Original scientific paper
DOI: /10.5513/JCEA01/21.1.2525
Journal of Central European Agriculture, 2020, 21(1), p.151-158

Isolation and characterization of endophytic bacteria from soybean (*Glycine max* L.)

Izolacija i karakterizacija endofitskih bakterija iz soje (*Glycine max* L.)

Sanja KAJIĆ (✉), Dora KRZNARIĆ, Ivana RAJNOVIĆ, Sanja SIKORA

University of Zagreb, Faculty of Agriculture, Department of Microbiology, Svetošimunska 25, 10000 Zagreb, Croatia

✉ Corresponding author: skajic@agr.hr

Received: May 15, 2019; accepted: August 23, 2019

ABSTRACT

Endophytic bacteria colonize the internal tissue of the plant usually forming beneficial association with their host. The main aim of this study was to genotypically and phenotypically characterize endophytic bacteria isolated from roots, stems and nodules of two soybean cultivars (AFZG Ana and Gabriela). A total of 29 endophytes were isolated from three different tissues of soybean. Genotypic characterization included *rrs* genes sequencing. Among soybean endophytes five different genera of bacteria were identified: *Pseudomonas* spp., *Sphingomonas* spp., *Bradyrhizobium* spp., *Rhizobium* spp., and *Agrobacterium* spp. Most of the isolates were Gram negative, rod-shaped, without capability of capsule production. The variability of the phenotypic characteristics of isolates was demonstrated, as well as the resistance to adverse environmental conditions such as high temperature, increased salt content, and low and high pH values. Most strains showed tolerance to the specific antibiotics. Most of the catalase positive isolates belong to *Bradyrhizobium* or *Rhizobium* spp., while the catalase negative isolates belong to the *Pseudomonas*, *Sphingomonas* and *Agrobacterium* spp. It was also shown that 85% of the tested isolates were oxidase negative while those that were positive belong to the *Pseudomonas* spp. Further characterization of endophytes is needed to determine their influence on plant growth.

Keywords: endophytic bacteria, soybean (*Glycine max* L.), 16S rRNA gene sequencing, phenotypic characterization

SAŽETAK

Endofitske bakterije koloniziraju unutrašnjost biljnih tkiva stvarajući pozitivne interakcije sa svojim domaćinom. Glavni cilj ovog istraživanja bio je genotipska i fenotipska karakterizacija endofitskih bakterija izoliranih iz korijena, stabiljke i nodula dva različita kultivara soje (AFZG Ana and Gabriela). Izolirano je ukupno 29 endofita iz tri različita tkiva soje. Genotipska karakterizacija uključivala je sekvenciranje *rrs* gena. Identificirano je pet skupina bakterija različitih rodova: *Pseudomonas* spp., *Sphingomonas* spp., *Bradyrhizobium* spp., *Rhizobium* spp. i *Agrobacterium* spp. Većina izolata je Gram negativna, štapićastog oblika, bez mogućnosti stvaranja kapsule. Dokazana je varijabilnost fenotipskih karakteristika izolata, ali i otpornost prema nepovoljnim uvjetima poput povišene temperature, povećanog sadržaja soli te niske i visoke pH vrijednosti. Većina sojeva pokazala je tolerantnost na istraživane antibiotike. Većina katalaza pozitivnih izolata pripadaju rodovima *Bradyrhizobium* ili *Rhizobium*, dok su katalaza negativni izolati identificirani kao *Pseudomonas*, *Sphingomonas* i *Agrobacterium* spp. Dokazano je da 85% ispitanih izolata ne reagira na enzim oksidazu, dok oni koji reagiraju pripadaju vrsti Pseudomonas spp. Potrebna je daljnja karakterizacija endofita kako bi se utvrdio njihov utjecaj na razvoj biljke.

