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Research Article

Effectiveness of *Bradyrhizobium japonicum* inoculation on nodulation dynamics in *Glycine max* (L.) roots

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Abstract

Bradyrhizobium japonicum bacteria on the intensity of nodulation during nine phenophases (R1-R7) in relation to the isoflavone content in soybean seeds. Nine domestic soybean varieties were sown in three replicates, with *Bradyrhizobium japonicum* inoculated and non-inoculated seeds. The different morphological characteristics of nodules, the number and mass of nodules were studied during plants' ontogenesis. The content of individual isoflavones in the sowing seed material was determined using high performance liquid chromatography mass spectrometry (HPLC-MS). The most abundant isoflavone was acetylgenistin in the variety Galeb (2741.4 μg/g), and the highest content of all isoflavones was recorded in the variety Gorštak (8117.7 μg/g). The variety Sava of the inoculated treatment exhibited the highest average values of the nodule mass (21.1 mg) and the highest number of nodules (23.3) in the phenophase, in which 10% of pods reached full length (R4). The average values of mass and number of nodules were calculated for the entire vegetation period, being higher in the inoculated treatment. There was no positive correlation between the content of isoflavones in seeds and the number and mass of nodules. The impact of quantity of isoflavone on nodulation intensity in soybeans was not significant,, which could be related to already saturated soil with nitrogen-fixing bacteria.

Keywords: *Bradyrhizobium japonicum*; isoflavones; nitrogen fixation; nodule; soybean

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Introduction

Soybean, *Glycine max* (L.) Merrill, is a legume crop of exceptional economic importance (Popović *et al*., 2019). In 2022, production areas in the world amounted to 130 million hectares, and in Serbia, it was planted on 220 thousand hectares (USDA, 2023). By sowing declared seeds and with the correct selection of varieties, stable ones are achieved yields (Kolarić *et al*., 2014; 2023). Soybean is well-known for its high nutrition value with a high protein content (Popović *et al*., 2012; 2013; 2016), ranging from 30 to 53% (Sediyama *et al*., 1999). The high protein content in the seeds and in other above-ground plant parts is related to the ability of soybean to perform symbiotic nitrogen fixation (SNF). The soybean forms nodules on its root that are the result of its symbiosis with the soil bacteria *Bradyrhizobium japonicum*. During the process of SNF in nodules, bacteria take food and energy in the form of carbohydrates from the plant (Biswas and Gresshoff, 2014), while in return the soybean plant receives the large amount of fixed nitrogen (N_2) necessary for plant development. The assumption is that 25% to 75% of the nitrogen (N) required for the development of soybean plants is obtained in the process of symbiotic nitrogen fixation. The process is dependent on environmental conditions (Popović *et al*., 2018; 2020), and the supply of soil with nitrogen nutrients (Stevanović *et al*., 2017). In cases of high nitrogen presence in the soil, usually over 90 kg/ha, the nodules will not be formed on the soybean roots (Belić *et al.,* 1987). It was estimated that legumes could fix a total of 18.5 million tonnes of nitrogen during one production year on a global scale, where soybean has the largest share, accounting for 16.4 million tonnes of the fixed nitrogen (Herridge *et al*., 2008). The formation of nodules on the soybean root and the process of symbiosis between the plant and the bacteria is realized gradually in several stages. First, the recognition of the plant and the bacteria in the soil occurs because the plant secretes various exudates that play a signalling role, mainly isoflavones such as daidzein, genistein, glycitein, and their glucosides (Sugiyama *et al*., 2007). Once the contact is established, isoflavones diffuse through the bacterial membrane, enabling entrance into the root cortex via root hair infection to establish the initial symbiosis (Nadzieja *et al*., 2018). The starting symbiosis contact is established thanks to the Nod factor of the bacteria recognized by NOD receptors on the root host plant cells (Fernandez-Göbel *et al*., 2019). After the bacterial infection through the cell wall of the root hair, nodule formation begins. First, is formed, which consists of bacteroides composed of bacterial cells and the peribacteroid membrane that surrounds the bacteroid on the outside and is composed of plant cells (Mylona *et al*., 1995; Oldroyd *et al*., 2011), after which the bacteroid is formed and the nitrogen fixation process begins (Jones, 2007). All metabolic processes and the exchange of nutrients are under the complete control of the plant.

The intensity of the nodulation process highly depends on the available soil nitrogen (Taylor *et al*., 2005). The consequence of nitrogen excess in the soil is low intensity in nodulation, i.e., in the formation of active nodules on the root of a host plant (Ohyama *et al*., 2012; Saito *et al*., 2014). It was shown that nodules on soybean roots actively begin to fix the atmospheric nitrogen in the phenophase of two (V2) or three developed tricots (V3), so that the number of nodules and the intensity of nitrogen fixation continuously increase until reaching a maximum in the phenophase of the beginning of pods formation (R5) (Lofton *et al*., 2017). The biological activity of a nodule lasts on average 35-45 days and the soybean plant can provide up to 2/3 of the nitrogen needed for development through the process of nitrogen fixation (Pérez-Pizá *et al*., 2020).

The aim of the present study was to identify the effect of *Bradyrhizobium* inoculation on the dynamics and intensity of N-fixing nodule formation in different soybean varieties and the possible dependence of this process on the initial isoflavone content in the sowing seed material. We hypothesised that nodulation dynamics might be a genetic trait, and therefore, different patterns of nodulation intensity may occur in different soybean genotypes. Moreover, it was intended to assess how strong an impact the inoculation could have on plots of previously cultivated soybean in a crop rotation system.

Materials and Methods

Plant material and inoculant

Nine domestic varieties of soybean were used, which were created in the Institute of Field and Vegetable Crops, Novi Sad, National Institute of the Republic of Serbia, and the company Delta Agrar Belgrade. The soybean varieties used in the experiment belong to the three different maturity groups: 0-early, I-medium, and II-late (Table 1).

Table 1. Soybean varieties and maturity groups

Variety	Galina	Dana	NS Princeza	Dukat	Sava	Galeb	NS Apolo	Gorstak	Trijumf
Maturity group	0				I			II	

Determination of individual isoflavones content

The extraction of phenolic compounds was performed after the removal of the lipids from the ground soybean seeds with *n*-hexane. The plant material (0.500 g) was weighed into centrifuge tubes, after which 10 ml of *n*-hexane was added with a vortex, and the entire medium was mixed with a magnet for 30 min on a magnetic stirrer. The test tubes were then centrifuged for 15 min at 12,857 g, after which the *n*-hexane was separated from the sample (decanted). The procedure was repeated twice, and the samples were left at room temperature, in a hood, until the remaining amount of *n*-hexane evaporated. To the defatted plant material was added 3 ml of a methanol solution with 1% (w/V) 2,6-di-tert-butyl-4-methylphenol, or BHT (BHT was added to prevent oxidation). Extraction was performed according to the protocol described by Mikulic-Petkovsek *et al.* (2016). Extraction of phenolic compounds from soybean sides was performed in an ultrasonic bath (1 h) (with the addition of ice to prevent heating), after which the extracts were centrifuged for 10 min (4 °C) at 12.857 g and filtered through polyamide filters (Chromafil AO-20/25 polyamide filter, Macherey-Nagel; Düren, Germany) into vials. Extraction was performed in five replicates for each soybean seed sample.

