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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 (4.9×10^6 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 influence 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 N_2 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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minimisation of unfavourable environmental influence (biotic and abiotic factors) [Wielbo and Skorupska 2003, Niewiadomska 2004].

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_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.

Table 1. Counts of bacteria from genus *Rhizobium* (in 1 ml of culture)
Tabela 1. Liczba bakterii z rodzaju *Rhizobium* (w 1 ml hodowli)

Density of inoculum – Gęstość inokulum, %	Number of microorganisms (CFU)·10^8 Liczba mikroorganizmów (CFU)·10^8	pH
10	4.9	6.5
25	17.7	5.9
50	38.1	5.6
100	106.6	5.2

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 180ºC, and then 20 ml of prepared medium, cooled to 20ºC was poured in a sterile way into each tube. The medium was cooled in order to obtain a proper slant with $CaCO_3$ 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 nMC_2H_4 employing a theoretical transfer coefficient $N_2 : C_2H_4 = 1 : 3$.

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.

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
Tabela 2. Stan fizjologiczny rośliny

Combination Kombinacja	Physiological condition of the host plant Stan fizjologiczny rośliny – gospodarza	Average number of nodules (of 10 plants) per plant: colour and size Średnia liczba brodawek korzeniowych (z 10 roślin) na roślinę: barwa i wielkość
Inoculated seed alfalfa *Sinorhizobium meliloti* Bp of 10% density Lucerna siewna szczepiona inokulatem o 10% gęstości	large, green plants, uniform leaves, single roots duże, zielone rośliny, równomierne liście, pojedyncze korzenie	7 pink (large), distributed in the central part of root 7 różowych (dużych), rozmieszczonych w środkowej części korzenia
Inoculated seed alfalfa *Sinorhizobium meliloti* Bp of 25% density Lucerna siewna szczepiona inokulatem o 25% gęstości	small plants with small yellow colourings, uniform leaves, complex roots małe rośliny z małymi żółtymi zabarwieniami, równomierne liście, złożone korzenie	9 pink (small), distributed in the central part of root 9 różowych, rozmieszczonych w środkowej części korzenia
Inoculated seed alfalfa *Sinorhizobium meliloti* Bp of 50% density Lucerna siewna szczepiona inokulatem o 50% gęstości	small plants with small yellow colourings, uneven leaves, complex roots małe rośliny z małymi żółtymi zabarwieniami, nierówne liście, złożone korzenie	pink (small), distributed in the central part of root różowe (małe), rozmieszczone w środkowej części korzenia
Inoculated seed alfalfa *Sinorhizobium meliloti* Bp of 100% density Lucerna siewna szczepiona inokulatem o 100% gęstości	large, green plants, uniform leaves, single roots duże, zielone rośliny, równomierne liście, pojedyncze korzenie	16 pink (small), distributed in the central part of root 16 różowych (małych), rozmieszczonych w środkowej części korzenia
(r)	–	0.993

(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 rośliny

Combination – Kombinacja	Weight of fresh mass, g·plant^{-1} Waga świeżej masy, g·roślina^{-1}		Length of root Długość korzenia cm
	aboveground parts części nadziemne	underground parts części podziemne	
Inoculated seed alfalfa *Sinorhizobium meliloti* Bp of 10% density Lucerna siewna szczepiona inokulatem o 10% gęstości	0.24	1.15	6.15
Inoculated seed alfalfa *Sinorhizobium meliloti* Bp of 25% density Lucerna siewna szczepiona inokulatem o 25% gęstości	0.13	0.12	4.75
Inoculated seed alfalfa *Sinorhizobium meliloti* Bp of 50% density Lucerna siewna szczepiona inokulatem o 50% gęstości	0.18	0.09	4.15
Inoculated seed alfalfa *Sinorhizobium meliloti* Bp of 100% density Lucerna siewna szczepiona inokulatem o 100% gęstości	0.16	0.08	4.4
(r)	-0.398	- 0.635	-0.679

(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 $nMC_2H_4 \cdot plant^{-1} \cdot hour^{-1}$, whereas in the remaining combinations it ranged from 1.59 to 2.59 $nMC_2H_4 \cdot plant^{-1} \cdot hour^{-1}$.

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 N_2 fixation in the case of plants inoculated with high inoculum concentrations.

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

Combination – Kombinacja	Nitrogenase activity, $nMC_2H_4 \cdot plant^{-1} \cdot hour^{-1}$ Aktywność nitrogenzay, $nMC_2H_4 \cdot roślina^{-1} \cdot godzina^{-1}$
Inoculated seed alfalfa *Sinorhizobium melilotii* Bp of 10% density Lucerna siewna szczepiona inokulatem o 10% gęstości	5.64
Inoculated seed alfalfa *Sinorhizobium melilotii* Bp of 25% density Lucerna siewna szczepiona inokulatem o 25% gęstości	2.59
Inoculated seed alfalfa *Sinorhizobium melilotii* Bp of 50% density Lucerna siewna szczepiona inokulatem o 50% gęstości	1.59
Inoculated seed alfalfa *Sinorhizobium melilotii* Bp of 100% density Lucerna siewna szczepiona inokulatem o 100% gęstości	1.72
(r)	- 0.720

(r) – correlation coefficient – współczynnik korelacji

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 (4.9×10^6 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.

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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 10^6 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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