Ključne riječi: endofitske bakterije, soja (*Glycine max* L.), sekvenciranje 16S rRNA gena, fenotipska karakterizacija

INTRODUCTION

Endophytic bacteria can be defined as bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Ryan et al., 2007). Bacterial endophytes colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents (Berg et al., 2005). Endophyte-plant associations have been found to improve plant health and may help host plant to avoid various biotic and abiotic stresses. They may also provide fitness benefits to host plants such as tolerance to herbivory, heat, salt, diseases, and drought and increased below and aboveground biomass etc. (Dalal and Kulkarni, 2013). Thus, endophytic colonization improves the ecological adaptability of the host plant. Hence endophytes may be regarded as a true companion of the host.

Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues. They were isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Hung and Annapurna, 2004). Owing to their plant growth promoting and disease control properties, endophytes can be used in the form of bioinoculants in agriculture as amendments to promote plant growth and health. A number of registered patents that are related to application of endophytic bacteria to enhance host tolerance to fungal pathogens as well as to promote plant growth demonstrate a potential for applications that would benefit development of sustainable agricultural production (Miliute et al., 2015). Rhizobia are perhaps the best known beneficial plant- associated bacteria because of the importance of the nitrogen fixation that occurs during the *Rhizobium-legume* symbiosis. Endophytic bacteria have been isolated from legume plants such as alfalfa (Stajkovic et al., 2009), lens (Dixit et. al., 2014)) and common bean (Lopes et al, 2015). Endophytic species mostly belong to the α-, β-, and γ-proteobacteria subgroups and are closely related to epiphytic species (Kuklinsky-Sobral et al., 2005). The γ-proteobacteria group is the most diverse and dominant. It has been reported that most of gram-negative endophytes act as agents of biological control, while among the gram-positive bacteria, the dominant endophytic species are primarily those belonging to the *Bacillus* species (Miliute et al., 2015).

Soybean is one of the most important legume crop, and extensive studies have been performed on their rhizobia. In general, diverse rhizobia belonging to *Bradyrhizobium* and *Sinorhizobium* are associated with soybeans, and typically, biogeographic patterns have been found among the soybean rhizobia (Yan et al., 2014). In comparison with other crops, studies on endophytes in soybean plants are still limited, especially those grown in Croatia. In North America 14 genera of fungal endophytes were isolated from the stems of soybeans (Impullitti and Malvick, 2013). In Brazil, 11 genera of fungal endophytes were identified in soybean leafs and stems. Fungal endophytic colonization in soybean plants was influenced by tissue type, with a greater number of endophytes isolated from leaves than from stems (Pimentel et al., 2016). Soybean is gaining popularity on account of its unique characteristics and adaptability to vary agro-climatic conditions (Dalal and Kulkarni, 2013).

The objective of this study was to isolate the culturable, endophytic bacteria from the root, nodule and stems of two differents soybean cultivars growing under controlled conditions and to characterize the community of culturable bacteria. To our knowledge, this is the first report on endophytic bacteria from the root, nodule and stems of different cultivars of the soybean.

MATERIALS AND METHODS

Isolation of endophytic bacteria

Host plants used in the experiment were two soybean cultivars (AFZG Ana and Gabriela). These plants were grown under controlled conditions; day temperature 26 °C, night temperature 22 °C, moisture was continuously 65%. Daylight period was setup for 16h, and night period for 8h.

The soybean tissue was collected at the flowering stage. Ten healthy plants were carefully removed, washed under tap water to remove vermiculite and they were

then separated into stems, roots and nodules. Stems and roots were cut into sections 2-3 cm long. The tissue was rinsed in 70% ethanol for 30 seconds and then sterilized with 3% NaClO 3 minutes for roots and nodules and 5 minutes for stems. The tissue was then washed ten times with sterile water (Hung and Annapurna, 2004). Surface-disinfected tissue was crushed with a sterile glass rod in a sterile test tube. One loop full of the nodule, stem or root content suspension was streaked on yeast mannitol agar (YEM) plates containing 0.0025% (w/v) Congo red. After incubation for 3 to 5 days at 28 °C, single colonies were selected and restreaked on YEM agar for purity (Vincent 1970). Pure cultures were preserved in 20% glycerol at -20 °C until further use.

