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Original scientific paper

ISOLATION OF RHIZOBACTERIA OF SESSILE OAK (*Quercus petraea* (Matt.) Liebl.) FROM THE FORESTS OF EASTERN SERBIA AND THEIR PRELIMINARY IDENTIFICATION

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Abstract: *Rhizobacteria are a heterogeneous group of bacteria inhabiting the rhizosphere of plants, whose dynamics and diversity of metabolism and activity are conditioned primarily by root exudates. Functionally, these bacteria contribute to the improvement of plant performances, and are often members of the genera Bacillus and Pseudomonas. The rhizobacteria of forest species in Serbia have not been sufficiently studied. Sessile oak is one of the most important oak species in Serbia and one of the most produced species in forest nurseries. In this study, the rhizobacteria of sessile oak from natural populations of eastern Serbia are isolated and the representatives of genus Bacillus, i.e., Pseudomonas are identified preliminarily. Out of a total of 94 isolates, 35 were preliminarily identified as Bacillus spp., and out of 29 Gram-negatives, some of the isolates belong to genus Pseudomonas. Further research is necessary in order to identify the isolates more precisely and examine their impact on sessile oak.*

Key words: rhizobacteria, sessile oak, identification, *Bacillus*, *Pseudomonas*, eastern Serbia.

IZOLACIJA RIZOBAKTERIJA HRASTA KITNJAKA (*Quercus petraea* (Matt.) Liebl.) IZ ŠUMA ISTOČNE SRBIJE I NJIHOVA PRELIMINARNA IDENTIFIKACIJA

Izvod: *Rizobakterije predstavljaju heterogenu grupu bakterija koje naseljavaju rizosferu biljaka, čiji su i dinamika i diverzitet metabolizma i aktivnosti uslovljeni pre svega*

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korenovim eksudatima. Funkcionalno, ove bakterije doprinose pospešivanju performansi biljke, i često su pripadnici rodova Bacillus i Pseudomonas. Rizobakterije šumskih vrsta u Srbiji nisu dovoljno istražene. Hrast kitnjak je jedna od najznačajnijih vrsta hrasta u Srbiji i najčešće proizvođenih u šumskim rasadnicima. U ovom radu izolovane su rizobakterije kitnjaka iz prirodnih populacija Istočne Srbije i preliminarno identifikovani predstavnici roda Bacillus, odnosno Pseudomonas. Od ukupno 94 izolata, 35 je preliminarno identifikovano kao Bacillus spp., a od 29 Gram negativnih, neki izolati pripadaju rodu Pseudomonas. Dalja istraživanja su neophodna radi preciznije identifikacije izolata i ispitivanja njihovog uticaja na hrast kitnjak.

Ključne reči: rizobakterije, hrast kitnjak, identifikacija, *Bacillus*, *Pseudomonas*, istočna Srbija

1. INTRODUCTION

Rhizobacteria are a heterogenous group of bacteria that inhabit plant rhizosphere. The availability of nutrients is the main reason of their dense population of this zone, where the abundance of bacteria is up to 100 times higher compared to surrounding land (Hajnal-Jafari et al., 2020; Vieira et al., 2020). The diversity and dynamics of metabolism and activity of rhizobacteria are conditioned by seasonality, availability of nutrients, type and physical and chemical characteristics of the soil, climate, plant genetics, as well as other biotic and abiotic factors. Plant species and its exudates secreted into the surrounding soil by means of its roots have a crucial role. Rhizodeposits of trees can contain up to 50% of carbon fixed by parent plant, and are incorporated into soil biomass (Baldrian, 2017). The changing environment affects the physiology of a plant, and in response to new circumstances, the plant changes the pattern of its exudates, spatially and temporally, realizing an effect on the associated microbial communities of the rhizosphere, in order to better adapt to new conditions. Plant Growth-Promoting Rhizobacteria (PGPR) stand out as a special functional group and they enhance plant growth. These bacteria are characterized by the ability of synthesis of phytohormones such as auxin, gibberellin, cytokinin, and inhibition of synthesis of ethylene, then dissolution of sparingly soluble forms of phosphates, mobilization of iron by production of siderophores, activation of induced systemic plant resistance, and various ways of exhibiting biocontrol activities (Vejan et al., 2016; Beneduzi et al., 2012). The beneficial effects of the above-mentioned mechanisms on the plant organism are reflected in the improvement of their performances – growth, fructification, resistance to biotic stressors, better adaptedness in relation to environmental conditions and survival in challenging habitats. It is considered that communication of PGPR with plants dates back to the arrival of first plants to land, when their co-evolution begins at the same time. (Lyu et al., 2021). In light of the fight against the pollution of the environment, climate changes and development of “green” technologies, PGPR have gained their place as a completely natural form of replacement of artificial fertilizers and pesticides, whose devastating effects on the environment have increasingly been examined (Backer et al., 2018), fully in line with the sustainability trends. Climate change problems are becoming more visible also in Serbia, so the need for their solution is more evident (Brašanac-

