THE INFLUENCE OF SOIL FUNGUS *Gliocladium* sp. ON WHITE LUPINE / *Bradyrhizobium lupini* SYMBIOTIC SYSTEM

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Abstract. The functioning of nodule bacteria/legumes symbiotic system significantly depends on some edaphic factors (pH level, soil moisture and temperature) as well as rhizosphere microbiota and saprophytic fungi being its important component. The new strain of saprophytic fungus *Gliocladium* sp. 278 was isolated from washed roots of white lupine, which is a traditional culture for Ukrainian Polissya. The *Gliocladium* fungi in lupins roots amounted to 49.0% of the total fungi content. The influence of *Gliocladium* sp. 278 on white lupine was investigate in pot experiments. The experimental plants, which growing on soil with *Gliocladium* sp. 278, were oppressed. Thus, their height decreased on 12.0%, the dry mass of sprout on 20.7%, the dry root mass on 25.6%. Without affecting the nodule mass accumulation, *Gliocladium* sp. 278 oppressed N_2-fixing activities in 1.4-2.2 times. As the results of electron microscopy indicated, it took place an early aging of cells in tissue of nodules of white lupine and bacteriodes form were changed.

Key words: bacteroides; *Bradyrhizobium lupini*; *Gliocladium*; lupine; nodules; symbiotic system.

INTRODUCTION

The process of biological N_2-fixation is unique natural phenomenon [3]. As well as photosynthesis, it helps to carry on the life processes on Earth. More than a half of fixed N_2 quantity on our planet are by legume-rhizobial symbiosis. Bean-rhizobial systems are able for N_2-fixation within the limits of 40 to 300 kg N_2 and more in 1 ha per year according to legumes type in Ukrainian soil-climatic zone. The amount of fixed N_2 in stationary field experiments were 40-70 kg per ha for vetch, 60-90 for soybean, 80-120 for lupine, 140-220 for alfalfa and goat's rue [31].

Lupine is an important legume culture at about 9000 ha sowing area in Ukraine [6]. The lupine seeds contain up to 40% of protein with balanced stuff of essential amino acids which are high solubile and nutritious. Such content of protein, fats (up to 20%) and also oligosaccharides, fiber, flavonoids, tannins and saponins [16] makes it very important for animal nutrition [29]. At the same time lupine is a good predecessor for the most of cultures being enriched the soil with nitrogen [14, 21].

The effective interaction between lupine and rhizobia allow them to use atmosphere nitrogen and to be independent from phospate and nitrogen fertilizers at favorable climatic conditions on poor sod-podzolic soils [25, 33]. This type of soil is the most wide-spread among arable lands of the Ukrainian Polissya [13]. The level of symbiotic N_2-nitrogen fixation can be enlarged by presowing inoculation with effective strains of nodule bacteria *Bradyrhizobium lupini* [4, 11].

However, the yield of lupine mostly depends on many factors. First of all, it may be the meteorological conditions [27], biological features of plant varieties and the strain of nodule bacteria [23, 30]. White lupine plants affection by root pathogens are often appeared to be the weighty argument for reducing the yields [8]. Besides, it can be the high number of fungi producing phytotoxic substances leading to soil toxicity [28].

The purpose of our work was to investigate the influence of saprophytic fungus *Gliocladium* sp. 278 on forming and functioning the system of white lupine / *Bradyrhizobium lupini* B-128.

MATERIALS AND METHODS

Strain

The strain of *B. lupini* B-128 was obtained from microorganisms collection of the Institute of Agricultural Microbiology And Agro-Industrial Production NAAS [32]. The strain of *Gliocladium* sp. 278 was collected from flushed roots of white lupine plants (*Lupinus albus* Shhedryj var., Ukraine, Institute of Agricultural Microbiology And Agro-Industrial Production NAAS).

