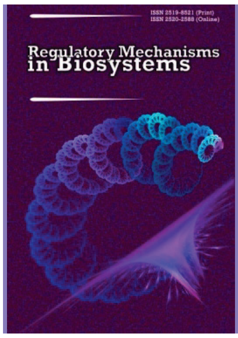


Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.,
2021, 12(2), 326–334
doi: 10.15421/022144

Role of nitrogen deficiency on growth and development near isogenic by E genes lines of soybean co-inoculated with nitrogen-fixing bacteria

A. S. Schogolev, I. M. Raievska

V. N. Karazin Kharkiv National University, Kharkiv, Ukraine

Article info

Received 18.04.2021
Received in revised form 15.05.2021
Accepted 16.05.2021

V. N. Karazin Kharkiv National University,
Sq. Svobody, 4,
Kharkiv, 61022, Ukraine.
Tel.: +38-050-634-99-92.
E-mail:
a.s.schogolev@karazin.ua

Schogolev, A. S., & Raievska, I. M. (2021). Role of nitrogen deficiency on growth and development near isogenic by E genes lines of soybean co-inoculated with nitrogen-fixing bacteria. Regulatory Mechanisms in Biosystems, 12(2), 326–334. doi:10.15421/022144

Nitrogen deficiency is a limiting factor in increasing efficiency of crop production in terrestrial ecosystems, and the transformation of inert nitrogen to forms that can be assimilated by plants is mediated by soil microorganisms. Symbiotic nitrogen-fixing bacteria and roots depend on each other and have developed various mechanisms for symbiotic coexistence. The aim of this work was to investigate the role of nitrogen deficiency on growth and development near isogenic by E genes lines of soybean (*Glycine max* (L.) Merr.): short-day (SD) line with genotype *E1e2e3(E4e5E7)*, and photoperiodic insensitive (PPI) line with genotype *e1e2e3(E4e5E7)* grown from seeds inoculated with active strains of *Bradyrhizobium japonicum* against the background of local populations of diazotrophs of the genus *Azotobacter* spp. and establish how the soybean – *Bradyrhizobium* symbiosis will develop as the genes of both microsymbionts and macrosymbionts are responsible for the formation of the symbiotic complex. Plants were grown in a vegetation chamber, in sand culture. To assess the quantitative composition of microorganisms in the rhizosphere and rhizoplanes, 6 plants were selected from each soybean line, then separation of the zones of the rhizosphere and rhizoplanes was performed using the method of washing and the resulting suspension was used for inoculation on dense nutrient media (mannitol-yeast agar medium and Ashby medium). The results of study showed that seed inoculation and co-inoculation provides faster formation of the symbiotic soybean – *Bradyrhizobium* complex. Differences in nodulation rates between the short-day line with genotype *E1e2e3(E4e5E7)*, and a photoperiodic insensitive line with genotype *e1e2e3(E4e5E7)* were identified. Determination of the amount of *B. japonicum* on the medium of mannitol-yeast agar in the rhizosphere and rhizoplane showed that inoculation by *B. japonicum* strain 634b caused a significant increase in the amount *B. japonicum* in the rhizosphere and rhizoplane in both soybean lines, comparison with non-inoculated seeds. Then, co-inoculation by *B. japonicum* strain 634b + *Azotobacter chroococcum* significantly increased the amount of *B. japonicum* only in the rhizoplane and decreased their number in the rhizosphere. Determination of the amount of *A. chroococcum* on the Ashby elective medium in the rhizosphere and rhizoplane showed that the inoculation by *B. japonicum* strain 634b caused a significant decrease in the amount of *A. chroococcum* both in the rhizosphere and in the rhizoplane of the PPI line of soybean, and in the rhizosphere the SD line, in comparison with non-inoculated seeds. That can testify to the competitive interaction of these microorganisms. However, the co-inoculation by *B. japonicum* strain 634b + *A. chroococcum* in the SD line significantly increased the number of *A. chroococcum* in the rhizoplane and decreased their number in the rhizosphere, in the PPI line their number decreased in the rhizoplane and increased in the rhizosphere, in comparison with non-inoculated seeds. Probably, the E genes (their dominant or recessive state) of soybean isogenic lines affect the regulation of the content and distribution of sugars. It was established that the nitrogen deficiency stimulated development of the root system of plants and the synthesized sugars were distributed predominantly to the root system growth. We suppose that the seeds' inoculation had extended sugar consumption to the symbiont, due to which it compensates the lack of nitrogen, but leads to a slower growth of the root system.

Keywords: *Bradyrhizobium japonicum*; *Azotobacter chroococcum*; *Glycine max*; isogenic lines; symbiosis; photoperiodic insensitive lines.

Introduction

Plants in the natural environment are surrounded by microorganisms, so they simultaneously interact with beneficial and pathogenic microflora. Today, one of the most important issues that need to be addressed is how plants control the interaction with beneficial microflora while at the same time inhibiting pathogenesis (Harris et al., 2020). In recent years, several studies have focused on the factors and mechanisms that regulate plant growth and development, as well as the functioning of signalling pathways in plant cells, unravelling the involvement of sugars in the processes regulating such growth and development. Saccharides play an important role in the life of plants: they are structural and storage substances, respiratory substrates, and intermediate metabolites of many biochemical processes. Sugars can also play an important role in the defence reactions of plants (Ciereszko, 2018). The signal transduction routes induced by sugars interact with other pathways in plant tissues (for example, hormonal pathways (Ferguson & Mathesius, 2014)) creating a complex communication and signalling network in plants which precisely controls interaction with microorganisms (Li et al., 2017). Many bacteria use membrane-diffusible small molecule quorum signals to coordinate gene transcription in response to changes in cell density, known as quorum sensing (QS) (Dong et al., 2020). Shi-Hui Dong recently reported the biochemical characterization of BjaI from *Bradyrhizobium japonicum*, which is involved in controlling the expression of virulence genes. In an open living system of interaction between a plant and microorganisms, plants provide the system with energy, while nitrogen fixing microorganisms provide plants with available forms of nitrogen. Many legumes can establish symbiotic relationships with specific soil bacteria collectively referred as rhizobia (Gano-Cohen et al., 2016), which possess the dinitrogenase enzyme complex capable of capturing atmospheric nitrogen (N_2) and fixing it into ammonium, which is incorporated into carbon skeletons to form nitrogenous organic acids that can be readily assimilated by plants (Hungria et al., 2015). Although 78% of earth's atmosphere consists of dinitrogen (N_2), plants cannot utilize N_2 for their growth. In fact, in an agro-ecosystem, after water, N becomes the second most limiting factor for plant growth. Under natural conditions plants absorb nitrogen in the form of nitrates, ammonium and urea from decaying biological matter, industrial fertilizers and ammonium released by prokaryotes capable of biological nitrogen fixation. However, it is not enough to meet the N demand in the intensely cultivated agro-ecosystem. Presently, to meet this demand industrial fertilizers are applied for plant growth. But leaching, volatilization and improper handling of the fertilizers lead to environmental pollution. Therefore, there is an urgent need to find an environmentally friendly strategy to reduce the application of chemical fertilizer and increase crop yield (Ferguson & Mathesius, 2014; Nag et al., 2019). Plant-growth-promoting rhizobacteria are free-living soil microorganisms that inhabit the rhizosphere or plant roots during plant growth and development (Han et al., 2020). Co-inoculation of seeds with *Azotobacter chroococcum* strains can positively influence plant growth and reduce nitrogen fertilization (Melnykova et al., 2002; Wang et al., 2020). Finding out the patterns of plant-microbial interactions is one of the most important areas in modern biology. Knowledge about the biological essence of nitrogen-fixing symbiosis, and developing methods to increase its efficiency for use in agriculture will help to increase the productivity of cultivated plant varieties.The efficiency of the soybean – *Bradyrhizobium* symbiosis is increased, on the one hand, by the selection of modern varieties capable of active nodule formation, and on the other – by the selection of new strains (created by different methods) of nodule bacteria with high nitrogen-fixing activity (Kots et al., 2014; Aranjuelo et al., 2014).

