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CHARACTERIZATION OF BRADYRHIZOBIA ASSOCIATED WITH SOYBEAN PLANTS GROWN IN UKRAINE

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ABSTRACT

The phenotypic and genotypic features of soybean nodule bacteria with different growth rates common in soils of Ukraine have been studied. The obtained results suggest high heterogeneity of soybean microsymbionts. It has been established that all studied strains are able to form active symbiotic relationships with soybean. When interacting with Chinese cowpea, intensive-growing strains form an ineffective symbiosis with a low nitrogen fixation level (phenotype Nod^+Fix^-), while slow-growing strains form complete nitrogen fixing systems (phenotype Nod^+Fix^+). Upon cultivation in the mannitol yeast medium, intensive-growing strain *B. japonicum* KC19 produces a significant amount of auxins and cytokinins, while the slow-growing strain *B. japonicum* KC23 is significantly superior by the amount of gibberellins. According to the organization of the genome defined in REP-PCR, studied strains of soybean rhizobia belong to two genetic groups, which corresponds to the division of strains into groups by the growth rate (slow- and intensive-growing). Based on the RFLP analysis of rpoB gene, using restriction enzymes MspI, HaeIII and NdeII, intensive-growing strains were combined into one rpoB type, while slow-growing strains belong to 2-3 rpoB types.

Keywords: *Bradyrhizobium japonicum*, phytohormones, *HPLC*, REP-PCR, RFLP analysis, rpoB gene, soybean

INTRODUCTION

Nodule bacteria (rhizobia) are the large group of microorganisms that are able to form a symbiotic relationship with leguminous plants, forming root nodules.
Recently, increasing attention has been paid to the study of soil populations of microsymbionts of different legumes (**de Bruijn, 2015; Chidebe *et al.*, 2017**). Under regular circulation between ecological niches (soil → nodules → soil) in populations of nodule bacteria, significant transformations of their spatial and genetic structures develop. Such changes can lead to the emergence of new genotypes of rhizobia (**Provorov, 2009**). To understand the mechanisms responsible for the formation of the genetic diversity of nodule bacteria in natural ecosystems and agrocenoses, detailed study of the representatives of the soil populations of these microorganisms is required.
The most common leguminous culture of world agriculture is soybean (*Glycine max* (L.) Merr.). Nodule bacteria of different genera: *Bradyrhizobium, Sinorhizobium (Ensifer)* and *Mesorhizobium* may to form a symbiotic relationship with the soybean plant. They are represented by species of bacteria with different growth rates: slow-growing *B. japonicum* (**Jordan, 1982**), *B. elkanii* (**Kuykendall *et al.*, 1992**), *B. liaoningense* (**Xu *et al.*, 1995**) and *B. yuanmingense* (**Yao *et al.*, 2002**) and fast-growing *Ensifer (Sinorhizobium) fredii*, *S. xinjiangensis* (**Peng *et al.*, 2002**) and *M. tianshanense* (**Chen *et al.*, 1995**).
It should be noted that the formation of local populations of soybean nodule bacteria in Ukrainian soils began relatively recently – since the intense introduction of soybeans in crop rotation and the use of biopreparations on the basis of specific microorganisms. Our studies have shown that soil soybean rhizobia populations differ in density and qualitative composition, and they are quite heterogeneous. As a result of the analysis of morphological, cultural, physiological, biochemical and chemotaxonomic properties of 180 strains of soybean rhizobia, isolated from soils of different regions of Ukraine, we have identified nodule bacteria, which according to genetic features belong to the species *B. japonicum*, but differ significantly from the typical slow-growing soybean microsymbionts (**Patyka *et al.*, 2010; Krutylo and Zotov, 2015**). Isolated strains are characterized by increased growth rates and conventionally called "intensive-growing strains". At day 5-6 after sowing, intensive-growing strains form large (2-4 mm diameter) translucent colonies with matte shading on the legume medium. Representatives of the second group of strains (slow-growing) accumulate bacterial mass at day 8-10 days and form small (up to 1 mm diameter) white colonies.
It has been established that intensive-growing soybean nodule bacteria are characterized by increased survival rate in the soil, but do not occur in all regions of Ukraine. The ratio between the representatives of the two groups of strains varies depending on the soil-climatic zone and the duration of soybean cultivation (**Patyka *et al.*, 2010**).
Considering the above, the objective of our work was to study the phenotypic and genotypic features of soybean nodule bacteria in soils of Ukraine.

