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EFFECT OF THE CO-INOCULATION OF LUCERNE (*MEDICAGO SATIVA* L.) WITH *SINORHIZOBIUM MELILOTI* AND *HERBASPIRILLUM FRISINGENSE* IN RELATION TO THE INTERACTIONS BETWEEN BACTERIAL STRAIN

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Abstract: The aim of the performed experiments was to analyse relationships occurring between endophytic bacteria from the *Herbaspirillum* genus and *Sinorhizobium meliloti* Bp nodule bacteria and to examine the condition of plants subjected to coinoculation with the above-mentioned strains in *in vitro* conditions. In experiments examining the impact of *Herbaspirillum frisingense* on *Sinorhizobium meliloti* BP, the stimulation of growth of inoculated bacteria from the *Sinorhizobium* genus was recorded in all three combinations (48-hour culturing, sediment and supernatant). On the other hand, the examination of interactions between the *Sinorhizobium meliloti* strain and *Herbaspirillum frisingense* strain revealed that in the case of culture and supernatant, an antagonistic action was recorded. Besides, it was found that such coinoculation exerted a beneficial influence on the process of seed lucerne symbiosis and yielding as confirmed by increased numbers of root nodules, higher nitrogenase activity and greater plant mass.

INTRODUCTION

Nitrogen is an essential plant nutrient, widely applied as N-fertilizer to improve yield of agriculturally important crops [12]. An interesting alternative to avoid or reduce the use of N-fertilizers could be the exploitation of plant growth-promoting bacteria (PGPB), capable of enhancing growth and yield of many plant species, several of agronomic and ecological significance [13].

It is worth drawing attention to the fact that microorganisms living in the natural environment frequently enter into various mutual relations not only with other microorganisms but also with plants. Some of these interactions are beneficial for both partners or are even, sometimes, indispensible for their proper functioning. Naturally, there are also situations when these relationships become harmful for one or even for both partners. Methods which assist microorganisms to enter into associations with higher organisms vary. Some of them derive from direct interactions between microorganisms and tissues of living organisms, while others are of indirect nature and are caused by modifications of the environment [2].

The phenomenon of frequent occurrence of bacterial populations accompanying plants appears to indicate that healthy plants contain complexes of the so called endophytic bacteria for which plants constitute nearly an indispensible niche. It is assumed that these organisms can enhance plant growth and also support biological control over diseases. Numerous investigations indicate that endophytic bacteria not only assist development of plant roots but, in addition, enter into relationships with other soil bacteria or species of organisms which belong to another systematic group. Sometimes, shared bacterial neighbourhood contributes to increased numbers of populations and, consequently, to the improvement of soil properties and by doing so create better growth conditions for plants, while on other occasions, mutual coexistence may lead to negative results [1].

The aim of the performed experiments was to analyse mutual relationships occurring between endophytic bacteria from the *Herbaspirillum* genus and *Sinorhizobium meliloti* Bp nodule bacteria and to examine the condition of plants subjected to coinoculation with the above-mentioned strains in *in vitro* conditions.

MATERIAL AND METHODS

Analysis of interactions between bacterial strains

The trial was carried out in laboratory conditions. In the performed analyses, the author employed the *Sinorhizobium meliloti* Bp strain which lives in symbiosis with lucerne and which is characterised by a very high level of activity of nitrogen fixation and which derived from the collection of the Section of Microbiology of IUNG in Puławy as well as a strain of *Herbaspirillum frisingense* derived from the collection of Microorganisms and Cell Cultures in Braunschweig. The following experimental combinations were applied to investigate interactions:

1. *Herbaspirillum frisingense* a *Sinorhizobium meliloti* Bp
2. *Sinorhizobium meliloti* Bp a *Herbaspirillum frisingense*

Each combination was investigated in the following three variants:

I variant: the impact of 48-hour culture of one strain to another;

II variant: the impact of sediment (obtained after centrifugation of the culture) one strain to another;

III variant: the impact of supernatant (obtained after centrifugation of the culture) one strain to another.

Prior to the investigations, bacterial strains used in the experiments were passaged onto slants with fresh medium appropriate for *Sinorhizobium meliloti* Bp and *Herbaspirillum frisingense*.