Eight genes, which influence time from planting to first flower, have been identified in soybean *Glycine max* (L.) Merr. to date: *E1* and *E2*, *E3*, *E4*, *E5*, *E6*, *E7* and *E8* (Cober et al., 2010). Two of these, *E3* and *E4* have been identified as phytochrome A genes while *E2* has been identified as a *GIGANTEA* homolog and *E1* as an inhibitor of *FT*. Alleles at these loci, in conjunction with photoperiod and temperature, regulate the timing of flowering and maturity of soybean lines. Soybean is a facultative short day plant, so under non-inductive long days (>14 h) and high temperatures (25–30 °C) dominant E alleles delay flowering, with the exception of the *E6* locus. Lower temperatures reduced the delaying effect of *E* alleles under non-inductive photoperiods. Isolines with dominant *E* alleles at two or three loci flowered earlier under low temperature (18 °C) and non-inductive photoperiods compared to higher temperature (28 °C) with non-inductive photoperiods, but the time to flowering in both instances was still greater than the time to flower under inductive short photoperiods. This result underscored the importance of the *E* genes in the adaptation of soybean lines to specific climates and highlighted the requirement for a thorough understanding of their function in controlling flowering (Cober et al., 2014; Liu et al., 2017). It is known that soybeans are able to use the nitrogen supply in the seeds in the early stages of vegetative development, but after formation of the symbiotic apparatus, it begins to use biogenic nitrogen obtained from the symbiont (Vorobey & Kots, 2018). The selection of the most effective pairs of nodule bacteria and a certain variety is essential to further increase the yield of soybean crops, as the genes of both microsymbiont and macrosymbiont are responsible for the formation of the symbiotic complex (Rumyantseva, 2019).

In view of the above, the aim of our research is to study the role of nitrogen deficiency on growth and development near isogenic by *E* genes lines of soybean (*Glycine max* (L.) Merr.): short-day (SD) line with genotype *E1e2e3(E4e5E7)*, and photoperiodic insensitive (PPI) line with genotype *e1e2e3(E4e5E7)* grown from seeds inoculated with active strains of *B. japonicum* against the background of local populations of diazotrophs of the genus *Azotobacter* spp. and establish how the soybean – *Bradyrhizobium* symbiosis will be developing as the genes of both microsymbiont and macrosymbiont are responsible for the formation of the symbiotic complex. The results may provide an insight into a long-term systemic interaction between host genes and strains of effective nitrogen fixers will lead to efficient production of crops and accelerate the development and application of these technologies for sustainable intensification of crop production.

Materials and methods

The objects of the study were near isogenic by E genes lines of soybean (*Glycine max* (L.) Merr.): of the cultivar "Clark" with different combinations of alleles. In the dominant state, these genes cause a short-day response, and in the recessive state – photoperiodic insensitive. The studies used the short-day (SD) line with genotype *E1e2e3(E4e5E7)*, and photoperiodic insensitive (PPI) line with genotype *e1e2e3(E4e5E7)*, which were provided by the National Center for Plant Genetic Resources of Ukraine. Plants were grown in a vegetation chamber, in sand culture, in three litre pots. Prior to the commencement of the experiment, coarse sand was obtained from the river sand, washed thoroughly to remove sediments, dirt and dissolved salts and then autoclaved at 120 °C and pressure of – 50 kPa. Each of four pots contained 10 plants.

The photoperiod was 16 h of light and 8 h of dark and temperature of 20–24/17–20 °C (day/night). Humidity was maintained at 60–70% of the total moisture content of sand, watered with distilled water. Lighting from white fluorescent lamps, light intensity of 300 μmol photons $m^{-2}s^{-1}$ (measured at just above the plant canopy). Inoculations was performed by *B. japonicum* (Kirchner) strain 634b, which was provided from the collection of cultures of nitrogen-fixing bacteria of the Department of Symbiotic Nitrogen Fixation of the Institute of Plant Physiology and Genetics of NASU. Co-inoculation was performed by *A. chroococcum* from the collection of microorganisms of the Department of Physiology and Biochemistry of Plants and Microorganisms of V. N. Karazin Kharkiv National University. The research scheme is presented in the diagram (Fig. 2).

Variants of the experiment: non-inoculated group – soybean seeds before sowing were sterilized with 70% ethanol for 15 min, washed under tap running water, inoculated group – soybean seeds before sowing were sterilized with 70% ethanol for 15 min, washed under running tap water and inoculated by aqueous suspensions of nodule bacteria *B. japonicum* strain 634b with a titer of 107 cells/mL (Turbidity Standard No. 5, Ukraine, 2014), co-inoculated group – soybean seeds before sowing were sterilized with 70% ethanol for 15 min, washed under running tap water and co-inoculation by *B. japonicum* strain 634b + *A. chroococcum* with a titer of 10^7 cells/mL (Turbidity Standard No. 5, Ukraine, 2014).

In order to restore physiological activity after storage in the conditions of the museum nodule bacteria were grown in biological tubes on the medium of mannitol-yeast agar (MDA), g/L: K_2HPO_4 (China, 2015) – 0.5, $MgSO_4$*$7H_2O$ (China, 2015) – 0.2, NaCl (Ukraine, 2017) – 0.1, mannitol (Ukraine, 2016) – 10.0, yeast extract – 0.5, agar (Spain, 2017) – 15.0, distilled water, pH 6.8–7.0 (MT, Russia, 2016) for 7–8 days at 28 °C. To prepare aqueous suspensions of nodule bacteria in the sterile conditions of a laminar box (Technogen, Ukraine, 2019) from the surface of agar mannitol-yeast medium was taken one microbiological loop of bacterial biomass, placed in a test tube with sterile water (5 mL), and suspended Vortex (Biosan, Latvia, 2016) until homogeneous in the same consistency. Soybean seeds were inoculated with aqueous suspensions of nodule bacteria with a titer of 10^7 cells/mL. *B. japonicum* strain 634b was grown in mannitol-yeast agar medium (MDA), and *A. chroococcum* were grown in Ashby medium (g/L: K_2HPO_4 (China, 2015) – 0.5, $MgSO_4$* $7H_2O$ (China, 2015) – 0.2, NaCl (Ukraine, 2017) – 0.1, mannitol (Ukraine, 2016) – 10.0, K_2SO_4 (Ukraine, 2016) – 0.1, $CaCO_3$ (Ukraine, 2014) – 5.0, agar (Spain, 2017)), both at 28 °C, thermostat (Medaparat, Ukraine, 2018) (full growth reached at seven days).

The following morphometric parameters were measured: shoot and root weight, and number and weight of nodules on plant roots. The plants were carefully removed from the pots, the subterranean and terrestrial parts were separated, the root system was washed, the nodules were separated and measurements were taken.

The contents of carbohydrates and total nitrogen content were measured in fixed (120 °C for 30 min) dry leaves of middle tier from 3–5 plants. Total nitrogen content in fixed leaves was performed by the Kjeldahl method (Yermakov et al., 1987). The content of soluble sugars was determined by the Shvetsov and Luk'yanenko micromethod (Yermakov et al., 1987). Starch content in leaves determined by the Yastrembovich and Kalinin method (Yermakov et al., 1987). Samples for analysis were taken during daylight hours at 9 am and noon during flowering phase.

Evaluation of the activity of the functioning of the symbiosis soybean – *Bradyrhizobium.* To assess the activity of the symbiosis soybean –*Bradyrhizobium* we studied the root systems of plants. Each plant was carefully removed from the soil, the remnants of the substrate washed away, and then we counted the number of nodules, which is a sign of the activity of this symbiosis.

