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Original Article

# Morphological and physiological properties of lupine nodule bacteria (*Bradyrhizobium lupini*) when grown on a typical urban soil

## Propriedades morfológicas e fisiológicas de bactérias nodulares de tremoço (*Bradyrhizobium lupini*) quando cultivadas em solo urbano típico

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**Abstract**

In the conducted studies, the moorphological and physiological properties of nodule bacteria of lupine were studied. Lupine plants were grown under the conditions of a microfield experiment on a typical medium loamy urban soil. In the study, a pure culture of *Bradyrhizobium lupini* was isolated. Then, the morphological properties of nodule bacteria cells and the chemical composition of cell membranes of nodule bacteria were determined. The acid resistance and physiological properties of lupine nodule bacteria were also determined, as well as the ratio of *Bradyrhizobium lupini* to antibiotics. All studies were carried out according to generally accepted methods. The results of the research showed that during the cultivation of lupine on a typical urban soil, nodule bacteria *Bradyrhizobium lupini* were isolated, which can be characterized as gram-negative, non-spore-forming rods that do not exhibit amylolytic activity. It was revealed that the rhizobia of nodule bacteria are not acid-resistant. Nodule bacteria turned out to be the least resistant to polymyxin, then to levomycetin, and Bradyrhizobium lupini showed the greatest resistance to tetracycline.

**Keywords:** lupine, nodule bacteria, morphological properties of nodule bacteria, physiological properties of nodule bacteria.

**Resumo**

Nos estudos realizados, foram estudadas as propriedades morfológicas e fisiológicas das bactérias nodulares do tremoço. As plantas de tremoço foram cultivadas nas condições de um experimento de microcampo em um típico solo urbano argiloso médio. No estudo foi isolada uma cultura pura de *Bradyrhizobium lupini*. Em seguida, foram determinadas as propriedades morfológicas das células das bactérias nodulares e a composição química das membranas celulares das bactérias nodulares. A resistência aos ácidos e as propriedades fisiológicas das bactérias do nódulo do tremoço também foram determinadas, bem como a proporção de *Bradyrhizobium lupini* em relação aos antibióticos. Todos os estudos foram realizados de acordo com métodos geralmente aceitos. Os resultados da pesquisa mostraram que, durante o cultivo do tremoço em solo urbano típico, foram isoladas bactérias nodulares *Bradyrhizobium lupini*, que podem ser caracterizadas como bastonetes gram-negativos, não formadores de esporos e que não apresentam atividade amilolítica. Foi revelado que os rizóbios das bactérias nodulares não são resistentes aos ácidos. As bactérias nodulares revelaram-se as menos resistentes à polimixina, depois à levomicetina, e o *Bradyrhizobium lupini* apresentou a maior resistência à tetraciclina.

**Palavras-chave:** tremoço, bactérias nodulares, propriedades morfológicas de bactérias nodulares, propriedades fisiológicas de bactérias nodulares.

## 1. Introduction

At present, the issue of providing livestock with balanced protein feed remains very relevant. Lupine is a promising leguminous crop. The grain is characterized by a high content of protein, balanced in quality, practically not inferior to soy. Therefore, lupine grain is used in compound feed in poultry and livestock (Ravkov and Malyshkina, 2020). The use of lupine flour as a feed additive improves the quality of poultry meat (Mierlita, 2015; Sorokin and Rutskaya, 2019; Seregina et al., 2021).

Of particular interest to bacteria of the genus Rhizobium is associated with their ability to fix atmospheric nitrogen during symbiosis with leguminous plants. The process

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occurs in root nodules, which are formed upon inoculation of root hairs with microorganisms (Schlegel, 1987). Nodule bacteria are classified depending on the host plant and some other features (Schlegel, 1987). Nodules form growth substances that have a beneficial effect on plants, mainly indole derivatives, cytokines and traces of gibberellic acid; culture itself forms such substances much less frequently. Also, some strains are able to secrete B vitamins (Sakhno and Trifonova, 2007). Symbiotic bacteria in exchange for the available receive from plants the products of photosynthesis and an ecological niche. A so-called mutually beneficial symbiosis is formed, which has adaptive properties (Zhukov et al., 2009).

The formation of symbiosis between legumes and nodule bacteria is controlled by a complex of complementary plant and bacterial genes. The main plasmid genes responsible for bacterial symbiosis are hos genes responsible for host plant recognition, nod genes contribute to the formation of nodules, and *nif*-genes determine the process of molecular nitrogen binding (Sakhno and Trifonova, 2007; Baĭmiev et al., 2012).

