EFFECT OF Nmin CONTENT ON NODULATION IN YELLOW LUPIN (*Lupinus luteus* L.) IN THE PRESENCE OF *Bradyrhizobium lupini* AND GENISTEIN

Study conducted as part of the research project N N310 148135: *Mineral nitrogen vs. molecular nitrogen in the cultivation of legumes*

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ABSTRACT

A two-factor pot experiment was carried out in the years 2009-2011 under a permanent plastic-covered tunnel greenhouse at the Mochełek Experimental Station of the University of Technology and Life Sciences in Bydgoszcz (POLAND). The subject of research was the traditional cultivar of yellow lupin, Mister. The design of the experiment included 5 levels of Nmin (mineral nitrogen content) content in the plant growth medium: from 4.45 mg Nmin·kg^{-1}·d.w.– very low content to 22.5 mg Nmin·kg^{-1}d.w. – very high content. Seeds of yellow lupin were inoculated with agar inoculum containing *Bradyrhizobium lupini* with or without the addition of genistein. Along with the plant growth, gradual decrease in the mean content of Nmin was observed, especially in the nitrogenous form until the stage of full bloom of lupin plants. No significant effect of the Nmin content was found on the initiation of the nodulation process, number and dry weight of nodules, independently of seed inoculation with *Bradyrhizobium lupini* and application of genistein. Average or higher content of Nmin in the medium was the reason of the lack of survival in symbiotic bacteria *Bradyrhizobium lupini* capable of nodulation in the medium after lupin harvest. Only in the case of low and very low Nmin content in the medium, the addition of genistein to bacterial inoculum did affect the increase in the survival of symbiotic bacteria after lupin harvest.

Key words – Nmin, yellow lupin, nodulation, *Bradyrhizobium lupini*, genistein

STRESZCZENIE

Ścisłe dwuczynnikowe doświadczenie wazonowe wykonano w latach 2009-2011 pod stałym tunelem foliowym w Stacji Badawczej Wydziału Rolnictwa i Biotechnologii UTP w Mochełku. Przedmiotem badań była tradycyjna odmiana łubinu żółtego Mister. Schemat doświadczenia obejmował 5 poziomów zawartości Nmin w podłożu wzrostu roślin: od 4,45 mg Nmin·kg^{-1}·sm – zawartość bardzo niska do 22,5 mg Nmin·kg^{-1}sm – zawartość bardzo wysoka. Nasiona łubinu żółtego odmiany Mister szczepiono agarową szczepionką zawierającą *Bradyrhizobium lupini* z dodatkiem lub bez genisteiny. Wraz z postępującym rozwojem roślin podczas okresu wegetacji obserwowano stopniowe zmniejszanie się średniej zawartości Nmin, szczególnie formy azotanowej do fazy pełni kwitnienia roślin łubinu. Nie stwierdzono istotnego wpływu zawartości Nmin na inicjację procesu nodulacji, liczbę i suchą masę brodawek, niezależnie także od szczepienia nasion *Bradyrhizobium lupini* i zastosowania genisteiny. Średnia lub wyższa zawartość Nmin w podłożu była przyczyną braku przeżywalności zdolnych do brodawkowania bakterii symbiotycznych *Bradyrhizobium lupini* obecnych w podłożu po zbiorze łubinu. Tylko w przypadku niskiej i bardzo niskiej zawartości Nmin w podłożu dodatek genisteiny do szczepionki bakteryjnej wpływał na zwiększenie przeżywalności bakterii symbiotycznych po zbiorze łubinu.

Słowa kluczowe – Nmin, łubin żółty, nodulacja, *Bradyrhizobium lupini,* genisteina

DETAILED ABSTRACT

Ścisłe dwuczynnikowe doświadczenie wazonowe zakładano 5., 1. i 5. kwietnia w latach 2009-2011 pod stałym tunelem foliowym w Stacji Badawczej Wydziału Rolnictwa i Biotechnologii UTP w Mochełku. Przedmiotem badań była tradycyjna odmiana łubinu żółtego Mister. Schemat doświadczenia obejmował następujące czynniki: A – zawartość Nmin w mg$\cdot$kg^{-1} suchej masy mieszaniny gleby płowej (37,5%) i piasku rzecznego (62,5%) oznaczona wg PN (Polska Norma 1997): a1 – 4,45 mg Nmin$\cdot$kg$\cdot$sm^{-1} – bardzo niska, a2 – 8,90 mg Nmin$\cdot$kg^{-1}sm – niska, a3 – 13,3 mg Nmin$\cdot$kg^{-1} sm – średnia, a4 – 17,8 mg Nmin$\cdot$kg^{-1}sm – wysoka, a5 – 22,5 mg Nmin$\cdot$kg^{-1}sm – bardzo wysoka I czynnik B – sposób przygotowania nasion do siewu: b1- kontrola – nasiona zaprawiane, nie szczepione, b2 - nasiona zaprawiane i szczepione agarową szczepionką zawierającą *Bradyrhizobium lupini i* b3 - nasiona zaprawiane i szczepione agarową szczepionką zawierającą *Bradyrhizobium lupini* z dodatkiem genisteiny (4',5,7-trihydroksyizoflawon). Każdą kombinację zakładano w 8 powtórzeniach (120 wazonów Kocha o średnicy 22,5 cm i wysokości 25 cm z pełnym dnem). Zawartość Nmin w kombinacjach a2 – a5 uzyskano poprzez dodanie stosowej ilości 34% saletry amonowej zawierającej po 50% $N\text{-}NO_3$ i $N\text{-}NH_4$. Do każdego wazonu wysiewano po 9 nasion, a po wschodach do dalszych analiz pozostawiano po 5 roślin. Po 7 (c1), 14 (c2), 21 (c3), 28 (c4) i 35 dniach (c5) od początku wschodów, a następnie w fazie pełni kwitnienia (c6) oraz w dojrzałości fizjologicznej (c7) i pełnej nasion (c8) likwidowano kolejno po 15 wazonów. Liczbę brodawek i ich suchą masę (po wysuszeniu w temperaturze 60^{o}C przez 24 godziny) oznaczono w fazie pełni kwitnienia i w dojrzałości fizjologicznej nasion. Po zbiorze nasion próby podłoża poddawano ocenie zawartości Nmin metodą spektrofotometrycznego pomiaru stężenia jonów azotanowych i amonowych w wyciągu glebowym 1% (*m*/*m*) roztworu siarczanu(VI) potasu. Występowanie oraz liczebność bakterii symbiotycznych w podłożu po zbiorze każdego wazonu określono metodą biotestu NPL (Najbardziej Prawdopodobnej Liczebności) po zbiorze nasion. Wraz z wegetacją roślin łubinu żółtego obserwowano stopniowe zmniejszanie się średniej zawartości obu form Nmin w podłożu do fazy pełni kwitnienia, po czym następował stopniowy i umiarkowany wzrost ich zawartości do końca okresu wegetacji. Pierwsze brodawki zaobserwowano 14 dnia po wschodach na wszystkich obiektach czynnika A, kiedy średnia zawartość Nmin w podłożu wynosiła aż 17,1 mg$\cdot$kg^{-1}, tj. 76,9 kg N$\cdot$ha^{-1}, niezależnie także od szczepienia nasion *Bradyrhizobium lupini* i zastosowania genisteiny. Średnio w latach badań liczba i sucha masa brodawek nie zależały istotnie od zawartości Nmin, a szczepienie nasion rizobiami z dodatkiem genisteiny nie zwiększało istotnie liczby i suchej masy brodawek. W fazie dojrzałości fizjologicznej średnia liczba brodawek na jednej roślinie łubinu żółtego była prawie 3-krotnie wyższa, a ich sucha masa nieco ponad 1-krotnie niż w fazie kwitnienia. Średnia lub wyższa zawartość Nmin w podłożu była przyczyną braku przeżywalności zdolnych do brodawkowania bakterii symbiotycznych *Bradyrhizobium lupini* obecnych w podłożu po zbiorze łubinu. W przypadku niskiej i bardzo niskiej zawartości Nmin w podłożu dodatek genisteiny do szczepionki bakteryjnej wpływał na zwiększenie przeżywalności bakterii symbiotycznych po zbiorze łubinu.

