Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. *85* (1) 111–123 (2020)
JSCS–5287

UDC 547.724+66.092:504.53.054+582.28
Original scientific paper

Degradation of carbofuran in contaminated soil by plant–microorganism combined technology

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(Received 1 March, revised 2 June, accepted 3 June 2019)

Abstract: With the development of modern agriculture, the pollution caused by the use of chemical fertilizers and pesticides has become a serious problem, posing a threat to human health and the living environment. Bioremediation technology is receiving more and more attention due to the safety of contaminated soil, non-secondary pollution, and low cost. In this study, white rot fungi were immobilized by the adsorption method, and the functional plants suitable for reducing carbofuran were screened by pot experiment. Based on a previous study, a combined remediation technique was established. The results showed that after 30 days, compared to the single bioremediation of carbofuran-contaminated soil, the degradation rate increased by 19 % through the corn–white rot fungi combined remediation, and by 17 % using the sorghum–white rot fungi combined remediation. The effect of the pesticide content in soil on the combined remediation is mainly reflected in the significant difference in the number of microorganisms ($p < 0.05$). Combined bioremediation may be a better alternative to mitigate the impact of high pollution on microorganisms at different pollutant concentrations compared to single microbial bioremediation or phytoremediation.

Keywords: fungal; combined bioremediation; immobilization; adsorption.

INTRODUCTION

Carbofuran (2,2-dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate) is toxic to humans, animals and fish and is widely used in agriculture.[1–3] It is extremely toxic to humans and animals and is reported to be teratogenic and embryotoxic. Hitherto, no reports have been made citing the effects of carbofuran toxicity on human cells. Carbofuran is easily hydrolyzed in the environment, but residues have been detected in groundwater due to its high fluidity and widespread use in the soil.[4,5] The World Health Organization (WHO) has mentioned

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https://doi.org/10.2298/JSC190301052L

that the oral lethal dose (LD_{50}) values for rabbits, cats, rats, guinea pigs, mice and dogs range from 3 to 19 mg/kg body weight.[6] The WHO classifies carbofuran as a "highly hazardous" insecticide.[7] It has been considered as a mutagen after metabolic activation.[8–10] The carbamate pesticide has a high solubility in water (351 mg L^{-1}).[11,12]

Currently, different methods are used to remove residual carbofuran from soil, such as photodegradation,[13] adsorption[14,15] and bioremediation.[16,17] Environmental biotechnologies are promising purification technologies that can restore a contaminated toxic environment to a safe state with the help of natural resources. As an environmentally friendly and cost-effective approach, environmental biotechnology has been used to reduce growing hazardous sites.[18–20] It describes how biological systems deal with ecological issues, such as environmental remediation, pollution prevention, as well as detection and monitoring of contaminants.[21]

Immobilization technology is often used to improve the repair efficiency. In an immobilization process, the microbial cells are encapsulated in a network of natural polysaccharides or synthetic polymer gels (entrapment, encapsulated) and attached to a solid carrier (adsorption or covalent bonding to the surface). Generally, immobilization limits or makes impossible or retards the free mobility of biocatalysts with substantial loss of catalytic activity and providing the possibility of reusing several times.[22–24] Immobilized microbial cells play a major role in the production of useful chemicals and the degradation of xenobiotics. The material of the immobilized cells is easy to prepare and has good activity. This method provides high cell density at the start of the bioreactor to prevent cells and biomass rinsing in a continuous process.[25] It also provides membrane stabilization to have a better rate of degradation in immobilized cells.[26]

As a green bioremediation technology, phytoremediation requires plants to remove organic pollutants (OCs).[27] Previous studies have found that plant growth has a positive impact on the removal of OCs such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAHs) and pesticides.[28,29] OC is absorbed by plant roots and can be converted into plant constituents. Non-toxic intermediates are stored in plant cells are converted to CO_2 and H_2O by volatilization or mineralization.[30] In addition, the composition of the rhizosphere community is also an important factor for successful rhizosphere restoration. Plants that are capable of selectively enriching OC at their rhizosphere perform better in enhancing pollutant degradation.[31]

In this study, white rot fungi were immobilized on straw and used for remediation of soil contaminated with carbofuran. In addition, six plants were selected and grown in soil modified with carbofuran to assess the efficiency of rhizosphere bioremediation. A combination of the above two techniques is used to study microbial remediation of pesticides.