Quantification of phenolic compounds was performed using high-performance liquid chromatography (HPLC) with a DAD detector (Diode Array Detector) on a Thermo Finnigan Accela HPLC system (Thermo Scientific, San Jose, USA), according to the conditions described in the work of Mikulic-Petkovsek *et al*. (2020). Spectra were recorded between 200-600 nm (isoflavones at 254 nm, phenolic acid derivatives and flavanols at 280 nm, flavonols and flavones at 350 nm and anthocyanins at 530 nm). A Gemini C18 column (150×4.6 mm 3 μm; Phenomenex, Torrance, USA) was used in the work, and the column temperature was 25 °C. The eluents were aqueous solution of 0.1% formic acid and 3% acetonitrile in twice distilled water (A) and a 0.1% solution of formic acid and 3% of water in acetonitrile (B). The samples were eluted according to a gradient decribed in Mikulic-Petkovsek *et al.* (2020). The injection volume was 20 μl and the flow rate was 0.6 ml/min. All phenolic compounds presented in our results were identified using an HPLC- MS detector and LCQ Deca XP MAX (Thermo Finnigan, San Jose, CA) instrument with an electrospray ionisation (ESI) interface operating in negative ion mode. Analyses were performed according to HPLC-MS conditions reported by Mikulic-Petkovsek *et al*. (2020). Spectral data were processed using Excalibur software (Thermo Scientific).

The identification of the compounds was confirmed by comparing the retention times and their spectra, as well as by adding a standard solution to the sample and fragmentation. The concentrations of phenolic compounds were calculated from the peak areas of the sample and corresponding standards and expressed in μg/g of dry weight (DW) of seed. For compounds without standards, quantification was performed using chemically similar compounds as standards.

Bradyrhizobium japonicum inoculant

The microbiological inoculant under the commercial name Azotofixin "S" was used for seed inoculation. The "Azotofixin S" was produced by the Institute of Soil Science, Belgrade, Serbia. Azotofixin "S" is applied by treating soybean seeds immediately before sowing or applying the inoculants in soil before or after sowing in furrows. The most common and cheapest method is the application of the inoculant on the seeds (seed inoculation) immediately before sowing and it should be carried out in a shaded place without direct sunlight. The action of UV rays leads to a decrease in the number of bacteria. One dose (package) of the product of 140 g is mixed with 2-3 dl of water and the obtained suspension is mixed with 50 kg of soybean seeds (Delić, 2014).

Description of the field experiment

The field experiment was set up in the area of southern Banat, on the experimental field of the Tamiš Research and Development Institute, Pančevo, Serbia, on the chernozem-type soil in the fields of the village of Kačarevo (N:4976420 E:478080) (*https://geosrbija.rs/*). The one-year trial was set up in a conventional way of soybean production in a four-year crop rotation.

The experiment was designed as a two-factorial (factors: inoculation and plant variety) with nine inoculated treatments and nine non-inoculated treatments (controls). Sowing was done manually at the beginning of April. The experiment was set up in two equal field parts (split plot design). In the first part, there were sown the inoculated varieties, and in the second, the non-inoculated soybean varieties. Nine inoculated varieties were sown in 5x2 m parcels in three replications, accounting for 27 small plots, i.e., 54 plots in total. Each plot was divided into four rows. The central two were used for sampling, whereas the first and fourth rows were used for isolation. The distance between rows within one plot was 0.5 m, with the same distance between the plots. The distance between replications was 1 m. The distance between plants in a row differed depending on the maturity group. In the 0 group, the spacing was 4 cm, accounting for the 125 plants in a row. In group I, the distance was 4.5 cm, with 113 plants in a row. In the II maturity group, the distance was 5 cm with 100 plants in a row. The total area of the plot was 35m x 18m, or 630 m^2. Sampling of nodules from the roots was done successively during different phenophases.

Physico-chemical and microbial analyses of soil samples

The experiment was performed on chernozem type soil. The value of soil acidity (pH) was determined by the potentiometer method. The content of total nitrogen was determined by the Kjejdal method. Average humus values were determined using the Turin Simakov modification method (ISO 14235: 1998). The soil carbonate,phosphorus, and potassium values were determined by the Al method of Egner-Riehm (Enger and Riehm, 1958). All laboratory analyses were performed at the Soil Institute, Belgrade.

The microorganisms were determined by the viable plate method using serial dilution techniques, where a dilution of the tested soil suspension is plated onto a selective solid or liquid media. The total number of microorganisms was determined on Soil Extract Agar Medium (Sarić, 1989) and the number of oligonitrophils on a nitrogen-free agar medium according to Fjodorov (Sarić, 1989). The number of *Azotobacter* spp. was determined in a liquid nitrogen-free media according to han method (Vojinović *et al.*, 1966). After serial dilution, the aliquots of the diluted sample are plated on an appropriate culture media and incubated at a temperature of 280 °C. To quantify soil bacteria we used colony forming unit (CFU) and most probable number (MPN) expressed as the number per gram of absolutely soil dry mass (g^{-1} dry soil mass).

Meteorological parameters

The meteorological parameters were monitored during the crop vegetation period (temperature and precipitation) for the Kačarevo location. The database of the Hydrometeorological Institute of the Republic of Serbia was used as a data source *www.hidmet.sr.gov.rs* (Figure 1).

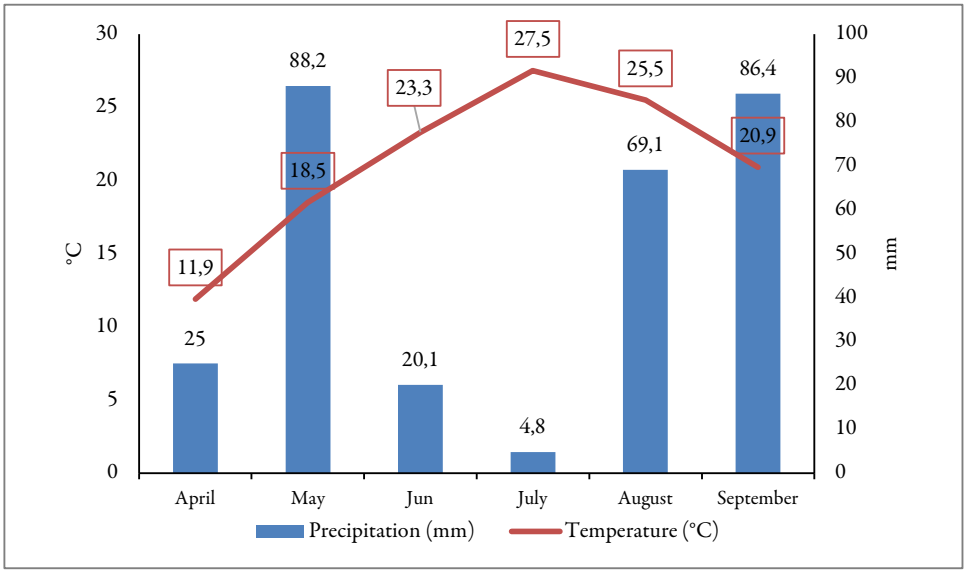

Figure 1. Basic meteorological data for the soybean vegetation period in 2015, Pančevo, Serbia