Genotypic characterization

The study involved 29 selected isolates. Total DNA was extracted using DNeasy® Blood & Tissue kite (QIAGEN, 2006, USA), according to manufacturer's instructions. The universal primers fD1 and rD1 were utilized used for PCR amplification of 16S rDNA (Sikora and Redzepovic, 2003). Amplification reactions were performed in a 25 µL volume, containing: 20 mmol/L Tris-HCl (pH=8.4), 50 mmol/L KCl, 2.0 mmol/L MgCl2, 200 µmol/L of dNTPs, 1 µmol/L of each primer, 30 ng of genomic DNA and 1.5 U of Taq DNA polymerase (TaKaRa Bio, USA). The temperature profile was as follows: initial denaturation at 95 °C for 3 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; and final extension at 72 °C for 3 min. The amplification protocol was accomplished according to Sikora and Redzepovic (2003). Five microliters of the PCR product were analyzed by electrophoresis on precast 6 % poly (NAT) gels run in SEA 2000 apparatus (Elchrom Scientific AG, Switzerland) for 2.5 h at 7 V/10mm and 20 °C. The PCR products were visualised under UV illumination after staining with ethidium bromide and photographed with Cannon Powershot A640 camera. A 1kb ladder (GenSura Laboratories, CA) was used as molecular size marker. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer and the primers fD1 and rD1. The identification of the isolates was performed using the Ribosomal Database Project (Cole et al., 2009) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in NCBI. We used the BLAST to verify the similarity of experimental sequences with the reference sequences in the databases and classified them at the genus level. Phylogenetic trees were constructed using the T-Rex program (Tree and Reticulogram Reconstruction).

Phenotypic characterization

Morphological and biochemical tests

Standard morphological and biochemical tests were performed; isolates were characterized by gram and capsule staining and biochemical tests like oxidase and catalase. They were identified according to Bergey's Manual of Systemic Bacteriology.

Fluorescence pigment production test

Isolates were also screened for fluorescent pigment production. Isolates were grown on King's B medium and incubated for 3 days, and then were checked for pigment production under UV light source.

Antibiotic resistance

The antibiotic sensitivity of endophytic bacteria was determined by disc diffusion method for the following antibiotics (ampicillin 10 µg/ml, streptomycin 10 µg/ml, erythromycin 15 µg/ml and kanamycin 30 µg/ml) and incubated for 5 days at 30 °C. The antibiotics resistance was recorded as positive if the test colony appeared on the plates, as compared to the control plate in which no antibiotic was added.

Salt, temperature and pH tolerance

The endophytic bacterial isolates were screened for their tolerance to salt on yeast extract mannitol agar (YMA) supplemented with 1, 2, 3 and 4% (w/v) NaCl (Adejumo and Orole, 2010). Temperature tolerance was tested by incubating the inoculated plates at 37 °C and 45 °C (Niste et al., 2015). Concerning the ability of the isolates to grow in acid and alkaline media, the isolates

were inoculated on YMA media, pH were adjusted to 4.5, 5.5, 8, and 9 by using sterile HCl or NaOH (Shamseldin and Werner 2005), the isolates were kept at 30 °C for 5 days.

RESULTS AND DISCUSSION

Genotypic characterization

The results of analysis conducted in this study show significant diversity among 29 isolated strains. Based on genotypic characterisation, five genera of bacteria were found as endophytes isolated from different soybean tissues; *Pseudomonas* spp., *Sphingomonas* spp., *Bradyrhizobium* spp., *Rhizobium* spp. and *Agrobacterium* spp. (Table 1).