INTRODUCTION

Negative effect of the high content of Nmin in the soil on the activity of rhizobia in *Fabaceae* has been known for a long time (Luciński et al. 2002), while part of them with high nitrate reductase activity may show a higher tolerance to Nmin content in the medium (Serrano and Chamber 1990). Infection of roots with rhizobia begins when the Nmin content in the soil decreases as a result of the uptake of N by the plants or its loss as a result of oxygenation or leaching (Buttery and Gibson 1990, Da Silva et al. 1993). Delay in the formation of root nodules leads to a significant decrease in the capability and effectiveness in N_2 fixation (Kipe-Nolt and Giller 1993). In the field experiments of Voisin et al. (2002), N_2 fixation, was possible only after decrease in the Nmin content below 56 kg $N \cdot ha^{-1}$.

However, Serrano and Chamber (1990) observed that in *Bradyrhizobium,* there may occur strains of bacteria capable of nodulation and effective N_2 fixation, even with its high content (12 mM) in the medium. According to Marek-Kozaczuk et al. (2006), the total nitrogen content in the soil does not affect the numbers of rhizobia, and the size of nodulation does not depend on the level of N in the soil nor on the population of autochthonic rhizobia specific for a given species. Tsai et al. (1993) state that in common bean, the low content of Nmin, synergistically affects the N_2 fixation through stimulation of nodule formation, however even with the high content, the stimulating effect of N on the assimilation of N_2 was constantly visible. In the research of Kocoń (1999) on faba bean, only a 90-fold increase in the amount of N applied to the plant growth medium (from 90 to 900 mg) did affect the significant decrease in the dry weight of root nodules at the stage of budding and at the beginning of flowering; however, at the stage of pod setting, these differences were not observed.

One of the methods of increasing infections, as well as the number and dry weight of root nodules, in lupin is application of isoflavonoids (e.g. genistein and hydroxygenistein) (D'Agostina et al. 2008). Genistein is a multi-functional isoflavonoid naturally secreted from roots of hydroponically grown legume plants (Kneer et al. 1999). Recognition between partners of symbiosis requires exchange of these specific molecular signals (isoflavonoids) as well as the bacterial factor, *Nod* (Spaink 2000). According to Subramanian et al. (2007), the effect of isoflavonoids on nodulation in legume plants consists in the stimulation of common *nod* gene expression. Isoflavonoids significantly shorten the time up to the nodule formation and active assimilation of N_2, and also increase the total content of N in the plant, especially in the soil with low Nmin content (Pan and Smith 2000).

Hypothesis of the author's own research assumes that high content of mineral nitrogen in the soil may negatively affect the size of nodulation in yellow lupin. The use of rhizobia and/or genistein should enable explanation of correlations between the Nmin content and the N_2 fixation activity and survival of *Bradyrhizobium lupini*.

MATERIAL AND METHODS

The two-factor pot experiment was set up on the 5., 1. and 5. April in the years 2009-2011 under a permanent plastic-covered tunnel greenhouse at the Experimental Station of the Faculty of Agriculture and Biotechnology of UTLS in Mochełek. The subject of research was the traditional cultivar of yellow lupin, Mister. The experiment design included the following factors:
A – Nmin content in $mg \cdot kg^{-1}$ of the dry weight of the mixture of lessive soil (37.5%) and river sand (62.5%), determined according to Polish Standards (Polska Norma 1997):

- a1 – 4.45 mg $Nmin \cdot kg^{-1}$ dw – very low,
- a2 – 8.90 mg $Nmin \cdot kg^{-1}$ dw – low,
- a3 – 13.3 mg $Nmin \cdot kg^{-1}$ dw – average,
- a4 – 17.8 mg $Nmin \cdot kg^{-1}$ dw – high,
- a5 – 22.5 mg $Nmin \cdot kg^{-1}$ dw – very high.