Cultural conditions

The nodule bacteria were cultivated on the lupine broth medium with the following components (g/L): K_2HPO_4 – 0.5, KH_2PO_4 – 0.5, $MgSO_4$ – 0.2, NaCl – 0.2, $CaSO_4$ – 0.1, $(NH_4)_2MoO_4$ – 0.01, mannitol – 20.0, lupine broth – 150.0, agar – 14.0. Medium pH was in amount of 6.8-7.0. The necessary sterilization mode was 0.5 atm within 20 minutes. The cultivation process continued for 5 days at 26 °C.

Sampling and isolation

The samples were picked up from the field with white lupine grown on the sod-medium-podzolic dusty-sandy soil. The samples of row-spacing soil and white lupine plants were transported in sterile plastic bags. There were detected the number of fungi and their genus stuff in row-spacing soil (without plants), rhizosphere soil and on the flushed roots.

The roots were shook off for ridding of the large soil lumps and leaving a small layer in 1-2 mm (rhizosphere soil). This soil was took off with a sterile

putty knife. It was put 1 g of soil into the flask with 100 mL of sterile water. Before detection the number of colony-forming units of fungi in roots, they were flushed within 15 minutes then rinsed twice with sterile water. After that 1 g of roots were grinded in a mortar with sterile sand and put into the flask with 100 mL of sterile water.

Colony-forming units (CFU) desorption from soil particles and roots were performed by deep rotations (180 rpm) within 15 minutes. It was made a tenfold dilution. Than obtained suspension inoculated on BMA (barley meal agar) with addition streptomycin (40-50 mg per 1 L) for bacterial growth inhibition. Petri plates were incubated at 26 °C. On the third day the fungal colonies were counted then on the 15-20-th day it were carried the identification of their generic composition. Soil moisture and counting the number of colony-forming units were remade per 1 g of dry soil. The number of CFU in roots were counted per 1 g of dry roots.

Morphological identification

The cultural and morphological characteristics were examined at BMA, Czapek's agar (CZA) and potato dextrose agar (PDA) in the darkness on Petri dishes at a temperature of 26 °C. Taxonomic identification by morphology of fungal isolates was mainly based on the identification keys in the 10th edition of Anisworth & Bisby's Dictionary of the fungi [10].

Pot Experiments

The influence of *Gliocladium* sp. 278 on white lupine (Shhedryj var.) symbiotic system was studied in a growing house with natural light and soil moisture within 60% of the total moisture content. The type of soil was sod-medium podzolic sulfur-sandstone; the humus contents – 1.02%; the nitrogen contents – 54.9 mg/kg; the moving forms of phosphorus contents – 110-120 mg P_2O_5; the exchangeable potassium contents – 120-130 mg K_2O per 1 kg of soil; pH salt – 5.2; pH water – 6.0; Ca – 5.8, Mg – 0.61 mg·eq per 100 g of soil. Plastic pots measuring 12×15 cm with 2.0 liters capacity were used. The seeds were sown to a depth of 2.0 cm, with 20 pieces of plants in each pots. The plants were grown for 50 days. The experiment was repeated five times.

The experiment was including such variants: 1) control (without microorganisms, the seeds were moistened with water at amount of 1% by their weight); 2) seed treatment with *B. lupini* B-128 (300 thousand cells per 1 seed); 3) introduction *Gliocladium* sp. 278 into the soil (100 thousand of CFU per 1 g of soil); 4) introduction *Gliocladium* sp. 278 into the soil (100 thousand CFU per 1 g of soil) + seed treatment with *B. lupini* B-128 (300 thousand cells per 1 seed).

The length and dry weight of plants were determined in the flowering phase. The mass of the nodules and their nitrogenase activity were determined in stalking, budding and flowering phases.

Determination of nitrogen fixation

Nitrogenase activity was determined with gas chromatographic method. The roots picked up from 3 plants were brought into 120 ml flask and sealed with rubber stoppers. Than were brought in acetylene (10% by volume), incubated for 1 hour at 27 °C and analyzed on a gas chromatograph Agilent Technologies 6850 using flame- ionization detector with H_2 carrier gas and He cooling.