MATERIAL AND METHODS

Microorganisms

180 strains of soybean nodule bacteria were isolated from different soil types of Ukraine (Regions of Chernihiv, Vinnytsia and Sumy) by sterile germ inoculation method. In the result of comparative analysis of morphological, cultural, physiological and biochemical properties, these strains were divided into two groups: intensive- and slow-growing strains. The objects of this study were strains – typical representatives of soybean nodule bacteria with slow (*B. japonicum* 46, KC23, KH10) and intensive (*B. japonicum* KB11, KC19, KC22) growth rates. The typical strain of slow-growing nodule bacteria *B. japonicum* VKM B-1967 = USDA 6^T = ATCC 10324^T also used in this work. Rhizobial strains are stored in the collection of the Laboratory of Plant-Microbial Interactions and in the Collection of useful soil microorganisms at the Institute of Agricultural Microbiology and Agroindustrial Manufacture of the National Academy of Sciences of Ukraine (*B. japonicum* 46 = B-23, KH10 = B-124).

Vegetation experiments conditions

In vegetation experiments, we studied the ability of soybean nodule bacteria to form a symbiotic relationship with soybean (*Glycine max* (L.) Merr.) and cowpea (*Vigna unguiculata* (L.) Walp.). Cultivation of nodule bacteria was carried out in flasks (750 ml) on liquid medium (for 72 hours), containing (g/l): K_2HPO_4 – 0.5,

KH_2PO_4 – 0.5, $(NH_4)_2SO_4 \cdot 7H_2O$ – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.2, NaCl – 0.2, $CaCO_3$ (sterile) – 0.1, sucrose – 2.0, mannitol – 3.0, glucose – 10.0, broth of peas (peas seeds – 50 g per 1 litre of water) – 100.0 ml/l; pH 7.0-7.2. The titre of bacteria was $2 \cdot 10^9$ CFU/ml. Inoculation load was 200–300 thousand cells per 1 seed. Experiments were conducted in the greenhouse in 2.5 L vessels. The sterile vermiculite was used as a nitrogen-free substrate. The repetition of the experiment was sixfold. Activity of symbiotic nitrogen fixation was measured in flowering phase by acetylene method (**Hardy *et al.*, 1968**).

Assay of phytohormones activity

Cultivation of bacteria was carried out in flasks (750 ml) on a rotary shaker (220 rev/min) at 26-28˚C on liquid mannitol–yeast digest medium with such composition (g/l): mannitol – 10.0; yeast extract – 2.0; calcium gluconate – 1.5; K_2HPO_4 – 0.5; $MgSO_4$ – $7H_2O$ – 0.2; NaCl – 0.1; $FeCl_3$ $6H_2O$ – 0.01; pH 7.2. Cultures of *B. japonicum* in the exponential growth phase were used as the inoculum. The amount of inoculum was 5% volume of the medium.

To separate the biomass, the cultural liquid of bacteria was centrifuged for 20 min at 9000 rpm and at + 4˚C. Cells of bacteria were washed with saline solution thrice in order to clean them from exopolymer residues, each time they were centrifuged under the same conditions. The supernatants were used for further research to extract phytohormonal compounds. The cells biomass was suspended in distilled water, then dried at 103-105˚C in a desiccator until constant weight. The amount of absolutely dry biomass (ADB) of microorganisms was determined gravimetrically.

Extracellular phytohormones were isolated from supernatants of nodule bacteria by the method described in (**Methodical recommendations by phytohormones definition, 1988**). Assay of phytohormones in the cultural medium was performed using high performance liquid chromatography (HPLC) on liquid chromatograph Agilent 1200 LC (USA) and mass spectral detector Agilent G1956B.

The standards were synthetic phytohormones *Sigma–Aldrich* (Germany) and *Acros Organic* (Belgium): *auxins* – indole-3-acetic acid, indole-3-butyric acid, indole-3-carboxylic acid, indole-3-carbinol, indole-3-acetic hydrazide, indole-3-carboxaldehyde; *cytokinins* – zeatin, *trans*-zeatin-riboside, kinetin, (**N^6-(2-Isopentenyl)adenine, N^6-(2-Isopentenyl)adenosine;** *abscisic acid*; *gibberellins* – GA_3, GA_4.

Chromatogram calculation was performed using the ChemStation software. Amount of phytohormones was calculated in µg per 1 g of absolutely dry biomass of producer.

DNA isolation

Soybean nodule bacteria were cultivated in agar medium TY (**Beringer, 1974**). The total DNA of the rhizobia strains was isolated from the fresh cultures (the exponential growth phase) by the lysis of the bacterial cells lysozyme-SDS with further phenol-chloroform extraction and precipitation with the use of isopropanol (**Laguerre *et al.*, 1992)**.

REP-PCR analysis

REP-PCR (repetitive extragenic palindromic sequences) was performed for genotyping and to detect the identity of soybean nodule bacteria strains. The amplification was conducted with the use of REP-primers: REP1R-I (5′-iiiicgicgicatciggc-3′) and REP2-I (5′-icgicttatciggcctac-3′). PCR were performed using the standard reaction mixture (**Versalovic *et al.*, 1994**). The temperature-time profile of amplification: denaturation at 94°C for 3 min, 40 cycles consisting of 30 sec at 94°C, 30 sec at 37°C, 3 min at 68°C, final elongation for 5 min at 68°C. The PCR products were separated by horizontal electrophoresis on 1.5% agarose. Gels were stained with ethidium bromide, visualized under UV radiation and photographed.

