EFFECT OF THE CULTURING DENSITY OF THE 
*Sinorhizobium meliloti* BP ON THE DEVELOPMENT 
OF LUCERNE (*Medicago sativa* L.) AND NITROGENASE 
ACTIVITY

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Abstract. The authors investigated the impact of the *Sinorhizobium* inoculum density on 
the plant development of alfalfa, nodulation and nitrogenase activity. It was found that 
plants inoculated with a 10% inoculant (*4.9* x *10⁶* CFU) were characterized by the best 
growth, more profuse fresh material and a very well developed root system and, additionally, they revealed higher nitrogenase activity.

Key words: inoculum density, lucerne, *Medicago sativa* L., nitrogenase activity, 
*Sinorhizobium meliloti*

INTRODUCTION

In recent years, the interest in the biological nitrogen fixation has been focused on 
the possibilities of improving the effectiveness of this phenomenon in agricultural 
practice. The fixed nitrogen is important not only because it exerts a yield-forming 
fluence on legume plants but, equally importantly, it increases yields of successive 
crop plants cultivated after harvesting legumes. These benefits, as well as the positive 
impact of the cultivation of legumes on the soil structure and its fertility, have been 
investigated and recognised thoroughly and there is no need to discuss them in detail 
here.

It is well known that both the quantity of the symbiotically fixed N₂ and the 
percentage quantity of N derived from the symbiosis depend on the genetic properties of 
the legume crop plant and its symbiont as well as on many environmental factors and 
agrotechnical treatments. The achievement of maximum nitrogen fixation requires the 
involvement of an effective bacterial strain and a suitable plant host as well as the

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Nodulation intensity is strongly influenced by the inoculum density of microorganisms of living cells. High strain activity and virulence increases the effectiveness of the symbiotic process and, consequently, supports the development of nodules and increases the quantity of the biological fixation of this element.

The objective of this study was to determine the influence of the density of the *Sinorhizobium meliloti* Bp culture on plant nodulation. Within the framework of the performed investigations, the authors also assessed the rate of nodulation, the shape, colour, nodule distribution as well as nitrogenase activity.

**MATERIAL AND METHODS**

In the course of laboratory experiments, one species of the crop plant – seed alfalfa (*Medicago sativa* L.), cv. Derby obtained from the Department of Genetics of the Agricultural University in Poznań was used. The authors used a strain of a high degree of virulence and N\textsubscript{2} fixation activity – *Sinorhizobium meliloti* Bp obtained from the Microbiology Department of the Institute of Soil Science and Plant Cultivation in Puławy.

The effectiveness of the symbiosis depending on the inoculum density of the *Sinorhizobium meliloti* strain was investigated on plants growing in test tubes. The inocula for plant inoculation were obtained from a three-day old culture of the *Sinorhizobium meliloti* Bp strain which developed on the agar slant on the Thorthon medium. The obtained slants were used to prepare a suspension by adding 5 ml of the YM liquid medium [Somesegeran and Hoben 1994]. Next, 1 ml of the suspension was sampled and used to inoculate 150 ml YM liquid medium which was then incubated on a shaker for 48 hours at the temperature of 28°C. The obtained sample provided 100% of the inoculum which was used to prepare 4 treatments obtained employing different dilutions:

- treatment I – 10 ml of 100% inoculum in 90 ml of the YM liquid medium,
- treatment II – 25 ml of 100% inoculum in 75 ml of the YM liquid medium,
- treatment III – 50 ml of 100% inoculum in 50 ml of the YM liquid medium,
- treatment IV – 100% inoculum in the YM liquid medium.

Using the Pelczer method [1957], medium turbidity was determined for each culture treatment. This was achieved by measuring the absorbance for a given treatment on the spectrophotometer at the wave lengths of 360 nm and 420 nm with the sterile medium serving as a model. Moreover, bacterial counts (CFU) were determined in 1 ml of the culture using the Koch plate method [Kunicki-Goldfinger 2001] (Table 1).