EXPERIMENTAL

Materials

Specific white rot fungi (*Phlebia* sp-C, *Lenzites betulinus*-Y) were domesticated and cultured in the Microbiology Laboratory of Shenyang University of Technology. Carbofuran (purity of 98 %) was purchased from Shandong Rongbang Pesticide Chemical Co., Ltd. (China). The experimented plant species are given in Table I. Plantt seeds were purchased from Shenyang East Asia Seed Co., Ltd. High-performance liquid chromatography (HPLC, Agilent 1260, Singapore) was used to determine the degradation rate of carbofuran.

TABLE I. The experimented plant species

No.	Name	Latin name
1	Soybean	*Glycine max (Linn.) Merr.*
2	Ryegrass	*Lolium perenne* L.
3	Corn	*Zea mays* L.
4	Sorghum	*Sorghum bicolor* (L.)
5	Paddy	*Oryza sativa*
6	Pacesetter	–

Soil sample collection

The soil without carbofuran came from the university campus. The soil contained trace element, such as nitrogen and phosphorus. The method of collection was as follows: Soil samples to a depth of 5cm were randomly selected from five different locations on the campus. After passing through an 8 mm mesh sieve, they were mixed for later use.

Immobilization procedure

Wheat straw was cut into a size of 3mm and used as a carrier for immobilizing white rot fungi.[32] Wheat straw (30 g) was accurately weighed and added to a culture dish. The culture dish was then sterilized in a high-pressure steam sterilizer (1.01325×10^5 Pa, 121 °C) for 30 min. The activated white rot fungus was attached to the carrier by adsorption, and then placed in an incubator (28 °C) for one week, and 5 mL of sterile water was added daily to retain moisture.

Microbial degradation experiment

Carbofuran (80 mg kg^{-1}) was added to a soil sample, sieved with a 0.35 mm sieve, sterilized by high-temperature steam, and then naturally dried. Free C, free C+Y, immobilized C, immobilized C+Y bacteria and only the carrier (as a blank) were added to the carbofuran--contaminated soil. It was then placed in an incubator (28 °C) and 5nmL of sterile water was added daily to maintain moisture. The pesticide components in the soil were analyzed using HPLC on days 2, 4, 6, 8, 10, 12, 14, and 18. All experiments were performed in triplet.

Plant degradation experiment

Carbofuran (80 mg kg^{-1}) was added to the soil sample, sieved with a 0.83 mm sieve, and then naturally dried. The experimental soil (300 g) was placed in a flower pot having a diameter of 10 cm, and seeds of the test plants were sown in the soil and marked. The pots were kept in a sunny place. After 10 days of culture, the seedlings became thinner, leaving 10 seedlings in each pot. The location of the potted plants was randomly exchanged within two days, and soil samples were collected for determination after 30 days. The experiments were performed in triplet for each treatment.

Combined remediation

Immobilization and plant culture of white rot fungi were the same as described above. The amount of carbofuran was maintained at 100 mg kg^{-1} but the number of white rot fungi C added to the soil was changed. (Detailed processing is given in Table II). To investigate the effect of different concentrations on remediation, 3 % immobilized white rot fungus C was added to 40, 80 and 120 mg mL^{-1} of the experimental soil. (Detailed processing is given in Table III).

