on the growth of bacteria of the genus *Rhizobium*, which are in symbiotic relationships with leguminous plants. The results obtained show that the introduction of nitrogen-containing compounds into the environment causes various effects, the form of which is determined by the nature of the nitrogen-containing compounds added and the amount of nitrogen introduced into the environment.Thus, while the addition of $(NH_4)_2SO_4$ and KNO_3 to the medium at a concentration of 60 mM does not affect bacterial growth compared to the control, the addition of KNO_2 at a concentration of 3.5 mM reduces growth by up to 50%. However, the addition of $Na_2[Fe(CN)_5NO]$ to the medium as an NO donor causes a reduction in growth of up to 25%, even at a lower concentration (0.02 mM).The decrease in growth continues as the concentration increases, and at a concentration of 0.90 mM, the reduction can reach up to 65%.

As is known, increasing the productivity of agricultural crops is determined by a number of factors, among which nitrogen is of particular importance (Wang *et al.*, 2024a). Although nitrogen makes up 71% of atmospheric air, plants cannot assimilate it because it is inert. For this reason, nitrogen-based fertilizers are used to address these issues in the agricultural sector(Ali *et al.*, 2025). Although its use leads to increased productivity, this process is sometimes accompanied by undesirable side effects(Navarro *et al.*, 2025). For example, such phenomena include the accumulation of nitrates in certain agricultural products, environmental pollution, disruptions to the soil salt balance, etc.

For this reason, perspectives on the symbiotic nitrogen-fixation process characterized as a unique biological phenomenon have taken on even greater significance (Chiurazzi *et al.*, 2025). Thus, the effective use of this process leads to an increase in both soil fertility and the productivity of the crops grown there, and the resulting produce better meets requirements regarding quantitative and qualitative indicators (Wang, T*et al.*, 2026). This is also one of the important issues for sustainable agriculture.

Legumes are one of the largest families of flowering plants and nitrogen-fixing ability has been detected in almost 90% of the studied species and the research conducted in this regard primarily covers the stage of complete formation of the nitrogen-fixing apparatus (the development of the mature root nodule)(Hughes *et al.*, 2025). However, insufficient attention has been paid to researching the initial stage of these issues, even though certain problems exist at this early phase of the process (for example, the mechanism by which the host plant and root nodule bacteria recognize each other as symbiotic partners in a dense microbial environment).

It should be noted that the formation of the symbiotic relationship between legumes and root-nodule bacteria is a complex, multi-stage process influenced by various ecological factors that regulate the development of this system(Tsyganova *et al.*, 2021). However, various environmental factors including mineral fertilizers can also be hinder the formation and functioning of this system in the natural environment (Bakhshaliyeva *et al.*, 2026). Because their application to the soil in high doses negatively affects the vital activity of both participants in the symbiotic relationship, either reducing the effectiveness of the process or causing it to stop completely(Wang *et al.*, 2024). Thus, when large amounts of nitrogen are applied to the soil, the formation of compounds such as NO and N_2O increases, and the nature of their impact on the process is not yet clearly understood.

Given the importance of this issue, the aim of the study presented was to investigate the effect of nitrogen compounds on the symbiotic relationships between leguminous plants and bacteria.

MATERIALS AND METHODS

The research was conducted in the territory of the Ganja-Tovuz economic region of the Republic of Azerbaijan. Sample collection, preparation for

laboratory analysis, isolation of pure microorganism cultures, and other procedures were carried out in accordance with established microbiological (Netrusov *et al.*, 2005) methods and approaches. Thus, for bacterial isolation, samples were collected from the plant rhizosphere, a suspension was prepared, appropriate dilutions (10- to 1000-fold) were made, and the samples were then cultured in a nutrient medium. Meat-peptone agar (MPA), meat-peptone broth (MPB), Endo medium, and Ashby medium were used for the isolation of bacteria.

The growth ability of bacterial colonies in the specified culture media was evaluated based on macroscopic (colony shape and size, edges, color, etc.) and microscopic (morphological features) characteristics (Pokhrel *et al.*, 2024). The isolated pure bacterial cultures were identified based on cultural, morphological, physiological, and biochemical characteristics, following the approaches described in the work of Li *et al.* (2014). The study of biochemical activity was determined based on standard test methods (Kyule *et al.*, 2022.).