Table 1. Endophytic isolates obtained from two soybean cultivars

Identified taxum	cv. Gabriela	cv. Ana
Alphaproteobacteria		
Rhizobium (R. nepotum)	1	2
Agrobacterium (A. tumefaciens; A. nepotum)	2	1
Bradyrhizobium (B.japonicum)	4	1
Sphingomonas (S. sanguinis)	4	2
Gammaproteobacteria		
Pseudomonas (P. flurescens; P. chlororaphis)	6	6
Total	17	12

cv = cultivar

For the most strains, 100% similarity with sequences of *Bradyrhizobium japonicum*, *Pseudomonas fluorescens*, *Pseudomonas chlororaphis*, *Sphingomonas sanguinis*, *Agrobacterium tumefaciens* was obtained, and for the other strains, the obtained similarity was 99%.

The largest group consisting of 12 strains (40%) isolated from different parts of the plant (nodules, roots and stems) belongs to the genus *Pseudomonas* spp. Three of these isolates were identified as *P. fluorescens*, and two as *P. chlororaphis*.

The next group belongs to the genus *Sphingomonas* spp. with a total of six isolates, four of which were identified as *S. sanquinis*. Five isolates belong to the genus *Bradyrhizobium* spp., and all are identified as *B. japonicum*. Four of the five bacteria belonging to this species are isolated from the nodules, while one is isolated from the root and most of them from the soybean variety Gabriela. All isolates belonging to the genus *Rhizobium* (GS11, AS2 and AS4) were isolated from the stem. Three isolates belong to the genus *Agrobacterium* spp. two of which belong to *A. tumefaciens* and one to *A. rubi*.

While most of *B. japonicum* strains were isolated from the nodules, *Pseudomonas* spp. was isolated from all parts of the soybean AFZG Ana and Gabriela, which proves its wide presence in those legumes.

In their study, Araujo et al (2002) isolated several endophytic bacteria from lemon root (*Citrus jambhiri*) and found that one of them (*Pseudomonas* spp.) has antipathogenic activity.

In the present work *S. sanguinis* was isolated from the nodules and stems of both soybean varieties. Only two isolates of *A. tumefaciens* were found, both from Gabriela variety. According to the study (Bai et al. 2002), three of the 14 endophytes improved the nodulation of soybean when inoculated with *B. japonicum* showing good plant growth promoting capability of these strains.

Woese et al. (1984) proposed the 16S rRNA (*rrs*) gene to be used as a universal marker for classification and identification of bacteria. This gene was also accepted as the basis for the classification of nodule bacteria from family *Rhizobiaceae* at genus level. In this study genera *Rhizobium* and *Agrobacterium* were identified using this gen (Kuykendall, 2005).

Based on the obtained sequences, a final dendrogram was constructed and then used to differentiate the strains and to study the genetic variability among them (Figure 1). The distribution of strains in the three main groups and two subgroups is evident from the dendrogram. Of all investigated strains, the most distinct strain is AN4 which belongs to the genus *Pseudomonas*.