Fig. 1. General view of the vegetation experiment: near isogenic by *E* genes lines of soybean (*Glycine max* (L.) Merr.) in the early flowering phase

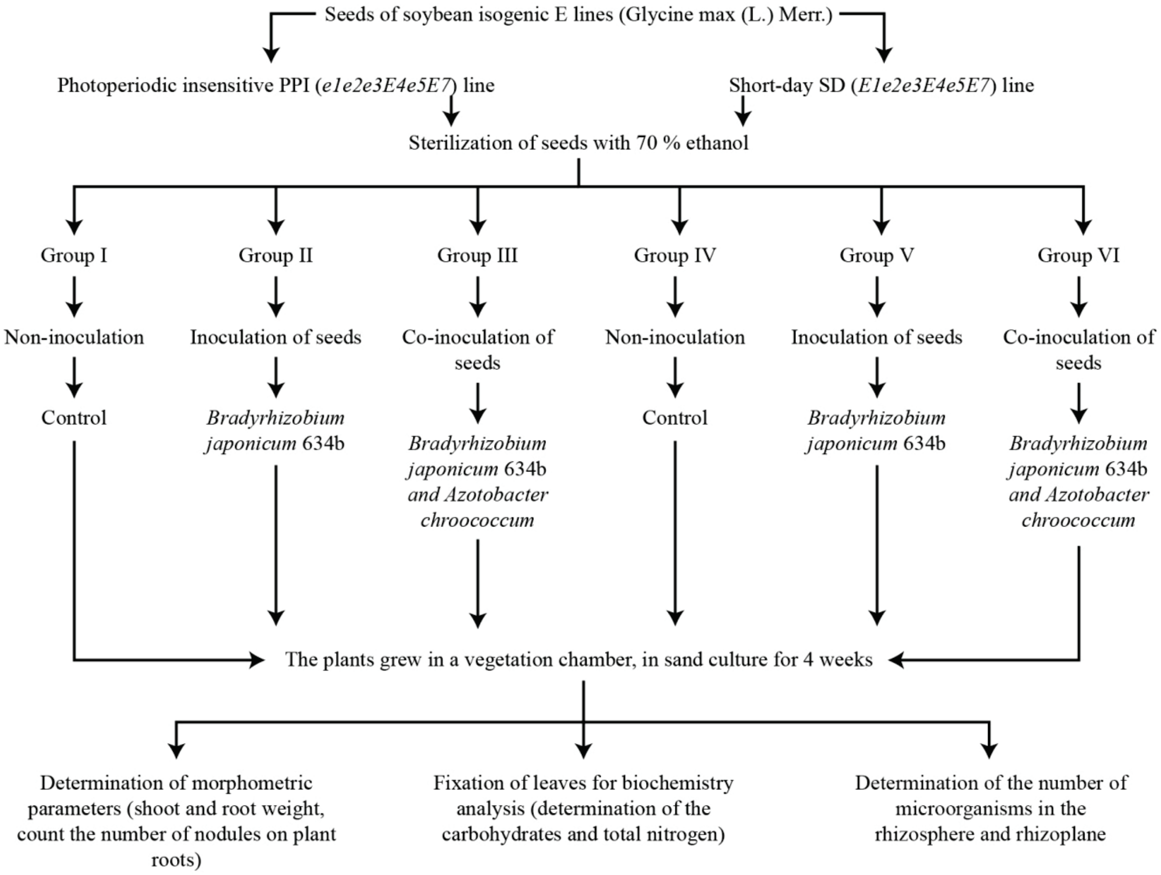


Fig. 2. The scheme of the research

Determination of the number of microorganisms in the rhizosphere and rhizoplane. To assess the quantitative composition of microorganisms in the rhizosphere, 6 plants were selected from each soybean line. Separation of the zones of the rhizosphere and rhizoplanes was performed using the method of washing. To do this, the roots with soil particles were placed in 100 mL of sterile water, stirred for several minutes and carefully removed with tweezers. The roots were then transferred to 100 mL flasks with sterile water and stirred for 40 minutes; soil that was washed from the roots was used to identify microorganisms in the rhizosphere. The resulting suspension was used for inoculation on dense nutrient media: MDA and Ashby. Their sterilization was performed in an autoclave (Medical equipment, Russia, 2017) for 20 minutes at 120 °C. They were poured into Petri dishes, which were sterilized (Medical equipment, Russia, 2006) at 180 °C for 3 hours. Sowing was performed according to the following scheme: pipette 0.1 mL of the dilution suspension onto the surface of the solidified dried medium with a pipette and distributed over the agar using a glass spatula. The experiment was performed in triplicate. The seeded Petri dishes were incubated in a thermostat (Medaparat, Ukraine, 2018) at a temperature of 26–28 °C for 5–7 days.

The data were statistically analysed using Statistica 7.0 software (StatSoft Inc., USA). We calculated standard mean values (x) and standard deviation (SD). Differences between groups were determined using Tukey's test, where the differences were considered reliable at $P < 0.05$ (with taking into account Bonferroni correction).

Results

It was established that the inoculation of seeds under conditions of nitrogen deficiency significantly reduces the mass of the root system in the PPI line by an average of 34.1% and in the SD-line plants by an average of 32.0% compared to the control variant of the experiment (Table 1). Co-inoculation by *B. japonicum* and *A. chroococcum* significantly reduces the mass of the root system in the PPI line by an average of 29.9% compared to the non-inoculated variant of the experiment and in the SD-line plants there was only a tendency to decrease in the mass of the root system compare to the control. There was no significant difference in the shoot biomass during flowering (Table 1).

Table1

Main effect on symbiotic properties during flowering near isogenic by *E* genes lines of soybean (*Glycine max* (L.) Merr.) under nitrogen-limiting conditions in response to seed inoculation consisting of single inoculation with *B. japonicum* strain 634b, or of co-inoculation with *B. japonicum* strain 634b and *A. chroococcum* (x ± SD, n = 6)

Isolines of soybean	Treatment	Shoot biomass, g/plant	Root biomass, g/plant	Nodule number, n/plant	Nodule weight, g/plant	Nodule average weight, g
Photoperiodic insensitive (PPI) line *e1e2e3(E4e5E7)*	Non-inoculated	1.49 ± 0.27^{a}	2.08 ± 0.54^{abc}	19.14 ± 4.08^{abc}	0.213 ± 0.033^{abc}	0.011 ± 0.0006^{abc}
	Inoculated with *B. japonicum*	1.43 ± 0.32^{b}	1.37 ± 0.28^{ab}	11.00 ± 3.46^{ab}	0.168 ± 0.018^{ab}	0.015 ± 0.0014^{ab}
	Co-inoculated with *B. japonicum* + *A. chroococcum*	1.29 ± 0.43^{c}	1.52 ± 0.13^{ac}	10.91 ± 2.46^{ac}	0.163 ± 0.027^{ac}	0.015 ± 0.0013^{ac}
Short-day (SD) line *E1e2e3(E4e5E7)*	Non-inoculated	1.27 ± 0.15^{a}	1.81 ± 0.46^{ab}	8.91 ± 1.22^{ab}	0.145 ± 0.018^{ab}	0.016 ± 0.0018^{ac}
	Inoculated with *B. japonicum*	1.29 ± 0.24^{b}	1.23 ± 0.28^{ab}	12.33 ± 1.27^{abc}	0.186 ± 0.036^{abc}	0.015 ± 0.0012^{bc}
	Co-inoculated with *B. japonicum* + *A. chroococcum*	1.36 ± 0.38^{c}	1.61 ± 0.42^{c}	7.36 ± 2.29^{bc}	0.131 ± 0.013^{bc}	0.018 ± 0.0024^{abc}

Note: comparisons were made within the isogenic line of soybean, means in each column followed by different letters are not significantly different one from another on the results of comparison using the Tukey test ($P < 0.05$) with Bonferroni correction.

The virulence of the studied rhizobia was determined by the number of initiated nodules of different sizes (Table 1). It was established that the inoculation of seeds under conditions of nitrogen deficiency significantly reduced nodule number per plant in both variants of the experiment with inoculation and with co-inoculation by 42.5% and 43.0%, respectively compared to the control (non-inoculated) variant of the experiment, but their nodule average weight was over 36.4% more than in control (Table 1).