Bosanac, 2014). Biopreparations on the basis of individual bacterial strains or consortiums which are commercially widely available on the world market have also entered the course of legal regulations within the European Union (DuJardin, 2015). For that purpose, bacterial strains from the genera *Bacillus* and *Pseudomonas* are best characterized, and thus the most frequently used. They are characterized by great metabolic potential and the ability to degrade and synthesize an enormous number of compounds, adaptation to various habitat conditions, and a special feature of members of the genus *Bacillus* is the ability to form endospores in adverse environmental conditions, ensuring long-term survival of organisms. (Kloepper et al., 2004; Bakker et al., 2007; Kashyap et al., 2019; Dorjey et al., 2017). Ćirković-Mitrović (2014) examined the effect of BactoFil® on black walnut seedlings and confirmed its phytostimulating effect.

The rhizobacteria of forest species in Serbia are not thoroughly researched. Sessile oak (*Quercus petraea* (Matt.) Liebl.) is an autochthonous species of the Republic of Serbia and in the growing stock it is a second most valuable oak species with the share of 5.9% in the total volume (Banković et al., 2009). It builds 23 types of forests within the distribution area and is highly valued due to its technical value, wide application, as well as ecological and cultural significance. The need for sessile oak is great, as shown by the fact that it is one of two most commonly grown species in nursery production in Serbia. (Popović et al., 2019). Today, sessile oak forests are in unsatisfactory condition in terms of quality, health, age, poor vitality, thinned canopies, affected by the process of forest decline, which is further reflected in the frequency of fructification, amount and quality of seeds and natural regeneration (Krstić, 2016).

The aim of this study was to isolate sessile oak rhizosphere bacteria from its natural stands as well as the preliminary identification of isolates belonging to the genera *Bacillus* and *Pseudomonas*, as common soil microorganisms with the ability to promote plant growth. The obtained results can serve as a basis for further research of also the potential for manifestation of beneficial abilities on plant organism.

2. MATERIAL AND METHODS

In August 2020 soil samples were taken for the isolation of bacteria from the rhizosphere of natural sessile oak stands of the management units Ravna Reka I and Grabova Reka, within Majdanpek Forestry Administration. In each of the forest management units 6 healthy trees were randomly selected, at the base of which, at 50 cm from the tree, litterfall was removed and soil samples were taken at the depth of 0-30 cm, which were then deposited in sterile bags. The data on the precise location of the trees and altitude are shown in Tables 1 and 2.

Until the moment of processing, the samples were kept in a refrigerator at a temperature of 4°C.

During processing, each individual soil sample was mixed, after which 1 g was taken and resuspended in 10 ml of distilled water. A 10⁻⁵ dilution was made and 1 ml was seeded in nutrient agar (Nutrient agar CM0003 Oxoid) substrate by pouring, in duplicate, after which the samples were incubated for 48 h at a temperature of 30°C.