When determining the concentrations of various amounts of nitrogenous compounds added to a bacterial culture, calculations were performed for nitrogen. When studying the effect of the nitrogenous compounds used on bacterial growth, the composition of the culture medium (g/L) was as follows: KH_2Po_4 – 0,3; Na_2HPo_4 -0,3; $MgSo_4$ x $7H_2o$ – 0,1; $CaC1_2x2H_2o$ – 0,005; H_3Bo_3 – 0,01; $ZnSo_4$ $x7H_2o$ – 0,001; $CuSo_4$ $x5H_2o$ - 0,0005; $MnC1_2x4H_2o$ - 0,0005; Na_2Moo_4 $x2H_2o$ - 0,0001; $FeCl_2$ $x6H_2o$ - 0,0001; NH_4NO_3 – 0,0025; sucrose – 1. The prepared medium is sterilized in 0.5-liter flasks for 0.5 hours at a pressure of 0.5 atm, and vitamins (thiamine – 0.1 mg/L, biotin – 0.1 mg/mL, and calcium pantothenate – 0.1 mg/mL) are added under sterile conditions. One of the prepared nutrent media were taken for the control group, and the other for the experimental, and 100 ml of nutrient medium was added to the latter flask, along with a suspension prepared from the biomass of the bacterium under study grown on agar medium. Water was added to the control, and both variants are placed in a thermostat at a temperature of 26–28°C for one day. After the completion of the period, the optical density of both variants was determined. Optical density is measured at a wavelength of 590 nm using an SF-2000 (Russia), measurements are taken every 8 hours after the initial 24-hour period, and the process continues until the increase in optical density ceases.

The adhesion of root nodule bacteria to pea seedlings was assessed using the methodology employed in the studies of D.J. Gage(2004), utilizing a microscope and 4,5-diaminofluorescein diacetate (DAF-2 DA) as the dye.

During the course of the research, all experiments were conducted with at least four replicates, and the obtained results were statistically analyzed (Welham *et al.*, 2014).

RESULTS AND DISCUSSION

Initially, in the research, studies were conducted to take pure culture of bacteria from the genus of *Rhizobium* which exist in a symbiotic relationship with legumes and on elucidating certain aspects of their cultural and morphological characteristics, as well as specific features of their mineral nitrogen metabolism. The data on the cultures obtained from the analysis of samples taken from the rhizosphere of leguminous plants are presented in Table 1.

As seen, although the bacteria selected for the studies were isolated from various plants and different biocenoses, their cultural and morphological characteristics generally exhibit the same features. The noted similarities are not limited to the data presented in the Table. Thus, they share similar characteristics such as the optimal growth temperature for the isolated bacteria is 26–28°C indicating that they are all mesophiles, growth occurs in a near-neutral pH range (6.5–7.5), and they do not have ability to form spores. However, this identity does not manifest itself at all times and in all characteristics. First of all, it should be noted that even strains belonging to the same species exhibit

distinct characteristics; in other words, the phenomenon of polymorphism is widespread among them. Thus, the *Rh. leguminosarum* bacterium isolated during the course of the research were found in the rhizosphere of virtually all the leguminous plants sampled from soils with salinity levels of up to 1%, and, as indicated (Table 1), it possesses characteristics common to all of them. Although many nodule-forming bacteria exhibit high specificity, their names are often linked to those of their host plants (e.g., *Rhizoblum japonicum , Rh. phaseoli, Rh.lupini* and so on). First of all, it should be noted that, unlike bacteria possessing a number of similar characteristics, *Rh. leguminosarum* does not exhibit strict specificity, and the plants with which it enters into symbiotic relationships include peas, lentils, alfalfa, and others. This point is supported by both our research and the scientific literature. Thus, root-nodule bacteria differ from one another based on their specificity to leguminous plants, that is, some can be characterized as "specific," while others as "universal."

Table 1. General characteristics of bacteria belonging to the genus *Rhizobium* that exist in a symbiotic relationship with plants

Sl	Name	The substrate from which it was separated	Cultural-morphological characteristics	Sampling place
1	*Rh. leguminosarum*	Alfalfa	Aerobic, Gram-negative, rod-shaped, 0.7–1.8 μm in length	Clean phytocoenoses
2	*Rh. lupini*	Pea		Irrigated areas
3	*Rh. loessense*	Alfalfa		Clean phytocoenoses
4	*Rh. gallicum*	Beans		Irrigated soils
5	*Rh. mongolense*	Alfalfa		Pasture
6	*Rh. lusitanum*	Alfalfa		Clean phytocoenoses

It should be noted that the polymorphism observed in root-nodule bacteria is not only morphological but also genetic in nature. Thus, a number of studies have established that, although the majority of bacteria currently engaged in symbiotic relationships with legumes belong phylogenetically to the species *Rhizobium leguminosarum,* they are all characterized by a high level of DNA polymorphism (Boivin *et al.*, 2021).