The inoculation of seeds under conditions of nitrogen deficiency significantly increased nodule number per plant, inoculated by *B. japonicum* strain 634b the SD soybean line by an average of 38.3% compared to the control variant of the experiment and 67.5% compared to the co-inoculation variant of the experiment. Furthermore, the average weight of nodules per plant in the SD soybean line was significantly higher in the variant with inoculation by *B. japonicum* strain 634b by an average of 28.3% compared to the control variant of the experiment and 42.0% compared to the co-inoculation variant of the experiment. Co-inoculation with *B. japonicum* and *A. chroococcum* caused reduction in the number of nodules in the SD soybean line roots, but nodule average weight was higher than in other variants in our experiment (Table 1).

The plant root system is crucial for anchorage and nutrition, and has a major role in plant adaptation, as well as in interactions with soil microorganisms. Figure 3 shows the formation of nodules under conditions of nitrogen deficiency on soybean roots depending on the variant of inoculation and the photoperiodic reaction of the soybean line, in the early flowering phase. Nodules were formed in all variants of experiment (Fig. 3). And they were situated in different part of the root system. In both soybean lines, on the roots of plants which were grown from non-inoculated seeds, single small nodules were situated only on the lateral roots (Fig. 3a, d).

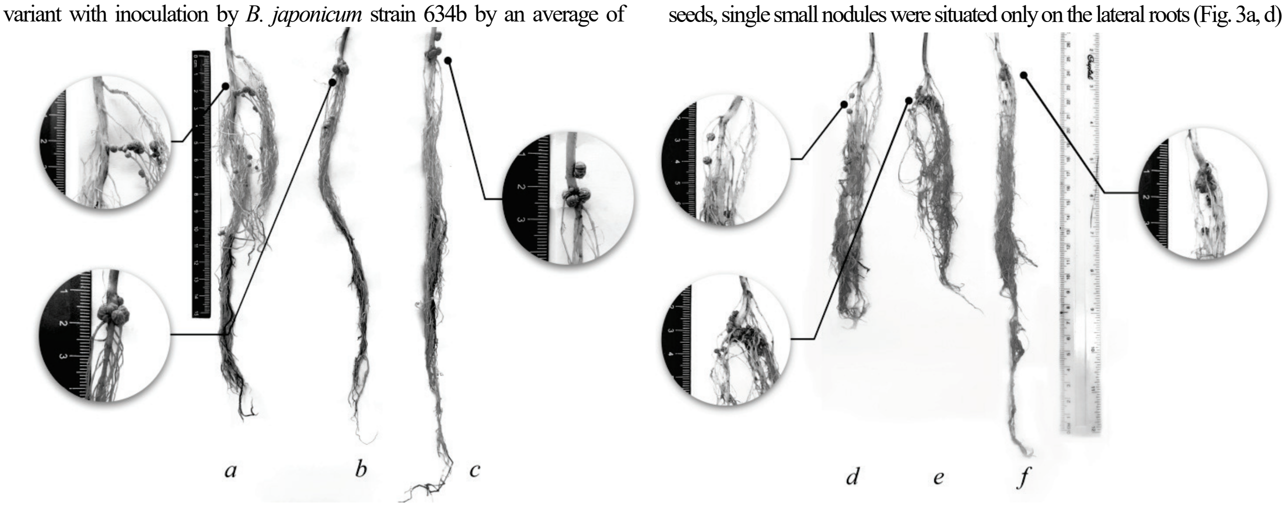


Fig. 3. The formation of nodules on soybean roots under nitrogen-limiting conditions depending on the variant of inoculation and the photoperiodic reaction of near isogenic by *E* genes lines of soybean (*Glycine max* (L.) Merr.) in the early flowering phase: *a* – photoperiodic insensitive line, non-inoculated group, *b* – photoperiodic insensitive line, single inoculation with *B. japonicum* strain 634b, *c* – photoperiodic insensitive line, co-inoculated with *B. japonicum* + *A. chroococcum*, *d* – short-day line, non-inoculated group, *e* – short-day line, single inoculation with *B. japonicum* strain 634b, f – short-day line, co-inoculated with *B. japonicum* + *A. chroococcum*

Table 2

Days from sowing to flowering, oligosaccharides, starch and total nitrogen content during flowering in leaves of near isogenic by *E* genes lines of soybean (*Glycine max* (L.) Merr.) under nitrogen-limiting conditions in response to seed inoculation consisting of single inoculation with *B. japonicum* strain 634b, or of co-inoculation with *B. japonicum* and *A. chroococcum* (x ± SD, n = 6)

Isolines of soybean	Treatment	Days from sowing to flowering	Oligosaccharides content in leaves, mg/g dry weight		Starch content in leaves, mg/g dry weight		Total N content in leaves, mg/g dry weight	
			8.00	12.00	8.00	12.00	8.00	12.00
Photoperiodic insensitive (PPI) line *e1e2e3(E4e5E7)*	Non-inoculated	32 ± 2^{a}	11.6 ± 2.0^{abc}	11.6 ± 1.3^{ab}	26.2 ± 0.8^{abc}	28.2 ± 0.7^{abc}	1.3 ± 0.06^{ab}	1.4 ± 0.05^{abc}
	Inoculated with *B. japonicum*	35 ± 3^{b}	23.3 ± 2.7^{abc}	16.5 ± 0.5^{abc}	18.8 ± 1.1^{ab}	15.5 ± 0.5^{abc}	1.5 ± 0.05^{ab}	1.7 ± 0.16^{ab}
	Co-inoculated with *B. japonicum* + *A. chroococcum*	35 ± 2^{c}	17.2 ± 0.5^{abc}	10.0 ± 0.9^{bc}	20.2 ± 1.2^{ac}	9.0 ± 0.7^{abc}	1.4 ± 0.05^{c}	1.6 ± 0.07^{ac}
Short-day (SD) line *E1e2e3(E4e5E7)*	Non-inoculated	34 ± 1^{abc}	20.0 ± 0.5^{abc}	24.7 ± 1.4^{abc}	18.5 ± 2.2^{abc}	14.4 ± 1.4^{ab}	1.5 ± 0.05^{a}	1.8 ± 0.05^{abc}
	Inoculated with *B. japonicum*	38 ± 2^{ab}	9.0 ± 0.4^{abc}	11.7 ± 0.7^{abc}	9.2 ± 2.2^{ab}	10.5 ± 0.9^{ab}	1.6 ± 0.07^{b}	2.1 ± 0.05^{ab}
	Co-inoculated with *B. japonicum* + *A. chroococcum*	38 ± 2^{ac}	17.0 ± 1.2^{abc}	16.0 ± 0.6^{abc}	8.0 ± 0.9^{ac}	12.0 ± 1.1^{c}	1.5 ± 0.07^{c}	2.0 ± 0.06^{ac}

Note: comparisons were made within the isogenic line of soybean, means in each column followed by different letters are not significantly different one from another on the results of comparison using the Tukey test ($P < 0.05$) with Bonferroni correction.

Single inoculation with *B. japonicum* strain 634b caused formation of big nodules located mainly on the main root, usually closer to its basal part (Fig. 3b, e). Co-inoculation with *B. japonicum* strain 634b + *A. chroococcum* caused formation of big nodules located mainly on the main root, their number were less than in the variant with a single inoculation (Fig. 3c, f). Co-inoculation slightly reduced the efficiency of the formation of the symbiotic apparatus, and stimulated the growth of the root system.

Table 2 shows that the effect of nitrogen deficiency reduced the number of days from sowing to flowering in both soybean lines by 3–5 days compared to plants grown from bacterized seeds.

The plants of the PPI line bloom earlier than SD line in the conditions of a 16-hour light period of the day. The content of oligosaccharides in the leaves of plants of the PPI line significantly increased (by 100.9% at 8 am – 42.2% at noon) under conditions of inoculation, compared with the control and 35.5–65.0% compared to the co-inoculation variant of the experiment. In SD plants of soybean line under conditions of inoculation with *B. japonicum*, the content of oligosaccharides decreased by 55.0–52.3% compared to the control and 47.1–26.9% compared to the co-inoculation variant of the experiment Also starch content in leaves of plants of the PPI was significantly lower (by 28.2% at 8 am – 45.0% at noon) under conditions of inoculation, compared with the control and significantly increased by 72.2% (at noon) compared to the co-inoculation variant of the experiment. Total N content in leaves significantly increased only in the variant with a single inoculation (Table 2).