TABLE II. Treatment of the experimental design

No.	Method
T0	Plant
T1	Plant + 1 % immobilized C
T2	Plant + 3 % immobilized C
T3	Plant + 5 % immobilized C

TABLE III. Treatment of experimental design

No.	Method
S	Carbofuran concentration of the soil 40 mg kg^{-1}
D	Carbofuran concentration of the soil 80 mg kg^{-1}
T	Carbofuran concentration of the soil 120 mg kg^{-1}
M1	Plant
M2	Immobilized C
M3	Plant + immobilized C

Determination of the number of bacterial colonies

Rhizosphere soil (1 g) and non-rhizosphere soil (1 g) were taken separately. The soil was placed in a sterile tube, thoroughly shaken with 9 mL of sterile water to make a soil suspension, and then diluted 100 times. The diluted sample (1 mL) was placed in an aseptic culture dish. The medium cooled to 50 °C was poured into a culture dish containing the diluted sample and shaken rapidly to completely mix the water sample and the medium. The culture dish was placed in an incubator at 37 °C. The number of bacterial colonies was determined after 48 h.

Determination of the root–shoot ratio

The root–shoot ratio refers to the ratio of fresh weight or dry weight of the underground and aerial parts of the plant, reflecting biomass allocation by plants.[33] In this article, fresh weight was used as a measure. The boundary is the soil surface, weighing the weight of the aboveground plants and underground soil roots.

Sample pre-treatment

A 1 g soil sample was placed in a centrifuge tube and diluted with dichloromethane to a final volume of 10 mL. The sample was then shaken for 5 min, ultrasonicated for 2 h and centrifuged for 5 min (5000 rpm). The organic phase obtained after extraction was collected in a beaker and completely evaporated. The dry content was dissolved in 3 mL of methanol. After a constant volume of solution had passed through a 0.22 μm organic phase filter membrane, it was injected into a high phase liquid bottle to be tested.

The conditions of HPLC

The test conditions for carbofuran were as follows: The mobile phase was prepared with alcohol and distilled water (55:45 volume ratio) at a rate of 1.0 mL min^{-1}, the column temperature was 25 °C, and the wavelength of the UV detector was set at 275 nm. The sample size was 10 μm and the retention time was about 11 min.

Data processing

Data analysis was performed by Origin software, and SPSS 19.0 software was used to test for differences with $p < 0.05$ been considered a significant difference.

RESULTS AND DISCUSSION

Degradation of carbofuran in soil by the immobilized white rot fungi

The degradation rate of carbofuran by different forms of white rot fungi is shown in Fig. 1. Whether it is free bacteria C or free bacteria C+Y, the degradation rate of carbofuran was about 50 %. However, the highest degradation rates of immobilized C and immobilized C+Y reached 78 and 84 %, respectively, which were 27 and 33 % higher than the free state, respectively. It could be observed that there is no significant difference between a single white rot fungus and mixed white rot fungi. From the trend of degradation rate, the free state gradually becomes stable, while the immobilized state shows an upward trend. Immobilized microorganisms adapt to the contaminated substrate as a source of nutrients. At the same time, the carrier can provide a stable environment for the growth of microorganisms, and increase the density and biological activity of the bacteria. In addition, it was found that the immobilized carrier has a certain adsorption effect on carbofuran. Kadakol *et al.*[34] separated *Klebsiella* sp. The strain ATCC 13883T was immobilized from the soil by the enrichment culture technique. The results showed that the rate of degradation of the immobilized cells was higher than that of the free cells, and the tolerance to changes in pH, temperature and concentration was better.

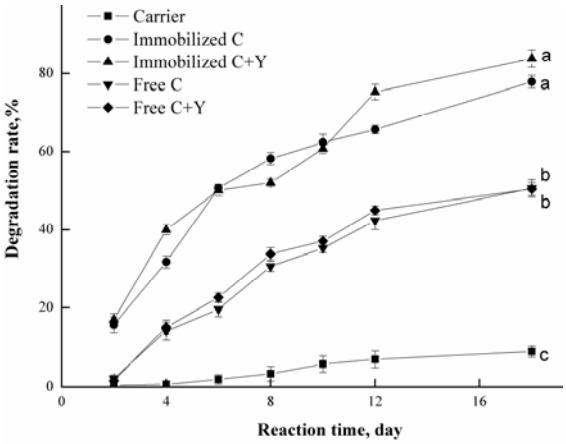

Fig. 1. Degradation of carbofuran in the investigated soil. Different lower case letters (a, b and c) represent significant differences of different forms of white rot fungi at the same time at the 0.05 level.