The fact that root-nodule bacteria exhibit both genetic and morphological polymorphism is due to their exposure to the influence of numerous abiotic and biotic factors in their environment (Ben-Laouane *et al.*, 2026) and the result of the individual as well as combined effects of those factors has caused the evolution of root-nodule bacteria to proceed in different directions. For example, abiotic factors affecting symbiotic nitrogen fixation include soil acidity, moisture, temperature, the initial nitrogen content in the root zone, and so on.

These indicators also manifest in varying degrees across different parts of the globe, and their impact affects both symbionts differently. Let's take the soil moisture. Compared to other bacteria, root-nodule bacteria are more sensitive to moisture, in other words, they require more moisture for their development. Signs of growth are observed in some legumes even when soil moisture is between 10% and 12% although an analogous state namely, the preservation of viability is observed in bacteria, they do not reproduce under such conditions. In other words, the movement of root-nodule bacteria in the soil occurs precisely due to moisture, and the intermittent nature of this movement is noted as an indicator that their movement has ceased. The result of the influence of various other factors, leading to diverse reactions, has shaped the current diversity.

External factors play a significant role in the formation and functioning of the symbiosis between plants and bacteria, primarily by hindering the establishment of the symbiosis. Given the importance of this matter, the present study aims to elucidate the effect of high levels of nitrogen compounds on the symbiotic relationships between legumes and bacteria, and for this purpose was investigated the effect of nitrogen compounds on the growth of six pure cultures belonging to the genus *Rhizobium* isolated during the course of the research.

The obtained results was presented for the sample *Rh. leguminosarum* (Table 2). As seen, the introduction of nitrogen compounds into the environment gives rise to various effects, the manifestation of which is determined by the nature and dosage of the added nitrogen compounds. Thus, while the addition of $(NH_4)_2SO_4$ and KNO_3 to the medium at a concentration of 60 mM does not affect bacterial growth compared to the control, the addition of KNO_2 at a concentration of 3.5 mM reduces growth by 19.6%. However, the addition of $Na_2[Fe(CN)_5NO]$ to the medium as an NO donor can reduce growth by up to 31.2%, even at a lower concentration (0.02 mM). As the concentration increases, the growth continues to decline, and at a concentration of 0.90 mM, the reduction can reach up to 70%.

Table 2. The effect of the inorganic form of nitrogen on the growth of bacteria belonging to the genus *Rhizobium*

Bacteria	Nitrogen compounds	Density (mM)	Optical density	
			Experiment	Compared to the control %
Rh. leguminosarum	Control (H_2O)		0,112	100
	KNO_3	60	0,112	100
	KNO_2	3,5	0,090	80,4
	$NaNO_3$	60	0,106	94,6
	NH_4NO_3	60	0,106	94,6
	$(NH_4)_2SO$	60	0,111	99,1
	$Na_2[Fe(CN)_5NO]$	0,02	0,077	68,8

It should be noted that, apart from minor quantitative differences, the effect of mineral nitrogen on the other five bacterial cultures isolated in pure culture during the study proceeds in virtually the same manner. For example, during the study of the growth of *Rh. loessense* isolated from the alfalfa rhizosphere, it becomes clear that adding KNO_2 to the medium at a concentration of 3.5 mM causes growth to decrease by up to 24%, the use of $Na_2[Fe(CN)_5NO]$ (0.02 mM) in this process results in a reduction of growth by up to 36In this case, increasing the concentrations is accompanied by a continued inhibition of growth, and at a concentration of 0.90 mM, the reduction can reach 69%. In short, although a certain amount of mineral nitrogen at a specific concentration does not significantly affect the growth of bacteria living in symbiotic relationships with plants, an increase in its quantity leads to reduced growth.

The results of the studies indicate that mineral nitrogen influences and regulates all stages of the symbiotic system. There are various hypotheses regarding the mechanism of this process, particularly concerning the inhibitory effect of nitrogen on it. Some attribute this to the plant's own response to "nitrogen stress," while others link it to the toxic effect of nitrogen compounds on the growth of root-nodule bacteria.