B – method of preparing seeds for sowing:

- b1 – control – dressed seeds, not inoculated,
- b2 – seeds dressed and inoculated with agar inoculum containing *Bradyrhizobium lupini,*
- b3 – seeds dressed and inoculated with agar inoculum containing *Bradyrhizobium lupini* with the addition of genistein (SIGMA Aldrich)

For seed dressing, Sarfun 50 WP (a.s. carbendazim) was used at a rate of 200 $g \cdot 100\ kg^{-1}$ of seeds. In combination b2, the agar suspension of *Bradyrhizobium lupini* bacteria (10^8 – 10^{10} bacteria cells in 1 ml) prepared each time by the Department of Agricultural Microbiology of IUNG Puławy, was solved in distilled water, and next, for 24 hours it was mixed in a vortex mixer at a room temperature. In combination b3, to inoculum prepared in this way, 60 µM genistein (4',5,7-trihydroxyisoflavone) was added which was earlier dissolved in 1 ml of methanol and mixed again for 24 hours. The seeds were sprayed with the suspension of *Bradyrhizobium lupini* (b2) or *Bradyrhizobium lupini* with genistein (b3), and after they dried up they were sown on the same day.

Each combination was set up in 8 replications (120 Koch pots of a diameter of 22.5 cm and height 25 cm with full bottom), which were dug into the soil in order to protect them against excessive drying, and placed in a plastic-covered tunnel greenhouse. The pots were filled with 9 kg of mixture of lessive soil of granulometric composition of light loamy sand and pH 6.1 – 6.4, sampled from the arable field at the Experimental Station Mochełek, of a mean content of P, K and Mg and river sand. Lessive soil after screening on a sieve with 1 cm holes, was subjected first to rinsing under running water, and next to thermal disinfection at a temperature of 70^oC for 24 hours. With such a proportion of lessive soil and river sand, the Nmin content was 4.45 $mg \cdot kg^{-1}$ dw. Other properties of the plant growth medium are shown in Table 1.

The Nmin content in combinations a2 – a5 was obtained through addition of a proper amount of 34% ammonium sulphate, each containing 50% of $N\text{-}NO_3$ and $N\text{-}NH_4$. The medium was also fortified with P and K, leading to their high content. 9 seeds were sown to each pot, and after their emergence, 5 plants in each one were left for further analysis. Constant moisture of the medium was maintained in the pots on the level of 60% field water capacity of soil. The initial content of N forms in the medium was measured in c0, i.e. on the day of medium preparation. After 7 (c1), 14 (c2), 21 (c3), 28 (c4) and 35 days (c5) since the beginning of emergence, and then at the stage of full bloom (c6), as well as at the stage of physiological maturity (c7) and full maturity of seeds (c8), 15 pots were eliminated by turns. The number of nodules and their dry weight (after drying at a temperature of 60^oC for 24 hours) was determined at the stage of full bloom and physiological maturity of seeds.

Table 1. Physical and chemical properties of the plant growth medium of yellow lupin (mixture of lessive soil and river sand in the proportion of 37.5% : 62.5%)
Tabela 1. Fizyczne i chemiczne właściwości podłoża wzrostu roślin łubinu żółtego (mieszanina gleby płowej i piasku rzecznego w proporcji 37.5% : 62.5%)

pH in KCl pH w KCl	Content % of d.w. Zawartość % psm		Content of granulometric fractions of a diameter, mm Zawartość frakcji granulometrycznych o średnicy, mm %			
	Corg.	Norg.		Silt – pył		
6.70	0.321	0.031	Sand piasek	coarse gruby 0.05-0.02	fine drobny 0.02-0.002	Clay ił < 0.002
			95.6	1.50	2.76	0.14

After harvesting the seeds (elimination of successive pots), 100 g samples were collected of a well-mixed medium from each pot, and they were frozen at a temperature of -18^oC. Each year in November, the samples were subjected to evaluation of the Nmin content according to the Polish Standard (Polska Norma 1997) at the Chemical and Agricultural Station in Bydgoszcz with the method of spectrophotometric measurement of concentration of nitrate and ammonium ions in the soil extract of 1% (*m*/*m*) potassium sulphate(VI) solution.

Occurrence and numbers of symbiotic bacteria in the medium after harvest from each pot were determined with the biotest method of MPN (the most probable number) after harvesting the seeds. In order to do this, seedlings of serradella (the test plant) were planted in two replications into plastic bags filled with sterile river sand, moisturized with Jensen medium. After 3 days since planting, the seedlings were inoculated with a water solution of tested media sampled from the pots, using the multiple dilution technique (from 10^{-1} to 10^{-6}). After four weeks, on the roots of tested plants, the number of nodules was evaluated, as well as the number of symbiotic bacteria *Bradyrhizobium lupini*, based on the total of plants from all dilutions, on roots on which there occurred nodules (Martyniuk et al. 2000).

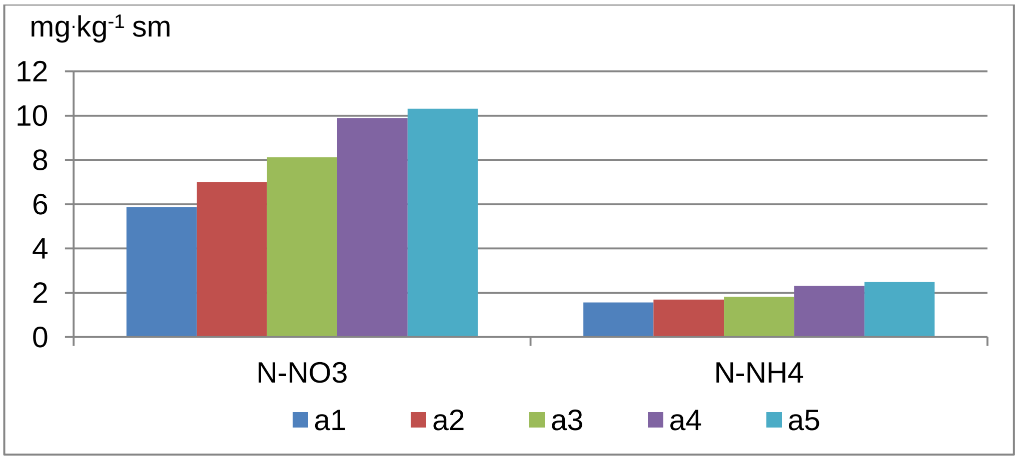
Obtained results were subjected to analysis of variance for the completely randomized design, and the significance of differences was verified with Tukey's test on the level of $\alpha = 0.05$. Means in charts denoted with the same letters did not differ significantly.

