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Genetic identification and assessment of the beneficial effect of selected rhizobacteria on plant growth and yield of common bean under the field conditions of West Bulgaria

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Abstract

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Soil and water pollution is increasing significantly, as a result of the active use of synthetic fertilizers and pesticides, which necessitates the application of nature-friendly approaches in modern agriculture. The aim of the present study is to identify an active and suitable strain(s), with potential for improvement the plant growth and yield of common bean *Phaseolus vulgaris*, in order to develop effective biofertilizers, under field conditions.

The subject of molecular investigation were eighteen clones isolated from legume plants based on 16S rRNA gene sequencing, used for identification of V1-V9 variable regions. The obtained Neighbor – Joining phylogenetic tree demonstrated high homology of the investigated clones with NCBI sequences, originated from genus *Bacillus* and *Rhyzobium*. Based on the genetic data, selected strains isolated from *Ph. vulgaris* were tested for their activity under field conditions in West Bulgaria. Indication for active N-fixation was demonstrated by significant elevation of the parameters root dry weight and number of nodules per plant. Additionally, the seed number and seed weight per plant were positively affected. The preliminary data obtained could be used as a prerequisite for future investigations, related to development of highly effective biofertilizers for common bean cultivation.

Keywords: common bean; *Phaseolus vulgaris*; *Rhizobium*; nodulation; 16S rRNA

Introduction

The common bean *Phaseolus vulgaris* belongs to the legume family *Fabacea*. Together with lentil, peas and chickpeas is part of cereal legumes group. The common bean is a popular crop worldwide and according to FAOSTAT data, is grown on more than 35 million ha per year (Mulas et al., 2011; FAOSTAT, 2019). *Ph. vulgaris* is important source of dietary protein for millions of people. Common bean is reach at minerals, as iron and zinc, and vitamins (White and Broadley, 2009). The common bean is a traditional crop for Bulgaria, especially for the population in mountainous areas. Climatic conditions in our country have led to the selection of local forms of common bean with excellent taste qualities and diverse in shape, size and colour range of the seeds (Sabeva and Stoilova, 2009). A special attention for this crop is given by the Institute of Plant Genetic Resources "Konstantin Malkov", Agricultural Academy, Sadovo, where a collection of approximately 2000 samples of common bean of various origins is maintained, evaluated and stored.

Like most legumes, the common bean is able to form symbiotic relationship with soil microorganisms, resulting in root nodules formation. This allows plants to assimilate the atmospheric nitrogen in the process called nitrogen fixation. In turn, the microorganisms benefit the organic compounds synthesized by the plants.

The microorganisms, which can establish symbiosis with common bean, belong to different genera and species of the *Alpha* and *Betaproteobacteria* group (Peix et al., 2015). Among the *Alphaproteobacteria*, the bacterial nodulating species are mainly from the genus *Rhizobium*, but may also belong to other closely related genera, such as *Ensifer* (*Sinorhizobium*) and *Pararhizobium* (Mousavi et al., 2015). The study of Dall'Agnol et al. (2013) reported the presence of 27 species of common bean nodulating rhizobia, which included both nitrogen-fixing and non-nitrogen-fixing strains. Based on the ability of the *Ph. vulgaris* to participate into symbiotic relationships with various non-rhizobia strains, it is determined as a promiscuous host under field conditions (Andrews and Andrews, 2017). Because of this promiscuity, the common bean is often nodulated by many ineffective native rhizobacterias. As a result, the level of biological nitrogen fixation (BNF) is low. According to the literature, common bean is defined as the lowest N-fixator in comparison with the other grain legumes (Martínez-Romero, 2003, cited by Pastor-Bueis et al. 2019).

The effective nitrogen fixation is extremely dependent on the diversity of microorganisms inhabiting a certain soil type. By appropriate selection and application of effective rhizobacteria strains, the plants would be able to produce an adequate amount of nitrogen by BNF. As a result, the application of mineral N fertilizers could be reduced. Inoculation of common bean seeds before sowing, with biological fertilizers containing active strains of rhizobacteria, especially in regions, where leguminous crops are not commonly grown, is highly recommended. Bacterial strain integration in the soil can improve the nitrogen balance for crop development and positively affect the yield quantity and quality (Chimdi et. al. 2022).

According to the European union directives, the growing of leguminous crops for feed and food has to be increased in a future, in order to reduce the greenhouses gas emissions (GHG) coming from the application of synthetic N fertilizers. In comparison with other crops, legumes cultivation resulted in 5 to 7 times less greenhouses gas emissions per unit area (Stagnari et. al. 2017).