Table 1. *Data on location of trees from Ravna Reka 1 FMU*

Number of the tree	Coordinates	Altitude
Tree 1.	49 21 793 N 75 78 166 E	587 m
Tree 2.	49 22 048 N 75 78 248 E	624 m
Tree 3.	49 22 067 N 75 78 267 E	604 m
Tree 4.	49 22 363 N 75 78 211 E	628 m
Tree 5.	49 22 704 N 75 78 532 E	630 m
Tree 6.	49 22 959 N 75 78 423 E	644 m

Table 2. *Data on location of trees from Grabova Reka FMU*

Number of the tree	Coordinates	Altitude
Tree 1.	49 16 994 N 75 70 844 E	351 m
Tree 2.	49 16 950 N 75 70 923 E	368 m
Tree 3.	49 16 899 N 75 70 964 E	349 m
Tree 4.	49 16 768 N 75 71 017 E	325 m
Tree 5.	49 16 825 N 75 70 905 E	323 m
Tree 6.	49 16 950 N 75 70 680 E	285 m

After incubation, the grown colonies were purified until pure cultures were obtained. Thereafter, a preliminary identification of bacterial isolates was performed.

Gram staining reaction was determined for all isolates by the method of Suslow et al., 1982. The presence of threads indicates Gram-negative reaction, while their absence indicates Gram-positive reaction.

The catalase test indicates the presence of the enzyme catalase and it was performed on a microscope slide, as a reaction of a bacterial culture sample with 3% hydrogen peroxide (Schaad et al., 2001). All isolates were tested.

The bacterial isolates that showed a Gram-negative reaction were tested for the presence of cytochrome c oxidase in the respiratory chain, which preliminarily identifies aerobic bacteria. The oxidase test was performed by the method of Shields and Cathcart, 2010.

The bacteria that proved to be Gram-positive were tested for the possibility of endospore formation in adverse environmental conditions, which is a characteristic of bacteria of the genus *Bacillus*. Overnight cultures of individual isolates in the nutrient broth medium were made. Thereafter, 4 ml of each culture sample was incubated for 10 minutes at a temperature of 80°C in a water bath (Memmert). After incubation, samples of each isolate were seeded on nutrient agar substrate in order to be tested for survival, and incubated for 48 h at a temperature of 30°C.

The ability of Gram-negative isolates to synthesize green fluorescent pigment on KB nutrient medium with the addition of glycerol, which is a

characteristic of members of *Pseudomonas* bacteria genus, was also studied. The presence of this characteristic was observed under a UV lamp.

3. RESULTS AND DISCUSSION

The obtained results of isolation and preliminary characterization of sessile oak rhizobacteria from its natural stands in eastern Serbia are presented in Tables 3 and 4.

Table 3. Preliminary characterization of bacterial isolates from sessile oak rhizosphere from the site of Grabova Reka FMU

Bacterial isolate	Gram reaction	Oxidase test	Catalase test	Fluorescent pigment production	Growth at 80° C
GR1.1	+	/	+	/	-
GR2.1	-	+	+	-	/
GR2.2	-	-	+	+	/
GR2.3	+	/	+	/	+
GR2.4	-	+	-	+	/
GR2.5	-	-	+	-	/
GR2.6	+	/	-	/	+
GR2.7	-	-	+	+	/
GR2.8	+	/	+	/	+
GR2.9	-	-	+	+	/
GR2.10	-	+	-	+	/
GR3.1	+	/	+	/	-
GR3.2	-	-	+	-	/
GR3.3	+	/	+	/	+
GR3.4	+	/	+	/	+
GR3.5	+	/	+	/	+
GR3.6	+	/	+	/	+
GR3.7	+	/	+	/	+
GR3.8	+	/	+	/	+
GR3.9	-	-	+	-	/
GR4.1	-	-	+	-	/
GR4.2	+	/	+	/	+
GR5.1	+	/	+	/	+
GR5.2	+	/	+	/	-
GR6.1	-	+	-	+	/
GR6.2	+	/	+	/	+
GR6.3	-	-	+	-	/

Legend:

(/) The bacterial isolate has not been tested for the relevant characteristic.

(-) There is no appropriate enzyme or growth in the bacterial isolate, or its Gram reaction is negative.

(+) There is appropriate enzyme or growth in the bacterial isolate, or its Gram reaction is positive.