RESULTS AND DISCUSSION

In the mixture of lessive soil and river sand, which constituted the plant growth medium for yellow lupin for further increase in Nmin content, according to the experiment design, 4.45 mg $Nmin \cdot kg^{-1}$ was observed including 0.56 $mg \cdot kg^{-1}$ $N\text{-}NO_3$ and 3.89 mg $N\text{-}NH_4$ $mg \cdot kg^{-1}$ (12.5:87.5%). In lessive soil this ratio is generally close to 1:1, however most probably as a result of rinsing it before mixing with river sand, the proportion of nitrate nitrogen greatly decreased (Spychaj-Fabisiak 2001), and its thermal disinfection caused increase in the content of the ammonium form (Fotyma 2000).

According to the experiment design, fortification of the medium with Nmin through addition of a proper amount of ammonium sulphate, affected the increase in the mean content of both forms in the growing season (Fig. 1a), while the average proportion of $N\text{-}NO_3$ was approximately 80% (Fig. 1b).

a)



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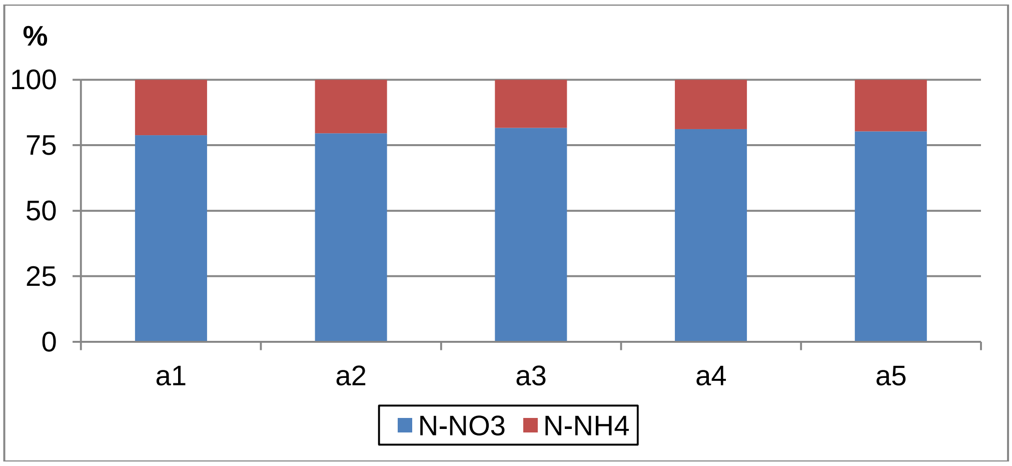
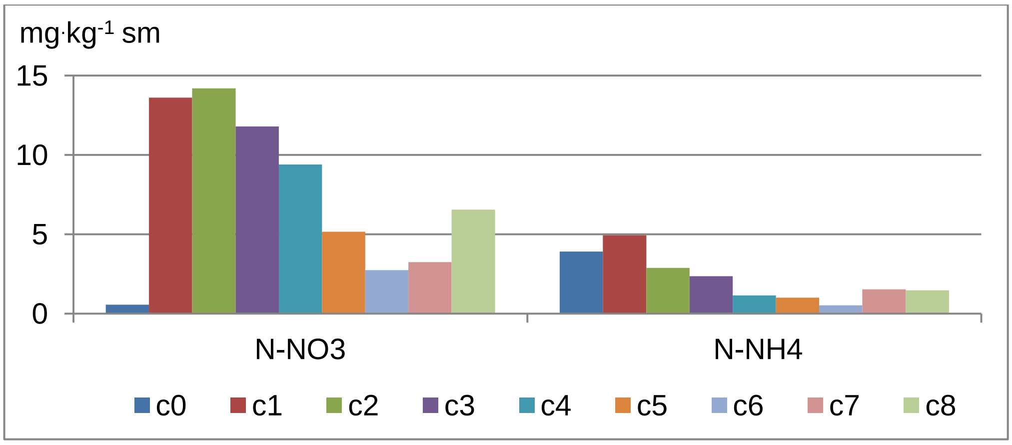


Fig. 1. Mean content (a) and proportion (b) of N-NO_3 and N-NH_4 forms in the plant growth medium of yellow lupin depending on the level of Nmin content (a1 – a5)
Rys. 1. Średnia zawartość (a) i udział (b) form N-NO_3 i N-NH_4 w podłożu wzrostu roślin łubinu żółtego w zależności od poziomu zawartości Nmin (a1 – a5)

The content of Nmin and its forms in the growth medium of lupin plants was analyzed 8 times. Even during the first analysis carried out at the stage of emergence, i.e. approximately 30-35 days since preparing the medium, in all years of research the Nmin content was higher than the assumed one (Fig. 2a), perhaps because of the increased soil temperature under the plastic-covered tunnel greenhouse and occurring nitrogen metabolism. The lowest content of Nmin and its both forms in the medium was observed at the stage of flowering (c6), after which its content started increasing, which is commonly known to be the result of supplying the medium with part of N assimilated by lupin plants. At the same time, it is worth noting that at this stage, a several-fold decrease in the content of N-NO_3 (to 27.9%) was observed again, as well as the increase in the proportion of N-NH_4 (to 72.1%) (Fig. 2b).

a)



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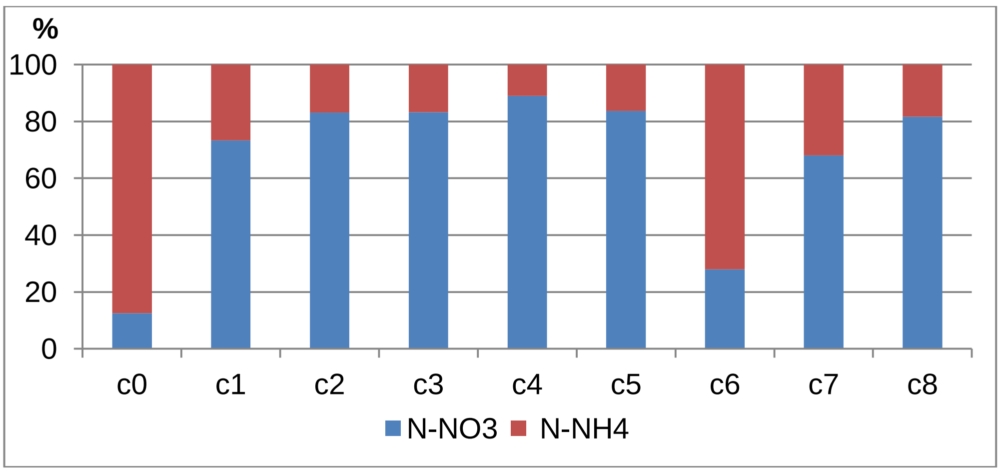