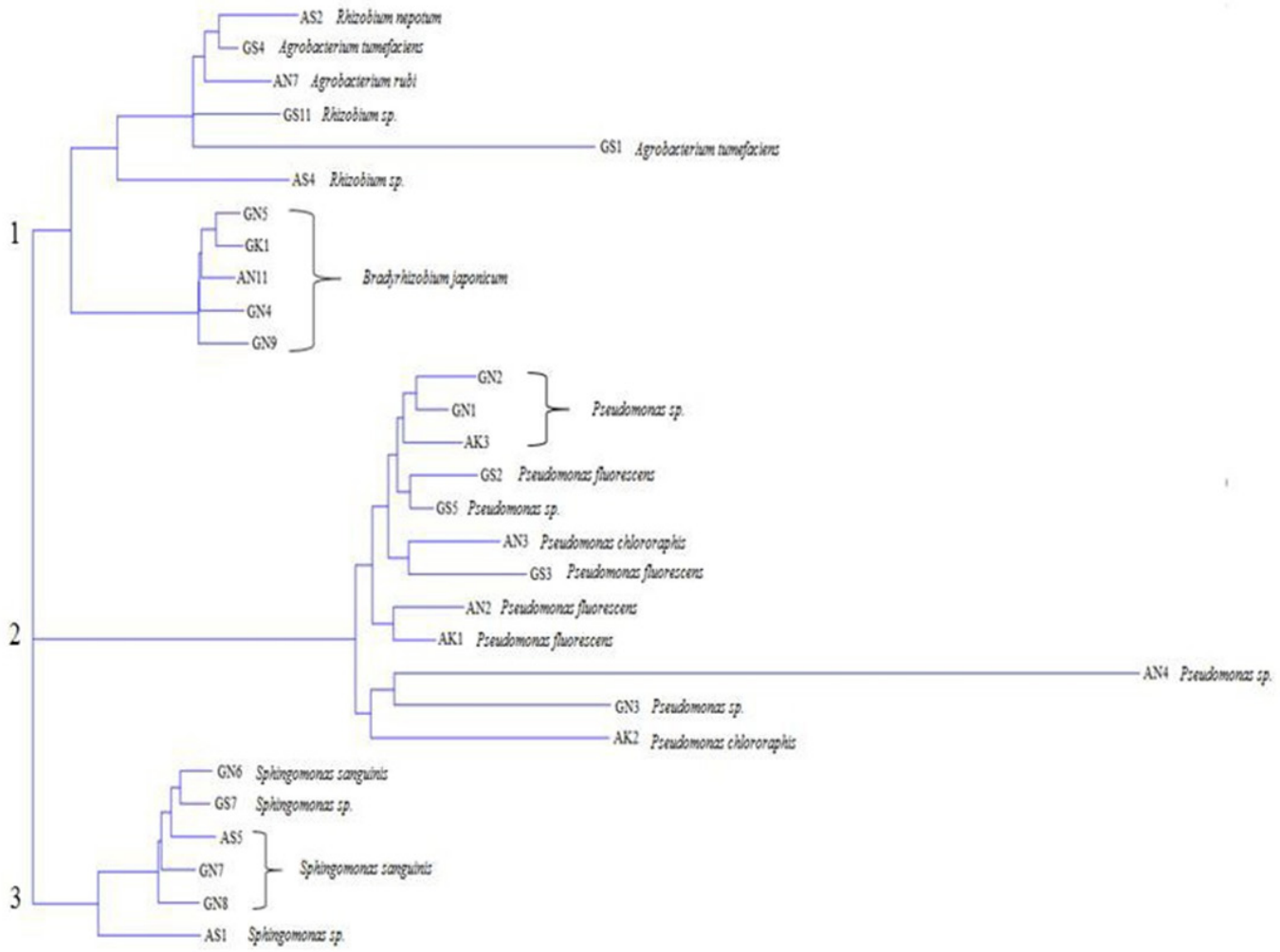

Figure 1. Phylogenetic tree showing the relationship between the 16S rRNA gene sequences from representative isolates of endophytic bacteria from two soybean cultivars

The first major group includes strains from three genera - *Rhizobium* spp., *Agrobacterium* spp. and *Bradyrhizobium* spp. The second major group comprises 12 strains, all belonging to the genus *Pseudomonas* spp.

The highest similarity was found between strains GN2 and AK3 and between the strains GS2 and AN2. GN2 and AK3 were identified as *Pseudomonas* spp., and GS2 strain was identified as *Pseudomonas fluorescens*, suggesting it is very likely that the AN2 strain belongs to the same species. The third group contains of the isolates from the genus *Sphingomonas*, four of them were identified at species level as *S. sanguinis*. The most different within this subgroup was strain AS1.