Despite the presence of nodules in non-inoculated plants of the experiment, the leaves of the lower tier visually showed pronounced signs of nitrogen deficiency, in inoculated and co-inoculated plants there were no signs of nitrogen deficiency (Fig. 4).

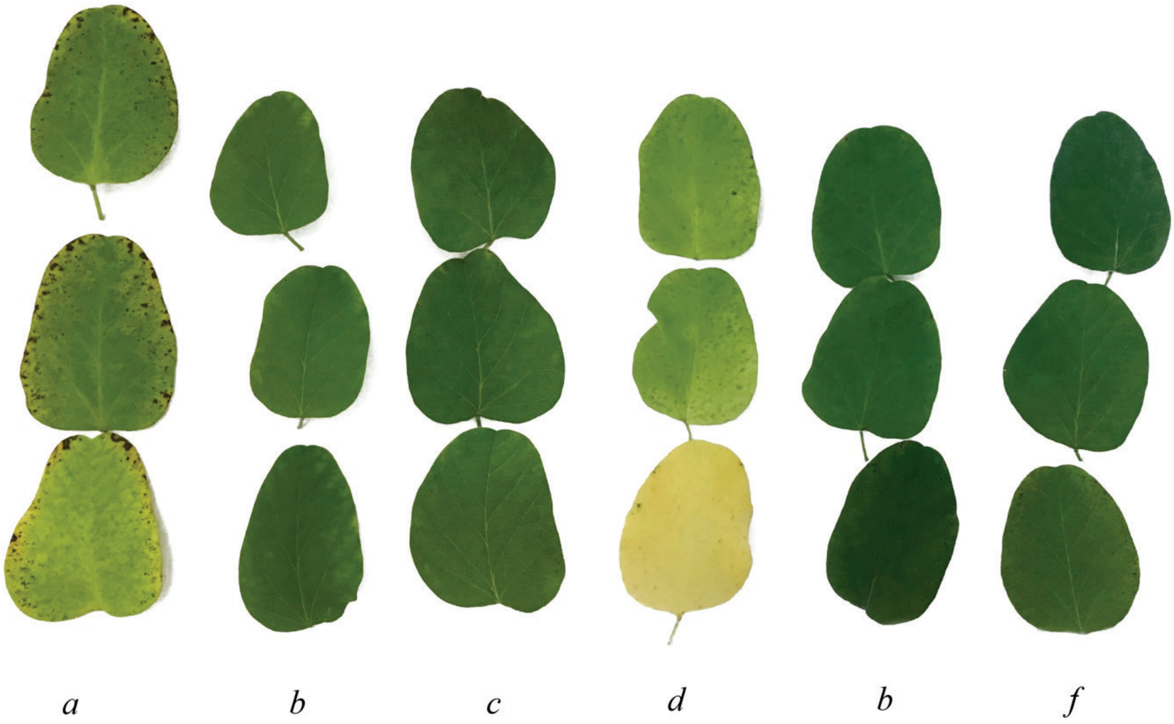


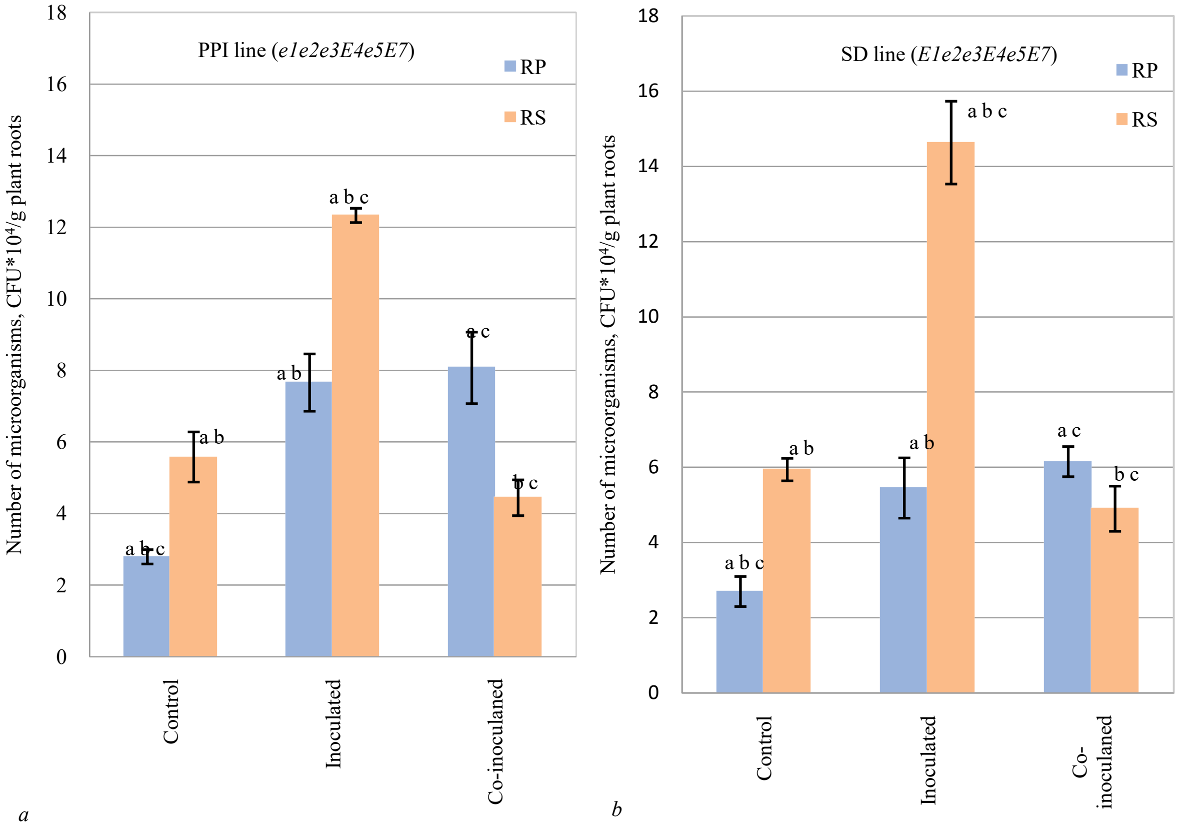
Fig. 4. Visual symptoms of nitrogen deficiency in leaves of near isogenic by *E* genes lines of soybean (*Glycine max* (L.) Merr.) in the early flowering phase grown from seeds inoculated with nitrogen-fixing bacteria: *a* – photoperiodic insensitive line, non-inoculated group, *b* – photoperiodic insensitive line, single inoculation with *B. japonicum* strain 634b, *c* – photoperiodic insensitive line, co-inoculated with *B. japonicum* + *A. chroococcum*, *d* – short-day line, non-inoculated group, *e* – short-day line, single inoculation with *B. japonicum* strain 634b, *f* – short-day line, co-inoculated with *B. japonicum* + *A. chroococcum*

Figure 5 shows influence of complex seed bacterization on the number of nitrogen-fixing bacteria in the rhizosphere and rhizoplane. Figure 5a shows that single inoculation seeds by *B. japonicum* strain 634b significantly increased the number of *B. japonicum* in the rhizoplane (on average by 174.5%) and rhizosphere (on average by 121.0%) in the photoperiodical insensitive soybean line. Seed co-inoculation increased the number of *B. japonicum* in the rhizoplane (by an average of 189.2%), but reduced the content of *B. japonicum* in the rhizosphere by 20.4% compared to the number of microorganisms in the control. Seed inoculation increased the number of *B. japonicum* in the rhizosphere (by an average of 177.7%) compared to the number of microorganisms in the co-inoculation variant of the experiment (Fig. 5a).