In the present study, a genetic identification was conducted by application of 16S rRNA sequencing of individual clones from bacterial strains isolated from leguminous species, including *Ph. vulgaris*. The selected clones 64.1, 65.2 and 66.2 isolated from common bean were investigated for their efficiency to improve plant growth and yield after pre-sowing seed inoculation. The field experiment was conducted under the conditions of Western Bulgaria.

Material and Methods

Molecular methods

Eighteen bacterial strains originated from the collection of Institute of Soil Science, Agrotechnologies and Plant protection "Nikola Poushkarov" were object of this study. They were isolated from a variety of plant species as *Phaseolus vulgaris*, *Pisum sativum, Lotus corniculatus, Vicia faba, Onobrihis sativa* and genus *Trigonela* and *Trifolium*.

Strains were grown at 28°C on *Yeast Extract Beef* (YEB) media (Fluka). Serial dilutions were applied in order to obtain a single colony from each strain, before DNA extraction.

Overnight bacteria cultures from single colonies were prepared for DNA extraction. The cells were pelleted after centrifugations, resuspended with 30 μl of TE buffer and boiled for 5 minutes at 95°C in a thermocycler. After centrifugation for 30 sec at 13000 rpm, two microliters of the supernatant were used for Polymerase Chain Reaction (PCR). PCR was performed using universal 16S rRNA gene primers 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´), (Heuer et al. 1997). The PCR amplification was carried out in a 25 µL volume and included 2 µL DNA, 1 µL of 10 pmol primers and 12.5 µL MyTaqHS Mix (Bioline, London, UK). The PCR conditions were: one step of denaturation at 95° C for 3 min, followed by 35 cycles of denaturation at 95° C for 15 s, annealing at 50°C for 30 s and elongation at 72°C for 45 sec, and finished with final elongation step at 72° C for 3 min. All PCR products were purified by using GeneJET™ Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA), and directly sequenced by Macrogene Inc. (Amsterdam, The Netherlands) with the universal primer 27F. The retrieved sequences with length of 928 bp and 1115 bp were manually edited using the Vector NTIv.10 software package. The distances were calculated according to Kimura (1980). Based on the Neighbor-Joining (NJ) method (Saitou and Nei, 1987), the phylogenetic tree was constructed using Mega 6.0 program (Tamura et al. 2007). Bootstrap analysis was based on 1000 resamplings.

Filed trial

During the growing season of year 2023, a field trial was carried out at the experimental field of the Institute of Ornamental and Medicinal Plants, Negovan village, Sofia region, Western Bulgaria (altitude 549 m). The experimental field (10 m^2) was treated with Alga Soil (1,7% – N; 1,7% - K_2O; 2,6 % – P_2O_5) in dose: 20 kg/ha. The cultivar "*Obraztsov*

Chiflik 12" was used in the experiment, from the selectin cataloque of the Institute of agriculture and seed science, Ruse, Bulgaria. The cultivar has an average yield of 174kg/da, good digestibility and excellent taste qualities. The mean plant height is 48.0 cm, and it is not necessary to be fix it on a sticks (https://izs-ruse.org/en/beans/).

The field trial is characterized by Alluvial – meadow soil with humus horizon thickness of 10 to 40 cm, below which follow alluvial sediments unaffected by soil formation. This soil type has medium water-holding capacity, high water permeability, and very good water regime.

The bacterial cultures of the sequenced clones No 64.1, 65.2 and 66.2 isolated from *Ph. vulgaris* (microbiological collection of the Institute of Soil Science, Agrotechnology and Plant Protection "Nikola Poushkarov") were used in the experiment. Directly before sowing, the bean seeds were treated with overnight bacterial culture with concentration of 1 x 10^8 CFU for 30 minutes in dark, following the scheme: Rows 1-3 – Control with untreated seeds; Rows 4-6 – seeds treated with strain 64.1; Rows 7 – 9 – seeds treated with strain 65.2; Rows 10 -12 – seeds treated with strain 66.2.

The seeds were sowed in a nested way with distance between the rows 70cm and 45cm between the plants. Plants were grown under non-irrigated conditions.

The following morphological parameters of each plant were monitored: at the stage active flowering (R6) – number of nodules, fresh and dry weight of nodules, root length, fresh and dry root weight; at the end of the vegetation (R9) – seed number and seed weight. The stages of development were evaluated according to Berrocal-Ibarra et al. (2002).