The number of bacteria in the soil with different plants

The number of bacteria in the rhizosphere and non-rhizosphere soil of each plant was determined, and the results are given in Table IV. The number of non-root colonies was 5–10 times lower than that of the root–soil in the soil containing carbofuran. Studies[35] have shown that plant rhizosphere bacteria species are 1.5 to 3 times more abundant than non-rhizosphere bacterial species, and that there were significant differences between the rhizosphere microorganisms associated with different plants. The number and population structure of rhizosphere microorganisms may differ due to differences in the substances secreted by different species. In general, the number of colonies of microorganisms associated with different plants from high to low is pancetta, corn, ryegrass, soybean, sorghum and rice. Different plants affect microbial density and composition due to the growth rate, root morphology, root exudate production, and oxygen transfer.[36] However, the extent to which plant species affect the composition of rhizosphere microbial community remains unclear.[37]

TABLE IV. Colony forming units (CFU) under different part

Plant	Colonies number in rhizosphere soil, CFU g^{-1}	Colonies number in non-rhizosphere soil, CFU g^{-1}
Soybean	$(3.1\pm1.5)\times10^5$	$(6.7\pm0.4)\times10^4$
Ryegrass	$(3.5\pm1.2)\times10^5$	$(9.1\pm1.2)\times10^4$
Paddy	$(6.0\pm0.9)\times10^4$	$(4.0\pm1.5)\times10^4$
Sorghum	$(2.9\pm2.2)\times10^5$	$(5.5\pm2.1)\times10^4$
Corn	$(4.7\pm2.2)\times10^5$	$(2.3\pm0.8)\times10^4$
Pacesetter	$(5.5\pm0.2)\times10^5$	$(1.1\pm0.6)\times10^5$

Degradation of carbofuran in soil by the plants

As shown in Fig. 2, the order of the root–shoot ratio is corn, rice, sorghum, ryegrass, soybeans and pacesetter. Sorghum has the highest degradation rate, followed by rice, with degradation rates of 59 and 54 %, respectively. Ryegrass has the lowest degradation rate with a degradation rate of only 30 %. Comparing the degradation rate and the root – shoot ratio, the relationship between them is basically positive, *i.e.*, the greater the root–shoot ratio is, the better is the degradation efficiency. Although the degradation rate of corn is not the highest, the ratio of root to shoot is the highest, which could provide more room for the growth of microorganisms. In general, sorghum and corn were selected as functional plants for the next step in plant–microbial remediation experiments. Roots are part of the direct contact between plants and soil, so root effects should be considered more when studying plant degradation. The roots secrete many inorganic acids, bases, and some organic compounds, which are beneficial to the conversion of soil contaminants and provide nutrients to the soil. The root zone (rhizosphere) is the most important and active reaction zone in the constructed wetlands. The

interaction between plants and soil, microorganisms and pollutants lead to physical, chemical and biological processes in the rhizosphere.[38] Plants absorb pollutants and provide organic matter and additional sources of oxygen for bacteria growth as biofilms on plant surfaces to remove pollutants.[39]