In addition, a growth curve of bacterial cultures according to Pelczar [14] was determined for each strain on the basis of spectrophotometric analysis, which made it possible to determine the moment in which the culture exhibited the highest metabolic activity and the moment of inhibition of its growth (Fig. 1). The performed analyses revealed that the 48-hour culture was appropriate for both experimental strains.

Suspensions were prepared from 3-day cultures of initial *Herbaspirillum* and *Sinorhizobium* strains passaged several times and developed on agar slants by the addition of 5 ml sterile distilled water to each test-tube.

The liquid YM medium (100 ml) for *Sinorhizobium meliloti* [16] and LB [3] for *Herbaspirillum frisingense* (five flasks for each strain) was inoculated with the obtained

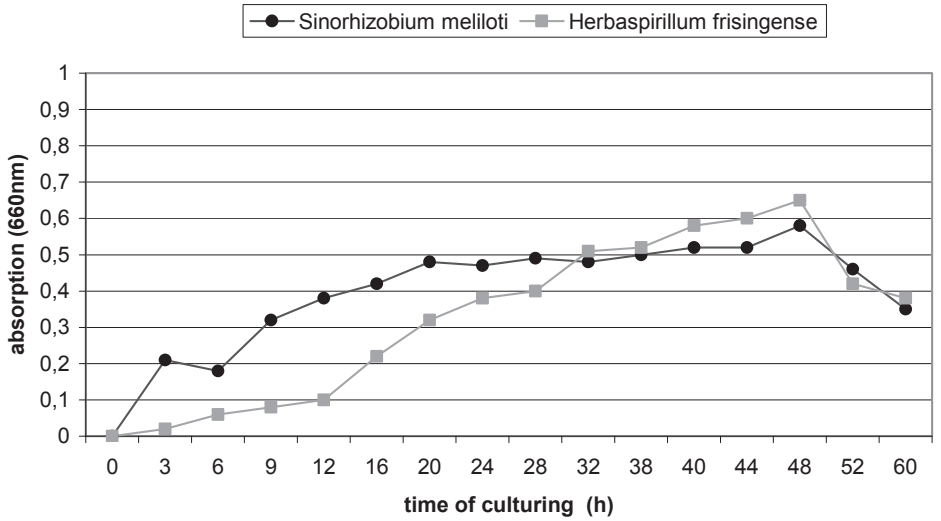

Fig. 1. The bacterial growth curve

suspension in the amount of 0.1 ml. The inoculated media were incubated on a shaker at the temperature of 28°C at 78 rpm for 48 hours. After the above period of time, one culture flask was taken for analyses and the remaining ones were centrifuged at 1000 rpm in order to obtain next combinations of sediments and supernatants.

Interactions between individual bacterial strains were investigated on Petri dishes using the ring method [3]. All analyses were performed in five replications.

First, 5 ml of 2% agar was poured onto appropriately labelled sterile Petri dishes and then, using pincers, sterile rings were placed aseptically onto solidified 2% agar; 3 rings per each dish. Once the rings were placed safely on the dish, the prepared mixture of the dissolved medium (10 ml) and appropriate bacterial dilution (10 x 10^{-5}) was carefully poured onto the dish so as not to let it get inside the ring. After cooling, rings were delicately and aseptically removed from Petri dishes.1 ml of the prepared combinations (culture, sediment, supernatant) was applied in two holes received after removed rings, while in the third, which served as control, physiological salt was applied. Then dishes were left for one hour to allow the suspension to be absorbed and, afterwards, Petri dishes were placed in a thermostat at the temperature of 28°C (dishes were not turned over).

Analysis of plant condition at their simultaneous inoculation

Parallelly to the above-described investigations, another experiment was performed the aim of which was to examine the condition of plants subjected to coinoculation with the above-mentioned bacterial strains in *in vitro* conditions. Plants of seed lucerne (*Medicago sativa* L.), cv. Socza were cultured on agar substrate for leguminous plants in the following four combinations:

- I combination – plant not inoculated (control),
- II combination – plant inoculated with *Sinorhizobium meliloti* Bp,
- III combination – plant inoculated with *Herbaspirillum frisingense,*
- IV combination – plant inoculated with *Sinorhizobium* and *Herbaspirillum*.

Each combination comprised the total of 25 samples.