Table 4. Preliminary characterization of bacterial isolates from sessile oak rhizosphere from the site of Ravna Reka 1 FMU

Bacterial isolate	Gram reaction	Oxidase test	Catalase test	Fluorescent pigment production	Growth at 80°C
RR1.1	+	/	+	/	+
RR1.2	+	/	+	/	+
RR1.3	+	/	+	/	-
RR1.4	+	/	+	/	+
RR1.5	-	+	+	+	/
RR1.6	+	/	+	/	+
RR2.1	+	/	+	/	+
RR2.2	+	/	+	/	-
RR2.3	-	-	+	-	/
RR2.4	+	/	+	/	+
RR2.5	+	/	+	/	+
RR2.6	-	+	+	+	/
RR2.7	+	/	+	/	-
RR3.1	+	/	+	/	-
RR3.2	+	/	+	/	-
RR3.3	+	/	+	/	-
RR3.4	+	/	+	/	-
RR3.5	+	/	+	/	-
RR3.6	+	/	+	/	+
RR3.7	+	/	+	/	-
RR3.8	+	/	+	/	-
RR3.9	+	/	+	/	+
RR3.10	-	+	+	+	/
RR3.11	-	+	+	+	/
RR3.12	+	/	+	/	-
RR3.13	+	/	+	/	+
RR3.14	+	/	+	/	-
RR3.15	+	/	+	/	-
RR3.16	+	/	+	/	-
RR3.17	-	+	+	+	/
RR3.18	+	/	+	/	+
RR3.19	+	/	+	/	-
RR3.20	-	+	+	+	/
RR3.21	+	/	+	/	-
RR3.22	+	/	+	/	+
RR3.23	-	+	+	+	/
RR3.24	-	+	+	+	/
RR4.1	+	/	+	/	-
RR4.2	-	-	+	-	/
RR4.3	-	-	+	-	/
RR4.4	-	-	+	-	/
RR4.5	+	/	+	/	+
RR4.6	+	/	+	/	+
RR4.7	+	/	+	/	-
RR4.8	+	/	+	/	-
RR4.9	+	/	+	/	-
RR4.10	-	-	+	+	/
RR4.11	+	/	+	/	-
RR5.1	+	/	+	/	+
RR5.2	-	+	+	+	/
RR5.3	-	+	+	+	/
RR5.4	+	/	+	/	-

Bacterial isolate	Gram reaction	Oxidase test	Catalase test	Fluorescent pigment production	Growth at 80° C
RR5.5	+	/	+	/	+
RR5.6	+	/	+	/	+
RR5.7	+	/	+	/	-
RR6.1	+	/	+	/	+
RR6.2	+	/	+	/	+
RR6.3	+	/	+	/	+
RR6.4	+	/	+	/	+
RR6.5	+	/	+	/	+
RR6.6	+	/	+	/	+
RR6.7	-	+	+	+	/
RR6.8	+	/	+	/	-
RR6.9	+	/	+	/	-
RR6.10	-	+	+	+	/
RR6.11	+	/	+	/	-
RR6.12	+	/	+	/	-

Legend:

(/) The bacterial isolate has not been tested for the relevant characteristic.

(-) There is no appropriate enzyme or growth in the bacterial isolate, or its Gram reaction is negative.

(+) There is appropriate enzyme or growth in the bacterial isolate, or its Gram reaction is positive.

A total of 94 bacterial isolates were isolated from rhizosphere samples of 12 sessile oak trees from their natural stands of eastern Serbia. Out of that number, 67 originate from the site of Ravna Reka 1 FMU, while 27 isolates originate from the site of Grabova Reka FMU.

Out of the total number of isolated bacteria, 29 showed a Gram-negative reaction and 12 of them originate from Grabova Reka FMU. The remaining 65 isolates had a Gram-positive reaction, out of which 15 rhizosphere samples were from the site of Grabova Reka FMU.

All 29 Gram-negative isolates were tested for the presence of cytochrome c oxidase, 16 of which were oxidase positive and 19 bacteria had the ability to synthesize a yellowish green fluorescent pigment. The highest fluorescence was observed in isolates RR1.5, RR3.10, RR3.17, RR5.2 and RR5.3. Out of the total number of Gram-negative bacteria, 26 isolates proved to be catalase positive.

Gram-positive bacteria were tested for the presence of the enzyme catalase and out of the total number, 63 isolates synthesized this enzyme. Following the exposure to a temperature of 80° C, 35 bacterial isolates were viable and grew on nutrient agar.