Analysis of morphological parameters of nodules

Nitrogen-fixing nodules were sampled during the following phenophases of soybean ontogenesis:

1. Phenophase - beginning of flowering (R1),
2. Phenophase - full flowering (R2),
3. Phenophase - final flowering (R2.5),
4. Phenophase - some pods reached full length (R3),
5. Phenophase - 10% of pods reached full length (R4),
6. Phenophase - 30% of pods reached full length (R5),
7. Phenophase - 50% of pods reached full length (R5.5),
8. Phenophase - all pods reached full length (R6),
9. Phenophase - 50% of the pods at full maturity (R7).

To examine morphological parameters, the following morphological characteristics were measured:

A number of nodules on the root of the plant, an average number of nodules during the vegetation period (PBN), and a mass of nodules on the root of the plant (mg), average nodule mass values during the vegetation period (PMN), and total average nodule mass values during the vegetation period (UPMN).

Statistical analysis

All measurements were performed in triplicate. To examine the influence of inoculation and variety on morphological parameters, a two-factor analysis of variance was used in a balanced design. This model is specified as follows: $X_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$, where X_{ijk} is the value of the morphological parameter in the i-th inoculation, j-th variety and k-th repetition; μ is the total average; αi is the main effect of presence/absence of inoculation; βj is the effect of the j-th variety; (αβ)ij is the first-order interaction effect of the i-th inoculation and the j-th variety; while ε_{ijk} is a random error assumed to have a normal distribution with zero mean and variance $\sigma\varepsilon^2$.In order to test the statistically significant differences between inoculated and non-inoculated plants, the *t* test was used. To analyse relationship between the concentration of isoflavones and the total average nodule mass values during the vegetation period and average number of nodules during the vegetation period, the Perason correlation coefficient was used. To assess the differences in isoflavones among different genotypes, the Duncan's test was used. All statistical analyses were conducted with SPSS 26.0 (SPSS, Inc., Chicago, IL) software.

Results and Discussion

Individual isoflavones content

The seed is one of the most important factors of successful agricultural production and that is why ensuring its high quality is the priority of modern seed production and a prerequisite for high yields of all plant species (Branković Radojčić *et al*., 2023). Soybean seeds contain a significant amount of isoflavones and other polyphenolic compounds (Deng *et al*., 2019). One gramme of dry matter contains 3 mg of isoflavones on average (Rostagno *et al*., 2004). Isoflavones are divided into four basic chemical groups: aglycones, glucosides, acetylglucosides, and malonylglucosides (Wang *et al*., 2013). According to Seguin *et al*. (2004), soybean seed yield is in positive correlation with the total content of isoflavones in the seeds.

The presence of isoflavones from three chemical groups was recorded in seeds used in the experimet ("starting seeds"), including the glycosides (genistin and daidzin), acetylglucosides (acetylgenistin, acetyldaidzin and acetylglycitin), and the malonylglucosides (malonylgenistin) (Table 2).

Table 2. Detected isoflavones in seeds of tested soybean varieties

N_0	Min	$[M]^-$ *(m/z)*	MS^2 $[M-H]^-$ *(m/z)*	*Tentative identification*
1	19.26	432	269	Genistein-hexoside
2	19.93	461	415, 253	Daidzin
3	20.77	491	445, 283	Acetylglycitin
4	23.93	477	431, 269	Acetylgenistin
5	24.74	457	253	Acetyldaidzin
6	28.61	459	269	Genistin
7	30.07	519	473, 269	Malonylgenistin

Table 3. Content of isoflavone compounds in sowing seed material (μg/g)

Variety	Galina	Dana	NS Princeza	Dukat	Sava	Galeb	NS Apolo	Gorstak	Trijumf
Isoflavones	X±Se	X±Se	X±Se	X±Se	X±Se	X±Se	X±Se	X±Se	X±Se
Daidzin	1277.3 ±55.4 ^{d}	1414.2 ±65.0 ^{d}	859.5 ±35.0 ^{e}	2181.8 ±79.8^{bc}	2242.1 ±109.0^{bc}	2475.3 ±119.6 ^{ab}	2219.6 ±118.2 ^{bc}	2639.5 ±53.5 ^{a}	2002.7 ±74.0 ^{c}
Acetyldaidzin	1056.0 ±66.6 ^{d}	822.4 ±49.2 ^{e}	478.1 ±22.5 ^{f}	1304.5 ±47.1^{bc}	1760.7 ±120.0 ^{a}	1114.8 ±68.2 ^{cd}	1421.2 ±69.1 ^{b}	1695.3 ±62.9 ^{a}	1395.6 ±89.3 ^{b}
Genistin	107.7 ±6.7 ^{c}	111.8 ±4.2 ^{c}	122.3 ±5.1 ^{c}	194.0 ±10.5^{ab}	194.7 ±14.6 ^{ab}	167.5 ±8.8 ^{b}	208.0 ±10.9 ^{a}	207.8 ±5.1 ^{a}	213.3 ±10.4 ^{a}
Genistein hexoside	19.8 ±0.5 ^{e}	22.9 ±0.6 ^{e}	48.1 ±2.4 ^{d}	155.8 ±9.2 ^{b}	36.0 ±4.3 ^{d}	92.9 ±8.0 ^{c}	37.2 ±2.7 ^{d}	173.4 ±10.2 ^{a}	34.7 ±2.4 ^{d}
Acetylgenistin	1576.2 ±60.5 ^{d}	1885.5 ±63.3 ^{c}	1899.4 ±51.6 ^{c}	2555.9 ±58.4 ^{a}	2370.4 ±64.3 ^{b}	2741.4 ±74.2 ^{a}	2637.9 ±69.7 ^{a}	2628.1 ±30.6 ^{a}	2452.0 ±53.1 ^{b}
Malonylgenistin	165.0 ±6.9 ^{d}	221.2 ±9.7 ^{d}	172.4 ±6.3 ^{d}	250.2 ±15.6 ^{c}	268.8 ±19.0 ^{c}	721.9 ±50.2 ^{a}	323.2 ±22.9 ^{b}	322.9 ±9.6 ^{b}	251.4 ±12.9 ^{c}
Acetylglycitin	101.9 ±4.9 ^{d}	74.9 ±0.9 ^{d}	389.8 ±21.4 ^{a}	436.3 ±19.8 ^{a}	289.7 ±32.7 ^{b}	221.1 ±22.1 ^{c}	175.1 ±9.3 ^{c}	450.7 ±33.9 ^{a}	78.0 ±8.4 ^{d}