The reason for high diversity of endophytic bacteria found in this study could be attributed to the soybeans long term history of breeding and selection in various geographical and climatic areas. The endophytic bacteria are significantly affected by the genotype and plant cultivar, since the host plant naturally selects its endophytic population. Other researchers have also find different species and genera of endophytes isolated from soybean (Kuklinsky-Sobral et al., 2005, Li et al. 2008)

Phenotypic characterization

Morphological and biochemical tests

Most isolates from this study were Gram-negative rods while only five out of 29 isolates (GN7, GN9, GK1, AS1, AS2) were cocci. The results show that none of the isolates formed the capsule. Most isolates showed a positive reaction to the catalase test. Approximately 30%

of other isolates were negative or weak positive to this test. It was also found that 85% of the tested isolates were oxidase negative. Strains with a positive oxidase test (AN2, AN3, AK2 and AK3) belong to the species *Pseudomonas* spp. (Table 2).

Fluorescence pigment production test

Nine out of 29 isolates produced fluorescence pigment and it has been shown that these isolates belong to *Pseudomonas* spp. (Table 2).

Antibiotic resistance

Different reactions were found while testing the isolates to antibiotic susceptibility. According to the results shown in Table 3, the highest resistance of the isolates to the ampicillin was observed, where all 29 isolates grew in its presence forming minimal halo around disc. Strains GN7 and GS7, identified as *Sphingomonas* spp. were the only strains susceptible to erythromycin but were resistant to all other tested antibiotics (Table 2).

A total of 17 isolates grew under the influence of all antibiotics. Considering the size of the zone around the disks, the tested strains showed least susceptibility to ampicillin, slightly less resistance for erythromycin, followed by streptomycin, and the highest sensitivity was observed for kanamycin.

Salt, temperature and pH

This study showed that most strains grew well at elevated salt concentrations. Li (2017) isolated 62 endophytic bacteria from the stems, leaves and roots of *Lepidium perfoliatum* L., plant growing in the extreme desert conditions. Research has shown that these bacteria tolerate 12% NaCl. This was the first finding of the existance of various endophyte species which can tolerate such extreme conditions.

Almost all isolates from our study grew in presence of 1% and 2% NaCl. More than 50% of the isolates showed constant growth at all salt concentrations, proving high resistance to elevated NaCl concentrations. Exception were three isolates (GN9, GK1 and AN11) that did not grow at any concentration of NaCl belonging to *B. japonicum* species. The highest tested concentration of salt was 4% were 17 isolates grew well. Considering the results of growth on different pH values a remarkable variety between isolates was observed. It was shown that most isolates grew at all tested pH values from 4,5 to 9. According to the results, the highest number of isolates were tolerant to pH values of 5.5 and 8 (Table 3). *Rhizobium* and *Sphingomonas* species showed the lowest tolerance to pH 4.5. Most of the strains that grew at all tested pH values belong to the *Pseudomonas* spp

Table 2. Morphological and biochemical characteristics of soybean endophytic bacteria

Tests	Cultivar Gabriela		Cultivar Ana	
	Positive	Negative	Positive	Negative
Catalase	12(70)	5(30)	10(84)	2(16)
Oxidase	0	17(100)	4(33)	8(67)
Fluorescence	6(41)	10(59)	3(25)	9(75)
Antibiotic resistance				
*Ampiciliin	17(100)	0	12(100)	0
*Streptomycin	13(77)	4(23)	10(84)	2(16)
*Erythromicin	13(77)	4(23)	11(92)	1(8)
*Kanamycin	12(70)	5(30)	9(75)	3(25)
Capsule formation	0	12(100)	0	12(100)
Gram staining	3(17)	14(83)	0	12(100)

Numbers in brackets express percentage

Table 3. Growth of strains at different temperature, pH and NaCl%

Tested parameters	Positive	Negative
Temperature		
37 °C	29(100)	0
45 °C	17(59)	12(41)
pH		
4,5	13(45)	16(55)
5,5	24(84)	5(16)
8	27(93)	2(7)
9	23(79)	6(21)
%(w/v) NaCl		
1	26(90)	3(10)
2	26(90)	3(10)
3	22(76)	7(24)
4	16(55)	13(45)

Numbers in brackets express percentage

species demonstrating their tolerance to acidic, neutral and alkaline soils since the optimal pH for their growth is between 4 and 8. Only two strains did not grow at pH 8, proving that tested strains prefer neutral to slightly alkaline reaction (Table 3).

