Journal of Agricultural Sciences
Vol. 65, No. 2, 2020
Pages 163-174

https://doi.org/10.2298/JAS2002163K
UDC: 633.327-186.7
Original scientific paper

BACTERIAL INOCULATION: A TOOL FOR RED CLOVER GROWTH PROMOTION IN POLLUTED SOIL

Vera M. Karličić[1*], Danka S. Radić[2], Jelena P. Jovičić-Petrović[1] and Vera B. Raičević[1]

[1]University of Belgrade, Faculty of Agriculture,
Nemanjina 6, Belgrade-Zemun, Serbia
[2]Educons University, Faculty of Ecological Agriculture,
Vojvode Putnika 85-87, Sremska Kamenica, Serbia

Abstract: Red clover (*Trifolium pratense* L.) seeds were inoculated with several plant growth-promoting bacteria (PGPB) and sown in the substrate contaminated with polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organometallic derivatives of tin (OT). The aim was to determine if selected PGPB strains can promote the growth of red clover in the substrate contaminated with several organic pollutants. The influence of bacteria on red clover growth (height, root length and biomass) was monitored during the three-month experimental period. The most significant improvements of seedling height were noted in the treatment with *Bacillus amyloliquefaciens* D5 ARV and *Pseudomonas putida* P1 ARV. Root growth was positively affected by *Serratia liquefaciens* Z-I ARV. The same isolates significantly affected biomass production. Those isolates caused total biomass increases of 70%, 48% and 33% compared to control. Bacterial strains used in this study were already confirmed as PGPB by biochemical testing, as well as by an *in vivo* test of mixed inoculums on several woody plants grown in the coal-mine overburden site. This work is the first-time record on their individual effects on one plant species. Obtained results confirmed that inoculation with several PGPB strains can enhance red clover growth in polluted soil.

Key words: organic pollutants, red clover, revegetation, plant growth-promoting bacteria.

Introduction

Organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organometallic derivatives of tin (OT) represent a global problem. The main anthropogenic sources of PAHs, PCBs and

[*]Corresponding author: e-mail: vera.karlicic@agrif.bg.ac.rs

OTs are industrial sector, agricultural practice, waste incineration and incomplete combustion of fossil fuels (Karličić et al., 2014; Skála et al., 2018). Broad use of those substances resulted in their accumulation in soil and water bodies representing a serious threat to wildlife, human health and the whole ecosystem. Jeelani et al. (2017) quote that 25% of global soils are highly degraded by the presence of PAHs and heavy metals, whereas 44% are referred to as moderately degraded. Those contaminants are characterized as toxic and persistent (Kummerová et al., 2012) and the remediation of contaminated soil is qualified as urgent.

Plant ability to take up organic and inorganic pollutants from soil is employed through the process of phytoremediation. They are capable to hyperaccumulate, immobilize and convert contaminants into simpler or volatile compounds (Ahmad et al., 2017). Plant mechanisms are strongly supported by the microbial activity in the root zone (Mesa et al., 2017). Some of the microorganisms inhabiting the root zone are capable to mobilize or immobilize contaminants and they are included in degradation and detoxification of pollutants through the process of bioremediation (Ahmad et al., 2017; Ite et al., 2019). Microbial-assisted phytoremediation takes advantage of the plant-microorganism partnership and coexistence under the same stressful conditions with one main goal – economical and effective soil purification (Pinto et al., 2018). Phyto/Bioremediation applies to a broad range of chemicals such as heavy metals, PCBs, PAHs, OTs, pesticides/insecticides (Rohrbacher and St-Arnaud, 2016; Jeelani et al., 2017).