Results marked with different Latin letters were significantly different at the significance level of $p<0.05$ (Duncan's test)

In eight of the nine analyzed soybean varieties, the most abundant isoflavone was acetylgenistin, followed by daidzin derivatives. The highest value of acetylgenistin was found in the variety 'Galeb' (2.7 mg/g). The highest daizdin content was present in the seed of the variety Gorstak (2.6 mg/g), which also had the highest value of the genistein hexoside. 'Trijumf' and 'Galeb' varieties had the highest content of genistin and malonylgenistin (213.3 and 721.9 μg/g, respectively), and the highest value of acetylglycitin was recorded in the

seeds of the 'Gorstak' variety, which had the highest total isoflavones content (1.08-2.04 times higher than other varieties) (Table 3). Similarly to our results, it was reported that glycitin derivatives account for the smallest part of isoflavones in the soybean seeds (5-10%) (Jung *et al.,* 2008). Contrary to other findings (Sun *et al.,* 2011; Riedl *et al.,* 2007), where the malonylglucoside group of isoflavones was the most abundant in the seeds with over 60%, tested soybean varieties showed abundance in acetyl glucoside derivatives.

Physico-chemical and microbial analyses of soil samples

Growing population pressures, industrialization and intensive use of soil exhaust natural resourcesand limit the performance of soil functions. The additional impacts of climate change and land usechanges affect the ability of soils to regenerate and even lead to degradation. The future capacityof soils to support life on Earth is in question. In light of these, soil science again became a majorcomponent of each environmental science courses, given that soil plays a key role in elementarynatural cycles (Životić and Gajić, 2023). The experiment was performed on chernozem-type soil, which is characterised by very good water and physical properties. The value of soil acidity (pH) was slightly alkaline (7.4). A high content of total nitrogen was recorded in the amount of 0.23, as well as high carbonate values of 12.1%, relatively high values of available phosphorus (P_2O_5: 19.7 mg/100 g), and medium values available potassium (K_2O: 16.4 mg/100 g). Average humus values were 3.43%.

The obtained results indicate quite favorable physical and chemical properties of the investigated soils, can help in a deeper understanding of soil ecology and the preservation of natural plant cover (Gajić *et al.,* 2023). A good representation of *Azotobacter* sp., as an indicator of soil fertility, was found (Rasulić *et al.,* 2023). In this research, soil microbial properties were determined according to number of total number of microorganisms, oligonitrophils and *Azotobacter* spp. as indicators of fertile soil (Radivojević *et al*., 2007; Quilliam *et al*., 2013; Živanov *et al*., 2020). The total number of microorganisms, the number of free nitrogen fixers, and *Azotobacter* spp. were determined in soil samples collected to the soil depth of 5-30 cm. In this soil layer, the number of these microorganisms and their activity are higher containing more organic matter and enough moisture and oxygen then deeper layers (Marinković *et al*., 2016).

The total number of microorganisms is the highest in fertile and healthy soil with a neutral pH reaction, higher content of humus and to the soil depth of 5-30 cm, average value is 10^8 -10^9 colony forming units/gram soil dry mass (CFU g^{-1} soil dry mass) (Govedarica and Jarak, 1995; Jarak and Čolo, 2007; Jarak *et al*., 2012; Živanov *et al*., 2020). Bacteria are generally the most numerous group of microorganisms in the soil, so their number is taken as the total number of microorganisms regardless of the characteristics or type of soil. Chernozem belongs to fertile soil due to its physical and chemical properties providing favorable conditions for the life of microorganisms, (average number 10^7 -10^8 CFU g^{-1} soil dry mass) (Govedarica and Jarak, 1995; Jarak *et al*., 2012) which is in agreement with our results (10.33×10^6 CFU g^{-1} soil dry mass). Since the total number of microorganisms is an indicator of potential soil fertility, the obtained results confirmed that our experiment was carried out on fertile soil (Jarak *et al*., 2007).

Free N-fixers are important microorganisms due to their ability to fix nitrogen from the air. They are under the influence of ecological factors of the environment. In soil with neural and slightly acid reaction, there are several hundred thousand of them per gram. According some results, their average number in fertile soil cannot be less than 10^5 CFU g^{-1} soil dry mass (Jarak *et al*., 2007) which is in agreement with our research 49.00×10^5 CFU g^{-1} dry mass. Among the free nitrogen fixers, the genus *Azotobacter* belongs to one of the most abundant and efficient bacteria in the fixation of atmospheric nitrogen. Bacteria of the genus *Azotobacter* are indicators of soil fertility because for their optimal growth and development in soil they require soil with a favorable water-air regime, good pH neutral reaction, chemical properties, moderately and well supplied with humus and nitrogen, readily available phosphorus and potassium, and certain microelements (Govedarica and Jarak, 1995; Marinković *et al*., 2016). These soil features characterize chernozem. Their average number in such soils is 10^2-10^4 MPN g^{-1} soil dry mass. They are sensitive to acidic soils, poorly aerated, with poor physical and

chemical properties and cannot be found in these kind of soils (Govedarica and Jarak, 1995; Jarak and Čolo, 2007; Jarak *et al*., 2007; Đorđević *et al*., 2014; Živanov *et al*., 2020). Results in this research showed satisfying number of genera *Azotobacter* spp. confirming that analyzed soil is fertile soil (173.75 MPN g^{-1} soil dry mass). Similar values were also obtained in some researches on chernosem soil (Govedarica *et al*., 1993; 2001).

Morphological parameters of nodules

A comparative analysis of the number of nodules on soybean roots shows a growing trend in the average value of the number of nodules from the first sampling, phenophase R1, to the last sampling R7. The growth trend exhibits an irregular shape, but continuity can be observed (Table 4). The plants from inoculated treatments had usually higher values of nodule numbers compared to the plants from the control. The highest individual value was observed in plants of 'Sava' variety with an average of 23.33 nodules in the R4 phenophase, followed by 'Galina' with 23 nodules in the R5.5 phenophase and Dukat with 20 nodules in the R4 phenophase (Table 4). However, based on the results of the measurements, it can be concluded that regardless of the higher values of the number of nodules on the roots of plants from the inoculated treatment compared to the plants from the control, in most varieties, the inoculation did not have a statistically significant effect.