Single inoculation seeds by *B. japonicum* strain 634b significantly increased the number of *B. japonicum* in the rhizoplane (on average by

101.9%) and rhizosphere (on average by 146.3%) in the short-day soybean line (Fig. 5b). Seed co-inoculation significantly increased the number of *B. japonicum* in the rhizoplane (on average by 127.8%), but reduced the content of *B. japonicum* in the rhizosphere by 17.5% compared to the number of microorganisms in the control. Also, seed inoculation increased the number of *B. japonicum* in the rhizosphere of the SD soybean line (by an average of 198.5%) compared to the number of microorganisms in the co-inoculation variant of the experiment (Fig. 5b).

The largest amount of *B. japonicum* was in the rhizoplane of the PPI soybean line, and the largest amount of *B. japonicum* in the rhizosphere of the SD soybean line (Fig. 5a, b). Figure 6a shows that single inoculation of seeds by *B. japonicum* strain 634b significantly reduced the number of *A. chroococcum* in the rhizoplane (on average by 43.3%) and rhizosphere (on average by 52.6%) in the photoperiodical insensitive soybean line and in the rhizosphere (on average by 37.4%) in the short-day soybean line (Fig. 6b). Seed inoculation by *B. japonicum* strain 634b significantly reduced the number of *A. chroococcum* in the rhizosphere (by an average of 60.5%) in the photoperiodical insensitive soybean line compared to the number of microorganisms in the co-inoculation variant of the experiment (Fig. 6a).



a *b*

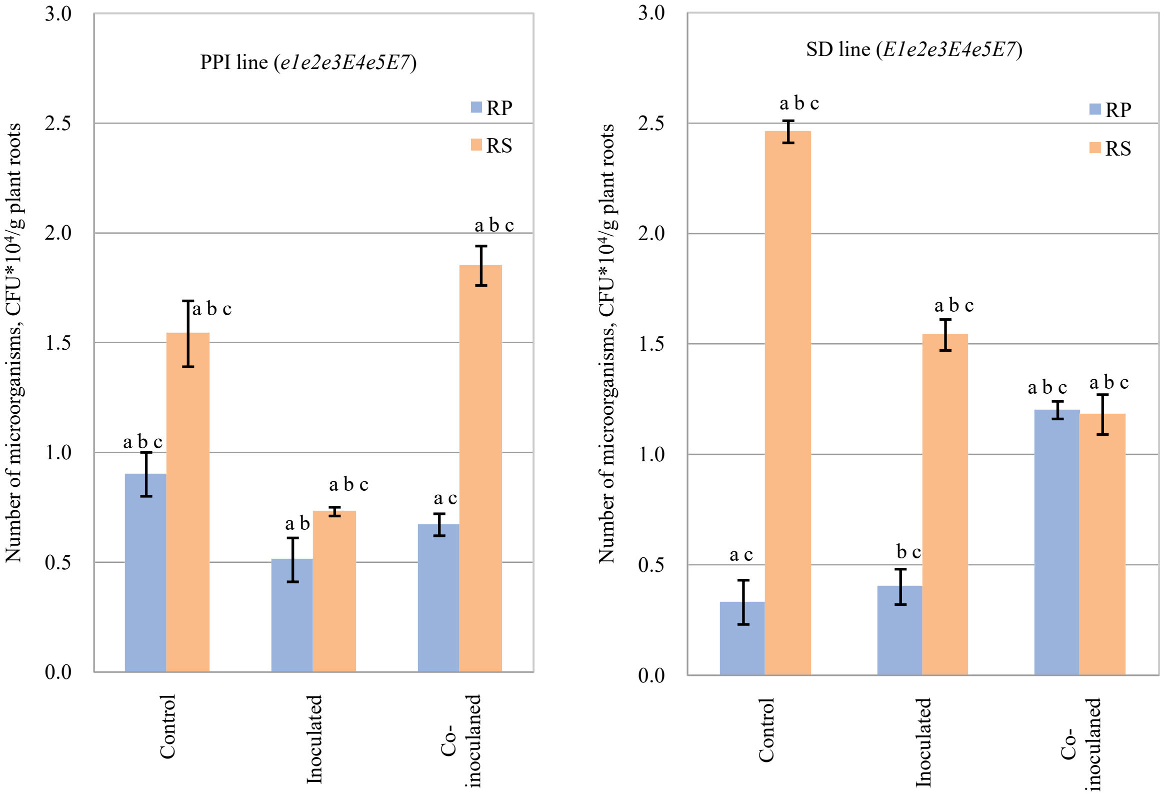
Fig. 5. The colony forming units (CFU) bacteria *B. japonicum* in the rhizoplane (RP) and rhizosphere (RS) of near isogenic by *E* genes lines of soybean (*Glycine max* (L.) Merr.) on the nutrient MDA medium, CFU*10^4/g plant roots: control – non-inoculated, inoculated – single inoculation with *B. japonicum* strain 634b, co-inoculated – co-inoculation with *B. japonicum* + *A. chroococcum*, comparisons were made within the isogenic line of soybean, means in each column followed by different letters are not significantly different one from another on the results of comparison using the Tukey test ($P < 0.05$) with Bonferroni correction, ($x \pm SD$, $n = 3$)

Seed co-inoculation significantly increased the number of *A. chroococcum* in the rhizoplane (on average by 263.5%), but significantly reduced the content of *A. chroococcum* in the rhizosphere (on average by 52.0%) in the short-day soybean line compared to the number of microorganisms in the control (Fig. 6b).

Discussion

The increasing world population and the awareness of potential impacts of human activities on global weather changes demand that agriculture becomes more efficient. Technologies must be developed that guarantee output and food supply, but cause the least, if any, alterations in the natural landscape, and make the area already claimed by agricultural activities more productive (O'Callaghan, 2016; Fagorzi et al., 2021). Fertilization has long been known to increase efficiency in agriculture, as a means to improve plant nutrition (Zhang et al., 2015). N deficiency is a limiting factor in many places of the world, demanding heavy fertilization, but the supply of N to plants can be increased by using biological N_2 fixation, especially when legumes are the main crop or take part in rotations with cereals (Hungria et al., 2015; Ohyama et al., 2017; Wang et al., 2020). Rhizobia are Gram-negative rod-shaped root nodule bacteria with a single polar flagellum. They are aerobic chemoorganotrophs, they grow on simple carbohydrate and amino acids in the presence of O_2. Very few strains of bacteria are able to fix free N, which needs a specialized structure in the cortical area of the leguminous plant containing rhizobia. These symbiotic nitrogen-fixing bacteria are hosted in nodules and they induce the roots of legumes and permit the plant to utilize atmospheric nitrogen. For over a century legume inoculation has been used to improve legume yield and productivity (Kots et al., 2011; Ohyama et al., 2017). The soil volume affected by roots – the rhizosphere – is one of the most important microbial hotspots determining the processes, dynamics and cycling of carbon (C), nutrients and water in terrestrial ecosystems. Rhizosphere visualization is necessary to understand, localize and quantify the ongoing processes and functions, but quantitative conclusions are very uncertain because of: (1) the continuum of the parameters between the root surface and root-free soil, i.e., there are no sharp borders, (2) differences in the distributions of various parameters (C, nutrients, pH, enzyme and microbial activities, gases, water etc.) across and along roots, (3) temporal changes of the parameters and processes with root growth as well as with water and C flows (Kuzyakov & Razavi, 2019). Furthermore, Moreau suggested that studying plants from N-poor habitats may assist in better understanding of plant traits directly controlling N-cycling microorganisms. Indeed, if we are to transition from high-input systems to low-input systems and exploit microbial properties that might be valuable in low-input sustainable systems, we need more information about those systems – not just conventional agricultural systems where large amounts of mineral

N inputs are commonly used. In addition, significant work is still required to better understand the spatial and temporal dynamics of N turnover and root N uptake in the rhizosphere and its association with plant traits (Moreau et al., 2019). The results of our studies show that the inoculation of seeds, under conditions of nitrogen deficiency, provides faster formation of the symbiotic soybean-*Bradyrhizobium* complex. This fact is known and shown by many studies (Melnykova et al., 2002; Kots et al., 2011; Ohyama et al., 2017) and is widely used when recommending the joint use of inoculants and the use of reduced doses of nitrogen fertilizers (Hungria et al., 2020). In the process of infection of the root system of legumes, the virulence of nodule bacteria is important. If the specificity determines the spectrum of rhizobia, the virulence characterizes the activity of their action within a certain spectrum. The term virulence, denotes the ability of nodule bacteria to penetrate into the root tissue, multiply there and cause the formation of nodules. The virulence of the studied rhizobia was determined by the number of initiated nodules of different sizes and *B. japonicum* strain 634b showed high activity of forming the symbiosis with both soybean lines.