Prior to the establishment of the culture, plant seeds were sterilised on a shaker for 25 minutes in 5% sodium hypochlorite and then rinsed several times with sterile water and placed on Petri dishes for sprouting for a period of 4 days at the temperature of 24°C. While the seeds were sprouting, agar slants for leguminous plants were prepared on which lucerne seeds were later transferred. After 24 hours, rootlets were infected with the suspension of three-day old bacterial strains in the amount of 0.1 ml/plant. Strains with which the seeds were inoculated were multiplied on slants on YMA medium for *Sinorhizobium* and on LB medium for *Herbaspirillum*.

Plants were cultivated in a vegetation facility at the temperature of 23°C and at 16-hour lighting period.

In the laboratory experiment, the following parameters were the object of analyses:

1. Nitrogenase activity,
2. Number of root nodules,
3. Mass of over-ground and under-ground parts,
4. Numbers of microorganisms colonising roots.

Analyses were conducted after 5, 14 and 28 days from inoculation.

Nitrogenase activity was determined by the acetylene method on a CHROM5 gas chromatographer [15] on the basis of the quantity of reduced acetylene and was expressed in nMC_2H_4. The mass of over-ground and under-ground parts was determined gravimetrically on a Sautorius weight. The numbers of bacteria settling lucerne roots were determined, in turn, by the flooded plate method according to Koch. For this purpose, after weighing plant roots, they were macerated in a mortar in 10 ml of sterile water and then consecutive dilutions of the obtained suspension were prepared. Appropriate dilutions were poured on dishes and YMA medium was added for bacteria from the *Rhizobium* genus [16] and LB medium for endophytes from the *Herbaspirillum* genus [3].

RESULTS AND DISCUSSION

Analysis of the results of interactions between bacteria used in experiments

In experiments examining the impact of *Herbaspirillum frisingense* on *Sinorhizobium meliloti* BP, a stimulation of growth of the inoculated bacteria from the *Sinorhizobium* genus was recorded in all three combinations (48-hour culturing, sediment and supernatant). Around rings on plates, increased numbers of bacterial colonies were recorded (Photos 1, 2 and 3). It is not possible to establish unequivocally causes of bacterial growth, although it is possible to assume with considerable certainty that this kind of impact of *Herbaspirillum* on the strain of nodule bacteria was the result of production by the above-mentioned endophytes of such phytohormones as: indoleacetic acid (IAA), gibberellins or cytokines [8]. In addition, also vitamins could have acted as compounds responsible for the observed stimulatory effect [9].

On the other hand, the examination of interactions between the *Sinorhizobium meliloti* strain and *Herbaspirillum frisingense* strain revealed that in the case of culture and supernatant, an antagonistic action was recorded. Growth inhibition of the inoculated endophytic bacteria took place and the developed halo was 4.5 mm wide in the case of the supernatant (Photo 4) and 3 mm wide in the case of the culture (Photo 5). A contrary situation occurred in the combination with the sediment where a stimulatory effect of the

Sinorhizobium on *Herbaspirillum* strain was observed. Colonies that developed around the ring were larger and more numerous (Photo 6).

The obtained results indicate that the nodule bacteria strain itself was neutral against the *Herbaspirillum* strain, whereas secondary metabolites accumulating in the medium during the strain incubation were toxic. The antagonistic effect could be explained by the synthesis of compounds of the nature of bacterial toxins which were secreted into the environment. Lorkiewicz emphasised certain phenotypic properties of nodule bacteria, e.g. production of antibiotics. Due to this trait, these bacteria defend the area occupied by them against attacks by other microorganisms [10].

Individual cultures possess capability to manufacture antibacterial or antifungal antibiotics. Some of them are referred to as beta-lactams or polyethers. Other strains are capable of synthesising exoenzymes [11]. *Sinorhizobium meliloti* is capable of producing and accumulating glutaminians as well as dipeptide-N-acetyloglutaminyl-glutamine. These compounds may exert an aseptic influence on other microorganisms because they become noxious at excessive quantities. It is also worth noting positive characters of these compounds, namely they fulfil important metabolic functions by providing a substrate in protein synthesis and, in addition, they assist in nitrogen transport.

Analysis of plant condition at their simultaneous inoculation

On the basis of the obtained results from the performed investigations in laboratory conditions on the impact of a simultaneous lucerne inoculation with the above-mentioned strains of *Sinorhizobium meliloti* and *Herbaspirillum frisingense* bacteria, it was found that such inoculation exerted a beneficial influence on the process of seed lucerne symbiosis and yielding as confirmed by increased numbers of root nodules, higher nitrogenase activity and greater plant mass.