Table 4. Analysis of the variance for the number of nodules on soybean roots

Treatment	Variety	Phenophase of development and sampling								
		R1 (1)	R2 (2)	R2.5 (3)	R3 (4)	R4 (5)	R5 (6)	R5.5 (7)	R6 (8)	R7 (12)
Noninoculated	Galina	3.00 ± 2.00^{aA}	8.00 ± 5.20^{aA}	5.00 ± 3.00^{aAB}	6.33 ± 2.89^{aA}	7.33 ± 4.16^{aA}	4.33 ± 2.31^{aAB}	6.00 ± 3.00^{aA}	7.33 ± 2.52^{aA}	10.33 ± 7.23^{aA}
	Dana	5.00 ± 1.73^{aAB}	10.33 ± 8.08^{aA}	4.33 ± 0.58^{aAB}	3.67 ± 2.08^{aA}	5.33 ± 2.08^{aA}	5.33 ± 3.06^{aAB}	4.33 ± 2.52^{aA}	7.00 ± 7.00^{aA}	5.00 ± 1.73^{aA}
	NS Princeza	5.33 ± 2.08^{aAB}	7.67 ± 6.43^{aA}	6.00 ± 3.46^{aAB}	4.67 ± 1.53^{aA}	7.67 ± 2.08^{aA}	5.33 ± 2.08^{aAB}	9.33 ± 5.13^{aA}	6.67 ± 4.51^{aA}	6.67 ± 1.53^{aA}
	Dukat	3.67 ± 1.53^{aAB}	6.33 ± 4.04^{aA}	3.33 ± 3.21^{aAB}	3.33 ± 1.53^{aA}	9.00 ± 10.58^{aA}	5.00 ± 0.00^{aAB}	9.00 ± 1.73^{aA}	7.00 ± 2.00^{aA}	9.33 ± 4.93^{aA}
	Sava	9.33 ± 5.13^{aB}	4.00 ± 1.00^{aA}	10.00 ± 7.94^{aB}	6.00 ± 4.36^{aA}	16.33 ± 12.34^{aA}	9.00 ± 3.61^{aB}	10.33 ± 9.24^{aA}	10.33 ± 5.13^{aA}	8.33 ± 6.03^{aA}
	Galeb	7.67 ± 4.93^{aAB}	3.00 ± 1.73^{aA}	6.33 ± 3.79^{aAB}	5.67 ± 0.58^{aA}	10.33 ± 2.52^{aA}	3.67 ± 1.53^{aA}	13.67 ± 5.86^{aA}	14.33 ± 11.15^{aA}	6.67 ± 2.52^{aA}
	NS Apolo	8.00 ± 2.65^{aAB}	10.67 ± 10.69^{aA}	7.33 ± 2.52^{aAB}	5.67 ± 4.04^{aA}	11.33 ± 6.66^{aA}	4.67 ± 3.06^{aAB}	15.00 ± 13.08^{aA}	6.00 ± 3.00^{aA}	7.00 ± 5.00^{aA}
	Gorstak	2.33 ± 2.52^{aA}	2.67 ± 0.58^{aA}	3.67 ± 1.53^{aAB}	2.67 ± 1.53^{aA}	5.00 ± 7.81^{aA}	4.00 ± 3.46^{aA}	10.33 ± 6.81^{aA}	10.00 ± 6.08^{aA}	9.00 ± 1.73^{aA}
	Trijumf	4.00 ± 0.00^{aAB}	2.33 ± 2.08^{aA}	1.33 ± 2.31^{aA}	2.33 ± 1.15^{aA}	5.67 ± 1.53^{aA}	2.67 ± 1.53^{aA}	14.33 ± 17.04^{aA}	4.00 ± 3.61^{aA}	5.00 ± 0.00^{aA}
Inoculated	Galina	5.67 ± 4.04^{aA}	8.00 ± 5.00^{aA}	8.67 ± 2.31^{aB}	10.67 ± 3.79^{aA}	9.00 ± 5.57^{aABC}	4.67 ± 0.58^{aA}	$23.00\pm6,56^{bA}$	8.67 ± 5.51^{aA}	11.00 ± 5.29^{aA}
	Dana	7.67 ± 4.04^{aA}	13.33 ± 5.86^{aA}	4.67 ± 1.15^{aAB}	5.33 ± 0.58^{aA}	5.33 ± 2.08^{aAB}	5.00 ± 2.65^{aA}	7.67 ± 2.89^{aA}	14.00 ± 7.55^{aA}	17.67 ± 7.51^{bA}
	NS Princeza	10.33 ± 3.06^{aA}	7.00 ± 1.73^{aA}	6.67 ± 3.79^{aAB}	11.33 ± 16.20^{aA}	17.00 ± 12.17^{aABC}	5.00 ± 4.00^{aA}	11.33 ± 6.43^{aA}	7.00 ± 2.65^{aA}	7.33 ± 2.31^{aA}
	Dukat	9.00 ± 6.93^{aA}	10.67 ± 4.93^{aA}	6.00 ± 2.00^{aAB}	5.33 ± 4.16^{aA}	20.00 ± 13.75^{aBC}	7.33 ± 4.51^{aA}	16.67 ± 2.52^{bA}	12.00 ± 6.24^{aA}	12.67 ± 6.43^{aA}
	Sava	13.67 ± 7.23^{aA}	11.33 ± 6.03^{aA}	4.67 ± 1.53^{aAB}	8.33 ± 6.81^{aA}	23.33 ± 10.97^{aC}	6.33 ± 1.53^{aA}	13.00 ± 6.08^{aA}	5.67 ± 2.52^{aA}	7.33 ± 3.51^{aA}
	Galeb	8.67 ± 1.53^{aA}	8.67 ± 5.69^{aA}	7.33 ± 2.52^{aAB}	7.00 ± 1.00^{aA}	14.33 ± 2.52^{aABC}	9.00 ± 4.58^{aA}	16.33 ± 10.41^{aA}	11.33 ± 4.51^{aA}	10.33 ± 1.53^{aA}
	NS Apolo	7.33 ± 5.86^{aA}	13.33 ± 10.07^{aA}	5.67 ± 2.52^{aAB}	7.00 ± 3.46^{aA}	13.00 ± 4.58^{aABC}	9.33 ± 6.43^{aA}	12.00 ± 3.61^{aA}	11.67 ± 3.79^{aA}	7.00 ± 3.00^{aA}
	Gorstak	8.00 ± 6.24^{aA}	3.33 ± 2.89^{aA}	3.33 ± 0.58^{aA}	8.00 ± 7.21^{aA}	4.67 ± 0.58^{aA}	3.00 ± 2.00^{aA}	13.33 ± 3.21^{aA}	10.00 ± 3.61^{aA}	6.00 ± 1.00^{aA}
	Trijumf	6.33 ± 2.52^{aA}	6.33 ± 4.93^{aA}	3.33 ± 0.58^{aA}	10.00 ± 0.00^{bA}	7.00 ± 2.65^{aAB}	4.33 ± 2.31^{aA}	9.00 ± 2.00^{aA}	7.33 ± 0.58^{aA}	5.33 ± 0.58^{aA}

Data are mean ± standard deviation during maturation. Average values marked with the same small Latin letters and the same capital letter do not differ significantly at $p<0.05$.