After the performed tests, 34 of 94 isolates were Gram-positive, catalase positive and manifested growth after the exposure to a temperature of 80° C. Sixteen isolates had Gram-negative reaction with the production of fluorescent yellowish-green pigment, while 12 out of this number were oxidase positive.

The members of the genus *Bacillus* and *Pseudomonas* are very represented in nature. Changing environmental conditions require the development of adaptive strategies of organisms, and *Bacillus* endospores have proven to be one of the best solutions. These bacteria can especially be found in soils with different characteristics – acidic, alkaline or deserts, whereby the frequency of occurrence of some species shows significant correlations with altitude (Liu et al., 2019). Gagelidze et al. (2018) examined the presence of *Bacillus* and *Pseudomonas* in different soil types of Georgia and found that they occurred equally in black soil,

that *Bacillus* genus was more dominant in chernozem, alkaline soils and marshes, while *Pseudomonas* genus occurred more frequently in brown forest soils. Both sites sampled in this study are located on the type of dystric brown soil.

Due to their special complexity, forest soils are characterized by a particularly large diversity of bacteria. One study showed a decrease in the diversity of bacteria of the genus *Bacillus* from forest to eroded soils (Zhang et al., 2003), while in other no significant correlation was found (Liu et al., 2016). Dukunde et al. (2019) examined the influence of tree species on the structure of soil bacterial communities in temperate deciduous forests. They found that in beech and oak monocultures the soil pH is low with a high C:N ratio, and in these communities the abundance of oligotrophic compared to copiotrophic bacterial taxa is greater. Also, compared to mixed stands, monocultures showed a larger number of indicator species highly associated with each stand. While Grabova Reka FMU represents typical oak forests, in Ravna Reka 1 FMU there are mixed oak and beech forests.

No molecular or biochemical methods by which bacteria identification down to the level of species could be carried out were performed in this study. Based on the performed preliminary identification tests 35 potential members of the genus *Bacillus* were isolated. On the other hand, out of 29 Gram-negative isolates, some belong to the genus *Pseudomonas*.

The results confirm the diversity of forest soil bacteria, as well as the abundance of the genera *Pseudomonas* and *Bacillus* in the rhizosphere of sessile oak. 94 bacteria were isolated, however, since the research of the composition of bacterial communities was performed using a breeding method, we can state with certainty that the total diversity is much greater. The reason for relatively small number of Gram-negative bacteria, oxidase-positive isolates of potential pseudomonads can be the way of the bacteria seeding, i.e., pouring method, as a result of which some aerobic organisms could not grow. Further research is needed in order to perform more precise characterization of isolates. Thereafter, their relationships with sessile oak, the host plant would be studied.

4. CONCLUSIONS

Rhizobacteria are a large group of diverse microorganisms that inhabit the root zone of plants and in previous research conducted mainly on agricultural species, many isolates from the rhizosphere have shown potential in promoting plant growth and replacing artificial fertilizers, whose negative effects on the environment are increasingly being revealed. Sessile oak is one of the most important species in the flora of Serbia, and as such it is the subject of numerous studies.

In this study, 94 pure bacterial cultures were isolated from sessile oak rhizosphere, from its natural populations of eastern Serbia. Gram, catalase and oxidase tests were applied, as well as testing for fluorescent pigment synthesis, i.e., survival at 80° C. While 35 isolates were preliminarily characterized as *Bacillus* spp., 29 Gram-negative isolates required additional research in order to confirm belonging to *Pseudomonas* genus. These results represent the first step in the study

of rhizosphere sessile oak communities and further studies are needed in order to determine the relationship with the parent plant.

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Summary

Rhizobacteria are a heterogeneous group of bacteria inhabiting the rhizosphere of plants, whose dynamics and diversity of metabolism and activity are conditioned primarily by root exudates. Functionally, these bacteria contribute to the improvement of plant performances, and are often members of the genera *Bacillus* and *Pseudomonas*. The beneficial effects are reflected in the improvement of their performances – growth, fructification, resistance to biotic stressors, better adaptedness in relation to environmental conditions and survival in challenging habitats. In light of the fight against the pollution of the environment, climate changes and development of “green” technologies, PGPR have gained their place as a completely natural form of replacement of artificial fertilizers and pesticides, whose devastating effects on the environment have increasingly been examined, fully in line with the sustainability trends.