The soil samples were collected before sowing, during the active flowering and after harvesting. The agrochemical analyzes of the soil samples includes determination of: mineral nitrogen content (NH_4 and NO_3), according to Bremner (1965); content of mobile phosphorus and potassium – calorimetrically, according to Ivanov (1984); content of organic carbon, by modified method of Tyurin (Kononova, 1963); pH – potentiometrically in KCI (Arinushkina, 1962).

Macroelement's content in plant biomass (leaves and stems) was measured during the phases R6 and R9. The following methods were used: N content by Kjeldahl, (Bremner et.al., 1996); content of Ca, K, Mg, P – by EPA – Method 3052, (Remeteiová et. al. 2020).

Statistical analysis

The effect of inoculation on monitored parameters was assessed by application of ANOVA: Single Factor (Microsoft, Excel, 10). The comparison of means (from 10 individual plants) ± SE was calculated. Differences were considered statistically significant at level of $p < 0.05$.

Results and Discussion

Genetics studies

PCR amplicons were produced successfully in all DNA samples. The obtained amplified products with universal primers for 16S rRNA were 1500 bp in length, respectively, which was the expected product size. Sixteen sequences (out of 18) were blasted against NCBI database sequences for identification in the constructed phylogenetic tree (Fig.1)..

The combined results from NCBI data base search and the phylogenetic tree shows that 8 out of 16 sequences showed identity from 98.89% to 99.90%, with known species from the Gene Bank. The other 8 sequences were unidentified to species level. Only 3 sequences showed lower identity from 98.24% to 98.89%. In the constructed phylogenetic tree, 4 main clusters were formed. The first cluster includes 8 sequences from the bacterial isolates numbered 325.1, 349.1, 72.1, 66.2, 321.1, 346.1, 333.1 and 48.1, extracted from 6 different plant species. The identity of these sequences ranged from 98.24% to 99.60% with NCBI annotated *Bacillus pumilus* and *Bacillus sp.* strains. Two sequences – 321.1 and 333.1 isolated from *Trifolium sp.* and *Trigonella sp*., showed 99.60 % identities to *Bacillus pumilus* strain 161.1.2 and *Bacillus sp*. strain MIS5, as well as 99.30% with *Mezorhizobium sp*. W39.

The second cluster includes 4 sequences numbered 65.2, 97.1, 345.1 and 64.1, clustered with 3 different *Bacillus subtilis* strains. The strain 345.1 isolated from *Onobrychis sativa* showed the highest identity (99.90%), with the annotated *Bacillus subtilis* strain MDR12, among all strains investigated here. The strains 64.1 and 65.2 isolated from *Ph. vulgaris* showed 99.6 %, identity with *B. subtilis* strains MDR12 and CMFRI/BS-29, respectively. Additionally, the strains 64.1 and 345.1 showed 99.4% – 99.70, identity with sequence KF008225.1 of *Rhizobium sp*. SOY5.

The third cluster includes one sequence – 73.1, which was close to *Paenibacillus polymyxa* strain SX303 with an identity of 99.64 %, but with the lowest identity of 95.83% to *Rhizobium sp.*rf002.

The fourth cluster includes three sequences – 348.2, 322.2 and 331.1, which were clustered next to the *Stenotrophomonas maltophilia* and *Stenotrophomonas geniculata* strains, while the strain *Rhizobium leguminosarum* was separated from them.

In the NJ tree, the annotated genome sequences of *Rhizobium legumisarum* and *Agrobacterium rhizogenes* formed an outgroup.

According to the distribution in the phylogenetic tree, a rich diversity among cultivated strains and low diversity in the detected genera was demonstrated. It was found that the genus