RFLP analysis of the rpoB gene

The amplification of the rpoB gene of soybean nodule bacteria was conducted with the use of primers rpoB83F: 5′-cctcatcgaggttcagaaggc-3′ and rpoB1061R: 5′-agcgtgttgcggatataggcg-3′ (**Martens *et al.*, 2008**). The temperature-time profile of amplification: denaturation at 94°C for 5 min, 4 cycles consisting of 2 min at 94°C, 2 min at 58°C, 1 min at 72°C, and them 31 cycles consisting of 30 sec at 94°C, 1 min at 58°C, 1 min at 72°C, final elongation for 7 min at 72°C.

The restriction analysis (RFLP) was carried out with the use of restriction endonuclease MspI, HaeIII, NdeII (Fermentas, USA) according to the manufacturer's instruction. DNA processed by restrictase was analyzed with the use of electrophoresis in 2.5% agarose gel.

Statistical data analysis

Microsoft Excel was used to analyze data on the average of four replicates (±SE). Comparison of *REP-PCR* fingerprints was performed using FPQuest software (Bio Rad, USA). The degree of similarity of the fingerprints was assessed by Pearson's correlation coefficient.

Fingerprint analysis of the RFLP profiles was carried out using Total Lab v. 2.01 software. Following installation of the gel images into the program, the bands were determined with their pixel positions and their molecular weights were scored according to the molecular size marker.

RESULTS AND DISCUSSION

Symbiotic characteristic of soybean nodule bacteria

In the work, strains of soybean nodule bacteria with slow and intense growth, common in soils of different regions of Ukraine were used (Tab 1). Under vegetative experiments, the specificity of the symbiosis of slow- and intensive-growing *B. japonicum* strains with two legume cultures was studied: soybean as a host plant, and Chinese cowpea. It is known that cowpea belongs to a symbiotically unspecialized plant species and can form nodules with representatives of several genera of nodule bacteria: *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* (**Fotev *et al.*, 2016; Tampakaki *et al.*, 2017**). Therefore, studied strains of soybean rhizobia may be potential microsymbionts of this plant.

The analysis of the results showed that all strains, regardless of the growth rate, formed effective symbiosis with soybean (phenotype Nod^+Fix^+), forming nodules with high nitrogen fixation activity (25.9-30.5 µg N per plant per 1 hour). We have detected significant differences between strains when they interact with Chinese cowpea. Slow-growing *B. japonicum* strains VKM B-1967, 46, KH10, KC23 formed well-developed nitrogen fixing nodules (phenotype Nod^+Fix^+) on the roots of plants, whereas intensive-growing strains of *B. japonicum* KB11, KC19, and KC22 predominantly formed inactive white nodules (phenotype Nod^+Fix^-). Nitrogenase activity of the cowpea nodules in the variants with inoculation using intensive-growing *B. japonicum* strains was 4.3-6.1 times lower compared to the slow-growing strains with Nod^+Fix^+ phenotype, which fixed the molecular nitrogen at 13.0-21.9 µg N per plant per 1 hour. We believe that revealed differences may indicate the formation of a nonspecific symbiosis between intensive-growing strains of soybean rhizobia and Chinese cowpea.

Table 1 Symbiotic characteristic of soybean nodule bacteria with different growth rate intensity

Strains	Geographical origin	Symbiotic phenotypes	
		Glycine max	*Vigna unguiculata*
B. japonicum VKM B-1967 *	Japan	Nod^+Fix^+	Nod^+Fix^+
B. japonicum 46*	Ukraine, Vinnytsia region	Nod^+Fix^+	Nod^+Fix^+
B. japonicum KH10*	Ukraine, Chernihiv region	Nod^+Fix^+	Nod^+Fix^+
B. japonicum KC23*	Ukraine, Sumy region	Nod^+Fix^+	Nod^+Fix^+
B. japonicum KB11	Ukraine, Vinnytsia region	Nod^+Fix^+	Nod^+**Fix**$^-$
B. japonicum KC19	Ukraine, Sumy region	Nod^+Fix^+	Nod^+**Fix**$^-$
B. japonicum KC22	Ukraine, Sumy region	Nod^+Fix^+	Nod^+**Fix**$^-$

Legend: * – slow-growing strains of soybean nodule bacteria, Nod^+Fix^+ – formation of nitrogen-fixing nodules; Nod^+Fix^- – formation of non-nitrogen fixing nodules.

Thus, studied soybean nodule bacteria with slow and intense growth differ in their symbiotic specificity.