The rhizobacteria of forest species in Serbia have not been sufficiently studied. Sessile oak is one of the most important oak species in Serbia and one of the most produced species in forest nurseries. At present, sessile oak forests are in unsatisfactory condition in terms of quality, health, age, poor vitality, thinned canopies, affected by the process of forest decline, which is further reflected in the frequency of fructification, amount and quality of seeds and natural regeneration.

The aim of this study was to isolate sessile oak rhizosphere bacteria from its natural stands and preliminary identification of isolates belonging to the genera *Bacillus* and *Pseudomonas*, as common soil microorganisms with the ability to promote plant growth.

Subsequent to performed Gram, catalase and oxidase reactions and examination of growth at 80°C and production of fluorescent pigment the rhizobacteria of sessile oak from natural populations of eastern Serbia were isolated and members of the genera *Bacillus*, i.e., *Pseudomonas* were preliminarily identified. Out of a total of 94 isolated bacteria, 35 potentially belong to the genus *Bacillus* and out of 29 Gram-negatives, some of the isolates belong to the genus *Pseudomonas*. Further research is necessary in order to identify the isolates more precisely and examine their impact on sessile oak.

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(*Quercus petraea* (Matt.) Liebl.) IZ ŠUMA ISTOČNE SRBIJE I NJIHOVA
PRELIMINARNA IDENTIFIKACIJA**

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Sažetak

Rizobakterije predstavljaju heterogenu grupu bakterija koje naseljavaju rizosferu biljaka, čiji su i dinamika i diverzitet metabolizma i aktivnosti uslovljeni pre svega korenovim eksudatima. Funkcionalno, ove bakterije doprinose pospešivanju performansi biljke, i često su pripadnici rodova *Bacillus* i *Pseudomonas*. Blagodetni efekti ogledaju se u

poboljšavanju njihovih performansi – rasta, plodonošenja, otpornosti na biotičke stresore, boljoj prilagođenosti na uslove sredine i opstanku na izazovnim staništima. U svetlu borbe sa zagađenošću životne sredine, klimatskim promenama i razvoju „zelenih” tehnologija, PGPR su dobile svoje mesto kao potpuno prirodan vid zamene veštačkih đubriva i pesticida, čiji se devastirajući efekti po okolinu sve više razmatraju, potpuno u skladu sa tendencijama održivosti.

Rizobakterije šumskih vrsta u Srbiji nisu dovoljno istražene. Hrast kitnjak je jedna od najznačajnijih vrsta hrasta u Srbiji i najčešće proizvođenih u šumskim rasadnicima. Šume kitnjaka su danas nezadovoljavajućeg stanja u pogledu kvaliteta, zdravstvenog stanja, starosti, slabe vitalnosti, razređenih krošnji, zahvaćeni procesom sušenja, što se dalje odražava na učestalost plodonošenja, količinu i kvalitet semena i prirodnu obnovu.

Cilj ovog rada je bio izolacija bakterija rizosfere hrasta kitnjaka iz njegovih prirodnih sastojina i preliminarna identifikacija izolata koji pripadaju rodovima *Bacillus* i *Pseudomonas*, kao čestim zemljišnim mikroorganizmima sa sposobnostima promocije rasta biljaka.

Nakon izvedene Gram, katalaza, oksidaza reakcije, zatim ispitivanja rasta na 80°C i proizvodnju fluorescentnog pigmenta, izolovane su rizobakterije kitnjaka iz prirodnih populacija Istočne Srbije i preliminarno identifikovani predstavnici roda *Bacillus*, odnosno *Pseudomonas*. Od ukupno 94 izolovane bakterije, 35 potencijalno pripada rodu *Bacillus*, a od 29 Gram negativnih, neki izolati pripadaju rodu *Pseudomonas*. Dalja istraživanja su neophodna radi preciznije identifikacije izolata i ispitivanja njihovog uticaja na hrast kitnjak.