Fig. 2. Dynamics of the content of N-NO_3 and N-NH_4 forms in the plant growth medium of yellow lupin in the growing season (c0 – on the day of growth medium preparation, c1 – 7 days, c2 – 14 days, c3 – 21 days, c4 -28 days, c5 - 35 days since the start of emergence, c6 – full bloom, c7 – physiological seed maturity, c8 – full maturity of seeds**)**

Rys. 2. Dynamika zawartości form N-NO_3 i N-NH_4 w podłożu wzrostu roślin łubinu żółtego w okresie wegetacji (c0 – w dniu przygotowania podłoża, c1 – 7 dni, c2 – 14 dni, c3 – 21 dni, c4 – 28 dni, c5 – 35 dni od początku wschodów, c6 – pełnia kwitnienia, c7 – dojrzałość fizjologiczna nasion, c8 – dojrzałość pełna nasion**)**

Nitrogen in the form of nitrate does not undergo sorption in the soil (Fotyma 2000), hence the necessity to maintain constant moisture in pots through watering them, which was the cause of the decrease in its content. However, beginning with physiological seed maturity (c7), the proportion of N-NO_3 started increasing at the cost of decreasing content and proportion of the ammonium form. Increase in the content of N after harvesting lupin may have resulted from two processes: distribution of root nodules and N releasing from plant roots (Fustec et al. 2009). However, despite significant differences in the uptake of both these forms, no symptoms resulting from prevalence of one of them were observed on plants. Leaf necrosis is characteristic in case of white lupin plants using only the ammonium form, while chlorosis only the nitrate form (Ciesiołka et al. 2003). In the research of Chaillou et al. (1986), bean plants absorbed nitrate form faster and in greater amounts than the ammonium form, which is also confirmed by the author's own research results on the dynamics of both forms.

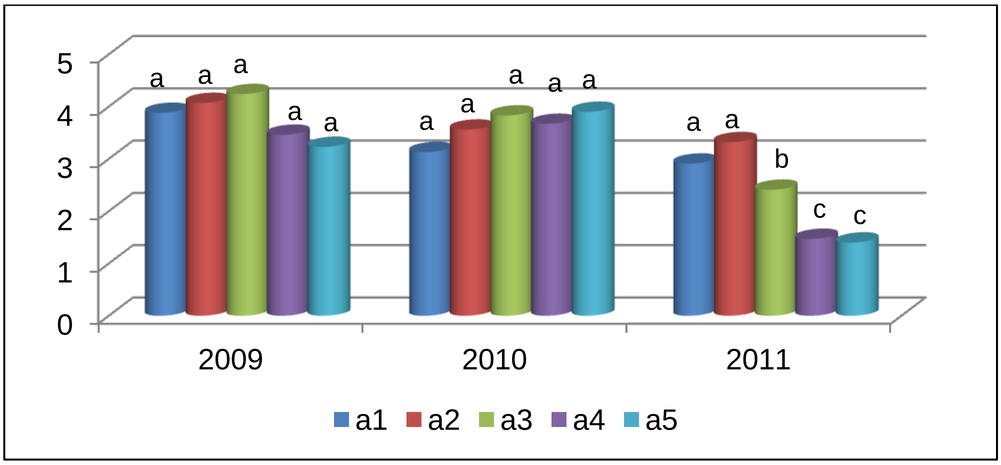
Nitrogen in the ammonium form negatively affects nodulation and symbiotic N_2 fixation, e.g. in bean, stronger than in the nitrate form (Chaillou et al. 1986), whereas according to Guo et al. (1992), any mineral form of nitrogen, especially the ammonium form, contributes to the reduction in the activity of nitrogenase, in the process of nodulation and dry weight of nodules in faba bean and white lupin.

Kipe-Nolt and Giller (1993) state that difficulties in initiating the infection of root hairs with rhizobia at the high Nmin content in the medium, cause delay in the formation of nodules in bean plants and their lower activity in N_2 fixation. The author's own research, does not confirm the effect of diversified and high Nmin content on the delay in the formation of root nodules in yellow lupin. First nodules were observed already on the 14th day after emergence (c2) in a form of a root thickening visible to the naked eye on all objects with factor A, when the mean content of Nmin in the medium was up to 17.1 $mg \cdot kg^{-1}$ (14.2 $mg \cdot kg^{-1}$ N-NO_3 and 2.88 N-NH_4 $mg \cdot kg^{-1}$), i.e. up to 76.9 kg $N \cdot ha^{-1}$. According to Merbach et al. (2008), the effect of inoculation of blue lupin seeds with rhizobia on the N_2 fixation without Nmin in the medium is significant, however when blue lupin growth occurs in the medium containing N, the effect remains insignificant. On the other hand, Voisin et al. (2002) states that after exceeding the level of 56 kg $Nmin \cdot ha^{-1}$ in the plough layer of soil, nodulation in field pea does not occur. Thus, it is possibile that *Rhizobium leguminosarum* does not show tolerance to high Nmin content in the medium, hence lack of initiation of nodulation with such a high Nmin content in the soil. High activity of nitrate reductase in *Bradyrhizobium lupinii* allows for the formation of root nodules with a significant Nmin content in the medium (Serrano and Chamber 1990). Nodulation in legume plants, independently of the Nmin content in the medium, is also confirmed by the results of the studies of Marek-Kozaczuk et al. (2006) under natural habitat conditions, as well as of Merbach et al. (2008) on blue lupin.