The inocula obtained in this way were used to inoculate the experimental plants (seed alfalfa) which were cultivated in the following 4 combinations:

- I – the plant inoculated with 10% inoculum,
- II – the plant inoculated with 25% inoculum,
- III – the plant inoculated with 50% inoculum,
- IV – the plant inoculated with 100% inoculum.

Each experimental combination was performed in 10 replications.
Effect of the culturing...

Table 1. Counts of bacteria from genus *Rhizobium* (in 1 ml of culture)

<table>
<thead>
<tr>
<th>Density of inoculum – Gęstość inokulum, %</th>
<th>Number of microorganisms (CFU)·10⁸</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4.9</td>
<td>6.5</td>
</tr>
<tr>
<td>25</td>
<td>17.7</td>
<td>5.9</td>
</tr>
<tr>
<td>50</td>
<td>38.1</td>
<td>5.6</td>
</tr>
<tr>
<td>100</td>
<td>106.6</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Prior to the establishment of the culture, seeds of the experimental plants were sterilised on a shaker for twenty minutes in 5% sodium hypochlorite and then rinsed with sterile water several times. After sterilization, the seeds were germinated on a layer of lignin with one layer of absorbent paper on Petri dishes, for 3-4 days. During seed germination, the slants with medium for leguminous plants were prepared. For this purpose, test tubes of dimensions 250 x 25 mm, sealed with a cotton plug, were sterilized in a sterilizer at 180°C, and then 20 ml of prepared medium, cooled to 20°C was poured in a sterile way into each tube. The medium was cooled in order to obtain a proper slant with CaCO₃ distributed evenly on the sufficiently thick slant. After preparing the slants, the germinated seeds were arranged on the medium in the tubes and after two days, they were infected with the appropriate combination of the inoculum in an amount of 0.1 ml.

The determined parameters of the effective symbiosis include:
– plant physiological status – nodulation, weight of the fresh matter of green parts,
– nitrogenase activity.

The weight of the plant fresh biomass of the green parts (mean from 10 replications for each combination) was determined on the analytical balance.

The nitrogenase activity in the examined strains was determined after 4 weeks of vegetation. The following parameters were adopted as indicators of activity: the number and colour of nodules, the size of the plant and the capability of nitrogenase to reduce acetylene to ethylene.

For this purpose, acetylene in the amount of 10% of the gaseous phase volume was injected into tightly sealed test tubes with experimental plants. After one hour, 1 ml gaseous phase was sampled from the inside of test tubes and subjected to analysis on a gas chromatographer CHROM 5, where the carrier gas was argon. The activity of nitrogenase was determined on the basis of the quantity of acetylene reduced to ethylene (mean from 5 samples) which was expressed in nMC₂H₄ employing a theoretical transfer coefficient N₂ : C₂H₄ = 1 : 3.

The obtained results were subjected to statistical analysis calculating the Pearson linear correlation coefficient (r) [Wysocki and Lira 2003].

RESULTS AND DISCUSSION

In the performed laboratory experiment with seed alfalfa cultured in test tubes, the authors observed a distinct influence of the inoculum density on the physiological status of plants, nodulation and nitrogenase activity.

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On the basis of the performed statistical analysis, employing the Pearson linear correlation coefficient, positive correlations were obtained only between individual inoculum densities and nodulation effectiveness where increased numbers of nodules developed on plants were observed together with the increase of the inoculum density (Table 2). However, it should be emphasized that the nodules developed at the applied inoculum concentrations of 25, 50 and 100% were smaller and pale pink, which indicated weakening of the virulence in the employed bacterial strain.