Ultrastructural samples processing

For electron microscopic studies the nodules were separated from plants of the control and the variant with *Gliocladium* sp. 278. Then the nodules were cut into layers up to 1 mm thick and fixed with glutaraldehyde 6% and OsO_4 1% solution. Dehydration of the fixed material was performed with 50, 70, 76 and 100% alcohols and filled in with Epon-812. Ultrathin sections were obtained with LKB ultramicrotome and contrasted with uranyl acetate 1% solution. The samples were viewed under BS-540 "Tesla" electron microscope.

Statistical methods

Calculations and statistical results processing were performed according to generally accepted methods. There had been used parametric criteria for normal distribution, arithmetic mean and standard deviation for significance level less than 0.05. To assess the differences significance between the variants it had been calculated LSD ($P \leq 0.05$) using Microsoft Excel.

RESULTS

The quantitative and generic stuff of sod-podzolic soil fungi in white lupine crops were studied. It was found, that fungi biota was represented by genera *Acremonium*, *Alternaria*, *Bipolaris*, *Cladosporium*, *Fusarium*, *Gliocladium*, *Mortierella*, *Mucor*, *Penicillium*, *Trichoderma* and Dematiaceae family. The representatives of *Acremonium*, *Bipolaris*, *Fusarium*, *Gliocladium*, *Mortierella*, *Mucor*, *Penicillium*, *Trichoderma* were found in rhizosphere soil. There were only representatives of *Acremonium*, *Fusarium*, *Gliocladium*, *Mucor*, *Penicillium*, *Trichoderma* on the washed roots (Table 1). The number of aisle fungi were 84.4 ± 7.8 thousand in 1 g of soil. Much more fungi were found in rhizosphere soil in the quantity of 383.1 ± 33.7 thousands. The least number of fungi were found on washed roots in the quantity of 28.6 ± 2.5 thousand per 1 g of roots.

It is necessary to note, that *Penicillium* fungi prevailed in soil without plants (40.7% of the total fungi number) and *Fusarium* fungi prevailed in rhizosphere soil (64.6% of the total fungi number). At the same time, on the washed roots there had been the most number of *Fusarium* (30.8%) and *Gliocladium* representatives (49.0% of the total fungi number). The high number of fungi of the genus *Gliocladium* on washed lupine roots is noteworthy. This suggests that these fungi perhaps are able to make an influence on plants in some way. The strain of *Gliocladium* sp. 278 was selected from the washed roots of white lupine. Its influence on plants was studied in pots experiment. Considering that an important method is presowing

treatment of the seeds with nodule bacteria when growing legumes it was used an effective strain of lupine nodule bacteria *B. lupins* B-128 in pots experiment.

Effects on plant growth

According to the results of pots experiment, the white lupine plants was growing with introduced *Gliocladium* sp. 278 significantly lagged behind the control (Fig. 1, Table 2) without any signs of the root system damage. Thus, the plant height decreased by 12.2%, the dry weight of sprout by 20.7%, the dry mass of roots by 25.6% compared to control. Inoculation of lupine seeds by nodule bacteria *B. lupins* B-128 of mitigated the negative effect of *Gliocladium* sp. 278 on lupine plants (Table 2).

Effects on nodule formation

As we had seen the inhibitory effect of *Gliocladium* sp. 278 on the height and weight of plants it was important for us to find out how the fungus is able for influence on formation and functioning of symbiotic system – white lupine / nodule bacteria.

Nodules analysis of on plant roots showed (Fig. 2) that the soil contained a local population of specific rhizobia, which formed nodules on plants without microorganisms (control). Against the background of this population inoculation of white lupine seeds with *B. lupins* B-128 contributed for an increase the mass of nodules up to 33-46% (depending on development phase) compared to control. We did not see the significant reduction of nodule mass during the introduction of *Gliocladium* sp. 278.