In the successive years of research, the average number of nodules per yellow lupin plant at the stage of flowering was 3.76, 3.55 and 2.30, respectively (Fig. 3a). Only in

2011, increase in the Nmin content to high (a4) and very high one (a5) was the cause of its significant decrease. However, in the studied three-year period, the average number of nodules per plant at the stage of flowering did not depend significantly on the content of Nmin in the plant growth medium. However, at physiological seed maturity the number of nodules per plant significantly increased – in 2009 to 9.29, and in 2010 to 11.9, while in 2011 because of the occurrence of strong symptoms of mosaic disease, at this stage there were almost no nodules at all (Fig. 3b). More nodules were observed on plants in pots with lower Nmin content (in 2009 with the lowest), while the fewest with a very high one (2010). Increasing, and similar to the author’s own research, number of nodules per plant of yellow lupin in the period from the formation of shoots to physiological seed maturity, was also observed by Pytlarz-Kozicka (2010).

a)



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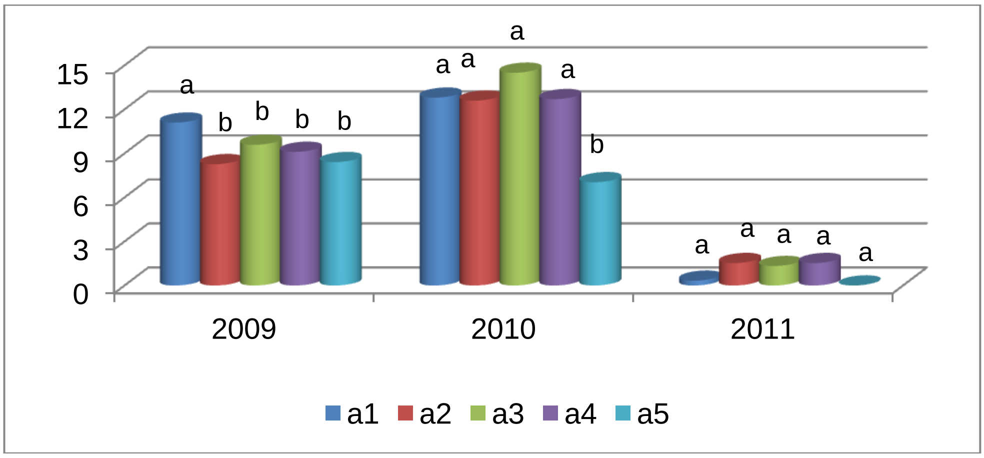
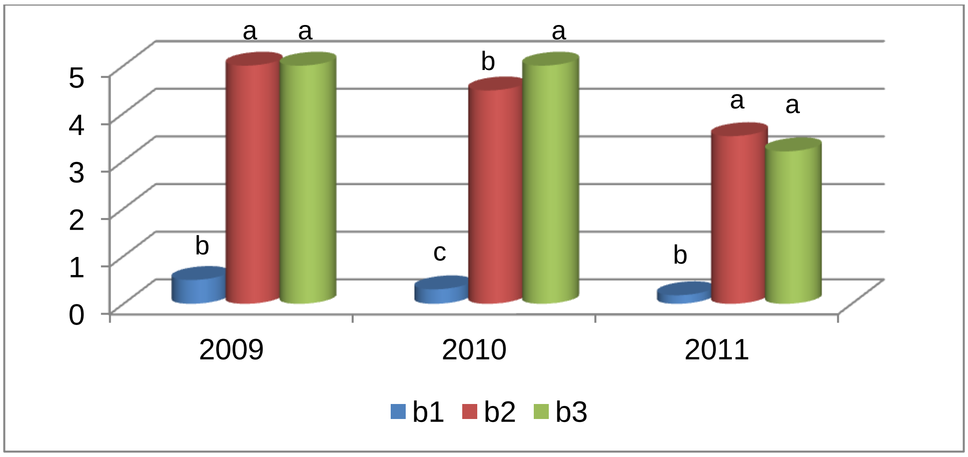


Fig. 3. Number of nodules per one yellow lupin plant at the stage of flowering (a) and physiological seed maturity (b) depending on the Nmin content in the medium (a1 – a5)
Rys. 3. Liczba brodawek na jednej roślinie łubinu żółtego w fazie kwitnienia (a) i dojrzałości fizjologicznej nasion (b) w zależności do zawartości Nmin w podłożu (a1 – a5)

Single nodules were observed in pots where the seeds were subjected only to dressing, which was most probably the result of their mechanical displacement during medium preparations (each year dressed seeds were sown first, then the ones which were inoculated with *Bardyrhizobium*, and finally the ones which were inoculated with the addition of genistein), and also perhaps as the result of the incomplete removal of living forms of *Bradyrhizobium* during thermal disinfection of the lessive soil used for preparations of the medium. The number of nodules per yellow lupin plant on plots b2 and b3, did not differ significantly either at the stage of flowering (Fig. 4a) or at the stage of physiological seed maturity (Fig. 4b), except 2010, which denotes lack of interaction between genistein and Nmin content in the formation of nodulation in yellow lupin.

a)



b)