CONCLUSION

Large genetic diversity has been determined within the natural population of endophytic soybean bacteria, with respect to the different plant tissues from which isolates have been obtained. Sequencing the *rrs* genes five different genera of bacteria were identified: *Pseudomonas* spp., *Sphingomonas* spp., *Bradyrhizobium* spp., *Rhizobium* spp., and *Agrobacterium* spp. Considering all phenotypic characteristics, it can be concluded that strains GS2 and GS3 (*P. fluorescens*) are the most tolerant to unfavourable conditions. *P. fluorescens* performs a competitive colonization of the plant root and therefore is considered to be the most promising PGPR bacteria. *Sphingomonas* spp. was isolated from the nodules and the stem of the soybean. The ability of some species of this genera to degradate pollutants in environment was found and therefore they could be studied for bioremediation purposes. Significant variability of tested isolates was determined in tolerance of different pH, temperature and salt concentration. Further research of isolated endophytic bacteria is needed in order to detect whether they have symbiotic properties or could be used in bioremediation or as biological agents for plant protection against nematodes and diseases.

REFERENCES

Adejumo, T.O., Orole, O. O. (2010) Effect of pH and moisture content on endophytic colonization of maize roots. Scientific Research and Essays, 5 (13), 1655- 1661.

Araújo, W.L., Marcon, J., Maccheroni, W. Jr., Van Elsas, J.D., Van Vuurde, J. W., Azevedo, J.L. (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Applied and Environmental Microbiology, 68 (10), 4906-14. DOI: https://doi.org/10.1590/1678-4685-gmb-2016-0056

Bai, Y.M., Pan, B., Charles, T.C., Smith D.L. (2002) Co-inoculation dose an root zone temperature for plant growth promoting rhizobacteria on soybean (*Glycine max* L.). Soil Biology and Biochemistry, 34, 1953-1957. DOI: https://doi.org/10.1016/S0038-0717(02)00173-6

Berg, G., Eberl, L., Hartmann, A. (2005) The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environmental Microbiology, 7 (11), 1673-85. DOI: https://doi.org/10.1111/j.1462-2920.2005.00891.x

Cole, J.R., Wang, Q., Cardenas, E., Fish, J.; Chai, B., Farris, R. J., Kulam-Syed- Mohideen, A.S., McGarrell, D. M., Marsh, T., Garrity, G. M., Tiedje, J.M. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research, 37: D141-145. DOI: https://doi.org/10.1093/nar/gkn879

Dalal, J., Kulkarni, N. (2013) Population Dyamics and Diversity of Endophytic Bacteria Associated with Soybean (*Glycine max* (L) Merril).British Microbiology Research Journal, 3 (1), 96-105. DOI: https://doi.org/10.9734/BMRJ/2013/2302

Dixit, S. Dubey, R. C., Seth, P. K. (2014) Characterisation of Plant growth Promoting Endophytic Bacteria Isolated from Lens culinaris medik with antagonistic Potential against *Fusarium Oxysporum*. International Journal of Bioinformatics and Biological Sciences, 2 (12), 95-109.

Hung, P. Q., Annapurna, K. (2004) Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). Omonrice, 12, 92-101.