As far as it's known N_2-fixation is the main function of bean-rhizobial symbiosis. It was important to find out the influence of *Gliocladium* sp. 278 on lupine N_2-fixing activity. According to our research the fungus not only negatively affected the plants growth and development but in addition it significantly reduced the symbiotic system activity. Thus, in vegetation experiment the nodule N_2-fixing activity decreased 1.4-2.2 times compared to control under the influence of *Gliocladium* sp. 278 (Fig. 2). Lower activity of N_2-fixing complex had been mentioned both in the variant with introduction of *Gliocladium* sp. 278 within spontaneous nodules formation and in the variant using *B. lupins* B-128 within the mentioned fungus. The fungus influence was less at the vegetation begginning. Obviously the fungus growth through the vegetation period led to gradual life products accumulation in soil. That's why the influence of *Gliocladium* sp. 278 was more significant in flowering phase.

Table 1. Table Generic composition of white lupine fungi on sod-podzolic soil crops

Samples	CFU per 1 g d.w. of soil (in thousands of colonies)	% of the total number of fungi											
		Acremonium	*Alternaria*	*Bipolaris*	*Cladosporium*	*Fusarium*	*Gliocladium*	*Mortierella*	*Mucor*	*Penicillium*	*Trichoderma*	*Dematiaceae*	*Inuui zpuõu*
Soil between rows	84.4±7.8	3.6	6.5	3.9	1.6	10.4	10.4	8.8	1.6	40.7	1.3	2.0	9.2
Rhizosphere soil	383.1±33.7	12.2	0	0.3	0	64.6	0.4	1.4	3.6	9.7	1.2	0	6.6
Washed roots	28.6±2.5	2.4	0	0	0	30.8	49.0	0	1.6	5.4	0.8	0	10.0

Figure 1. White lupine plants (pots experiment): without of microorganisms - control (1), introduction into the soil *Gliocladium* sp. 278 (2).

Table 2. Influence of microorganisms on white lupine plants (pots experiment)

Variant	Height		Dry mass			
			sprout		roots	
	sm	± to control, %	g per plant	± to control, %	g per plant	± to control, %
Without microorganism (control)	53.5	-	1.64	-	0.39	-
Inoculation of seeds of *B. lupini* B-128	55.5	+3.7	1.91	+16.5	0.44	+12.8
Inoculation to the soil of *Gliocladium* sp. 278	46.9	-12.2	1.30	-20.7	0.29	-25.6
Inoculation to the soil of *Gliocladium* sp. 278 + inoculation of seeds of *B. lupini* B-128	50.2	-9.5	1.56	-18.3	0.34	-22.7
LSD ($P \leq 0.05$)	2.4		0.10		0.03	

To find the reason for lower activity of lupine – *Bradyrhizobium lupins* symbiotic system it was necessary to investigate an ultrastructure of white lupine (Shchedryi var.) nodules in flowering phase. Electron microscopic studies revealed the large number of bacteroids in bacteroid zone of rhizobia-infected cells in flowering phase (Fig. 3a). Bacteroids were rod-shaped, long, thin, not branched, fill most of the plant cell except the central zone of the nucleus (Fig. 3a, 3b). They surround the nucleus of infected cells compressing its membrane on all sides. As a result, the cell nucleus changes its forms. In the most cells of bacteroid zone the nucleus was absent.