Phytohormones activity

It is known that phytohormonal substances have a significant influence on the symbiosis of microorganisms and macroorganisms. Study of the spectrum phytohormones synthesised by the strains of nodule bacteria is important in

evaluating their symbiotic potential and identifying the features of interaction with host plants (**Spaepen *et al*., 2007; Leonova *et al*. 2013; Held *et al*., 2014**). In our work, we studied the ability of soybean rhizobia strains with slow (*B. japonicum* KC23) and intense (*B. japonicum* KC19) growth to synthesise biologically active substances.

HPLC analysis showed that during cultivation in the mannitol yeast medium, *B. japonicum* strains synthesised phytohormones of auxin, cytokinin and gibberellic nature (Tab 2).

Table 2 Production of extracellular phytohormones by *Bradyrhizobium japonicum* strains with different growth rate

Phytohormones	Amount of phytohormones, µg/g of absolutely dry biomass	
	slow-growing strain *B. japonicum* KC23	intensive-growing strain *B. japonicum* KC19
Auxins, including:	**1.6**	**44.7**
indole-3-acetic acid (IAA)	0.1	5.0
indole-3-*carbinol (IC)*	–	4.3
indole-3-butyric acid (IBut)	–	–
indole-3-*acetic hydrazide (*IAA-hydr.*)*	–	28.3
indole-3-carboxaldehyde(ICal)	0.8	–
indole-3-carboxylic acid (ICA)	0.7	7.1
Cytokinins, including:	**1.4**	**17.0**
kinetin (Kin)	–	12.5
zeatin (Z)	0.1	1.3
trans-zeatin-riboside (ZR)	–	–
N^6-(2-Isopentenyl)adenine (IPA)	–	–
N^6-(2-Isopentenyl)adenosine (IPAr)	1.3	3.2
***Gibberellins*, including:**	**205.5**	**85.0**
GA_3	201.2	85.0
GA_4	4.3	–
Abscisic acid	**–**	**–**

Legend: «–» phytohormones not detected.

The quantitative and qualitative differences between strains on the level of synthesis stimulating action phytohormones have been detected. Intensive-growing strain *B. japonicum* KC19 synthesised significantly more auxins (44.7 µg/g ADB) and cytokinins (17.0 µg/g ADB) compared to the slowly-growing strain *B. japonicum* KC23 (1.6 and 1.4 µg/g ADB, respectively). A supernatant of the intensive-growing strain presented a wider range of phytohormonal substances of auxin and cytokinin nature. However, the slow-growing strain was significantly superior by gibberellin synthesis (205.5 µg/g ADB) compared to intensive-growing strain of *B. japonicum* KC19 (85.0 µg/g ADB).

Both strains *B. japonicum* KC23 and *B. japonicum* KC19 did not produce abscisic acid, which is a phytohormone able to inhibit plant growth and development. It is known that some representatives of *B. japonicum* species are capable of producing abscisic acid (**Kots et al., 2010**).

Therefore, the studied strains of soybean nodule bacteria differ in the level of the synthesis of extracellular phytohormones: intensive-growing strain *B. japonicum* KC19 produces a higher amount of auxins and cytokinins, whereas the slow-growing strain *B. japonicum* KC23 is significantly superior by the amount of gibberellins. The revealed differences can play an important role in the processes of root colonization by the rhizobia and formation of nodules.

REP-PCR amplification

It is known that REP elements (short repetitions of 32 bp) are widely used as targets for the identification of microorganisms. The result of the amplification reaction of the bacterial genome sites between the REP elements is the formation of a set of DNA fragments separated in agarose gel, which reflect the organization of the genome of a particular microorganism (**Judd *et al.*, 1993; Kozyrovskaya and Kovtunovich, 1994**).

For determination of genetic heterogeneity and detection of related groups in strains of soybean nodule bacteria, their genotyping was performed using REP-PCR. The genomic fingerprints of seven *B. japonicum* strains with slow and intense growth, obtained by amplification of their DNA with RER primers, were analysed (Fig 1).

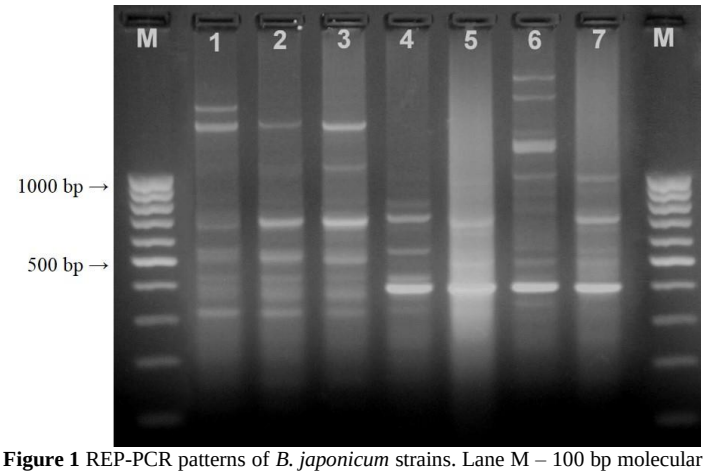

Figure 1 REP-PCR patterns of *B. japonicum* strains. Lane M – 100 bp molecular weight marker (GeneRuler 100bp DNA Ladder, Fermentas); lanes 1, 2, 3 – strains with intensive growth rate (KB11, KC19, KC22); lane 4 – type strain *B. japonicum* VKM B-1967; lanes 5, 6, 7 – strains with slow growth rate (46, KC23, KH10).