The obtained research results showed that the highest nitrogenase activity was obtained in plant combinations in which *Sinorhizobium* – *Herbaspirillum* coinoculation was employed, both on the second and third day (Fig. 2). It amounted to, respectively, 30.4 nMC_2H_4/plant/h after 14 days of plant culturing, whereas after 28 days – to 35.63 nMC_2H_4/plant/h and was, on average, by 23% higher in comparison with the activity of nitrogenase in plants inoculated exclusively by the *Sinorhizobium meliloti* Bp strain.

On the other hand, biological nitrogen fixation in the lucerne inoculated with endophytes from the *Herbaspirillum* genus was found to be on a relatively low level at all dates of analysis and it was most effective on the third date of analyses reaching 10.13 nMC_2H_4/plant/h (Fig. 2).

Similarly to nitrogenase activity, also plant nodulation was the highest in the combination in which simultaneous inoculation with symbiotic bacteria and endophytes was applied both on the 14^{th} and 28^{th} day of plant culturing (Fig. 3).

Nodules had pink pigmentations indicating the presence of leghemoglobin which constituted the evidence of the nitrogen fixation process taking place.

Despite the absence of nodules in lucerne plants after 5 days of culturing from plant inoculation, nevertheless slight nitrogen fixations did occur in all combinations with the exception of the control plant, which was not subjected to inoculation (Fig. 3).

Plants subjected to any inoculation (especially on the second and third day) distinguished themselves by greater mass of roots and over-ground parts in comparison with the control (Fig. 4).

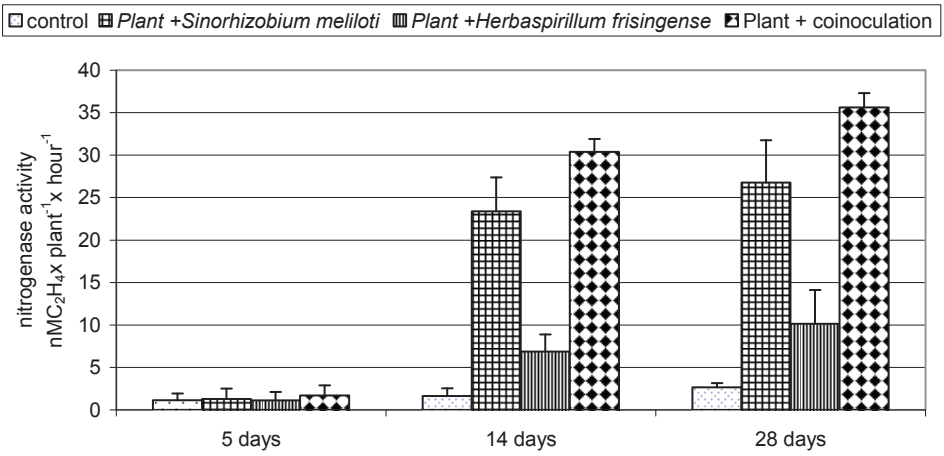

Fig. 2. The effect of the co-inoculation on nitrogenase activity in vitro cultures