On the other hand, organic pollutants are well known as phytotoxic. Plants are especially susceptible to the presence of pollutants during the germination and root formation stages (Kummerová et al., 2012). Such effects complicate the establishment of vegetation cover which is a precondition of phytoremediation. That is why contaminant-tolerant plant species, with flexibility in stress conditions, ability to grow quickly and produce high biomass are highly recommended (Hou et al., 2015; Eskandary et al., 2017). Similarly, the root zone microorganisms play a critical role in the plants' survival under stressful conditions (Backer et al., 2018). Among rhizosphere inhabitants, plant growth-promoting bacteria (PGPB) attracted special attention. This group of bacteria is capable to promote plant growth in contaminated soils, to increase plant resistance to environmental stresses (De Souza et al., 2015), to change the availability of organic and metal pollutants (Ahmad et al., 2017). They also play a great role in nutrient acquisition by nitrogen fixation, solubilization of phosphorus, or other unavailable forms of nutrients and siderophore production (Pii et al., 2015). PGPB produce plant hormones (auxins, cytokinins, gibberellins) which are in charge of growth regulation and stress response (Backer et al., 2018). A wide range of plant stimulating mechanisms of PGPB ensured them a significant place in current agricultural practice. They are considered as a "green and clean" technology (Ramakrishna et al., 2019) which can be a proper alternative for mineral fertilizers and pesticides, two main pollutants of conventional agriculture. Even though the main field of their study and application is food production, PGPB find their place in numerous fields related to plant production (forestry, horticulture) or remediation. Those bacteria possess the natural potential to alleviate the impacts of toxic contaminants in soil (Backer et al., 2018), and help in the establishment of vegetation.

Red clover (*Trifolium pratense* L.), fast-growing legume suitable for remediation of contaminated soils (Gkorezis et al., 2016), was chosen as a test plant in the study. The objective was to estimate the effects of bacterial inoculation on red clover growth in the substrate contaminated with PAHs, PCBs and OT substances. Several bacterial strains were used for inoculation and we searched for the most effective ones in terms of red clover growth promotion.

Materials and Methods

The substrate used in the experiment was soil from Tivat City Park, Montenegro. Chemical analyses of this substrate were performed by a gas chromatography-mass spectrometry method and they revealed the presence of PAHs, PCBs and OT substances (Table 1).

Table 1. Concentrations of PAHs, PCBs and OT (mg/kg) in the soil of Tivat City Park (Jovičić Petrović et al., 2014, Karličić et al., 2014).

Contaminant	Soil sample	MAC* mg/kg
PAH	5.97	0.6
PCB 28	<0.005	
PCB 52	0.016	
PCB 101	0.030	
PCB 118	0.030	0.004
PCB 138	0.024	
PCB 153	0.018	
PCB 180	0.010	
ORGANOTINS		
MONOBUTYLTIN	0.005	
DIBUTYLTIN	0.008 ± 0.0009	
MONOFENIL TIN	< 0.004	0.005
TRIBUTILTIN	0.026 ± 0.0032	
DIPHENILTIN	< 0.004	
TRIPHENILTIN	< 0.004	

Maximum allowed concentration (MAC)* statutory values in Montenegro (Official Gazette of RCG, No. 18/97).

Bacterial material. Strains used in this study were: *Psedomonas putida* P1 ARV (agricultural soil), *Serratia liquefaciens* Z-I ARV (isolated from the soil of

Tivat City Park), *Ensifer adhaerens* 10_ARV (isolated from the soil of Tivat City Park), and *Bacillus amyloliquefaciens* D5 ARV (isolated from the overburden site of coal-mine Kolubara). The strains were identified by molecular methods, and characterized as PGPB based on indoleacetic acid, siderophore production, phosphate solubilization ability and *in vivo* tests on London plane (*Platanus x acerifolia*), black locust (*Robinia pseudoacacia*) and Scots pine (*Pinus sylvestris*) grown in the coal-mine overburden used as a substrate (Karličić et al., 2015; Karličić et al., 2017).

Seed sterilization. The seeds of red clover were surface sterilized by emersion in 70% ethanol for 2 min followed by a 15-min exposure to 2% NaOCl. Seeds were washed properly with sterile distilled water (5-10 x) and soaked for the next half an hour to antibiotics solution (600 mg L^{-1} penicillin, and 250 mg L^{-1} streptomycin). Seeds were washed, and the final step was drying in aseptic conditions.

Inoculum preparation. Separate bacterial strains were grown in nutrient broth aerobically at 28±2°C/48h/100 rpm (BIOSAN, Latvia). *Ensifer adhaerens* 10_ARV was grown in Fjodorov medium (Anderson, 1958) at 28±2°C/72h/100 rpm. The bacterial suspensions were centrifuged at 6000 *x g* for 10 min (5804 R, Eppendorf, Germany), and diluted in distilled water to achieve 10^8 CFU mL^{-1}. The mixed inoculum was prepared by mixing bacterial strain-specific inoculums in the 1:1:1:2 (*Ensifer adhaerens* 10_ARV) ratio.