Table 2. Plant physiological condition

<table>
<thead>
<tr>
<th>Combination</th>
<th>Physiological condition of the host plant</th>
<th>Average number of nodules (of 10 plants) per plant: colour and size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated seed alfalfa</td>
<td>large, green plants, uniform leaves, single roots</td>
<td>7 pink (large), distributed in the central part of root</td>
</tr>
<tr>
<td><em>Sinorhizobium meliloti</em> Bp of 10% density</td>
<td>duże, zielone rośliny, równomierne liście, pojedyncze korzenie</td>
<td>7 różowych (duży), rozmieszczonych w środkowej części korzenia</td>
</tr>
<tr>
<td>Lucerna siewna szczepiona inokulatem o 10% gęstości</td>
<td>small plants with small yellow colourings, uniform leaves, complex roots</td>
<td>9 pink (small), distributed in the central part of root</td>
</tr>
<tr>
<td></td>
<td>male rośliny z małymi żółtymi zabarwieniami, równomierne liście, złożone korzenie</td>
<td>9 różnych, rozmieszczonych w środkowej części korzenia</td>
</tr>
<tr>
<td>Inoculated seed alfalfa</td>
<td>small plants with small yellow colourings, uneven leaves, complex roots</td>
<td>pink (small), distributed in the central part of root</td>
</tr>
<tr>
<td><em>Sinorhizobium meliloti</em> Bp of 50% density</td>
<td>male rośliny z małymi żółtymi zabarwieniami, nierówne liście, złożone korzenie</td>
<td>różowe (małe), rozmieszczone w środkowej części korzenia</td>
</tr>
<tr>
<td>Lucerna siewna szczepiona inokulatem o 50% gęstości</td>
<td>large, green plants, uniform leaves, single roots</td>
<td>16 pink (small), distributed in the central part of root</td>
</tr>
<tr>
<td></td>
<td>duże, zielone rośliny, równomierne liście, pojedyncze korzenie</td>
<td>16 różnych (małych), rozmieszczonych w środkowej części korzenia</td>
</tr>
</tbody>
</table>

(r) – correlation coefficient – współczynnik korelacji

Negative correlations were obtained between the density of the inoculum and the weight of the aboveground and underground plant parts and the root length (Table 3). The plant which was inoculated with 10% inoculum was characterized by a considerably bigger growth, more profuse green matter and a very well developed root system (Table 2). In the culture treatments where alfalfa plants were treated with higher concentrations of the inoculum (25, 50 and 100%), a distinct inhibition of the development of lateral roots was recorded and the plants themselves were characterized by weaker growth, yellow cotyledons and true leaves, which affected the weight of the discussed parameters.
Table 3. Weight of the aboveground and underground plant parts
Tabela 3. Masa części nadziemnych i podziemnych roślin

<table>
<thead>
<tr>
<th>Combination – Kombinacja</th>
<th>Weight of fresh mass, g·plant⁻¹</th>
<th>Length of root, cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aboveground parts</td>
<td>underground parts</td>
</tr>
<tr>
<td>Inoculated seed alfalfa <em>Sinorhizobium meliloti</em> Bp of 10% density</td>
<td>0.24</td>
<td>1.15</td>
</tr>
<tr>
<td>Inoculated seed alfalfa <em>Sinorhizobium meliloti</em> Bp of 25% density</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>Inoculated seed alfalfa <em>Sinorhizobium meliloti</em> Bp of 50% density</td>
<td>0.18</td>
<td>0.09</td>
</tr>
<tr>
<td>Inoculated seed alfalfa <em>Sinorhizobium meliloti</em> Bp of 100% density</td>
<td>0.16</td>
<td>0.08</td>
</tr>
</tbody>
</table>

(r) – correlation coefficient – współczynnik korelacji

The greater plant weight observed in treatments in which plants were inoculated with 10% inoculum can be attributed to such factors as the additional carbon source found in the medium on which the bacteria were cultured as well as a low concentration of toxic products of its own metabolic processes accumulating in the medium in the course of static culturing.

The applied inoculum densities exceeding 10% resulted in a decreased nitrogenase activity as shown by the negative correlation between the inoculum density and the fixation effectiveness of the molecular nitrogen (Table 4). The nitrogenase activity in the combination where the plant was inoculated with 10% inoculum amounted to 5.64 nMC₂H₄plant⁻¹·hour⁻¹, whereas in the remaining combinations it ranged from 1.59 to 2.59 nMC₂H₄plant⁻¹·hour⁻¹.