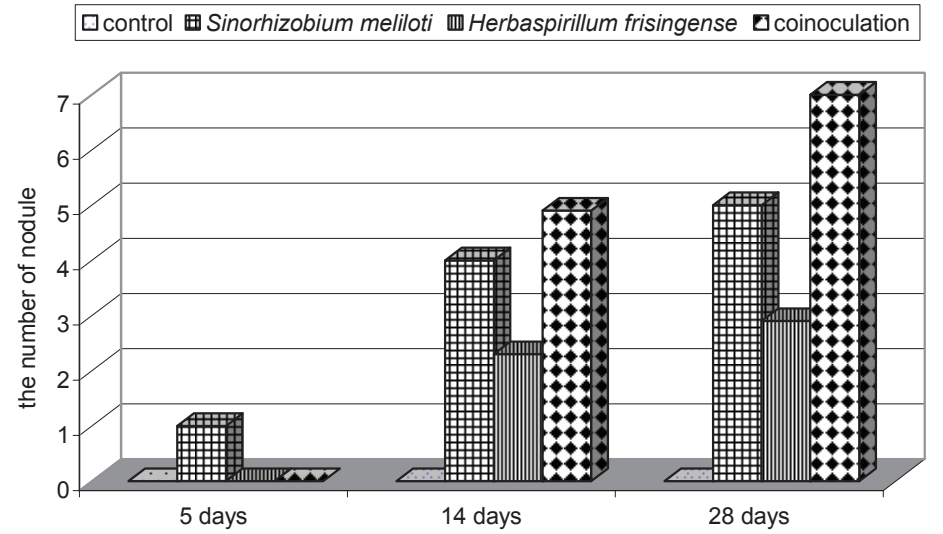

Fig. 3. The effect of the co-inoculation on the nodulation

However, a conspicuous impact was noticeable of the applied simultaneous inoculation with *Sinorhizobium meliloti* and *Herbaspirillum frisingense* strains on the root mass. At each date of analysis, it was higher not only when compared with the root mass of the control plant but also in relation to plants from combinations inoculated with bacterial strains of either the *Sinorhizobium* or *Herbaspirillum* genera (Fig. 4).

A similar effect of the simultaneous inoculation with *Sinorhizobium* and *Herbaspirillum* strains was recorded in the case of the mass of the over ground parts. Plants from this treatment were characterised by significantly higher mass of green parts which, after 28 days, was almost 10 times higher in comparison with plant infected with only *Sinorhizobium meliloti* Bp or *Herbaspirillum frisingense* strain (Fig. 4).

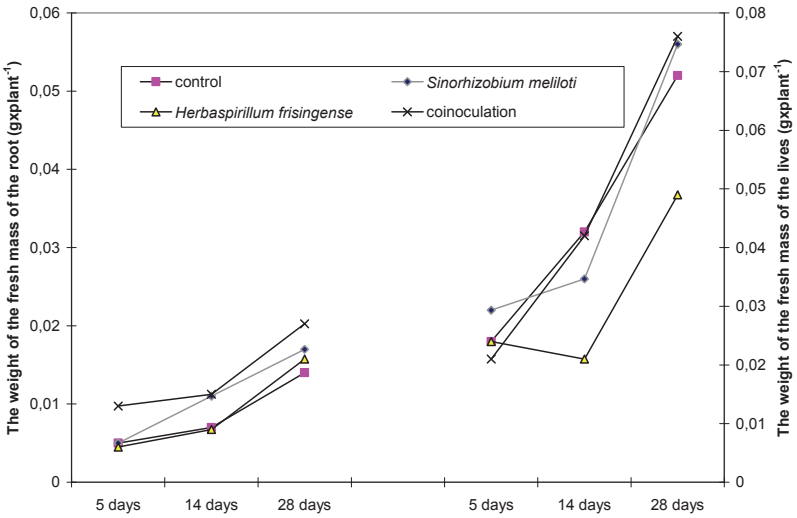

Fig. 4. The effect of co-inoculation on the weight of the fresh mass of green plant

The obtained results can be attributed to the capability of these microorganisms to synthesise and secrete into the environment of biologically active substances called phytohormones. It is believed that these substances exert a beneficial influence on the development, growth and branching of root hairs which, in turn, causes that the plant becomes more sensitive to *Rhizobium* infections as well as to the development of symbiotic bacteria in this combination [9]. This was also confirmed in experiments carried out by Bashana [2] and Chebotar [5] in which they employed *Azospirillum* and *Bradyrhizobium* bacteria. Also Głowacka [7] and Burdman [4] reported a favourable influence of endophytes from the *Azospirillum* genus on plants resulting from the secretion of such phytohormones as auxins and cytokinins stimulating the development of root hairs in leguminous plants.

Also Itzigshon reported a beneficial impact of coinoculation with *Rhizobium – Azospirillum* on numbers of nodules, seed yields of leguminous plants, increase of root length, weight, number of root hairs and root diameter.

An additional parameter of the performed investigations was the determination of the numbers of symbiotic bacteria and endophytes on roots of lucerne. Its objective was to find out if the *Herbaspirillum* strain was competitive in relation to nodule bacteria in settling roots when applied in co-inoculation.

The highest root colonisation with nodule bacteria was observed after 28 days of plant culturing both in the case of single inoculation as well as coinoculation (Fig. 5).