Currently, scientists are developing methods to optimize the use of microbiological preparations in conjunction with agrochemicals. This issue is of high relevance and practical significance. The effective functioning of the legume-rhizobium symbiosis is mainly affected by temperature, water and air conditions, the acidity of the environment, the content of micro- and macroelements in the soil, and the illumination of plants. Solar radiation, in the form of PAR, is accumulated during photosynthesis and acts as the energy expended in the process of nitrogen fixation (Tikhonovich and Kruglov, 2005; Sakhno and Trifonova, 2007; Annicchiarico et al., 2018; Gataulina et al., 2021).

It is known that the yield of legumes depends on their effective interaction with nodule bacteria. Effective symbiosis of legumes and nodule bacteria depends on virulence, competitiveness, as well as complementarity (specificity) of microsymbiont strains to a particular plant species and variety. The use of active strains as inoculants ensures intensive nitrogen fixation, enhances plant photosynthesis and leads to an increase in plant productivity (Kuznetsova et al., 2018). At the same time, the number of nodules increases significantly, the soil is enriched with environmentally safe nitrogen, which can be used by subsequent crops. The use of strains of nodule bacteria for seed inoculation is of great importance when cultivating legumes in new territories where the necessary microorganisms are absent in the soil (Zhakeeva et al., 2015).

Nodule bacteria of lupine (*Bradyrhizobium lupini*) are slow-growing, develop on 7-10 days after incubation. *Bradyrhizobium lupini* are small to medium (0.5-0.9 × 1.2-3.0 µm), non-spore-forming, gram-negative rods, less often they can occur in the form of cocci. Motile due to peritrichous flagella, but only in well-moistened soil. The culture belongs to aerobic heterotrophs, it can be grown on various media containing yeast extract to improve growth, there are also fully synthetic media suitable for their development. For their better cultivation, the environments of Isvaran and Lazarev are used (Milto, 1982). *Bradyrhizobium lupini* is able to survive at low temperatures, while being at temperatures above 50 °C for several hours is detrimental to them. They grow best in a neutral and slightly acidic environment (Milto, 1982).

Currently, one of the current areas of research is the production of environmentally friendly crop products. Creating favorable conditions for the good development of symbiotic interactions of nodule bacteria with legumes can reduce the cost of using nitrogen fertilizers is a pressing issue that is of high practical importance.

The scientific literature contains many studies devoted to the study of the life activity of nodule bacteria. However, there have been insufficient studies devoted to the study of the morphological and physiological properties of lupine nodule bacteria (Bradyrhizobium lupini). To do this, it is necessary to conduct research to identify the types of microorganisms that predominate in the soil when growing lupine plants. Then it is necessary to study the development of the isolated microorganisms and study their main characteristics and morphological and physiological properties to assess the influence of external conditions on their development. In this regard, the goal of our research was to isolate a pure culture of the nodule bacteria Bradyrhizobium lupini and study their morphological and physiological properties to assess the influence of external conditions on their development. These studies are of great relevance and practical significance for improving the technology of growing lupine plants while obtaining environmentally friendly products and reducing the costs of using nitrogen fertilizers.

## 2. Research Methods

To solve the questions posed, lupine plants were grown in the field at the experimental site of the Department of Agronomic, Biological Chemistry and Radiology, RGAU-MSHA named after. K.A. Timiryazev.

The object of the study was white lupine plants (*Lupinus albus L.*) variety Degas. The white lupine variety Degas was included in the State Register of Breeding Achievements in 2004. This lupine variety is relatively early ripening and highly productive. Plants of this variety are resistant to fusarium and lodging due to severe overmoistening. The growing season of white lupine plants is 120 days, provided there is no waterlogging. According to research data, the Degas variety grows well in the northern parts of the Central Black Earth region (Gataulina et al., 2020; Seregina, 2018; Seregina et al., 2021). The seeds of this lupine variety are resistant to cracking of beans and shedding of seeds on the root.The height of lupine plants reaches 80-90 cm (Gataulina et al., 2020; Seregina et al., 2021). White lupine plants were grown under microfield experiment conditions at the experimental site of the Russian State Agrarian University - Moscow Agricultural Academy named after K.A. Timiryazev. Field research was carried out according to generally accepted methods (Kobzarenko et al., 2015).

The soil of the experimental plot is a typical medium loamy urbanozem. Agrochemical characteristics of the soil of the experimental plot: humus content (according to the Tyurin method) was 3.3% (Russia, 1992), soil exchange acidity (pHKCl) - 6.0 (Russia, 2023), soil hydrolytic acidity was 0.9 mg -eq/100 g of soil (Russia 2024), the sum of exchangeable bases was 24.3 mg-eq/100 g of soil (Russia, 2022), the content of the alkaline hydrolysable form of nitrogen in the soil was 90 mg/kg of soil (according to Kornfield cited in Moscow, 1985). The availability of mobile forms of phosphorus was 125 mg/kg of soil (class IV). The availability of mobile forms of potassium was 120 mg/kg of soil (class IV) (according to Kirsanov cited in Russia, 2013).