a *b*

Fig. 6. The colony forming units (CFU) bacteria *A. chroococcum* in the rhizoplane (RP) and rhizosphere (RS) of near isogenic by *E* genes lines of soybean (*Glycine max* (L.) Merr.) on the nutrient Ashby medium, CFU*10^4/g plant roots: control – non-inoculated, inoculated – single inoculation with *B. japonicum* strain 634b, co-inoculated – co-inoculation with *B. japonicum* + *A. chroococcum*, comparisons were made within the isogenic line of soybean, means in each column followed by different letters are not significantly different one from another on the results of comparison using the Tukey test ($P < 0.05$) with Bonferroni correction ($x \pm SD$, $n = 3$)

Fagorzi et al. (2021) showed that the response to root exudates involved hundreds of changes in the rhizobium transcriptome. Of the differentially expressed genes, 35% were influenced by the strain genotype, 16% were influenced by the plant genotype, and 29% were influenced by strain-by-host plant genotype interactions. Differences in nodulation rates between the photoperiodic insensitive (PPI) line with genotype *e1e2e3 (E4e5E7)* and the short-day (SD) line with genotype *E1e2e3(E4e5E7)* were identified and it probably shows that *E1* alleles interact with pathways which take part in control of forming nodules. Ferguson & Mathesius (2014) show that nodules are highly organized root organs that form in response to Nod factors produced by rhizobia, and they provide rhizobia with a specialized niche to optimize nutrient exchange and nitrogen fixation. Nodule development and invasion by rhizobia is locally controlled by feedback between rhizobia and the plant host. In addition, the total number of nodules on a root system is controlled by a systemic mechanism termed 'autoregulation of nodulation'. Both the local and the systemic control of nodulation are regulated by phytohormones (Ferguson & Mathesius, 2014). But the results of our studies show that the inoculation of seeds under conditions of nitrogen deficiency significantly reduces the mass of the root system in the PPI line and SD-line plants compared to the control non-inoculated variant of the experiment. Co-inoculation slightly reduced the efficiency of the formation of the symbiotic apparatus, and stimulated the growth of the root system. This may indicate that soybean plants can control the number of nodules formed, based on the need for nitrogen and the amount of carbohydrates formed. In addition, starch content in leaves was significantly lower under conditions of inoculation, compared with the control. That may indicate a higher level of use of carbohydrates, since assimilative starch does not accumulate in the leaves.

In the current study we established that the nitrogen deficiency stimulated development of the root system of plants and the synthesized sugars were distributed predominantly to the root system growth (Table 1). Sucrose from the shoot is converted to malate in the plant and imported across the symbiosome membrane and into bacteroids, where it fuels nitrogen fixation. The product of the nitrogen fixation is then exported back to the plant, where it is assimilated into ureides for export to the shoot (Udvardi & Poole, 2013). We suppose that inoculation of seeds had extended sugar consumption to the symbiont due to which it compensates the lack of nitrogen, but leads to a slower growth of the root system. Rhizobial colonization of developing nodules takes just a few days and a visible root nodule begins to be seen (1 to 2 weeks after infection). Within the root nodule, the rhizobia enlarge and elongate to perhaps five times the normal size of rhizobia and change physiologically to forms known as bacteroids. Inoculation of seeds under conditions of nitrogen deficiency caused formation of large nodules located mainly on the main root, the low amount of microorganisms in the control caused formation of single small nodules which were situated only on the lateral roots (Fig. 3). Root nodules are lateral root organs, and like lateral roots they influence the architecture of the root system, then when they are exposed to fluctuations in the soil environment such as drought or nitrate or phosphate availability, roots activate compensating mechanisms ranging from changes in nutrient uptake ability to changes in root architecture (Desbrosses & Stougaard, 2011; Herrbach et al., 2014). Total N content in leaves significantly increased only in the variant with a single inoculation (Table 2). Furthermore, in non-inoculated plants of the experiment, the leaves of the lower tier visually showed pronounced signs of nitrogen deficiency, but in inoculated and co-inoculated plants there were no signs of nitrogen deficiency (Fig. 4) and these results are consistent with previous reports showing that the effect of nitrogen deficiency reduces the number of days from sowing to flowering (Kots et al., 2011). In our research non-inoculated plants flower 3–5 days earlier than plants grown from bacterized seeds.

Determination of the amount of *B. japonicum* on MDA elective medium in the rhizosphere and rhizoplane showed that inoculation by *B. japonicum* strain 634b caused a significant increase in the amount of *B. japonicum* in the rhizosphere and rhizoplane in both soybean lines, in comparison with non-inoculated seeds (Fig. 5a, b). Then, co-inoculation by *B. japonicum* strain 634b + *A. chroococcum* significantly increased the amount of *B. japonicum* only in the rhizoplane and decreased their number in the rhizosphere. Scarcity of natural nitrogen fixers in the rhizosphere may constrain the diazotrophic contribution of fixed nitrogen to plants. Determination of the amount of *A. chroococcum* on the Ashby elective medium in the rhizosphere and rhizoplane showed that the inoculation by *B. japonicum* strain 634b caused a significant decrease in the amount of *A. chroococcum* both in the rhizosphere and in the rhizoplane of the PPI line of soybean, and in the rhizosphere of the SD line, in comparison with non-inoculated seeds (Fig. 6a, b). However, the co-inoculation by *B. japonicum* strain 634b + *A. chroococcum* in the SD line significantly increased the number of *A. chroococcum* in the rhizoplane and decreased their number in the rhizosphere, in the PPI line their number decreased in the rhizoplane and increased in the rhizosphere, in comparison with non-inoculated seeds. We observed that the inoculant *B. japonicum* strain 634b suppresses the growth of *A. chroococcum* both in the rhizosphere and in the rhizoplane (Fig. 6a, b). That can testify to the competitive interaction of these microorganisms.

Conclusion

In conclusion, it was established that the nitrogen deficiency stimulated development of the root system of plants and the synthesized sugars were distributed predominantly to the root system growth. Probably, the E genes (their dominant and/or recessive state) of soybean isolines affect the regulation of the content and distribution of carbohydrates. We suppose that inoculation of seeds had extended sugar consumption to the symbiont, due to which it compensates the lack of nitrogen, but leads to a slower growth of the roots. Nitrogen deficiency reduced the number of days from sowing to flowering in both soybean lines by 3–5 days compared to plants grown from bacterized seed. The obtained results proved the potential of the *B. japonicum* strain 634b inoculant for soybean in terms of increased symbiotic performance, especially under nitrogen deficiency conditions. This study indicated that inoculants could provide faster formation of the symbiotic soybean – *Bradyrhizobium* complex. Differences in nodulation rates between photoperiodic insensitive line with genotype *e1e2e3 (E4e5E7)* and short-day line with genotype *E1e2e3(E4e5E7)*, were identified and it probably shows that *E1* alleles interact with the pathway which take part in control of forming nodules. As a result of the performed studies, we determined antagonistic activity of *B. japonicum* strain 634b towards local population of diazotrophs of the genus *Azotobacter* spp. Futhermore, determination of the amount of *B. japonicum* on the medium of mannitol-yeast agar in the rhizosphere and rhizoplane showed that inoculation by *B. japonicum* strain 634b caused a significant increase in the amount of *B. japonicum* in the rhizosphere and rhizoplane in both soybean lines, in comparison with non-inoculated seeds. In addition, the studies carried out under controlled conditions will need to be extended into the field to test yield. The search for systemic interactions between host genes and strains of effective nitrogen fixers will lead to efficient production of crops and accelerate the development and application of these technologies for sustainable intensification of crop production.