Some other studies (Gitonga *et al*., 2010) showed significantly higher values of the number of nodules on the soybean roots after the inoculation with *Bradhyrhizobium japonicum* inoculum USDA 110, three to eight times higher values of the examined parameters in the R2 and R5.5 phenophases. In addition, some other reports highlighted the significantly higher nodule number in phenophase R2 in plants inoculated with

particular strains of genera *Bradhyrhizobium* (Tahir *et al.,* 2009; Bekere *et al*., 2012; Getu *et al*., 2019; Rahim *et al*., 2016), contrary to the results obtained in current experiment.

Reviewing the results for the morphological parameter of mass of nodules on soybean roots, it was observed that the mass of nodules increases from the first sampling (phenophase R1), to the last sampling (R7) (Table 5). The trend of nodule mass growth was not regular, but certain uniformity was observed when nodule mass of plants form control and inoculated treatmentat the same phenophases of soybean development was compared. The highest individual value was recorded in plants of the Sava variety from treatment with an average value of 21.1 mg in the R4 phenophase, followed by 'Galina' and 'Dana' with a nodule mass of 18.3 and 17.5 mg in the R5.5 phenophase, respectively (Table 5). Although, in certain phenophases of development, some of the observed varieties of soybean had higher values for the nodule mass in inoculated plants, throughout the entire vegetation period, there was no significant difference when treated plants were compared to control plants.

Similarly to our findings, in the experiment by Bekere and Hailemariam (2012), there were no significant differences between the control and plants inoculated with commercial effective *Bradhyrhizobium* strain TAL 379, regarding the nodule mass at R2 phenophase. In contrast to this research, differences in nodule biomass were reported for the R2 phenophase (Getachew and Dagnaw, 2020) inoculated with rhizobial strain MAR-1495, and for the R5 phenophase (Mathenge *et al.,* 2019) inoculated whit Legumefix (*Bradyrhizobium japonicum* strain 532c).

Table 5. Analysis of the variance of nodule mass (mg) on soybean roots

Treatment	Variety	Phenophase of development and sampling								
		R1 (1)	R2 (2)	R2.5 (3)	R3 (4)	R4 (5)	R5 (6)	R5.5 (7)	R6 (8)	R7 (12)
Noninoculated	Galina	3.30±0.30^{aB}	8.00±0.80^{aG}	14.13±1.15^{aE}	12.30±1.20^{aD}	11.87±1.00^{aB}	15.80±1.40^{aC}	18.30±1.70^{aE}	7.53±0.65^{aA}	8.10±0.90^{aAB}
	Dana	2.10±0.10^{aA}	5.40±0.50^{aE}	6.50±0.60^{aC}	10.83±0.58^{aC}	7.27±0.75^{aA}	14.50±1.30^{aBC}	17.50±0.50^{aE}	17.07±3.00^{aC}	15.07±5.00^{aC}
	NS Princeza	2.10±0.20^{aA}	1.50±0.10^{aA}	9.70±0.80^{aD}	6.20±0.60^{aA}	13.53±1.25^{aB}	15.30±1.30^{aC}	13.10±1.20^{aD}	7.27±0.75^{aA}	5.80±0.50^{aA}
	Dukat	3.70±0.30^{aBC}	4.60±0.40^{aCD}	5.00±0.50^{aA}	12.40±1.20^{aD}	12.00±1.00^{aB}	12.50±0.50^{aB}	12.00±1.00^{aD}	10.10±0.90^{aB}	10.00±1.00^{aB}
	Sava	6.00±0.50^{aD}	6.50±0.50^{aF}	7.00±0.50^{aC}	8.80±0.70^{aB}	16.90±1.60^{aC}	13.70±1.30^{aBC}	10.10±0.90^{aB}	7.50±0.70^{aA}	10.20±0.80^{aB}
	Galeb	4.13±0.35^{aC}	5.10±0.40^{aDE}	5.70±0.50^{aAB}	6.30±0.60^{aA}	10.70±0.60^{aB}	10.82±1.80^{aD}	10.60±0.90^{aB}	9.70±0.80^{aB}	8.60±0.80^{aAB}
	NS Apolo	3.43±0.31^{aB}	3.70±0.20^{aB}	4.60±0.40^{aA}	5.90±0.50^{aB}	11.80±1.00^{aB}	9.90±0.90^{aA}	9.87±0.90^{aB}	10.33±0.25^{aB}	8.00±0.20^{aAB}
	Gorstak	2.30±0.20^{aA}	4.17±0.35^{aBC}	6.30±0.60^{aBC}	8.40±0.70^{aB}	6.53±1.74^{aA}	12.50±1.10^{aB}	9.20±0.70^{aB}	8.30±0.80^{aA}	8.10±0.60^{aAB}
	Trijumf	2.50±0.20^{aA}	3.60±0.30^{aB}	5.70±0.50^{aAB}	8.70±0.30^{aB}	10.87±0.78^{aB}	9.13±0.15^{aA}	7.50±0.30^{aA}	9.77±0.15^{aB}	8.23±0.35^{aAB}
Inoculated	Galina	3.63±0.15^{aB}	8.13±0.75^{aD}	9.50±1.00^{aC}	12.20±1.20^{aC}	17.20±1.60^{bF}	12.30±1.20^{aE}	9.50±0.90^{aBC}	9.10±0.80^{aC}	8.33±0.80^{aBC}
	Dana	5.10±0.40^{bC}	5.50±0.50^{aB}	6.10±0.60^{aAB}	7.00±0.70^{aA}	7.30±0.70^{aA}	9.10±0.90^{aBC}	11.43±0.49^{aC}	10.47±0.46^{aD}	9.90±0.80^{aD}
	NS Princeza	3.20±0.30^{bB}	4.50±0.40^{bA}	7.33±0.80^{aB}	9.87±0.80^{bB}	12.70±1.20^{aDE}	11.00±1.00^{bDE}	10.33±0.95^{bC}	9.07±0.85^{aC}	8.00±0.80^{aBC}
	Dukat	4.87±0.31^{bC}	6.40±0.20^{bC}	7.20±0.70^{bB}	10.10±0.90^{aB}	11.00±1.00^{aCD}	15.30±1.50^{bF}	11.50±1.10^{aC}	10.60±0.90^{aD}	10.50±0.90^{aD}
	Sava	5.00±0.50^{aC}	6.33±0.70^{aC}	10.60±0.90^{bC}	14.70±1.30^{bD}	21.10±1.90^{bG}	11.50±1.00^{aDE}	12.40±1.20^{aC}	10.73±0.75^{bD}	9.40±0.80^{aCD}
	Galeb	3.60±0.40^{aB}	4.20±0.40^{aA}	6.30±0.60^{aAB}	12.00±1.00^{bC}	12.50±1.00^{aDE}	9.90±0.80^{aCD}	6.62±4.83^{aAB}	8.40±0.70^{aBC}	8.10±0.80^{aBC}
	NS Apolo	3.70±0.20^{aB}	5.43±0.25^{bB}	10.50±1.00^{bC}	11.50±1.00^{bBC}	13.70±1.30^{aE}	10.50±1.00^{aCDE}	9.10±0.70^{aABC}	11.80±1.00^{aD}	10.10±0.80^{bD}
	Gorstak	2.40±0.30^{aA}	4.50±0.20^{aA}	6.70±0.60^{aAB}	10.10±0.90^{aB}	9.90±0.80^{aBC}	7.50±0.70^{aB}	5.80±0.50^{aA}	6.50±0.60^{aA}	4.30±0.40^{aA}
	Trijumf	2.50±0.20^{aA}	3.80±0.30^{aA}	5.50±0.50^{aA}	7.50±0.70^{aA}	8.20±0.80^{aAB}	5.10±0.50^{aA}	5.90±0.50^{aA}	7.30±0.70^{aAB}	7.10±.0.70^{aB}