The total area of the experimental plot is 1 $m^2$. The placement of plots in the experiment was carried out using the Latin rectangle method with a systematic row arrangement of plots within repetitions. The repetition of the experiment is fourfold.

The level of mineral nutrition was created by scattering ammonium nitrate ($NH_4NO_3$), ammophos ($NH_4H_2PO_4$) and monopotassium phosphate ($KH_2PO_4$). In all variants, nitrogen was added at the rate of 20 kg/ha, phosphorus and potassium at the rate of 115 and 145 kg of the element per ha, respectively. Fertilizers were applied with a hand cultivator to a depth of 10-15 cm.

High quality seeds were used for sowing. Laboratory germination was 93%. Sowing was carried out with dry seeds to a depth of 3-4 cm. The row spacing was 15 cm, the distance between seeds in a row was 4 cm (Annicchiarico et al., 2014; Gataulina et al., 2020).

Cleaning was carried out by a continuous method. A number of microbiological experiments were carried out to carry out diagnostic studies of a pure culture of nodule bacteria. Lupine nodule bacteria (*Bradyrhizobium lupini*) were grown on Isvaran medium (Tepper et al., 2004).

Isolation of a pure culture of *Bradyrhizobium lupini* was carried out according to the Shilnikova method modified by Sigute (Tepper et al., 2004; Netrusov et al., 2005).

To obtain a pure culture of nodule bacteria of lupine (*Bradyrhizobium lupini*), multiple transfers were carried out by the streak method on the Isvaran nutrient medium (Tepper et al., 2004).

To determine the morphological properties of *Bradyrhizobium lupini*, fixed stained preparations were prepared. The technique for preparing fixed stained preparations is generally accepted (Tepper et al., 2004; Netrusov et al., 2005). To determine the chemical composition of cell walls of nodule bacteria, cells were stained according to Gramm (Tepper et al., 2004; Netrusov et al., 2005).

The acid resistance of *Bradyrhizobium lupini* was determined by the Ziehl-Neelsen method (Tepper et al., 2004; Netrusov et al., 2005).

In addition, the physiological properties of lupine nodule bacteria were determined, that is, the amylolytic activity of *Bradyrhizobium lupini* was determined, as well as the degree of their inhibition in relation to antibiotics (Tepper et al., 2004; Netrusov et al., 2005).

To determine the ratio of *Bradyrhizobium lupini* to antibiotics, the method of paper disks impregnated with antibiotics polymyxin, levomycetin, and tetracycline was used (Tepper et al., 2004; Netrusov et al., 2005).

## 3. Research Results

It is known that nodule bacteria enter into symbiosis with leguminous plants, depending on their genetic compatibility. During inoculation, nodules are formed, in which the process of atmospheric nitrogen fixation takes place. As a result of legume-rhizobium symbiosis, plants grow and develop better, macro- and microelements are absorbed much more efficiently, and they are less susceptible to disease and stress. Nodule bacteria receive carbohydrates, oxygen and other necessary substances for effective nitrogen fixation. The research results showed that the lupine yield was 400 g/$m^2$. At the same time, the mass of the valves and the mass of the stems were 430 g/$m^2$ and 380 g/$m^2$ (Figure 1).

A pure culture is a population of bacteria of one species or one variety, grown on a nutrient medium. Many types of bacteria are subdivided according to one characteristic into biological variants - biovars. Biovars that differ in biochemical properties are called chemovars, in antigenic properties - serovars, in sensitivity to phage - phage vars. Cultures of microorganisms of the same species, or biovar, isolated from different sources or at different times from the same source, are called strains, which are usually denoted by numbers or symbols.

A colony is a visible isolated accumulation of individuals of one type of microorganisms, which is formed as a result of the reproduction of one bacterial cell on a dense nutrient medium (on the surface or in its depth). Colonies of bacteria of different species differ from each other in their morphology, color and other characteristics.

A pure culture of bacteria obtained by the stroke method in Petri dishes (Figure 2) is used for identification, which is achieved by determining the morphological, cultural, biochemical and other signs of microorganisms. The Bradyrhizobium lupini strain was identified according to the 8th edition of Bergi's key to microorganisms (Milto et al., 1990).

Table 1 describes the characteristics of a pure culture of bacteria.

In the plates with nodule bacteria, good slimy growth was observed, as can be seen in Figure 2.