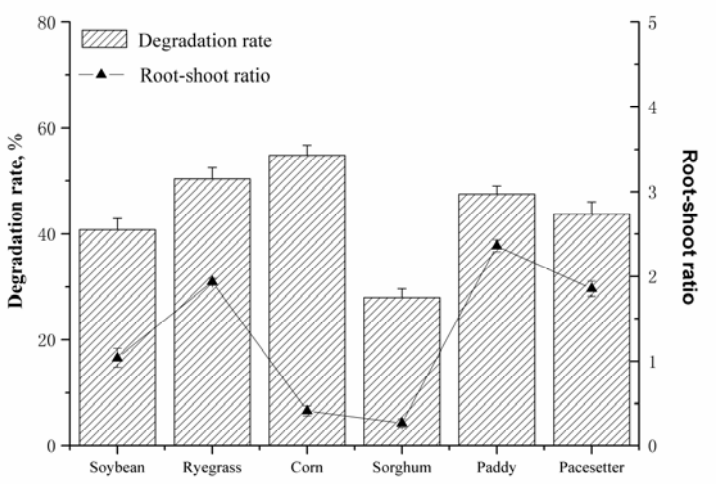

Fig. 2. Degradation of carbofuran in the investigated soil by different plants.

The degradation rate of carbofuran by the amount of immobilized white rot fungi

The effect of different doses on the degradation of carbofuran is shown in Fig. 3. The combined remediation of corn–white rot fungi significantly increased the degradation rate of carbofuran by adding microbial agents ($p < 0.05$). When the fungi were not added to the bacteria, it increased by 18 % (the bacterial dose was 3 %), to 45 %.

When the dose of the bacteria was 1 %, the combination of sorghum and microorganisms did not show much advantage in the degradation of carbofuran compared to the control of the blank plants. However, when the number of bacteria reached 3 %, the degradation rate of the composite repair increased from 55 to 67 %. However, regardless of whether corn or sorghum was used, there was no significant increase in the investment as the number of bacteria increased. After two days of planting, all sorghum seeds had germinated and were growing well, but only some of the corn seeds germinated. After 10 days, the corn grew well. Among them, the corn grown with 3 % white rot fungus was the best. At this point, some sorghum appeared yellowing, and there was no significant difference in different doses. Sixteen days after planting, 1 % corn was withered. The other doses were very good. It shows that microbes provide some help for plants to absorb nutrients. After 20 days of planting, the roots of sorghum became rotten, greatly reducing the absorption of water by the soil. Plants have been shown to

induce and stimulate the growth of specific bacterial populations and produce distinct bacterial communities around the rhizosphere, resulting in enhanced biomass and activity of the rhizosphere microorganisms compared to microorganisms in the bulk soil.[40]

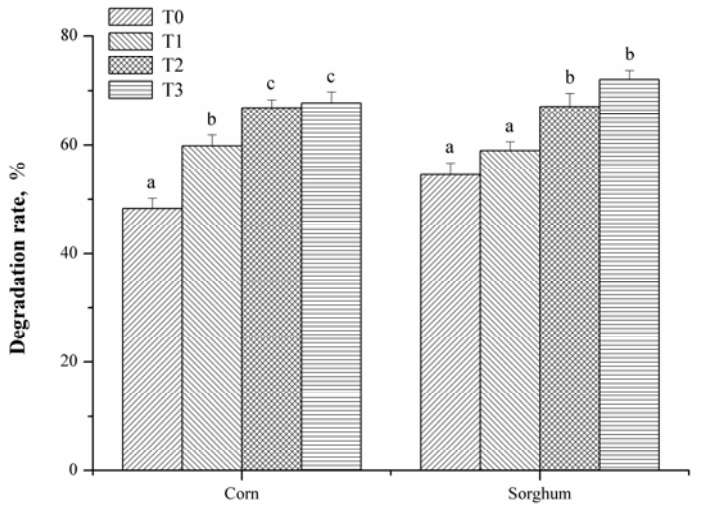

Fig. 3. The influence of dosage to combined bioremediation degradation of carbofuran. Different lowercase letters (a, b and c) represent significant differences of different methods at the 0. 05 level, the same as below. T0, T1, T2 and T3 are methods mentioned in Table II.