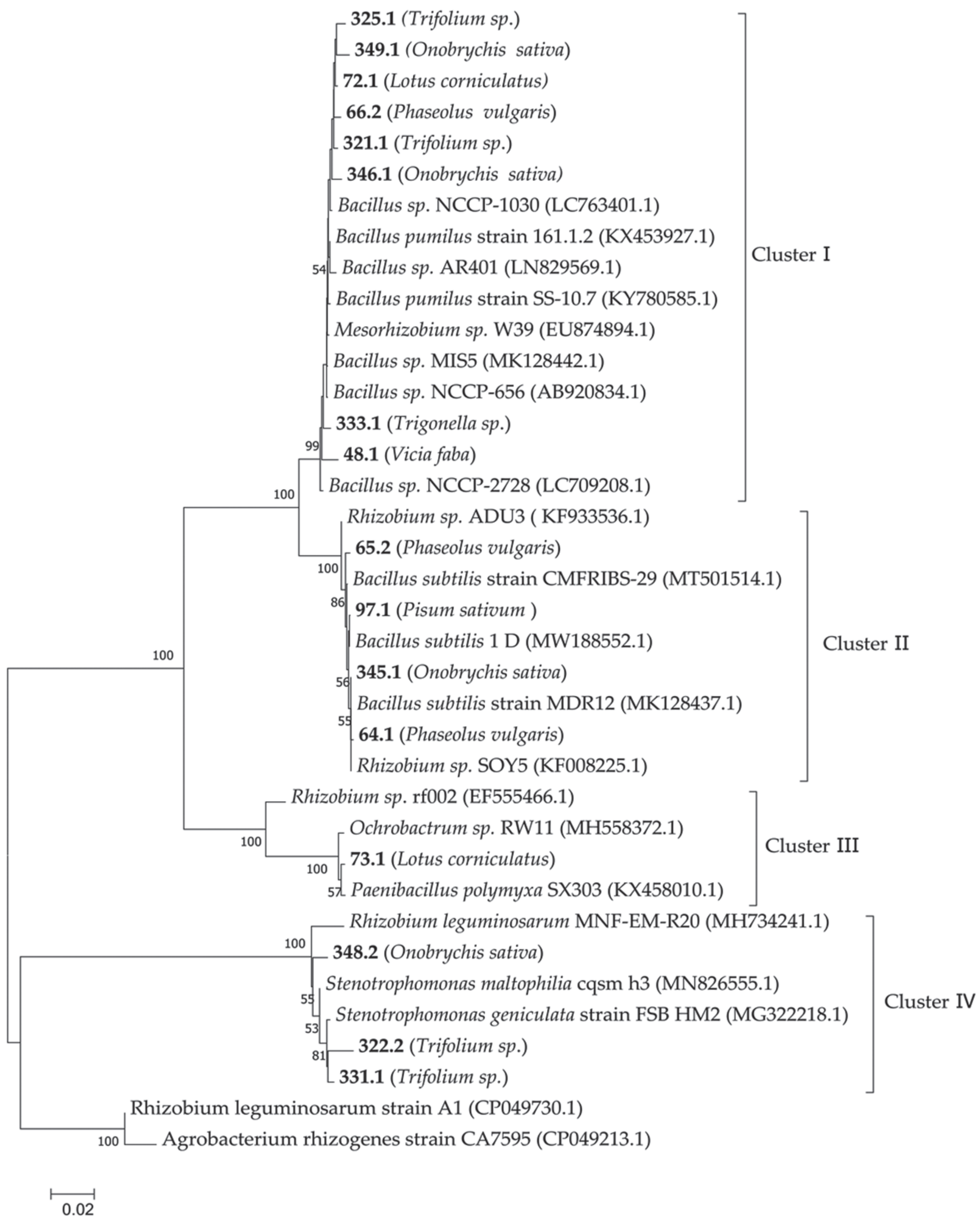

Fig. 1. Neigbour – Joining (NJ) phylogenetic tree, constructed with 16S rRNA nucleotide sequences and nearest high homology sequences, obtained after Blast search in NCBI database. In the brackets next to the corresponding number of each sequence is given the name of the plant species from which the strain was isolated. The analysed sequences were with length between 928 bp and 1115 bp.

Source: Authors' own elaboration

Bacillus was detected in 12 (out of 16 strains), and could be assessed as the most abundant cultivable genus, followed by *Stenotrophomonas*, detected in 3 of the strains. The presence of *Bacillus* strains in the studied collection was not surprising. During the recent years, there were large number of publications, describing the isolation and detection of non-rhizobia endophytes or nodule associated bacteria. The strains belong to *Bacillus* were presented as a largest group (De Meyer et.al. 2015). In the literature, this wide variety of microorganisms from the rhizosphere are grouped together as plant growth promoting rhizobacteria (PGPR). This group include microorganisms from the genus *Aerobacter*, *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Chryseomonas*, and is known to improve plant growth following different mechanisms, related to nutrient availability, such as nitrogen fixation, mineral solubilisation, overcoming plant stress etc. (Ravinder et al., 2024).