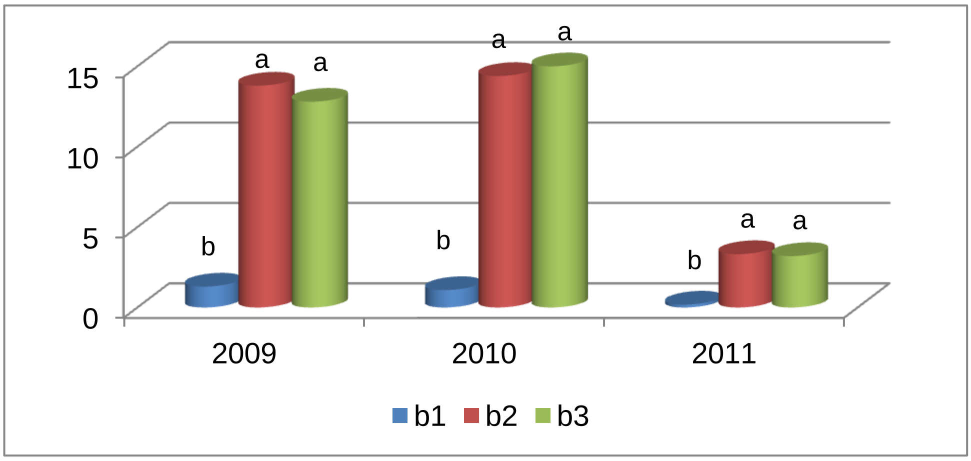
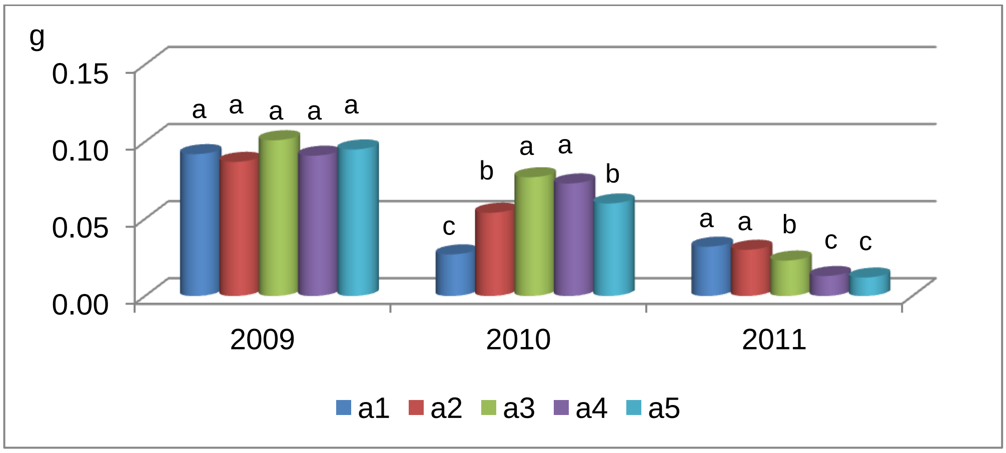


Fig. 4. Number of root nodules per one yellow lupin plant at the stage of flowering (a) and physiological seed maturity (b) depending on the method of preparing seeds for sowing (b1 - b3)

Rys. 4. Liczba brodawek korzeniowych na jednej roślinie łubinu żółtego w fazie kwitnienia (a) i dojrzałości fizjologicznej nasion (b) w zależności od sposobu przygotowania nasion do siewu (b1 - b3)

In the successive years of research, the dry weight of root nodules per lupin plant was 0.091, 0.058 and 0.022 g (Fig. 5a), while at the stage of physiological maturity 0.117, 0.064 and 0.006 g, respectively (Fig. 5b). No significant effect of Nmin content on the dry weight of nodules was observed at the stage of flowering in 2009. In 2010 the dry weight of nodules increased to the mean (a3) content of Nmin, whereas in 2011 it was the highest at this stage with very low and low N content in the soil. At the stage of physiological maturity in these years, decrease in the weight of nodules was observed from the average Nmin content, while in 2009 it was really unstable. Arrese-Igor et al. (1990) found that the Nmin content in the medium may affect the decrease in the dry weight of nodules, unless rhizobia are characterized, as in the case of *Bradyrhizobium*, by a substantial activity of nitrate reductase – in such case the N content may also increase in the aboveground parts.

a)



b)

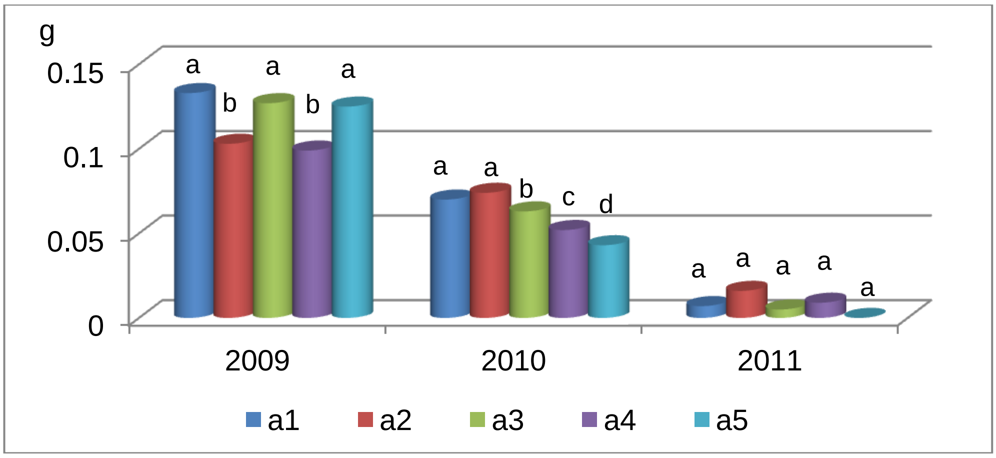
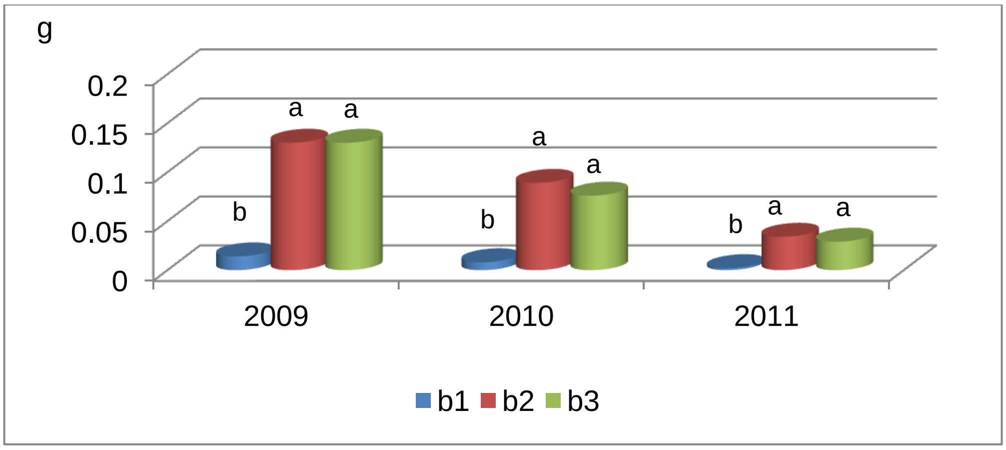


Fig. 5. Dry weight of root nodules per one yellow lupin plant at the stage of flowering (a) and physiological seed maturity (b) depending on the Nmin content in the medium (a1 - a5)
Rys. 5. Sucha masa brodawek korzeniowych na jednej roślinie łubinu żółtego w fazie kwitnienia (a) i dojrzałości fizjologicznej nasion (b) w zależności od zawartości Nmin w podłożu (a1 - a5)