We used the method of fixing the Bradyrhizobium lupini stain with fuchsin (Figure 3) to determine the shape and cells of the bacteria. Figure 3 shows that nodule bacteria

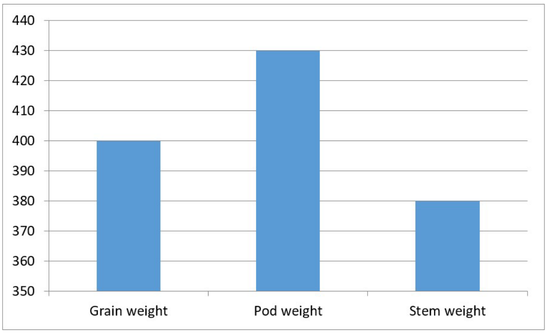


**Figure 1.** Productivity of lupine plants.

**Table 1.** Description of the *Bradyrhizobium lupini* colony.

| Signs of culture | ***Bradyrhizobium lupini*** |
|---|---|
| The shape of the colonies | Round |
| Size, mm | 4-9 |
| Color | Milk cream |
| Shine | Eat |
| Surface | Smooth |
| Edge | Smooth |
| Structure | More dense in the middle, slimy rim around the edges |
| Consistency | Mucous |

**Figure 2.** Colonies of the *Bradyrhizobium lupini.*

**Figure 3.** Staining the preparation with nodule bacteria with fuchsin.

have a rod-shaped cell. The shape of the ends of the cells is rounded, and the arrangement of the cells is single. The ability to form spores is not observed.

When stained by Gram, it can be said that *Bradyrhizobium lupini* stained gram-negatively (Figure 4).

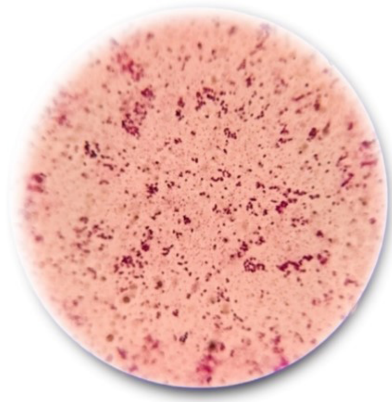

**Figure 4.** Staining of nodule bacteria using the Gram method.

**Figure 5.** Result of amylolytic activity.

When determining amylolytic activity, after cultivation, the agar plate must be treated with Lugol's solution. In this case, the medium containing starch turns blue, and the hydrolysis zone remains colorless. As can be seen in Figure 5, there is no hydrolysis zone, which indicates the absence of amylolytic activity in *Bradyrhizobium lupini.*

When determining acid resistance according to the Ziehl-Neelsen method, a blue color of the preparation was observed, therefore, *Bradyrhizobium lupini* are non-acid-resistant bacteria (Figure 6).

To determine the sensitivity of *Bradyrhizobium lupini* to antibiotics after their cultivation, the diameters of the zones of growth inhibition were measured, taking into account the diameters of the disks. Figure 7 shows that *Bradyrhizobium lupini* is most sensitive to the action of polymyxin (diameter 21 mm), to levomycetin in rhizobia the average sensitivity (diameter 10 mm), and low sensitivity to tetracycline (diameter 7 mm).

Thus, based on the totality of all the previously mentioned facts, we can conclude that *Bradyrhizobium lupini* are gram-negative, non-spore-forming rods that do not exhibit amylolytic activity, and rhizobia are also non-acid-resistant. Nodule bacteria turned out to be the least resistant to polymyxin, then to levomycetin, and *Bradyrhizobium lupini* showed the greatest resistance to tetracycline.

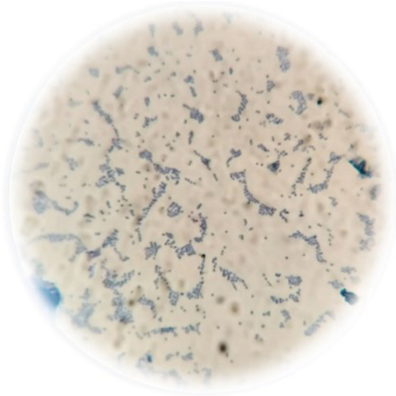

**Figure 6.** Acid resistance of *Bradyrhizobium lupini*.

**Figure 7.** The result of the sensitivity of nodule bacteria to antibiotics.

## 4. Conclusions

As a result of the research carried out, the following conclusions can be drawn:

1. In the course of microbiological experiments, a pure culture of Bradyrhizobium lupini was isolated. After diagnosing the morphological and physiological properties, we can conclude that *Bradyrhizobium lupini* are gram-negative, non-spore-forming rods that do not show amylolytic activity, and rhizobia are also non-acid-resistant;
2. Nodule bacteria turned out to be unstable to polymyxin, to chloramphenicol they showed medium resistance, and to tetracycline *Bradyrhizobium lupini* showed the greatest resistance;
3. The research results showed favorable conditions for the formation of active symbiosis between lupine plants and nodule bacteria.

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