In the future, additional investigation will be perform in order to confirm the origin of the strains isolated from *Ph. vulgaris* – 64.1, 65.2 and 66.2 to genus *Bacillus* or *Rhizobium*. Recently, MALDI-TOF MS method has become a popular technique for microbiological identification of various microorganisms in water sample, soil and vegetables, including family *Rhizobiaceae* (Mulas et al. 2011; Ferreira et al., 2011).

Effect of rhizobacteria inoculation on the soil parameters

The analysis of soil samples showed increase in the organic carbon from 2.1% before sowing to 2.5–3% during both studied phases (Table 1). The content of total nitrogen was 10,4 mg/kg before sowing and increase in double during the active flowering in the control rows. In the rows treated with strains 64.1 and 66.2, the N-content was reduced. In the end of vegetation phase, statistically significant elevation of N-content was measured after treatment with strain 66.2 ($p < 0.05$) (Table 1). A differential answer of the treatment with strains 64.1 and 66.2 related to the P-content was observed. The application of strain 64.1 did not change the P-content in comparison with the control during the active flowering and at the end of vegetation phase. Whereas, the treatment with strain 66.2 showed a significant enhancement of P values in the soil samples, collected during the both stages of plant development (Table 1). The research of Chimdi et al. (2022) demonstrated positive influence of the total nitrogen and available P content on the soil, as well as on the yield parameters of *Vicia faba* plants under filed conditions. The effect was achieved after combined application of P fertilizer and rhizobium inoculation.

Additional experiments will be conducted in order to confirm the relation between the activity of strain 66.2 and accumulation of P in the rhizosphere.

Effect of rhizobacteria inoculation on macroelements content in plant biomass

Macroelements in plant biomass were monitored during active flowering and at the end of the growing season. The presented values from Table 2 were averaged from 10 individual plants. A positive effect was obtained after treatment with all 3 tested strains concerning the nitrogen accumulation during active plant flowering and at the end of the growing season. A statistically significant increase was demonstrated after treatment with strains 64.1 ($p < 0,05$) and 66.2 ($p < 0,001$) during the active flowering (Table 2). Same tendency was observed in the end of the growing season, following inoculation with all 3 strains investigated ($p < 0,001$; $p < 0,01$) (Table 2).

Table 1. Agrochemical analyses of soil samples. Asterisks indicate statistically significant differences compared to the control: $p < 0.05$*; $p < 0.01$; $p < 0.001$*****

Parameters/ variants	pH		∑ $N\text{-}NH_4 + NO_3$	P_2O_5	K_2O	Organic C
	H_20	KCL	mg/kg	mg/kg	mg/kg	%
	Before sowing					
Negovan	6.93	6.16	10.36	44.36	48.32	2.12
	Active flowering					
Control	6.8	6.3	21.89	12.01	46.81	2.47
64.1	6.9	6.4	14.40	13.01	27.26	1.60
66.2	7.0	6.6	13.82	40.31***	55.68	3.26
	End of plant vegetation					
Control	6.8	6.3	18.43	13.30	36.06	2.87
64.1	7.0	6.5	12.10	15.62	51.70	2.81
66.2	7.0	6.6	24.19*	36.11***	58.36	2.87

Source: Authors' own elaboration

Table 2. Macroelements content in the aboveground biomass. Asterisks indicate statistically significant differences compared to the control: $p < 0.05$*; $p < 0.01$; $p < 0.001$*****

Stages	Active flowering				End of vegetation			
Macroelements (%)	Control	64.1	65.2	66.2	Control	64.1	65.2	66.2
N	1.17	1.77*	1.63	1.93***	2.27	2.97**	3.1***	2.87**
Ca	1.86	3.46***	2.37	2.78*	2.18	2.79	2.88*	2.42
K	1.01	0.67	0.81	0.82	0.93	0.85	1.18	1.28
Mg	0.54	1.08***	0.77*	0.87**	0.67	0.86	0.73	0.71
P	0.13	0.14	0.12	0.15	0.21	0.21	0.21	0.22

Source: Authors' own elaboration

Under greenhouse conditions, an enhanced nodulation resulting in high N-content in plant tissue of *Ph. vulgaris* cultivar Carioca after treatment with *Rhizobim* strains PRF 81 and CIAT 899, were described by Hungria et al. (2000). The same authors demonstrated the increase of the yield up to 906 kg/ha under field condition by application with same strains. Stajkovic et al. (2011) reported about N-content increase in common bean plants after co-inoculation of *R. phaseoli* with *Pseudomonas* sp. LG.