Cluster analysis of REP-PCR profiles allowed to detect significant differences in the genetic organization of soybean nodule bacterial strains with different growth rates. For example, by the DNA profiles, the studied strains were reliably divided into two clusters (Fig 2). Genetic group I included intensive-growing strains similar by RER profiles. Genome group II includes all the slow-growing strains, including the typical strain of *B. japonicum* VKM B-1967.

Within the identified genetic groups, we have also noted the variability of the nodule bacteria. For example, intensive-growing strain of *B. japonicum* KB11 differed from intensive-growing strains of *B. japonicum* KC19 and *B. japonicum* KC22, which had identical fingerprint patterns. It has been proved that the number of DNA fragments studied slow-growing strains are genetically different from the typical strain of *B. japonicum* VKM B-1967. The same REP profiles were typical for slow-growing strains of *B. japonicum* 46 and *B. japonicum* KH10.

Therefore, according to the results of genotyping, the studied strains of soybean rhizobia are genetically heterogeneous. Two separate groups of related nodule bacteria were identified, which corresponds to the division of strains into groups by the growth rate (slow and intense growth).

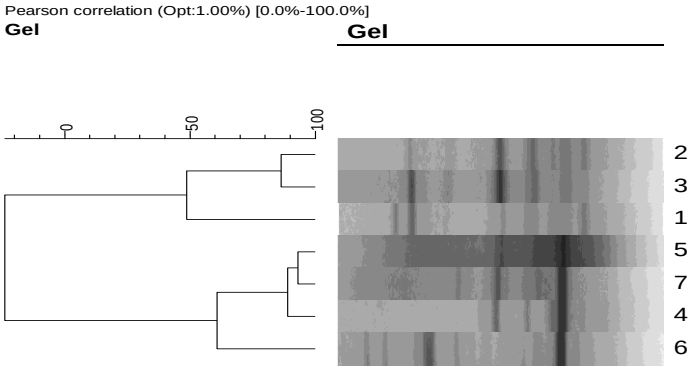

Figure 2 Dendrogram showing genetic relationships among *B. japonicum* strains with different growth rate using REP profiles: 1, 2, 3 – strains with intensive growth rate (KB11, KC19, KC22); 4 – type strain *B. japonicum* VKM B-1967; 5, 6, 7 – strains with slow growth rate (46, KC23, KH10).

PCR-RFLP analysis of the rpoB gene

Recently, in addition to 16S rRNA and 16S-23S rRNA intergenic spacer region (ITS), the analysis of functionally important genes (housekeeping genes) is widely used to identify microorganisms and assess the diversity. One of these genes is the rpoB gene encoding RNA polymerase β-subunit and used as a highly conserved marker (**Martens *et al.*, 2008; Rivas *et al.*, 2009**).

We have studied a restriction fragments length polymorphism of the rpoB gene in five strains of soybean nodule bacteria with different growth rates. By amplification of the gene in all strains, one fragment approximately 900 bp long was formed, which was then separately cleaved with restriction enzymes MspI, HaeIII and NdeII.

The restriction analysis of rpoB gene amplification products allowed to reveal a high degree of heterogeneity of studied soybean rhizobia, and as the result they were classified as different rpoB types (Tab 3).

Table 3 RFLP types and restriction fragments determined by PCR-RFLP analysis of rpoB gene of *B. japonicum* strains

Strains	Restricted fragments size (pb)	rpoB types
	MspI	
B. japonicum KB11	140–170–260	MI
B. japonicum KC19	140–170–260	MI
B. japonicum VKM B-1967*	140–160–170–260	MII
B. japonicum 46*	140–170–260	MI
B. japonicum KC23*	140–170–260	MI
	HaeIII	
B. japonicum KB11	65–90–130–160–370	HI
B. japonicum KC19	65–90–130–160–370	HI
B. japonicum VKM B-1967*	65–90–100–160–525	HII
B. japonicum 46*	65–90–130–160–525	HIII
B. japonicum KC23*	80–160–200–525	HIV
	NdeII	
B. japonicum KB11	120–170–500	NI
B. japonicum KC19	120–170–500	NI
B. japonicum VKM B-1967*	120–170–200–300	NII
B. japonicum 46*	120–170–200–300	NII
B. japonicum KC23*	100–120–130–170–200–300	NIII

Legend: * – slow-growing strain of soybean nodule bacteria

Upon the use of the MspI enzyme, intensive-growing strains (*B. japonicum* KB11, KC19) and slow-growing strains (*B. japonicum* 46, KC23) had the same restriction profiles and formed one rpoB type (MI). A typical strain of *B. japonicum* VKM B-1967 differed from the rest of the strains, and was classified as MII rpoB type.