The effect of high nitrogen levels on the adhesion and internal penetration of root nodule bacteria in pea (*Pisum sativum* L.) seedlings was also investigated. For this purpose, nitrogen compounds such as KNO_3, KNO_2, $(NH_4)_2SO_4$, and $Na_2[Fe(CN)_5NO]$, as well as the *Rhizobium leguminosarum* N-04 strain isolated from the rhizosphere of alfalfa grown in clean soil, were used; both of the aforementioned processes (adhesion and cell penetration) were evaluated over a 24-hour period. From the obtained results became clear that high concentrations of certain nitrogen compounds (KNO_2 and $(NH_4)_2SO_4$), compared to the control, either stimulate the processes, have no effect (KNO_3), or significantly inhibit them ($Na_2[Fe(CN)_5NO]$) (Fig. 1). For example, the presence of $(NH_4)_2SO_4$) in the medium causes growth to increase to 18%, while the presence of KNO_2 leads to an increase of up to 10%. An increase in growth relative to the control was observed in the presence of KNO3, amounting to 3%, however, considering the accuracy of the method used to evaluate this process as well as the fact that the degree of authenticity in similar studies falls within the 0–5% range this can also be characterized as a stimulation effect with an undetermined degree of authenticity.

In our opinion, it would be more appropriate to assess this indicator as having no effect.

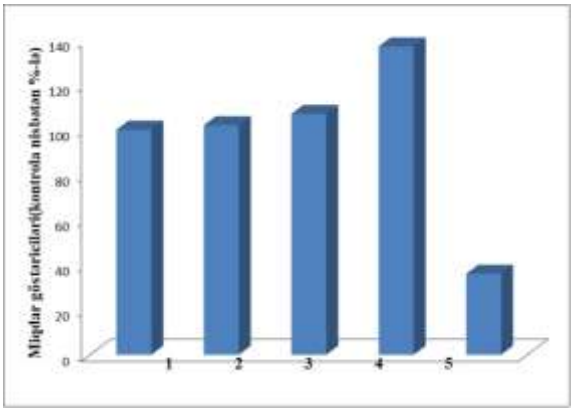


Fig. 1. The effect of various nitrogen compounds on the adhesion of the *Rhizobium leguminosarum* S-17 strain to pea plant roots
1–Control (H2O), 2-KNO3, 3-KN02, 4-(NH4)2S04 and 5-Na2[Fe(CN)5NO].

Unlike stimulation, the inhibitory effect is characterized by a higher quantitative value, and this phenomenon, as noted, is observed when a compound such as $Na_2[Fe(CN)_5NO]$ is present in the medium. In this case, the inhibitory effect can reach up to 65% compared to the control.

It seems the reason for the occurrence of the inhibitory effect should be sought in NO, thus, one of the substances released during the use of $Na_2[Fe(CN)_5NO]$ in biological systems is precisely nitric oxide.

It would be appropriate to note that, apart from minor quantitative differences, the same pattern of effects from the above-mentioned nitrogenous compounds was observed among other bacteria(*Rh.lupini, Rh.loessense, Rh.gallicum, Rh.mongolense* and *Rh.lusitanum*) isolated from the rhizosphere of leguminous plants during the course of the research, some exert a positive influence on the formation of symbiosis, while others have a negative effect. All this indicates that the essence of the matter is determined not by the characteristics of the bacterial symbionts, but by the nature of the nitrogen compounds.

Thus, research has shown that mineral nitrogen significantly affects both the growth of root-nodule bacteria that form symbiotic relationships with legumes and all stages of the establishment of this symbiosis (including the adhesion of rhizobia to the plant root surface and their penetration into the root) and whether the effect is negative or positive is determined by both the nature of the mineral nitrogen compounds used and the dosage applied to the environment. Nevertheless, the presence of a compound such as $Na_2[Fe(CN)_5NO]$, which acts as an NO donor, in the medium invariably leads to observed negative effects on both the growth of the individual symbionts and the formation, development, and activity of the symbiotic relationships between them.

CONCLUSION

Research has shown that the introduction of nitrogen compounds into the environment leads to various effects, the nature of which is determined by the type and dosage of the added compounds. Thus, while the addition of $(NH_4)_2SO_4$ and KNO_3 to the medium at a concentration of 60 mM does not affect bacterial growth compared to the control, the addition of KNO_2 at a concentration of 3.5 mM reduces growth by up to 50%. Adding $Na_2[Fe(CN)_5NO]$ to the medium as an NO donor can reduce growth to 25%, even at a lower concentration (0.02 mM). As the concentration increases, the reduction in growth continues, and at a concentration of 0.90 mM, the reduction can reach up to 65%. All this allows us to note the importance of a more cautious approach to the use of nitrogen compounds in agrocenoses where symbiotic relationships exist.

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