Impullitti, A. E., Malvick, D. K. (2013) Fungal endophyte diversity in soybean. Journal of Applied Microbiology, 114, 1500-1506. DOI: https://doi.org/10.1111/jam.12164

Kuklinsky-Sobral,J., Luiz Araujo, W., Mendes, R., Pizzirani-Kleiner, A. A., Azevedo, J.L. (2005) Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. Plant and Soil, 273, 91–99. DOI: https://doi.org/10.1007/s11104-004-6894-1

Kuykendall, L. D. (2005) Family I. Rhizobiaceae. In The Alpha-, Beta-, Delta and Epsilonproteobacteria, The Proteobacteria, Part C. In: Brenner, D.J., Krieg, N.R., Staley, J.T., Garrity, G.M. (eds.), Bergey's Manual of Systematic Bacteriology. New York: Springer, 324-362.

Li, Y., Cheng, C., An, D. (2017) Characterisation of endophytic bacteria from a desert plant *Lepidium perfoliatum* L. Plant Protection Science, 53, 32-43. DOI: https://doi.org/10.17221/14/2016-PPS

Lopes, R. B., Costa, L. E., Vanetti, M. C., de Araújo, E. F., de Queiroz, M. V. (2015) Endophytic Bacteria Isolated from Common Bean (*Phaseolus vulgaris*) Exhibiting High Variability Showed Antimicrobial Activity and Quorum Sensing Inhibition. Current Microbiology, 71 (4), 509-16. DOI: https://doi.org/10.1007/s00284-015-0879-6

Miliute,I., Buzaite,O., Baniulis, D., Stanys, V. (2005) Bacterial endophytes in agricultural crops and their role in stress tolerance: a review. Zemdirbyste- Agriculture, 102 (4), 465–478.

Niste, M., Vidican, R., Rotar, I., Pop, R. (2015) The Effect of Temperature Stress on *Rhizobium trifolii* and *Sinorhizobium meliloti* Strains *In Vitro*. Bulletin UASVM Agriculture, 72 (1).

Pimentel, I.C., Glienke-Blanco, C., Gabardo, J., Makowiecky Stuart, R., Azevedo, J.L. (2006) Identification and Colonization of Endophytic Fungi from Soybean (*Glycine max* (L.) Merril) under Different Environmental Conditions. Brazilian Archives of Biology and Technology, 49 (5), 705-711.

Ryan, R. P., Germaine,K., Franks, A., Ryan, D.J., Dowling, D. N. (2007) Bacterial endophytes: recent developments and applications. Federation of European Microbiological Societies, FEMS Microbiol Letter, 278, 1–9.

Shamseldin A. & Werner D (2005) High salt and high pH tolerance of new isolated *Rhizobium etli* strains from Egyptian soils. Current Microbiology, 50, 11-16.

Sikora, S., Redzepovic, S. (2003) Genotypic Characterisation of Indigenous Soybean Rhizobia by PCR-RFLP of 16S rDNA, rep-PCR and RAPD Analysis. Food Technology and Biotechnology, 41 (1), 61-67.

Stajkovic, O., De Meyer, S., Bogic Milicic, S., Willems, A., Delic, D. (2009) Isolation and characterization of endophytic non-rhizobial bacteria from root nodules of alfalfa (*Medicago sativa* L.). Botanica Serbica, 33 (1), 107-114.

Vincent, J.M. (1970) A Manual for the Practical Study of Root Nodule Bacteria. IBP, Handbook No 15 Blackwell Scientific Publications, Oxford.

Woese, C.R., Stackebrandt, E., Weisburg, W.G., Paster, B.J., Madigan, M.T., Fowler, V.J., Hahn, C.M., Blanz, P., Gupta, R., Nealson, K.H., Fox, G. E. (1984) The phylogeny of purple bacteria: the alpha subdivision. Systematic and Applied Microbiology, 5, 315-326.

Yan, J., Han, X.Z., Jun Ji, Z., Li, Y., Wang, E.T., Xie, Z. H., Chen, W. C. (2014) Abundance and Diversity of Soybean Nodulating Rhizobia in Black Soil are Impacted by Land Use and Crop Managements. Applied and Environmental Microbiology, 80 (17), 5394-402.