Seed inoculation and pot experiment. Sterilized seeds were inoculated with selected strains by 1h immersion at 100 rpm/h (BIOSAN, Latvia) and the treatments were as follows:

Z-I ARV: Seeds inoculated with *S. liquefaciens* Z-I ARV;
10_ARV: Seeds inoculated with *E. adhaerens* 10_ARV;
D5 ARV: Seeds inoculated with *B. amyloliquefaciens* D5 ARV;
P1 ARV: Seeds inoculated with *P. putida* P1 ARV;
MIX: Seeds inoculated with mixed inoculum;
CONPS: Noninoculated seeds grown in polluted soil.

Prepared seeds were sown in 0.5-dm^3 plastic pots filled with the substrate. Afterwards, the plants were cultivated under controlled conditions in the growth chamber, exposed to sunlight for 12 h daily (14 000 lux; MH Philips 600W) with a maximum temperature of 30°C and a minimum temperature of 20°C. Soil moisture was kept at 60% of the soil field capacity.

The experimental period lasted for three months and the aboveground biomass of plants was harvested three times, about 3 cm above soil level, approximately every 25^{th} day. The height of the seedlings was measured prior to every cut. Mown grass was dried at 60°C until constant mass and dry matter (DM) of the harvests was determined. Root length was measured at the end of the experiment. Fifty seeds were sown on each pot and the experiment was set up as a completely randomized design with three replications.

Statistical analyses. Data were analyzed by two-way analyses of variance (ANOVA) followed by the Fisher's Least Significant Difference (LSD) test. The analyses were conducted using the SPSS 22 software package (SPSS Inc., Chicago, IL, USA).

Results and Discussion

This work examines the potential of several PGPB to promote red clover growth in soil polluted with organic contaminants. Red clover seeds were inoculated and sown in the substrate with an increased content of PAHs, PCBs and OTs according to the regulation of the Republic of Montenegro. Even though the regulation of The Republic of Serbia is less rigorous on this issue, concentrations of total PAHs and several PCBs were marked as elevated. According to the 2010 Statute (Official Gazette of RS, No. 88/2010), MAC of total PAHs is 1 mg kg^{-1}. In the case of PCBs, the MAC value is 0.02 mg kg^{-1}.

The effect of applied treatments on red clover seedlings was monitored through seedling height (Table 2), root length (Table 3) and biomass (Table 4) in the three-month experimental period.

After the first mowing, the highest values of height were noted on seedlings inoculated with *B. amyloliquefaciens* D5 ARV, followed by *P. putida* P1 ARV treatment. All other treatments did not induce significantly better growth compared to CONPS. The second mowing showed that the treatment with *B. amyloliquefaciens* D5 ARV kept the highest values of height. Other treatments gave the same results as in the time of the first cut. The third mowing showed that *P. putida* P1 ARV seedlings were significantly higher compared to others. The remaining treatments did not induce the promotion of this growth parameter.

Table 2. Height (cm) of red clover seedlings grown on the soil contaminated with organic pollutants.

Treatment	Red clover seedling height (cm)			$LSD_{0.01}$
	Mowing			
	I	II	III	
Z-I ARV	7.90 ± 1.21^{b}	6.50 ± 1.54^{b}	8.60 ± 0.96^{b}	0.191
10 ARV	7.83 ± 1.13^{b}	6.75 ± 1.47^{b}	9.00 ± 0.66^{c}	
D5 ARV	9.53 ± 1.59^{d}	7.95 ± 0.97^{c}	8.50 ± 0.50^{ab}	
P1 ARV	8.50 ± 1.31^{c}	5.75 ± 0.44^{a}	9.70 ± 1.19^{d}	
MIX	7.30 ± 0.98^{a}	6.75 ± 1.35^{b}	8.30 ± 0.40^{a}	
CONPS	7.68 ± 1.16^{b}	6.75 ± 1.11^{b}	9.00 ± 0.45^{c}	
$LSD_{0,01}$		0.292		

± shows standard deviation; different small caps represent significant statistical differences between treatments (p=0.01).

The data of the root length were recorded at the end of the experiment (Table 3). The only isolate that significantly influenced root length was *S. liquefaciens* Z-I ARV. The presence of all others isolates and their mixed inoculum did not induce significant root development.