However, in the combination in which only the *Sinorhizobium meliloti* Bp strain was applied, lower populations of symbiotic bacteria occurred than in the combination in which coinoculation was employed (Fig. 5).

In the case of endophytes, the highest root colonisation was also recorded on the 28th day of plant culturing where, in a single inoculation, the amount of bacteria from the *Herbaspirillum* genus on the root amounted to 51.53 x 10^5cfu, whereas in the combination where the coinoculation was employed – 160 x 10^5cfu (Fig. 5). This effect could have resulted from the investigated interactions between strains used in these investigations.

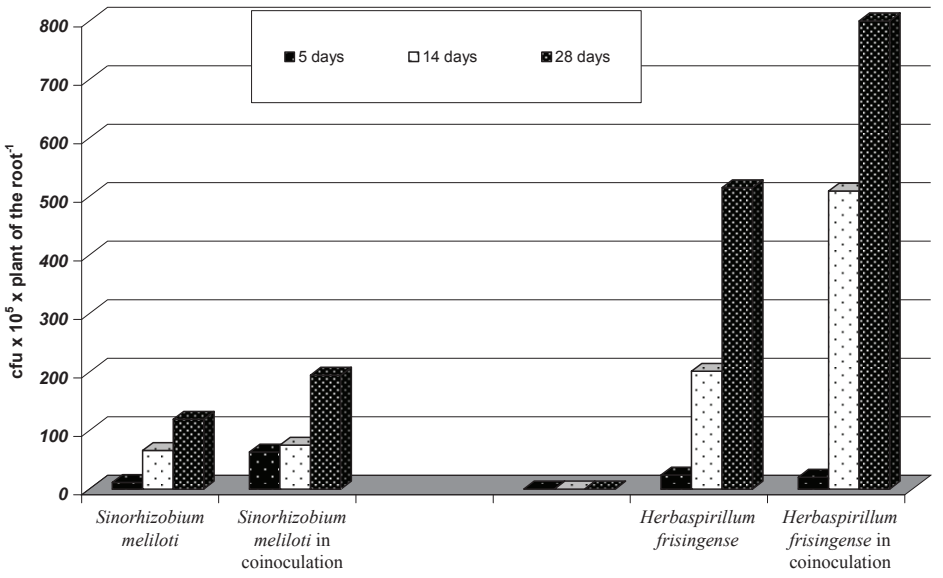

Fig 5. The number of the nodulation and endophytic bacteria

CONCLUSION

In experiments examining the impact of *Herbaspirillum frisingense* on *Sinorhizobium meliloti* BP, a stimulation of growth of the inoculated bacteria from the *Sinorhizobium* genus was recorded in all three combinations (48-hour culturing, sediment and supernatant).

The examination of interactions between the *Sinorhizobium meliloti* strain and *Herbaspirillum frisingense* strain revealed that in the case of culture and supernatant, an antagonistic action was recorded.

Coinoculation with the above-mentioned strains of *Sinorhizobium meliloti* and *Herbaspirillum frisingense* bacteria, it was found that such inoculation exerted a beneficial influence on the process of seed lucerne symbiosis and yielding as confirmed by increased numbers of root nodules, higher nitrogenase activity and greater plant mass. It was found that coinoculation with the above-mentioned strains of *Sinorhizobium meliloti* and *Herbaspirillum frisingense* bacteria exerted a beneficial influence on the process of seed lucerne symbiosis and yielding as confirmed by increased numbers of root nodules, higher nitrogenase activity and greater plant mass.

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WPŁYW JEDNOCZESNEJ INOKULACJI LUCERNY (*MEDICAGO SATIVA* L.) SZCZEPAMI *SINORHIZOBIUM MELILOTI* I *HERBASPIRILLUM FRISINGENSE* W STOSUNKU DO ZACHODZĄCYCH INTERAKCJI POMIĘDZY SZCZEPAMI BAKTERII