Data are mean ± standard deviation during maturation. Average values marked with the same small Latin letters and the same capital letter do not differ significantly at $p<0.05$

Research by Zhang *et al*. (2003) revealed that in the R1 and R3 phenophases, all varieties of plants from the inoculated treatment with three *Bradhyrhizobium japonicum* strain (USDA 30, USDA 31, and 532 C) had significantly higher nodule mass values compared to plants from the control, from one and a half to two times

higher value. In addition, six tested soybean varieties inoculated with three isolates of *Bradyrhizobium* sp. (UK-isolate, TAL-379 isolate and local-isolate) in the phenophase R4, exhibited 50-70% higher values of the nodule mass, in comparison to uninoculated control (Argaw, 2014) which is similar with our results at varieties NS Apolo and Sava.

Testing the effectiveness of Azotofixin S in the experiment of Nenadić *et al*. (2002), showed that the number of nodules was the highest in the treatment with the Azotophysin S, then Bioselfix and finally Nitragin (39.77; 23.86 and 18.84, respectively), while the efficiency in terms of the mass of nodules it was the highest for the preparation Nitragin, then Bioselfixin and finally Azotofixin S (9.34 mg, 9.22 mg and 5.46, respectively). The highest values were achieved in the years with the best climatic conditions, i.e. with optimal amounts of precipitation and the absence of dry conditions, while in dry years the effect of inoculation was not significantly affected, which is similar to our research.

The effectiveness of Azotofixin S according to some experiments, showed an increase in the yield of inoculated soybeans by more than 90% compared to the untreated control, and a 20% higher yield compared to the fertilized control (Delić *et al*., 2011)

According to Miljković *et al*. (2022), the increase in the number of nodules in the plot where soybeans inoculated with *Bradhyrhizobium japonicum* were grown for the first time compared to the plot where previously inoculated soybeans were grown was 111% and 5%, respectively, and the increase in nodule mass was 49% and 9%, respectively. Similar to our research, the influence of inoculation on the number and mass of nodules on soils previously sown with soybean seeds inoculated with *Bradhyrhizobium japonicum* was shown to be significantly lower.

Reviewing the results for the total nodule mass (total average mass value for the entire vegetation period, i.e., "mass * number") for all investigated varieties, it can be seen that the plants from the inoculated treatment had significantly higher values than the control plants, but there were no statistically significant differences (Table 6).

Table 6. Results of testing differences between inoculated and non-inoculated plants in terms of total average nodule mass (*t*-test)

Variety	Galina		Dana		NS Princeza		Dukat		Sava	
Inoculated	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
PMN	11.03	9.98	10.8	7.98	8.27	8.44	9.14	9.72	9.63	11.31
PBN	6.41	9.92	5.59	8.96	6.59	9.22	6.22	11.07	9.29	10.41
UPMN	70.70^{ns}	99.02^{ns}	60.37^{ns}	71.5^{ns}	54.5^{ns}	77.81^{ns}	56.85^{ns}	107.60^{ns}	89.46^{ns}	117.74^{ns}
Variety	**Galeb**		**NS Apolo**		**Gorstak**		**Trijumf**			
Inoculated	No	Yes	No	Yes	No	Yes	No	Yes		
PMN	7.96	7.96	7.5	9.59	7.31	6.41	7.33	5.88		
PBN	7.92	10.33	8.41	9.59	5.52	6.63	4.63	6.55		
UPMN	63.04^{ns}	82.22^{ns}	63.07^{ns}	91.96^{ns}	40.35^{ns}	42.50^{ns}	33.94^{ns}	38.51^{ns}		

S-variety; I-inoculation; PMN-average nodule mass values during the vegetation period; PBN-average number of nodules during the vegetation period; UPMN-total average nodule mass values during the vegetation period; ns-not significant

The highest total nodule mass values were recorded for the varieties from the "0" maturity group ('Galina', 'Dana', 'NS Princeza', and 'Dukat') and from the "I" maturity group ('Sava', 'Galeb' and 'NS Apolo'). The lowest values were recorded for soybeans from the "II" maturity group ('Trijumf' and 'Gorstak' varieties). Individually, the highest values were recorded for inoculated plants of the varieties 'Sava' (117.74 mg), 'Dukat' (107.6 mg), 'Galina' (99.02 mg), and 'NS Apolo' (91.96 mg) (Table 6). The lowest average values were measured for control plants of the "II" maturity group: noninoculated plants of 'Trijumf' (33.94 mg) and noninoculated plants of 'Gorstak' (40.35 mg) (Table 6).

Concerning the parameter average number of nodules, it could be seen that the inoculated plants of all tested varieties showed significantly higher values compared to the control plants (Table 7). The highest

average value of the number of nodules was observed for plants of soybean varieties from the "0" and "I" maturity groups, and the lowest values were recorded for plants of soybean varieties from the "II" maturity group. Individually, the highest values were recorded in the varieties of the inoculated treatment: 'Dukat' (11.07), 'Sava' (10.41), 'Galeb' (10.33), and 'Dana' (9.59). The lowest average values were measured for the control plants of the "II" maturity group: 'Trijumf' (4.63) and 'Gorstak' (5.52) (Table 7).

Table 7. The differences between inoculated and non-inoculated plants in average number of nodules (*t*-test)

Variety	**Galina**		**Dana**		**NS Princeza**		**Dukat**		**Sava**	
Inoculated	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
PBN	6.41^{ns}	9.92^{ns}	5.59^{ns}	8.96^{ns}	6.59^{ns}	9.22^{ns}	6.22^{ns}	11.07*	9.29^{ns}	10.41^{ns}
Variety	**Galeb**		**NS Apolo**		**Gorstak**		**Trijumf**			
Inoculated	No	Yes	No	Yes	No	Yes	No	Yes		
PBN	7.92^{ns}	10.33^{ns}	8.41^{ns}	9.59^{ns}	5.52^{ns}	6.63^{ns}	4.63^{ns}	6.55^{ns}		

PBN-average number of nodules during the vegetation period; ns-not significant; * - significant differences ($p \leq 0.05$)

Regardless of the differences in absolute values in favour of plants from the inoculated treatment, significant differences were not observed for the tested parameters. Inoculation did not have a statistically significant effect, neither on number nor on nodule mass. The assumption for such results is that the soybean was previously grown on the experimental plots for several years and the soil had a significant amount of N-fixing bacteria, including *Bradyrhizobium japonicum*, so that the recommended concentration of commercial inoculum had no effect. Our results support the idea of possible "soil saturation" with nitrogen-fixating bacteria, in contrast to studies highlighting the high inoculation impact on nodulation intensity in soybeans cultivated on nitrogen-deficient soils.