Significant differences between strains were detected under the use of restriction endonucleases HaeIII and NdeII.

Following cleavage of rpoB gene by HaeIII restriction enzymes, four to five fragments of different sizes were formed in the studied rhizobia, which allowed the strains to be combined into four restriction types – HI-HIV. One fragment of 160 bp in size was found in all nodule bacteria. Intensive-growing strains of *B. japonicum* KB11 and KC19 had identical set of fragments and formed rpoB type HI. Strains with a slow growth of *B. japonicum* VKM B-1967, 46, KC23 were characterized by unique restriction profiles. Despite the presence of two common fragments of 525 and 160 bp in size, they are classified into different rpoB types (HII, HIII, and HIV, respectively).

Under processing of amplificates with restriction enzyme NdeII, strains with intense growth formed three DNA fragments of 500, 170 and 120 bp in size, they are combined into one rpoB type – NI. The slow-growing rhizobia of *B. japonicum* VKM B-1967 and *B. japonicum* 46 are classified as NII rpoB type with four fragments in restriction profiles. The largest number of fragments (6 units) was observed in a pattern of slow-growing strain of *B. japonicum* KC23 (NIII rpoB type).

Therefore, using RFLP analysis of rpoB gene, we revealed significant differences between the strains of soybean nodule bacteria with different growth rates. With the use of restriction enzymes, MspI, HaeIII and NdeII, intensive-growing strains were combined into one rpoB type, while the slow-growing strains were more heterogeneous, they were classified in two to three rpoB types (depending on restriction enzymes).

It should be noted that the results we have obtained regarding the heterogeneity of *B. japonicum* strains are confirmed by literary data. The authors pay attention to the high degree of polymorphism of the studied nodule bacteria by different taxonomic markers. Using genome fingerprint techniques, S. Sikora et al. found that soybean nodule bacteria, common in Croatian soils, have significant differences and belong to three genetic groups (**Sikora and Redzepović, 2003**). Significant diversity of soybean microsymbionts has been noted in Kenyan agrocenoses. The study of ITS region of nodule bacteria has allowed to classify them into thirteen ITS types. At the same time, only five ITS types representatives were dominant soybean microsymbionts (Wasike ***et al.*, 2009**). Y. *Saeki* (***Saeki,* 2011**) using different experimental approaches (denaturing gradient gel electrophoresis (DGGE), T-RLFP analysis, automated ribosomal intergenic spacer analysis (ARISA), 16S rDNA and 16S-23S rDNA sequences) described soil populations of soybean rhizobia in Japanese soils. It has been established that the diversity of local nodule bacteria depends on the geographical and climatic characteristics of the region, and also associated with the diversity of host plant genotypes. Our previous studies have shown that intensive-growing strains of *B. japonicum* KB11, KC19, KC22 belong to the genetic group USDA 123 by the structure of the intergenic region of 16S-23S rRNA (**Krutylo and Zotov, 2015**). According to many authors, the representatives of this gene group are characterized by increased saprophytic competence – they can live for a long time in the soil without the host plant (**Madrzak *et al.*, 1995; Godoy *et al.*, 2008**). Intensive-growing soybean rhizobia were also characterized by increased survival in the soil. The typical *B. japonicum* strain and the slow-growing strains that we have isolated are representatives of two genetic groups – USDA 6 (*B. japonicum* VKM B-1967, 46, KH10) and USDA 4 (*B. japonicum* KC23).

CONCLUSION

In our study we have shown that slow and intensive-growing soybean nodule bacteria, common in Ukrainian soils, differ significantly in phenotypic and genotypic features.

All studied rhizobia can form active symbiotic relationships with soybean. Upon interaction with Chinese cowpea, intensive-growing strains form an ineffective symbiosis with a low nitrogen fixation (phenotype Nod^+Fix^-), while slow-growing strains form complete nitrogen fixing systems (phenotype Nod^+Fix^+).

Upon cultivation in the mannitol-yeast medium, *B. japonicum* strains are capable of synthesizing phytohormonal substances of auxin, cytokinin and gibberellic nature. Intensive-growing strain of *B. japonicum* KC19 synthesizes a large number of auxins and cytokinins, while the slow-growing strain of *B. japonicum* KC23 is significantly superior by the amount of gibberellins.