Celem doświadczenia była analiza wzajemnych relacji zachodzących pomiędzy bakteriami endofitycznymi z rodzaju *Herbaspirillum frisingense* i bakteriami brodawkowymi *Sinorhizobium meliloti* Bp oraz zbadanie kondycji roślin poddanej koinokulacji wyżej wymienionymi szczepami w warunkach *in vitro*. W badaniu oddziaływania *Herbaspirillum frisingense* na *Sinorhizobium meliloti* BP we wszystkich trzech kombinacjach (hodowla 48h, osad i supernatant) zanotowano stymulację wzrostu wysianych bakterii z rodzaju *Sinorhizobium.* Z kolei badanie oddziaływania pomiędzy szczepem *Sinorhizobium meliloti* a szczepem *Herbaspirillum frisingense* wykazało, że w przypadku hodowli i supernatantu zanotowano antagonistyczne oddziaływanie. Na podstawie uzyskanych wyników z przeprowadzonych badań w warunkach laboratoryjnych nad efektem jednoczesnej inokulacji lucerny wyżej omawianymi szczepami bakterii *Sinorhizoium meliloti* i *Herbaspirillum frisingense*, stwierdzono korzystne oddziaływanie takiego szczepienia na proces symbiozy i plonowanie lucerny siewnej.

Photo 1. Interaction between *Herbaspirillum frisingense* (culturing) and *Sinorhizobium meliloti*

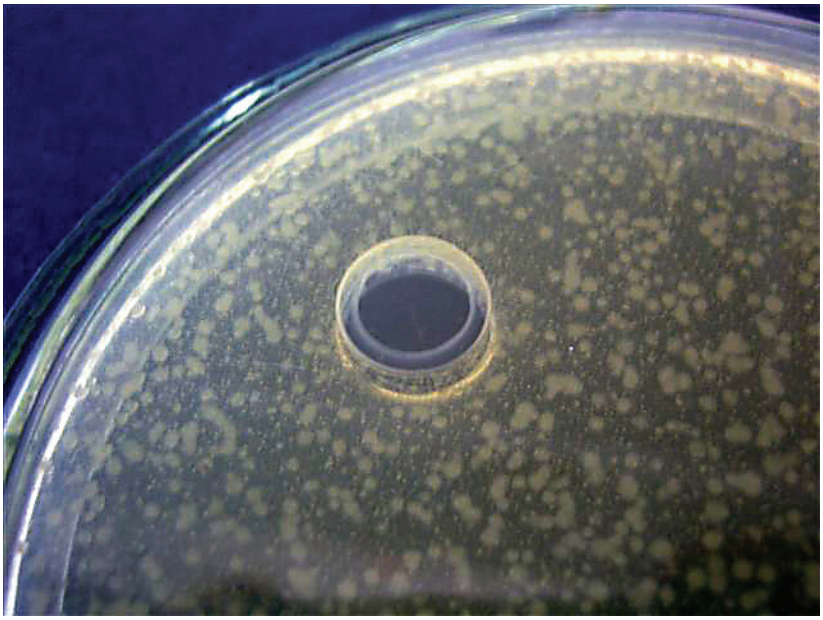

Photo 2. Interaction between *Herbaspirillum frisingense* (sediment) and *Sinorhizobium meliloti*

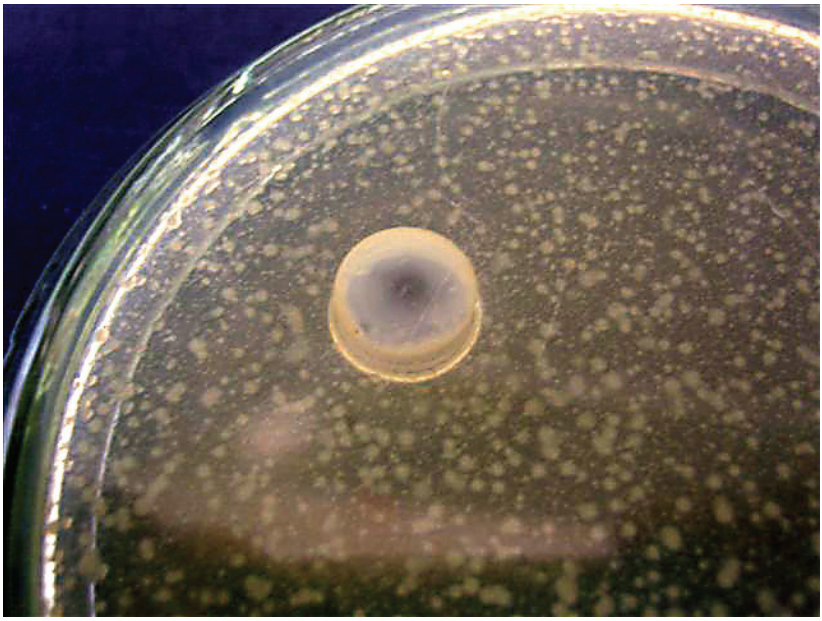

Photo 3. Interaction between *Herbaspirillum frisingense* (supernatant) and *Sinorhizobium meliloti*