Inoculation of seeds with rhizobia with the addition of genistein did not significantly increase the dry weight of nodules at the stage of flowering nor at physiological seed maturity in yellow lupin (Fig. 6a and b). Voisin et al. (2002) state that decrease in the intensity of N_2 fixation at the end of plant development results primarily from nodule senescence, and then from a slower rate of plant growth.

a)



b)

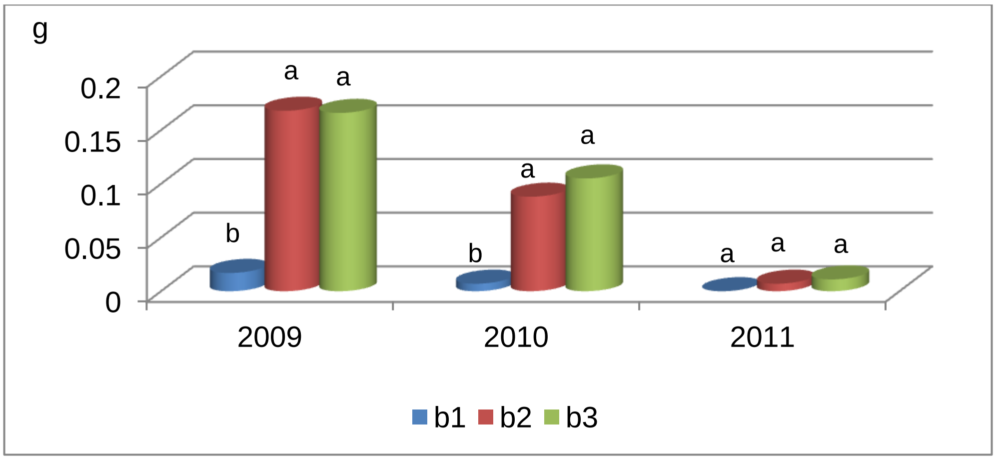


Fig. 6. Dry weight of root nodules per one yellow lupin plant at the stage of flowering (a) and physiological seed maturity (b) depending on the method of preparing seeds for sowing (b1 - b3)

Rys. 6. Sucha masa brodawek korzeniowych na jednej roślinie łubinu żółtego w fazie kwitnienia (a) i dojrzałości fizjologicznej nasion (b) w zależności od sposobu przygotowania nasion do siewu (b1 - b3)

In the research of Vargas et al. (1999), inoculation of seeds did not cause any improvement in nodulation with the content of symbiotic bacteria of 7 x 10^2 cells per 1 g of dry weight of soil, whereas when it was less than 10 cells per 1 g of dry weight of soil^{-1}, the inoculation favourably affected nodulation. Biotest of MPN (the most probable number) enables both finding the presence and estimating the numbers of rhizobia under fabaceous plants (Martyniuk et al. 2000). In the author's own research, only in the pot media containing initial very low and low Nmin content, occurrence of nodule bacteria was observed after collecting the seeds (Table 2).

Table 2. Mean number of symbiotic bacteria (in g^{-1} d.w. of medium) after harvesting yellow lupin seeds depending on the Nmin content and method of preparing seeds for sowing

Tabela 2. Średnia liczebność bakterii symbiotycznych (w g^{-1} sm podłoża) po zbiorze nasion łubinu żółtego w zależności od zawartości Nmin i sposobu przygotowania nasion do siewu

Nmin content in $mg \cdot kg^{-1}$ d.w. of plant growth medium Zawartość Nmin w $mg \cdot kg^{-1}$ sm podłoża wzrostu roślin	Method of preparing seeds for sowing Sposób przygotowania nasion do siewu		
	Control Kontrola Sarfun 50WP	Sarfun 50WP + *Bradyrhizobium lupini*	Sarfun 50WP + *Bradyrhizobium lupini* + genistein (genisteina)
Very low - Bardzo niska	2.0 x 10	2.0 x 10^2	2.0 x 10^4
Low - Niska	6.9	1.7 x 10^2	1.7 x 10^4
Average - Średnia	0	0	0
High - Wysoka	0	0	0
Very high - Bardzo wysoka	0	0	0

At the same time, it is worth noting that in both these cases rhizobia and genistein favourably affected the increase in the numbers of symbiotic bacteria remaining after harvesting lupin. Repeated tests of the medium with mean and higher Nmin content after harvesting lupin, again did not confirm occurrence of *Bradyrhizobium lupini* bacteria, capable of symbiosis. De Luca et al. (1997) state that population and activity of free-living symbiotic bacteria seems to be significantly decreased by the decreasing pH of the soil, and through high concentration of mineral N in the soil after legumes cultivation, which is also confirmed by observations of Martyniuk et al. (2000), according to whom the number of symbiotic bacteria, e.g. *Rhizobium leguminosarum*, is positively correlated with the content of organic carbon and total N, and negatively correlated with pH of the soil.

CONCLUSIONS

Along with yellow lupin growth, gradual decrease in the mean content of both Nmin forms was observed in the medium from the stage of full bloom, after which there occurred a gradual and moderate increase in their content till the end of the growing season. First nodules were observed on the 14th day after emergence on all plots with factor A, when the mean content of Nmin in the medium was up to 17.1 $mg \cdot kg^{-1}$, i.e. 76.9 kg $N \cdot ha^{-1}$), also independently of seed inoculation with *Bradyrhizobium lupini* and the use of genistein. In the years of research, on average, the number and dry weight of nodules did not depend significantly on the Nmin content, while seed inoculation with rhizobia with the addition of genistein did not significantly increase the number and dry weight of nodules. At the stage physiological maturity, the mean number of nodules per yellow lupin plant was almost 3 times higher, and their dry weight slightly over 1-fold lower than at the stage of flowering. Average or higher content of Nmin in the medium was the cause of lack of survival in symbiotic bacteria *Bradyrhizobium lupini* capable of nodulation and present in the medium after harvesting lupin. In the case of low and very low Nmin content in the medium, the addition of genistein to bacterial inoculum affected the increase in survival of symbiotic bacteria after harvesting lupin.

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