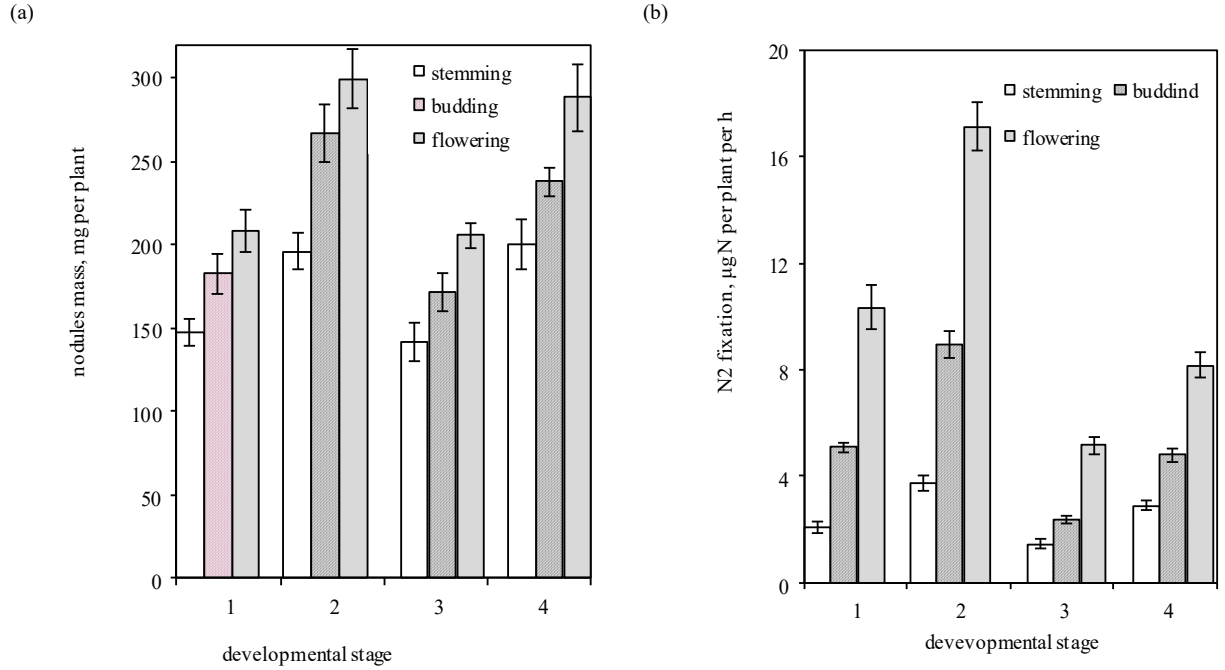

Figure 2. Influence of microorganisms on the accumulation of nodules mass (a) and N_2-fixation (b) of white lupine (pots experiment): 1) without microorganism (control); 2) inoculation of seeds of *B. lupini* B-128; 3) inoculation to the soil of *Gliocladium* sp. 278; 4) inoculation to the soil of *Gliocladium* sp. 278 + inoculation of seeds of *B. lupini* B-128