The nodulation intensity

The high average values of the nodule number in inoculated plants corresponded to the high content of detected isoflavones in most of the tested varieties. The highest average values of the nodule number (Table 4) were observed for the varieties 'Dukat' (10.15), 'Galeb' (9.85), Sava (9.36), and 'NS Apolo' (8.91) and these varieties also had high values of the total isoflavones (Table 3). The variety 'Gorstak' with the highest value of isoflavones didn't exhibit high nodulation intensity (Table 7). The content of isoflavones in control seeds of varieties from the "0" maturity group: 'Galina' (4.3 mg/g) and 'Dana' (4.5 mg/g) was significantly lower than the content of isoflavones in the control seeds of varieties from the "II" maturity group: 'Gorstak' (8.1 mg/g) and 'Trijumf' (6.4 mg/g). However, the average number of nodules during the vegetation period for inoculated plants of the 'Galina' (9.94) and 'Dana' (8.61) varieties was much higher than the number of nodules of plants from the inoculated treatments of the varieties 'Gorstak' (7.09) and 'Trijumf' (6.03) (Table 4).

Similarity, the high average value of the nodule mass did correspond to the high content of detected isoflavones. Varieties with a high content of isoflavones in the initial seed material exhibited a high average nodule mass, which was particularly expressed in the 'Sava' (11.31 mg), 'Dukat' (9.72 mg), and 'NS Apolo' (9.59 mg) varieties (Table 5).

The results of Pearson's correlation values (Table 8) showed that there was no correlation between the content of the different isoflavone compounds and the average value of the nodule number and mass during the vegetation period (R1-R7) in both experimental groups.

The peculiarity of the vegetation season was very low precipitation and high daily air temperatures (Figure 1). It is assumed that the varieties of the maturity group "II" were not favoured by such climatic conditions, because of a longer vegetation period compared to the maturity groups "0" and "I". Unfavourable agro-climatic factors were not conducive to the intensive development of root nodules. In most varieties of the "0" and "I" maturity groups, the content of isoflavones in the starting seed material was more-less proportional

to the intensity of nodulation. Contrary to this, the varieties of the "II" maturity group haven't achieved their full potential under specific climatic conditions, independently of the relatively high isoflavone content in the seeds.

According to (Subramanian *et al.,* 2006; Dong and Song, 2020; Biała-Leonhard *et al*., 2021) the intensity of nodulation directly depends on the amount of secreted isoflavones in the rhizosphere. Exogenous addition of isoflavones (daidzein, glycitein, genistein and coumestrol) was performed in the inoculums of three beans varieties resulting in nodule number increase for 11.8%, 21%, 28% depending on a tested cultivar (Abd-Alla, 2011). Also, the exogenous addition of isoflavones, daidzein and genistein, produced statistically significantly higher values of number and dry weight of soybean root nodules by 29.20 and 7.78%, respectively (Lyu *et al*., 2022).

Table 8. Pearson's correlation coefficient (*r*) between concentration of isoflavones and the total average nodule mass and the nodule numberfor nine soybean varieties

Isoflavones		UPMN (I)	UPMN (C)	PBN (I)	PBN (C)
Genistein hexoside	*r*	-0.258	-0.357	-0.087	-0.193
	Sig. (2-tailed)	0.502	0.345	0.823	0.618
Daidzin	*r*	-0.132	-0.399	-0.105	0.239
	Sig. (2-tailed)	0.734	0.288	0.788	0,535
Acetylglycitin	*r*	0.079	-0.45	0.108	0.105
	Sig. (2-tailed)	0.84	0.225	0.782	0.789
Acetylgenistin	*r*	-0.223	-0.522	-0.091	0.237
	Sig. (2-tailed)	0.564	0.149	0.816	0.539
Acetyldaidzin	*r*	0.076	-0.40	-0.182	0.245
	Sig. (2-tailed)	0.845	0.285	0.64	0.525
Aenistin	*r*	-0.18	-0.628	-0.279	0.128
	Sig. (2-tailed)	0.642	0.07	0.468	0.744
Malonyl genistin	*r*	-0.181	-0.212	0.159	0.35
	Sig. (2-tailed)	0.641	0.583	0.682	0.355

PBN-Average number of nodules during the vegetation period, UPMN-Total average nodule mass during the vegetation period, (I)-inoculated, (C)-control

The presented experiments clearly indicate a direct connection between high isoflavone content in the root and high intensity of nodulation. In our experiment, this tendency was observed for a larger number of varieties, despite the fact that the intensity of nodulation was adversely affected by agro-climatic conditions. We tested the possible effect in the isoflavones of the starting seed material rather than the isoflavone content in the rhizosphere.

Conclusions

The results of the current study show that the inoculation with *Bradyrhizobium japonicum* bacteria, in the used concentration recommended for commercial soybean production, did not have a statistically significant effect on the intensity of nodulation, i.e., on the number and mass of formed nodules. The soil on which the experiment was carried out contains a significant amount of microorganisms as a result of several years of soybean cultivation with inoculated seeds. Because of "soil saturation" with N-fixing symbiotic bacteria, the effect of inoculation on the number and mass of nodules was not much expressed. Analysis of isoflavone content showed that tested soybean varieties had a high content of acetylglucosides in their seeds. No positive correlation was recorded between the isoflavone content in the seeds and the number and mass of root nodules.

Future studies will be focused on monitoring the total isoflavone content and related concentrations of some individual isoflavone compounds in the rhizosphere during key developmental stages, particularly R1-R4 phenophases. The activity of the nodule nitrogenase should also be assessed to determine the possible links between the enzyme production dynamics and the isoflavone exudation.

Authors' Contributions

Conceptualization: V.M.; V.P.; Z.D.S.; V.U; Formal analysis: M.M.P.; D.D.; Statistical analysis: S.K.; B.K.; V.P.; Funding acquisition V.M.;V.U; V.P.; Investigation V.M.;V.U; Methodology: V.M.;V.U.,M.M.P.; Resources V.U; Z.D.S; Writing - original draft: V.M.; V.P.; Z.D.S.; B.K.; Writing - review and editing: V.M.; V.P.; Z.D.S.; D.D.; B.K, V.U. and M.M.P.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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