References

Aranjuelo, I., Arrese-Igor, C., & Molero, G. (2014). Nodule performance within a changing environmental context. Plant Physiology, 171(12), 1076–1090.

Bezrutczyk, M., Yang, J., Eom, J., Prior, M., Sosso, D., Hartwig, T., Szurek, B., Oliva, R., Vera-Cruz, C., White, F. F., Yang, B., & Frommeret, W. B. (2018). Sugar flux and signaling in plant-microbe interactions. The Plant Journal, 93, 675–685.

Ciereszko, I. (2018). Regulatory roles of sugars in plant growth and development. Acta Societatis Botanicorum Poloniae, 87(2), 1257–1268.

Cober, E. R., Curtis, D. F., Stewart, D. W., & Morrison, M. J. (2014). Quantifying the effects of photoperiod, temperature and daily irradiance on flowering time of soybean isolines. Plants, 3(4), 476–497.

Cober, E. R., Molnar, S. J., Charette, M., & Voldeng, H. D. (2010). A new locus for early maturity in soybean. Crop Science, 50, 524–527.

Desbrosses, G. J., & Stougaard, J. (2011). Root Nodulation: A paradigm for how plant-microbe symbiosis influences host developmental pathways. Cell Host Microbe, 10(4), 348–358.

Dong, S. H., Nhu-Lam, M., Nagarajan, R., & Nair, S. K. (2020). Structure-guided biochemical analysis of quorum signal synthase specificities. ACS Chemical Biology, 15(6), 1497–1504.

Fagorzi, C., Bacci, G., Huang, R., Cangioli, L., Checcucci, A., Fini, M., Perrin, E., Natali, C., diCenzo, G. C., & Mengoni, A. (2021). Nonadditive transcriptomic signatures of genotype-by-genotype interactions during the initiation of plant-rhizobium symbiosis. mSystems, 6(1), e00974-20.

Ferguson, B. J., & Mathesius, U. (2014). Phytohormone regulation of legume-rhizobia interactions. Journal of Chemical Ecology, 40(7), 770–790.

Gano-Cohen, K. A., Stokes, P. J., Blanton, M. A., Wendlandt, C. E., Hollowell, A. C., Regus, J. U., Kim, D., Patel, S., Pahua, V. J., & Sachs, J. L. (2016). Nonnodulating *Bradyrhizobium* spp. modulate the benefits of legume-rhizobium mutualism. Applied and Environmental Microbiology, 82(17), 5259–5268.

Han, Q., Ma, Q., Chen, Y., Tian, B., Xu, L., Bai, Y., Chen, W., & Li, X. (2020). Variation in rhizosphere microbial communities and its association with the symbiotic efficiency of rhizobia in soybean. The ISME Journal, 14, 1915–1928.

Harris, J. M., Balint-Kurti, P., Bede, J. C., Day, B., Gold, S., Goss, E. M., Grenville-Briggs, L. J., Jones, K. M., Wang, A., Wang, Y., Mitra, R. M., Sohn, K. H., & Alvarez, M. E. (2020). What are the top 10 unanswered questions in molecular plant-microbe interactions? Molecular Plant-Microbe Interactions, 33(12), 1354–1365.

Herrbach, V., Remblière, C., Gough, C., & Bensmihen, S. (2014). Lateral root formation and patterning in *Medicago truncatula*. Journal of Plant Physiology, 171(3–4), 301–310.

Hungria, M., Nogueira, M. A., & Araujo, R. S. (2015). Alternative methods of soybean inoculation to overcome adverse conditions at sowing. African Journal of Agricultural Research, 10(23), 2329–2338.

Hungria, M., Nogueira, M. A., Campos, L. J. M., Menna, P., Brandi, F., & Ramos, Y. G. (2020). Seed pre-inoculation with *Bradyrhizobium* as time-optimizing option for large-scale soybean cropping systems. Agronomy Journal, 112(6), 5222–5236.

Kots, S. Y., Morgun, V. V., Patyka, V. F., Malichenko, S. M., Mamenko, P. M., Kiriziy, D. A., Mikhalkiv, L. M., Beregovenko, S. K., & Melnykova, N. M. (2011). Biologicheskaya azotfiksatsiya: Bobovo-rizobial'nyy simbioz [Biological nitrogen fixation: Legume-rhizobial symbiosis]. Logos, Kiyv (in Russian).

Kuzyakov, Y., & Razavi, B. S. (2019). Rhizosphere size and shape: Temporal dynamics and spatial stationarity. Soil Biology and Biochemistry, 135, 343–360.

Li, J., Wu, L., Foster, R., & Ruan, Y.-L. (2017). Molecular regulation of sucrose catabolism and sugar transport for development, defence and phloem function. Journal of Integrative Plant Biology, 59, 322–335.

Liu, X., Wu, J.-A., Re, H., Qi, Y., Li, C., Cao, J., Zhang, X., Zhang, Z., Cai, Z., & Gai, J. (2017). Genetic variation of world soybean maturity date and geographic distribution of maturity groups. Breeding Science, 67(3), 221–232.

Melnykova, N. N., Bulavenko, L. V., Kurdish, I. K., Titova, L. V., & Kots, S. Y. (2002). Formation and function of the legume-rhizobium symbiosis of soybean plants while introducing bacterial strains from the genera *Azotobacter* and *Bacillus*. Applied Biochemistry and Microbiology, 38(4), 368–372.

Moreau, D., Bardgett, R. D., Finlay, R. D., Jones, D. L., & Philippot, L. (2019). A plant perspective on nitrogen cycling in the rhizosphere. Functional Ecology, 33, 540–552.

Nag, P., Shriti, S., & Das, S. (2019). Microbiological strategies for enhancing biological nitrogen fixation in nonlegumes. Journal of Applied Microbiology, 129, 186–198.

O'Callaghan, M. (2016). Microbial inoculation of seed for improved crop performance: Issues and opportunities. Applied Microbiology and Biotechnology, 100, 5729–5746.

Ohyama, T., Tewari, K., Ishikawa, S., Tanaka, K., Kamiyama, S., Ono,Y., Hatano, S., Ohtake, N., Sueyoshi, K., Hasegawa, H., Sato, T., Tanabata, S., Nagumo, Y., Fujita, Y., & Takahashi, Y. (2017). Role of nitrogen on growth and seed yield of soybean and a new fertilization technique to promote nitrogen fixation and seed yield. In: Soybean – the basis of yield, biomass and productivity. IntechOpen.

Rumyantseva, M. L. (2019). Kluben'kovyye bacterii: Perspectivy monitoringa simbioticheskikh svoystv i stressoustoychivosti s ispol'zovaniem geneticheskikh markerov [Root nodule bacteria: Perspectives of monitoring symbiotic properties by applying genetic markers]. Sel'skokhozyaistvennaya Biologiya, 54(5), 847–862 (in Russian).

Udvardi, M., & Poole, P.S. (2013). Transport and metabolism in legume-rhizobia symbioses. Annual Review of Plant Biology, 64, 781–805.

Vorobey, N. A., & Kots, S. Y. (2018). Ctrategiya otbora klubenkovykh bakteriy *Br. japonicum* po uluchshennomu simbioticheskomu fenotipu [Selection strategy for improved symbiotic phenotypes of *Br. japonicum*]. Fiziologiya Rasteniy i Genetika, 50(4), 344–357 (in Russian).

Wang, J., Li, R., Zhang, H., Wei, G., & Li, Z. (2020). Beneficial bacteria activate nutrients and promote wheat growth under conditions of reduced fertilizer application. BMC Microbiology, 20(38), 1–12.

Yermakov, A. I., Arasimovich, V. V., & Yarosh, N. P. (1987). Metody biokhimicheskogo issledovaniya rastenii [Methods of biochemical research of plants]. Agropromizdat, Leningrad (in Russian).

Zhang, X., Davidson, E., Mauzerall, D. L., Searchinger, T. D., Dumas, P., & Shen, Y. (2015). Managing nitrogen for sustainable development. Nature, 528, 51–59.