Table 3. Root length (cm) of red clover seedlings grown on the soil contaminated with organic pollutants.

Treatment	Root length (cm)
Z-I ARV	8.63 ± 2.86^{c}
10 ARV	6.87 ± 1.07^{b}
D5 ARV	7.15 ± 1.48^{b}
P1 ARV	5.73 ± 1.30^{a}
MIX	6.02 ± 1.46^{ab}
CONPS	6.26 ± 1.43^{ab}
$LSD_{0,01}$	0.896

± shows standard deviation; different letters represent significant statistical differences between treatments based on the LSD test (p=0.01).

After the first mowing, the highest biomass production (Table 4) was noted in the case of seedlings inoculated with *B. amyloliquefasiens* D5 ARV, *P. putida* P1 ARV and *S. liquefaciens* Z-I ARV. The presence of other isolates did not induce significantly different results compared to the CONPS treatment. The second mowing showed similar results. At the end of the experiment, inoculation did not show any effects on seedling biomass production.

Table 4. Dry aboveground biomass (g) of red clover seedlings grown on the soil contaminated with organic pollutants.

Treatment	Dry aboveground biomass (g)			$LSD_{0.01}$
	Mowing			
	I	II	III	
Z-I ARV	1.03 ± 0.12^{bC}	0.85 ± 0.09^{bB}	0.60 ± 0.09^{aA}	0.133
10 ARV	0.80 ± 0.13^{aB}	0.47 ± 0.10^{aA}	0.72 ± 0.03^{aB}	
D5 ARV	1.33 ± 0.21^{cC}	1.07 ± 0.19^{cB}	0.77 ± 0.06^{aA}	
P1 ARV	1.07 ± 0.19^{bB}	0.89 ± 0.09^{bcA}	0.80 ± 0.23^{aA}	
MIX	0.74 ± 0.10^{aB}	0.53 ± 0.08^{aA}	0.61 ± 0.17^{aAB}	
CONPS	0.67 ± 0.06^{aB}	0.50 ± 0.03^{aA}	0.69 ± 0.08^{aB}	
$LSD_{0,01}$		0.202		

± shows standard deviation; different small caps represent significant statistical differences between treatments; different large caps represent significant statistical differences between mowing based on the LSD test (p=0.01).

Analyses of data within a treatment obtained after every mowing showed that the first mowing gave the highest yield. The yields obtained in the other two mowings were significantly lower in most cases (Table 4). Plant growth can cause nutrient depletion considering limited pot resources. Besides, the main reason to apply bacterial inoculants on seeds is to support the plant growth in the earlier stages of plant ontogenesis. Those microbes affect germination and early growth which is critical for the success of revegetation. Further research is needed to evaluate the potential effects of subsequent application of the bacterial inoculants in soil, in later phases of plant growth.

Red clover inoculated with *B. amyloliquefasiens* D5 ARV produced the highest aboveground dry biomass through the whole experimental period. Also, *P. putida* P1 ARV and *S. liquefaciens* Z-I ARV significantly elevated biomass yield (Figure 1). This effect is highly desirable in the case of phytoremediation based on phytoextraction where biomass represents the storehouse of pollutants.

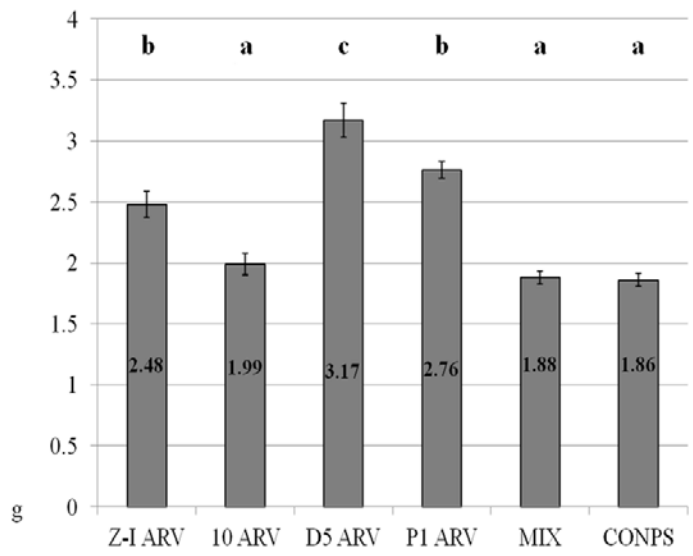

Figure 1. Total dry aboveground biomass (g) of red clover seedlings grown on the soil contaminated with organic pollutants. Different letters represent significant statistical differences between treatments based on the LSD test ($p=0.05$).