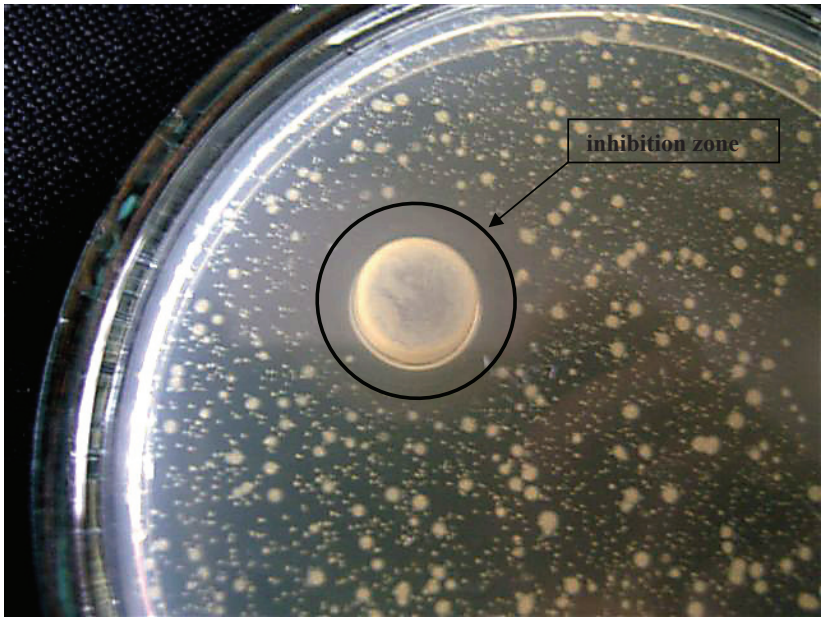

Photo 4. Interaction between *Sinorhizobium meliloti* (supernatant) and *Herbaspirillum frisingense*

Photo 5. Interaction between *Sinorhizobium meliloti* (culturing) and *Herbaspirillum frisingense*

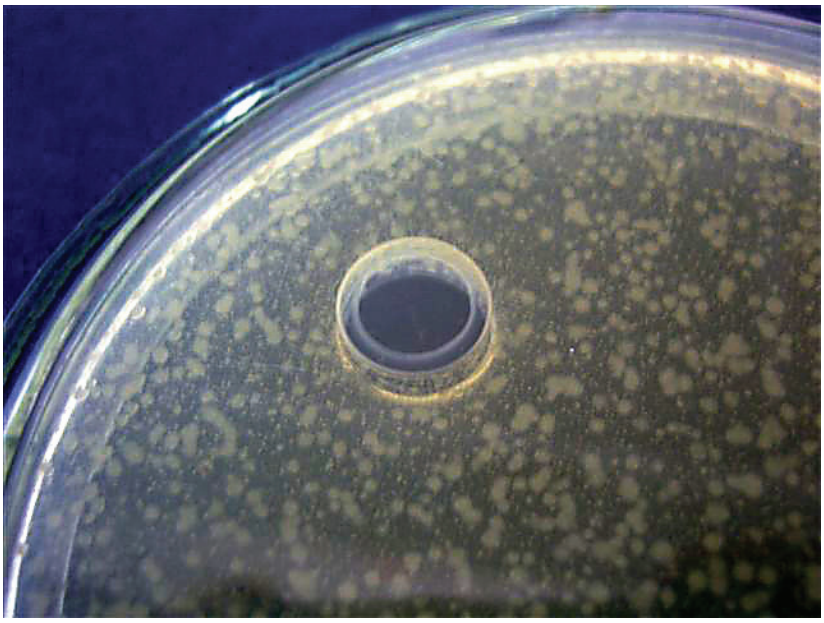

Photo 6. Interaction between *Sinorhizobium meliloti* (sediment) *and Herbaspirillum frisingense*