The influence of initial concentration on the degradation rate of carbofuran

Corn–white rot fungi degradation of carbofuran. The degradation rate of the combined repair system under different carbofuran levels is shown in Fig. 4. As the concentration increased, the combined repair system significantly improved the degradation of carbofuran. Compared to the blank plants, the degradation rate of the composite repair was increased by 26 % at a concentration of 120 mg kg^{-1}, and the degradation rate of the combined repair was increased by 17 % at a concentration of 80 mg kg^{-1}.

At low concentrations, the degradation rate of white rot fungi was basically the same in the different treatments, but there was a significant difference in high concentrations ($p < 0.05$). With the addition of highly efficient microbial agents, the soil is rich in microorganisms. In addition, immobilized fungi can gradually adapt white rot fungi to the original environment and help their growth and reproduction in the soil. The formation of a rich micro-ecological environment in the roots of plants makes the joint repair system stand out in high concentration polluted environments. The immobilized carrier used in this experiment was not only used for soil organic matter, but also for the growth of microorganisms to provide nutrition. Zhang *et al.*[41] combined *Pennisetum* and *Arthrobacter* sp.

strain DNS10 to repair atrazine-contaminated soil. The results show that the interaction between plants and microorganisms has a good degradation effect. Planting has a positive effect on improving soil microbial growth and activity, leading to higher microbial diversity.

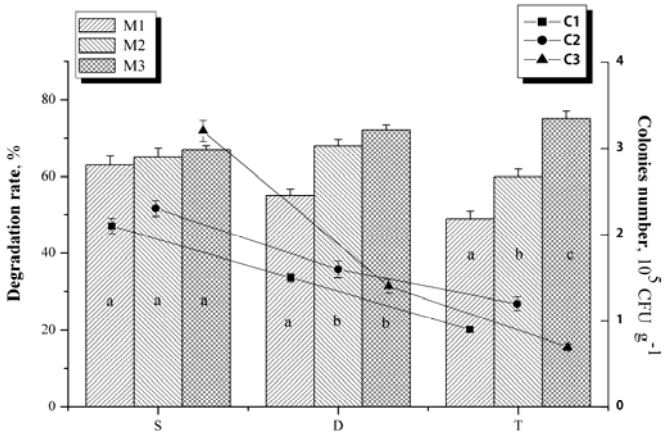

Fig. 4. The influence of the initial concentration to corn–white rot fungus degradation of carbofuran. S, D, T, M1, M2 and M3 are methods mentioned in Table III; C1, C2 and C3 are colony numbers of M1, M2 and M3 (10^5 CFU g^{-1}), the same as below.

Sorghum–white rot fungi degradation of carbofuran. The degradation rate of sorghum at different concentrations of carbofuran was not significant ($p > 0.05$) (Fig. 5), which was about 60 %. At a concentration of 120 mg kg^{-1}, the highest degradation rate can reach 66 %. The effect of concentration on bacterial colonies was not as pronounced as that with corn. When the concentration was 80 mg kg^{-1}, the degradation rate and the number of colonies were relatively stable, indicating that 80 mg kg^{-1} is the best repair concentration when using sorghum to repair carbofuran. Through the study of bacterial colonies under different treatment conditions, it was found that an increase in the pollutant concentration had a great impact on the biomass.

Although organic pollutants bring about the carbon and nitrogen sources needed for bacterial growth, they are toxic to microorganisms when the concentration is increased to 80 mg kg^{-1}. Due to the increase in concentration to accelerate the metabolic rate of microorganisms, some secondary metabolites have a certain inhibitory effect on the metabolism of microorganisms. The reduction in the number of soil microorganisms has a negative impact on the degradation rate. A study by Ribeiro *et al.*[42] aimed at assessing the potential of plant–microbial associations for the dissipation of petroleum hydrocarbons in estuarine salt marshes. The results showed that plants promoted the development of the microbial populations of the rhizosphere hydrocarbon degradation, aiming to affect

the microbial communities in salt marshes. Studies have shown that plant–microbial combination technology has a positive impact on hydrocarbon removal in estuarine environments.