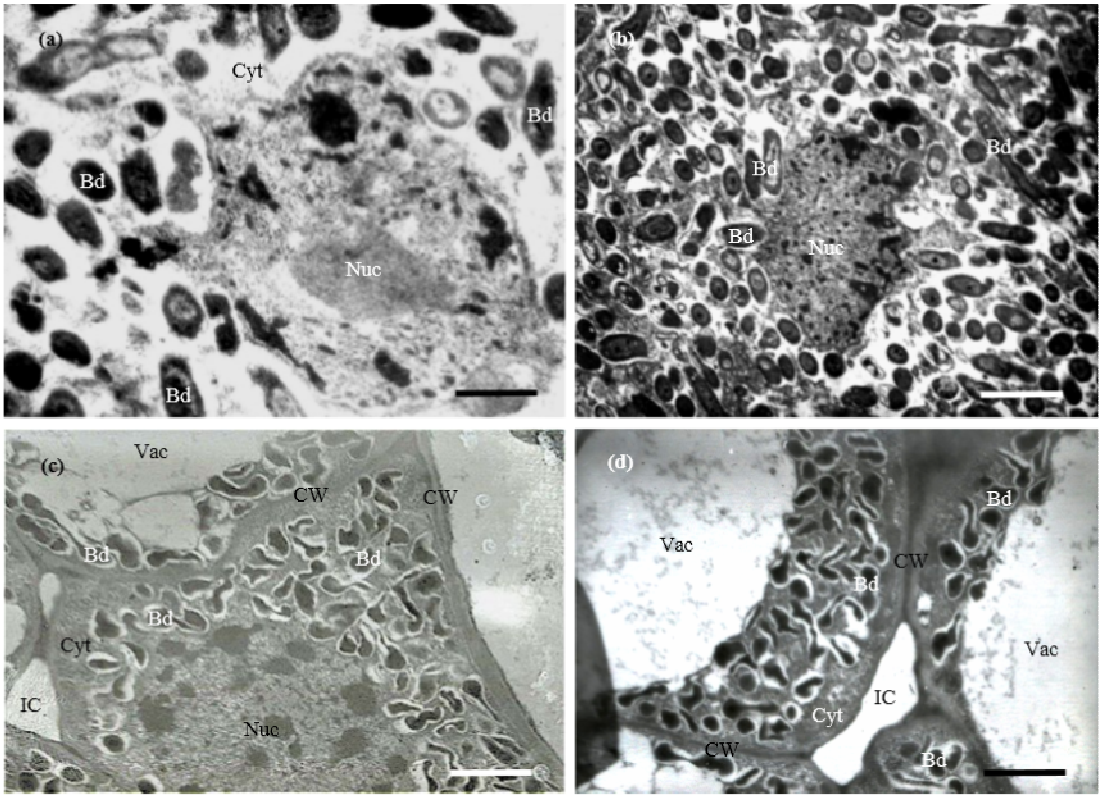

Figure 3. Bacteria-infected tissue of lupine root nodules cultured without *Gliocladium* sp. 278. Bar – 1.5 µm (a) and Bar – 2.1 µm (b). Bacteria-infected tissue of lupine root nodules cultured with *Gliocladium* sp. 278. Bar 1.9 µm (c-d). CW, cell wall; Bd, bacteroid; IC, intercellular space; Nuc, nucleos; Cyt, cytoplasm; Vac, vacuole.

The ultrastructure of nodule cells grown using *Gliocladium* sp. 278 was differed from earlier discribed (Fig. 3c, 3d). Only the individual cells in the center of bacteroid tissue had the nucleus inside and the cell were filled with bacteroids, but the number of the cells was small. Distorted form of bacteroids were noted for all the obtained sections. The some cells of bacteroid zone didn't have the nucleus and the vacuoles were observed merged into one central one. In this case bacteroids were pushed to the cell periphery near cell wall and the central vacuole occupied most of the cell part (Fig. 3d). The formation of vacuoles indicates the cessation of cell division and the beginning of early aging of infected cells.

DISCUSSION

Phytotoxins are secondary metabolites of bacteria and fungi. They are able to have a toxic effect on plants inhibiting germination, seed germination energy and the mass of shoots and roots [34]. Phytotoxins belong to different classes of organic compounds and they may induce the disease development [5]. They are even considered as potential herbicides for weeds control [1].

The most phytotoxins are produced as usual by five fungal genera: *Aspergillus*, *Penicillium*, *Cladosporium*, *Alternaria* and *Fusarium* [7, 17]. Generally the questions of microorganism toxins influence on plant growth and development including Fabaceae plants have been studied widespread for nowadays. Thus it had been shown that m-methoxyphenylacetic acid (m-OMePAA), being a derivative of m-hydroxyphenylacetic acid (m-OHPAA) with the same chemical composition as phytotoxic substance, prosuced by soybean phytopathogen *Rhizoctonia solani* reduced plants' growth and symbiotic apparatus, manifested a strong tetragenic effect leading to root hypertrophy [18]. It is known an antimicrobial activity of *Gliocladium* metabolites. Gliotoxin isolated from *G. virens* limited the growth of phytopathogenic fungi *Pythium* and *Rhizoctonia* [12]. Another investigation revealed *G. virens* 39 antibiotic action concerning to gram-negative bacterium *Agrobacterium tumefaciens* 8464 (inhibition zones diameter reached up to 18.0 mm). But the same time it didn't reveal any phytotoxic effect to the test object *Chlorella vulgaris* 190 [24]. Our investigations of *Gliocladium* sp. 278 antagonistic activity to nodule bacteria *B. lupins* B-128 didn't show up antimicrobial fungus action on the studied nodule bacteria.