Even though red clover is used in bioremediation frequently (Gkorezis et al., 2016; Nazarov et al., 2017), Sverdrup et al. (2003) recorded its sensitivity on PAHs presence which resulted in the fresh and dry weight decrease. Such a problem can be successfully alleviated by PGPB. Bacterial strains used in this study were already confirmed as growth promoters of several woody species. Previously those strains were used in the form of mixed inoculums and this is the first time that their individual effects were studied on one plant species.

The overview of the presented results showed that, in the presence of *B. amyloliquefasiens* D5 ARV and *P. putida* P1 ARV, seedlings were higher and produced more biomass in comparison to control. Karličić et al. (2017) referred to the ability of those two isolates to produce ammonia, siderophores and indoleacetic acid (1.5 µg ml^{-1} and 1.2 µg ml^{-1} respectively). *P. putida* P1 ARV was capable to solubilize inorganic phosphates, too (Karličić et al. 2017). *S. liquefaciens* Z-I ARV induced higher biomass production and supported root growth. This isolate showed similar PGP characteristics as the previous two, but it possessed higher indoleacetic acid production (8.4 µg ml^{-1}) abilities (Karličić et al., 2017).

Production of indoleacetic acid is one of the most important characteristics of PGPB due to the great role of this hormone in plant-microbe interactions. Even a small amount of indoleacetic acid produced by rhizobacteria is enough to induce a positive effect on plant growth (Teixeria et al., 2007). The higher amount does not guarantee a positive plant response and may be the reason for plant growth inhibition (Gamalero and Glick, 2015). This could be the explanation for disappointing results obtained in the treatment with *E. adhaerens* 10_ARV. Biochemical testing marked this isolate as the most promising one. It produced a high amount of indoleacetic acid (44.5 µg ml^{-1}), solubilized inorganic phosphates, produced ammonia and siderophores (Karličić et al., 2017). This emphasizes the significance of numerous factors (amount of endogenous auxins, plant species and phase of plant growth) that need to be taken into account for successful PGPB application.

Higher biomass accumulates higher amounts of pollutants and speeds up the soil purification process (Jiang et al., 2015). Ficko et al. (2010) assert that plants with the ability to storage low PCB concentration could still extract a valuable quantity of PCBs with large shoot biomass. This is the aspect that can be significantly improved by PGPB. Rostami et al. (2017) showed that *Sorghum bicolor* inoculation with *Pseudomonas aeruginosa* resulted in higher shoot (21.27%) and root biomass (14.5%) at the pyrene concentration of 150 mg kg^{-1}. They also showed that such a combination reduced 66–82% of pyrene after the 90-day experimental period. In our study, the presence of *S. liquefaciens* Z-I ARV induced higher biomass production (an increase of 33%), *P. putida* P1 ARV induced an increase of 48%, while *B. amyloliquefaciens* D5 ARV caused an increment of biomass that reached 70%. Eskandary et al. (2017) reported the shoot and root biomass increase of *Festuca arundinacea* inoculated with *B. licheniformis* ATHE9 and *B. mojavensis* ATHE13 under polluted soil conditions. Those authors related 95% PAH degradation to the synergistic effect of *B. mojavensis*, *B. licheniformis* and the plant.

The contaminated soils are the main pools of microorganisms suitable for bioremediation (Karličić et al., 2016). In this study, *S. liquefaciens* Z-I ARV and *E. adhaerns* 10_ARV originated from the soil that was the object of interest. Among them, the presence of *S. liquefaciens* Z-I ARV gave promising results. We also used *P. putida* P1 ARV and *B. amyloliquefaciens* D5 ARV of a completely different origin, but they showed the capacity to improve red clover growth under given circumstances.