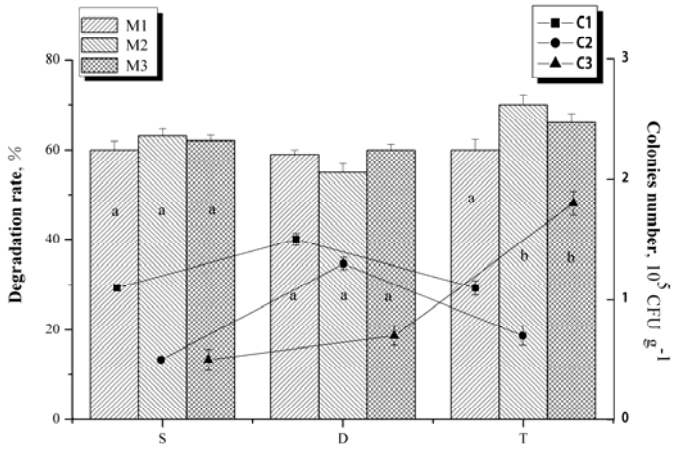

Fig. 5. The influence of the initial concentration on sorghum–white rot fungus degradation of carbofuran.

CONCLUSIONS

This study indicates that the application of combined bioremediation technology in the bioremediation of carbofuran contaminated soil is a successful strategy. Based on the results of this study, it could be concluded that the immobilized microorganisms increased the removal rate of carbofuran from the soil system by 30 % compared to the un-immobilized system. Corn and sorghum are able to take carbofuran from root soil to improve the quality of the soil environment. They could, therefore, be used to remediate soil contaminated with carbofuran. In summary, the rate of bioremediation is higher than that of phytoremediation. However, both processes are very susceptible to the environmental conditions. The effect of phytoremediation on soil is stable, but is affected by factors such as plant growth, geography, *etc.* Two types of remediation techniques can be combined to maximize the remediation effect. Due to the complexity of the soil environment, various factors, such as cross-contamination of different pesticides, geographical location, and climatic conditions, should be considered in soil pollution control. Despite these limitations, environmental biotechnology is becoming an attractive approach for widespread pollution mitigation.

Acknowledgements. The research was funded by the National Natural Science Foundation of China (Grant No. 31670515) and the National Key R&D Program of China (Grant No. 2017YFD0800702).

ИЗВОД

РАЗГРАДЊА КАРБОФУРАНА У КОНТАМИНИРАНОМ ЗЕМЉИШТУ КОМБИНОВАНОМ ТЕХНОЛОГИЈОМ БИЉКА–МИКРООРГАНИЗАМ

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Развојем савремене пољопривреде, загађење изазвано коришћењем хемијских ђубрива и пестицида постало је озбиљан проблем, који угрожава здравље људи и животну средину. Све већа пажња поклања се ремедијацији, због сигурности земљишта од контаминације, избегавања секундарног загађења и економичности. У овој студији, гљивице беле трулежи су биле имобилисане адсорпцијом, а функционалне биљке погодне за разградњу карбофурана, испитане су скринингом, експериментом у посуди. На основу претходне студије установљена је комбинована метода ремедијације. Резултати су показали да је након 30 дана брзина разградње, у односу на једностране биоремедијацију земљишта контаминираног карбофураном, била већа за 19 % у случају комбиноване ремедијације кукуруз–гљиве беле трулежи, а за 17 % у случају кинеска шећерна трска–гљиве беле трулежи. Утицај садржаја пестицида у земљишту на комбиновану ремедијацију углавном се огледа у значајној разлици у броју микроорганизама ($p < 0,05$). Комбинована биоремедијација може бити боља алтернатива за ублажавање ефеката високог загађења на микроорганизме при различитим концентрацијама полутанта него једностране микробиолошка биоремедијациа или фиторемедијација.

(Примљено 1. марта, ревидирано 2. јуна, прихваћено 3. јуна 2019)

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