According to the results of REP-PCR, the studied strains are classified into two separate groups of nodule bacteria, which corresponds to their division into groups by the growth rate (slow and intense growth).

The RFLP analysis of rpoB gene revealed significant differences between *B. japonicum* strains with different growth rates. Under the use of MspI, HaeIII and NdeII restriction enzymes, intensive-growing strains were combined into one rpoB type, while the slow-growing strains were more heterogeneous and classified as two to three rpoB types.

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REFERENCES

BERINGER, J.E. 1974. R1 transfer in *Rhizobium leguminosarum*. *Journal of general microbiology*, 84(1), 188-198. https://doi.org/10.1099/00221287-84-1-188

CHEN, W., WANG, E., WANG, S., LI, Y., CHEN X. 1995. Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, people's republic of China. *International Journal of Systematic and Evolutionary Microbiology*, 45(1), 153-159. https://doi.org/10.1099/00207713-45-1-153

CHIDEBE IN, JAISWAL SK, DAKORA FD. 2017. Distribution and phylogeny of microsymbionts associated with cowpea (*Vigna unguiculata*) nodulation in three agroecological regions of Mozambique. *Applied and Environmental Microbiology*, 84(2), 1-25. https://doi.org/10.1128/AEM.01712-17

DE BRUIJN, F.J. 2015. *Biological nitrogen fixation.* (Vol. 2). Hoboken, New Jersey: Wiley Blackwell.

FOTEV, YU.V., SIDOROVA, K.K., NOVIKOVA, T.I., BELOUSOVA, V.P. 2016. Study of nodulation and nitrogen fixation in two cowpea [Vigna unguiculata (L.) Walp.] cultivars inoculated with different strains of *Bradyrhizobium* sp. *Vavilov Journal of Genetics and Breeding*, 20(3), 348-354 (in Russian). https://doi.org/10.18699/VJ16.099

GODOY, L.P., VASCONCELOS, A.T.R., CHUEIRE, L.M.O., SOUZA, R.C., NICOLÁS, M.F., BARCELLOS, F.G., HUNGRIA, M. 2008. Genomic panorama of *Bradyrhizobium japonicum* CPAC 15, a commercial inoculant strain largely established in Brazilian soils and belonging to the same serogroup as USDA 123. *Soil Biology and Biochemistry*, 40(11), 2743-2753. https://doi.org/2743–2753.10.1016/j.soilbio.2008.07.016

HARDY, R.W.F., HOLSTEN, R.D., JACKSON, E.K., BURNS, R.C. 1968. The acetylene-ethylene assay for nitrogen fixation: laboratory and field evaluation. *Plant Physiology*, 43(8), 1185-1207. https://doi.org/10.1104/pp.43.8.1185

HELD, M., HOU, H., MIRI, M., HUYNH, CH., ROSS, L., HOSSAIN, M.S. SATO, S., TABATA, S., PERRY, J., WANG, T.L., SZCZYGLOWSKI, K. 2014. *Lotus japonicus* cytokinin receptors work partially redundantly to mediate nodule formation. *Plant Cell*, 26(2), 678-694. https://doi.org/10.1105/tpc.113.119362

JORDAN, D. 1982. Notes: Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *International Journal of Systematic Bacteriology*, 32, 136-139. https://doi.org/10.1099/00207713-32-1-136

JUDD A.K., SCHNEIDER M., SADOWSKI M., DE BRUIJN F.J. 1993. Use of repetitive sequences and polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* seroclaster 123 strains. *Applied and Environmental Microbiology*, 59(6), 1702-1708.

KOTS, S.YA., VOLKOGON, N.V., GRISCHUK, E.A. 2010. Ability of strains and Tn5-mutants of *Bradyrhizobium japonicum* to synthesize IAA and ABA in vitro. *Physiology and biochemistry of cultivated plants*, 42(6), 491-496 [in Russian].

KOZYROVSKAYA, N.A., KOVTUNOVICH, G.L. 1994. Molecular biological methods of detection and identification of microorganisms in the environment. *Biopolymers and cells*, 10(3–4), 5-23 (in Russian).

KRUTYLO, D.V., ZOTOV, V.S. 2015. Genotypic analysis of nodule bacteria nodulating soybean in soils of Ukraine. *Russian Journal of Genetics: Applied Research*, 5(2), 102-109. https://doi.org/10.1134/S2079059715020057

KUYKENDALL, L., SAXENA, B., DEVINE, T., UDELL, S. 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Canadian journal of microbiology*, 38, 501-505. https://doi.org/10.1139/m92-082

LAGUERRE, G., MAZURIER, S.I., AMARGER, N. 1992. Plasmid profiles and restriction fragment length polymorphism of *Rhizobium leguminosarum* bv. viceae in field populations. *FEMS Microbiology Ecology*, 101, 17-26. https://doi.org/10.1016/0378-1097(92)90693-I

LEONOVA, N.O., DANKEVICH, L.A., DRAGOVOZ, I.V., PATYKA, V.F., IUTYNSKA, G.O. 2013. Synthesis of extracellular phytohormones-stimulators by nodule bacteria and bacteria phytopathogenic for soybean. *Reports of the NASU*, 3, 165-171. (in Ukrainian).