The low nitrogenase activity recorded in plants inoculated with the culture characterized by a higher inoculum density can be attributed to the weakening of the strain *Sinorhizobium meliloti* following the depletion of nutrient components and the decrease of pH in the culture medium (Table 1). Strains from the genus *Sinorhizobium* are the most sensitive to the reaction decline [Glenn and Dilworth 1994, Kurek 2002]. Low pH of the culturing medium affects exopolisacharides (EPS) synthesis. Bacteria from the family *Rhizobiaceae*, defective during the EPS synthesis, induce ineffective nodules, so called abortive nodules, deprived of infection threads and bacteroids, sometimes referred to as empty nodules [Skorupska 1995]. It is therefore possible that the above mentioned factors may have resulted in the poor effectiveness of N₂ fixation in the case of plants inoculated with high inoculum concentrations.

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Table 4. Nitrogenase activity in test tube nodule bacteria cultures
Tabela 4. Aktywność nitrogenazy w testach probówkowych bakterii brodawkowych

<table>
<thead>
<tr>
<th>Combination – Kombinacja</th>
<th>Nitrogenase activity, nMC$_2$H$_4$·plant$^{-1}$·hour$^{-1}$</th>
<th>Aktywność nitrogenazy, nMC$_2$H$_4$·roślina$^{-1}$·godzina$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated seed alfalfa Sinorhizobium melilotii Bp of 10% density</td>
<td>5.64</td>
<td></td>
</tr>
<tr>
<td>Lucerna siewna szczepiona inokulatem o 10% gęstości</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculated seed alfalfa Sinorhizobium melilotii Bp of 25% density</td>
<td>2.59</td>
<td></td>
</tr>
<tr>
<td>Lucerna siewna szczepiona inokulatem o 25% gęstości</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculated seed alfalfa Sinorhizobium melilotii Bp of 50% density</td>
<td>1.59</td>
<td></td>
</tr>
<tr>
<td>Lucerna siewna szczepiona inokulatem o 50% gęstości</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculated seed alfalfa Sinorhizobium melilotii Bp of 100% density</td>
<td>1.72</td>
<td></td>
</tr>
<tr>
<td>Lucerna siewna szczepiona inokulatem o 100% gęstości</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(r) – correlation coefficient – współczynnik korelacji</td>
<td>-0.720</td>
<td></td>
</tr>
</tbody>
</table>

CONCLUSION

1. In the performed laboratory experiment with seed alfalfa cultured in test tubes, the authors observed a distinct influence of the inoculum density on the physiological status of plants, nodulation and nitrogenase activity.

2. It was found that plants inoculated with a 10% inoculant (4.9 x 10$^6$ CFU) were characterized by the best growth, more profuse fresh material and a very well developed root system and, additionally, they revealed a higher nitrogenase activity.

REFERENCES

Kurek E., 2002. Microbiological activity in acid soils. 36$^{th}$ Microbiological Symposium The activity of microorganisms in different environments, AR Krakow, 97-103.

**WPŁYW GĘSTOŚCI HODOWLI Sinorhizobium meliloti BP NA ROZWÓJ LUCERNY SIEWNEJ I AKTYWNOŚĆ NITROGENAZY**

**Streszczenie:** Badano wpływ gęstości inokulum *Sinorhizobium* na rozwój lucerny siewnej, brodawkowanie i aktywność nitrogenazy. Zanotowano, że roślina szczepiona 10% inokulatem (4,9 x 10⁶ CFU) charakteryzowała się najlepszym wzrostem, bujniejszą masą zieloną i bardzo dobrze rozwiniętym systemem korzeniowym oraz wykazywała większą aktywność nitrogenazy.

**Słowa kluczowe:** gęstość inokulum, lucerna, *Medicago sativa* L., aktywność nitrogenazy, *Sinorhizobium meliloti*

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