Nodule is a specific root organ responsible for N_2 fixation with rhizobia in legume-rhizobial symbiosis. Like the rest of other organs they have limited life period and over time they are getting old. Aging is characterized by less N_2-fixing level and coordinated death both bacteria and a plant cell. This phenomenon can be initialized prematurely due to adverse conditions, including toxins [9].

Among the unfavorable conditions can be noted water stress factor and exposure to certain metals and fungicides. Thus, Ramos et al. exploring the effects of water stress observed severe damage to infected tissue with vacuolation of host cells and loss of peribacterioid membrane [22]. The host cell degradation and bacteroid aging with their ejection into the intercellular spaces occurred. In addition they revealed both nitrogenase activity and nodules mass reduction caused by influence of water stress factor.

Regarding the metals influence, Baig et al. revealed raw mass nodules reduction by 17 and 47% and dry nodules mass by 6 and 30% growing to Pb and Hg soybean plants treatment [2]. Hg soybean treatment led to vascular bundles and bacteroids damage. Shahid & Khan investigating the toxic effects of the fungicide hexaconazole showed nodule bacteria *Bradyrhizobium japonicum* cells damages with it [26]. Treatment of plants with *B. japonicum* reduced fungicide toxic effects and increased the overall plants productivity. This can be explained by persistent root surface and nodule tissues colonization with rhizobia, growth regulators synthesis and reducing the level of antioxidant system enzymes.

As for the toxic substances synthesized by microorganisms, Orellana & Mandava revealed the influence of *R. solani* phytotoxic substance led to cytopathological and histopathological disorders of the nodule central tissue, nucleoli extrusion and abnormal nuclei [18]. It is known that fungi are able for a strong phytotoxic effect on plant protoplasts, even to complete cell death [19, 20]. However there are no available literary data regarding to *Gliocladium* fungi toxins influence on symbiotic apparatus of legumes particularly white lupine.

In our investigations with *Gliocladium* sp. 278 introduced into soil it was observed N_2-fixing activity reduction without nodule mass changing. Also early aging of nodule bacteroid tissue cells and changing the shape of bacteroids in the cell were observed. According to the literary data it is known that similar effects can be caused by stress factors. Stress factors may lead to bacteroids degradation but it is not irreversible. A regeneration point may appear in its nuclear field. The alternation of suppression and regeneration may be repeated several times within a single bacteroid giving it distorted form. Soil toxicity caused by *Gliocladium* sp. 278 metabolites can be considered as a stress factor in our experiments.

Thus introduced into the soil *Gliocladium* sp. 278 is able to inhibit height and dry mass of sprout and roots of white lupine. *Gliocladium* sp. 278 inhibitand activity of white lupine/nodule bacteria symbiotic system and reduced N_2-fixing activity 1.4-2.2 times compared to control in different phases of plant development. Inoculation of lupine seeds by nodule bacteria *B. lupins* B-128 of mitigated the negative effect of *Gliocladium* sp. 278 on growth of lupine plants. Lower activity of N_2-fixing complex had been mentioned in the variant with introduction of *Gliocladium* sp. 278, but

inoculatuon of seeds *B. lupins* B-128 on background *Gliocladium* sp. 278 contributed to increasing the mass of nodules and nitrogen fixation at various stages of plant development. We did not see the significant reduction of nodule mass during the introduction of *Gliocladium* sp. 278. It was observed the early aging of bacteroid tissue cells and the changes of bacteroids form. As a result all of it led to less N_2-fixation activity.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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