MADRZAK, C.J., GOLINSKA, B., KROLICZAK, J., PUDELKO, K., LAZEWSKA, D., LAMPKA, B., SADOWSKY M.J. 1995. Diversity among field populations of *Bradyrhizobium japonicum* in Poland. *Applied and Environmental Microbiology*, 61(4), 1194-1200.

MARTENS, M.; DAWYNDT, P.; COOPMAN, R.; GILLIS, M.; DE VOS, P.; WILLEMS, A. 2008. Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). *International Journal of Systematic and Evolutionary Microbiology*, 58, 200-214. https://doi.org/10.1099/ijs.0.65392-0

Methodical recommendations by phytohormones definition. (1988). Kyiv: Institute of Botany AS of USSR (in Russian).

PATYKA, V.F., KRUTYLO, D.V., NADKERNYCHNA, O.V., KOVALEVSKA, T.M., SPYRIDONOV V.G., VOLKOVA I.V. 2010. Phenotypic and genotypic signs of soybean nodule bacteria widespread in soils of Ukraine. *Reports NAS of Ukraine*, 8, 167-172 (in Ukrainian).

PENG, G.X., TAN, Z.Y., WANG, E.T., REINHOLD-HUREK, B., CHEN, W.F., CHEN W.X. 2002. Identification of isolates from soybean nodules in Xinjiang Region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. *International Journal of Systematic and Evolutionary Microbiology*, 52, 457-462. https://doi.org/10.1099/ijs.0.01921-0

PROVOROV, N.A. 2009. Evolution of plant-microbial symbioses: phylogenetic, population, genetic and selection aspects. *Extended abstract of doctoral dissertation by speciality* 03.00.15 – genetic. St.-Petersburg (in Russian).

RIVAS, R., MARTENS, M., DE LAJUDIE, P., WILLEMS, A. 2009. Multilocus sequence analysis of the genus *Bradyrhizobium*. *Systematic and Applied Microbiology*, 32, 101-110. https://doi.org/10.1016/j.syapm.2008.12.005

SAEKI, Y. Characterization of soybean-nodulating rhizobial communities and diversity. In: Aleksandra S., editor. *Soybean-Molecular Aspects of Breeding*. Intech; Rijeka, Croatia: 2011. pp. 163-184. https://doi.org/10.5772/14417

SIKORA, S, REDZEPOVIĆ, S. 2003. Genotypic characterization of soybean rhizobia. *Food Technology and Biotechnology*, 41, 61-67.

SPAEPEN, S., VANDERLEYDEN, J., REMANS, R. 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiology Reviews*, 31, 425-448. https://doi.org/10.1111/j.1574-6976.2007.00072.x

TAMPAKAKI, A.P., FOTIADIS, C.T., NTATSI, G., SAVVAS, D. 2017. *Phylogenetic multilocus sequence analysis* of *indigenous slow-growing rhizobia nodulating cowpea* (*Vigna unguiculata L.*) *in Greece*. *Systematic and Applied Microbiology*, 40(3), 179-189. https://doi.org/10.1016/j.syapm.2017.01.001

VERSALOVIC, J., SCHNEIDER, M., DE BRUIJN, F.J., LUPSKI, J.R. 1994. Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Methods in Molecular and Cellular Biology*, 5(1), 25-40.

WASIKE, V.W., LESUEUR, D., WACHIRA, F.N., MUNGAI, N.W., MUMERA, L.M., SANGINGA, N., MBURU, H.N., MUGADI, D., WANGO, P., VANLAUWE, B. 2009. Genetic diversity of indigenous *Bradyrhizobium* nodulating promiscuous soybean [*Glycine max* (L) Merr.] varieties in Kenya: impact of phosphorus and lime fertilization in two contrasting sites. *Plant Soil*, 322, 151-163. https:// doi.org/10.1007/s11104-009-9902-7

XU, L.M., GE, C., CUI, Z., LI, J., FAN, H. 1995. *Bradyrhizobium liaoningense* sp. nov. isolated from the root nodules of soybeans. *International Journal of Systematic Bacteriology*, 45, 706-711. https://doi.org/10.1099/00207713-45-4-706

YAO, Z.Y., KAN, F.L., WANG, E.T., WEI, G.H., CHEN, W.X. 2002. Characterization of rhizobia that nodulate legume species of the genus Lespedeza and description of *Bradyrhizobium yuanmingense* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 52, 2219-2230. https://doi.org/10.1099/ijs.0.01408-0