Revegetation is a process that occurs naturally and slowly (Panchenko et al., 2018), but it can be speeded up by taking measures that reconcile *in situ* requirements. Two crucial factors that need to be optimized are plant species, adapted to the presence of high levels of contaminants, and microbial community capable to survive and influence plant growth and tolerance under given circumstances (Hou et al., 2015). This can be achieved by plant inoculation with proper PGPB. The right combination enhances metabolic processes in the soil, causing faster and more complete soil recovery. The results of the presented work show that some of the tested combinations may be qualified as the “right” since applied PGPB stimulated growth and biomass production of red clover grown in the substrate burden with a high presence of organic pollutants. Obtained data imply that red clover used in combination with PGPB can achieve better growth in polluted soil, and thus it has a better potential for use in revegetation of contaminated soil.

Conclusion

The wide use of different organic and inorganic pollutants causes deterioration of soil. The most vulnerable are plant species, tightly attached to the substrate, which accumulate toxic substances in their biomasses. Some of them are very resistant and highly appreciated for revegetation and remediation of contaminated soils. Soil microorganisms are ever-present residents well known as plant helpers. The obtained results pointed out at three isolates, *B. amyloliquefaciens* D5 ARV, *Pseudomonas putida* P1 ARV and *S. liquefaciens* Z-I ARV, whose individual application resulted in significant plant growth promotion. Among them, the most effective was *B. amyloliquefaciens* D5 ARV, which substantially raised red clover biomass production. The biochemical tests, especially those for indoleacetic acid production, pointed out at *E. adhaerens* 10_ARV, but this strain failed *in vivo*. This shows the complexity of factors that modulate plant-microbe interactions, and indicate the necessity for additional selection through *in vivo* tests, particularly in such specific substrates. A significant rise of biomass production caused by the presence of PGPB strains will certainly encourage further research in the estimation of remediation utility of such combinations.

Acknowledgements

This research was supported by the Ministry of Education, Science and Technological Development, the Republic of Serbia (contract number 451-03-68/2020-14/200116).

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Received: November 6, 2019
Accepted: May 20, 2020

BAKTERIJSKA INOKULACIJA: POSTUPAK ZA STIMULACIJU RASTA CRVENE DETELINE GAJENE U ZAGAĐENOM ZEMLJIŠTU

Vera M. Karličić[1*], Danka S. Radić[2], Jelena P. Jovičić-Petrović[1] i Vera B. Raičević[1]

[1]Univerzitet u Beogradu, Poljoprivredni fakultet, Nemanjina 6, Beograd-Zemun, Srbija
[2]Educons Univerzitet, Fakultet zaštite životne sredine, Vojvode Putnika 87, Sremska Kamenica, Srbija

R e z i m e

Seme crvene deteline (*Trifolium pratense* L.), inokulisno sa nekoliko bakterija stimulatora biljnog rasta (PGPB), posejano je u supstrat kontaminiran policikličnim aromatičnim ugljovodonicima (PAHs), polihlorovanim bifenilima (PCBs) i organometalnim derivatima kalaja (OT). Cilj je bio da se utvrdi da li selektovane PGPB mogu promovisati rast crvene deteline u supstratu kontaminiranom sa nekoliko organskih zagađujućih materija. Uticaj bakterija na rast crvene deteline (visina, dužina korena i biomasa) praćen je tri meseca. Najveća visina je zabeležena kod biljaka inokulisanih sa *Bacillus amyloliquefaciens* D5 ARV i *Pseudomonas putida* P1 ARV. Rast korena je stimulisan od strane *Serratia liquefaciens* Z-I ARV. Ovi izolati su značajno uticali i na produkciju biomase. Ukupna biomasa dobijena tokom celog ogleda je za 70%, 48% i 33% veća u odnosu na kontrolu. Bakterijski sojevi korišćeni u ovoj studiji su prethodno potvrđeni kao PGPB kroz biohemijske i *in vivo* testove mešanog inokuluma na nekoliko drvenastih vrsta gajenih u jalovini. Ovaj rad prvi put beleži njihove pojedinačne efekte na jednu biljnu vrstu. Dobijeni rezultati potvrđuju da inokulacija sa nekoliko PGPB sojeva može ubrzati rast crvene deteline u zagađenom zemljištu.

Ključne reči: organske zagađujuće materije, crvena detelina, revegetacija, bakterije stimulatori biljnog rasta.

Primljeno: 6. novembra 2019.
Odobreno: 20. maja 2020.

[*]Autor za kontakt: e-mail: vera.karlicic@agrif.bg.ac.rs