According to our data, the application of strain 64.1 induce the accumulation of higher content (p < 0,001) of Mg and Ca in the plant biomass during active flowering (Table 2). A significant value of Mg (p < 0.01) and Ca (p < 0,05) were measured in plant biomass after inoculation with strain 66.2 during the same phase. Additionally, the seeds inoculation with strain 65.2 resulted in increased Ca-content in plant biomass at the end of the growing season.

The results will be confirmed in further experiments under different climatic and soil conditions. The mineral uptake in seeds will be evaluated as well.

Effect of rhizobacteria inoculation on root development, nodulation and yield

The morphological indicators related to root development, nodulation and yield were monitored under the field conditions of West Bulgaria (Sofia region).

In Figure 2 is shown the positive effect of inoculation with the strains 65.2 and 66.2 on root dry weight (p < 0.05). Additionally, the inoculation with strain 66.2 induce the formation of more nodules per plant (p < 0.05). With respect to the yield parameters, a significant result was obtained after inoculation with strain 64.1 (p < 0.05) concerning seeds number. The inoculation with strain 65.2 resulted in significant increase of seed weight per plant (p < 0,05) (Figure 2).

The value of the parameter root dry weight after inoculation with rhizobia is additional indicator for identification of the most effective N-fixing strains (Hefny et al., 2001). The research of Stajkovic et al. (2011) demonstrated better growth performance of common bean plants resulting in

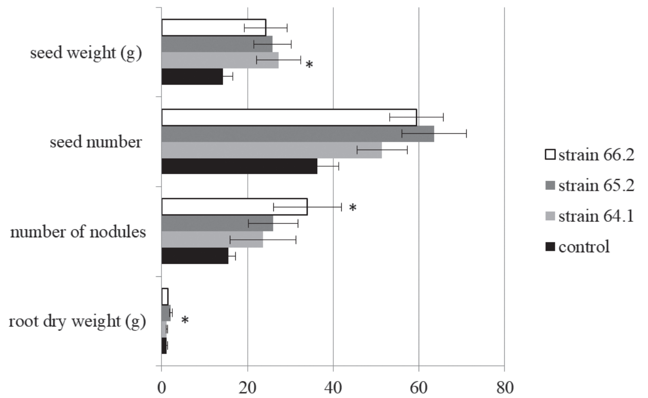

Fig. 2. Biometric parameters of common bean plants. Asterisks indicate statistically significant differences compared to the control: $p < 0.05$*; $p < 0.01$; $p < 0.001$*****

Source: Authors' own elaboration

high level of root dry weight after treatment with *R. phaseoli* and *Pseudomonas sp.* LG.

Figueiredo et al. (2008) presented increased nodule numbers and higher levels of accumulated nitrogen after combined inoculation of common bean seeds with *Rhizobium tropici* strain (CIAT899) and *Paenibacillus polymyxa* (DSM 36), which is another example for stimulation of nodulation. A positive effect on the yield from inoculation of common bean, variety *Rinon*, with selected local strains under filed conditions was published by Mulas et al. (2011). The authors suggest that common bean biofertilization with selected strains have a great potential to replace the conventional N fertilization.

Conclusion

Genetic analysis of 16 fast-growing rhizobia strains based on 16S rRNA region showed high similarity to NCBI sequences from the genus *Bacillus*, *Stenotrophomonas* and

Rhizobium. The majority of strains (12 out of 16 investigated), including the strains isolated from *Phaseolus vulgaris* – 64.1, 65.2 and 66.2, were clustered together with annotated *Bacillus* strains. At the same time, the strain 64.1 demonstrated high homology with *Rhizobium* strain. Additionally, more analyses need to be perform to confirm the strain identify

Under the field conditions in West Bulgaria, Negovan village (Sofia region), a positive effect of common bean inoculation with strains 64.1, 65.2 and 66.2 before sowing was demonstrated. Significant enhancement of nitrogen content was detected in the plant biomass during the phases of active flowering and the end of vegetation. The higher nitrogen level contributed to positive impact on root development. The parameters root dry weight and number of nodules per plant after inoculation were enhanced, which is indication for active N-fixation. The yield parameters number of seeds and seed weight per plant were significantly increased after inoculation with strains 64.1 and 65.2, respectively.

Additional studies will be performed in order to confirm the capacity of the investigated strains 64.1, 65.2 and 66.2, as a biofertilizers for cultivation of common bean. The different combinations among these strains, as well as testing their activity under different soil and climatic conditions, has to be conducted in the future.

Conflict of Interest

The presented manuscript has no conflict of interest.

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