Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. Novi Sad, № 129, 45—54, 2015

UDC 633/635:631.445.466]:581.11
DOI: 10.2298/ZMSPN1529045D

Gordana M. DANILOVIĆ[1*], *Nataša Ž. ĆURČIĆ*[1], *Mira M. PUCAREVIĆ*[1], *Ljubinko B. JOVANOVIĆ*[2], *Csaba VÁGVÖLGYI*[3], *László KREDICS*[3], *Dejana M. PANKOVIĆ*[1]

[1] Faculty of Environmental Protection, Educons University, 21208 Sremska Kamenica, Serbia
[2] Faculty of Ecological Agriculture, Educons University, 21208 Sremska Kamenica, Serbia
[3] Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, H-6726 Szeged, Hungary

DEGRADATION OF LINURON IN SOIL BY TWO FUNGAL STRAINS

ABSTRACT: Two fungal strains were applied to soil polluted with herbicide in order to determine their degradation potential. Three experimental setups were used. In the first setup, the soil in pots was contaminated by linuron in final concentration of 1 ppm. Suspensions of *Phanerocheate chrysosporium* and *Trichoderma asperellum* were applied separately or in combination. Tomato plantlets were transplanted and chlorophyll content in their leaves was determined at two time points during plant growth. In the second setup in pots, the final concentration of linuron was lower, 0.45 ppm. In the third setup 0.1 ppm of linuron was applied in the field plot. Plantlets of lettuce were transplanted and chlorophyll content was measured as indicator of plant stress. The content of linuron in soil was determined by HPLC. The applied fungal strains significantly reduced toxic effect of 0.45 ppm linuron on plants, which was not the case for 1 ppm linuron. Both fungi, applied separately or in combination, were effective in decreasing the linuron content in the soil. However, in field conditions the combination of both fungi was the most effective.

KEYWORDS: *Trichoderma asperellum*; *Phanerochaete chrysosporium*; bioremediation, herbicide, linuron

INTRODUCTION

The demand for food supply increases constantly throughout the world due to the increase of human population. It is predicted that by the year 2050, agricultural production may need to be increased by 60–110%, which can only be achieved through targeted increase in crop yield [Ray *et al.*, 2013].

* Corresponding autor e-mail: gordana.racic@educons.edu.rs

Intensive agriculture is highly dependent on the use of chemical pesticides to control plant pathogens. However, these methods are time-consuming and environmentally harmful. As chemicals build up in soil they become toxic to microorganisms and plants, therefore the cleaning of the soil using the remediation is of the primary importance [Verma *et al*., 2014]. Among different remediation technologies biological methods are very promising as they are easy to operate, do not produce secondary pollution and show higher efficiency in cleaning the soil [Beškoski *et al*., 2011]. The use of microbes for pesticide removal/degradation from the agricultural soils is widely known, mostly through the enzymatic degradation [Vidali 2001].

Various microorganisms are used for bioremediation purposes, although indigenous species are the ones with best remedial potential.

Among different microorganisms, *Phanerochaete* spp. and *Trichoderma* spp. are recognized as fungi capable for degradation of organic pollutants, such as PAH and POPs. *Phanerochaete chrysosporium*, a basidiomycetous filamentous fungus, is proven to be effective in biodegradation of these compounds. *P. chrysosporium*is is a white rot fungus which has a highly efficient lignin degrading enzyme system. With this enzyme system the fungus can also break down different xenobiotic pollutants. In these types of degradation processes, the lignin peroxidase and the manganese peroxidase have a great significance [Vágvölgyi *et al*., 2014].

Beside these enzyme systems, laccases also oxidize various organic and inorganic compounds such as diphenols, polyphenols, substituted phenols, diamines and aromatic amines with concomitant reduction of molecular oxygen to water [Körmöczi *et al*., 2013a].

Trichoderma spp. is a widely present cosmopolitan soil borne fungi [Kredics 2014]. Strains of *Trichoderma* spp. exert a number of different capabilities as they are genetically quite diverse [Harman *et al*., 2004a]. Some of the strains are recognized as promising biocontrol agents, as well as plant growth promoters [Harman *et al*., 2004b; Kormoczi *et al*., 2013b]. Moreover, they are known for their potential in bio- and phytobioremediation of toxic compounds, such as pesticides and heavy metals [Woo *et al*., 2014; Jovičić Petrović 2014]. Like *Phanerochaete* spp*., Trichoderma* spp. is also able to produce laccase enzymes. Strains known for laccase production are *T. atroviride, T. harzianum* and *T. assperellum* [Körmöczi *et al*., 2013a].

Linuron (IUPAC: 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) is a nonselective herbicide used worldwide for the control of grasses and broadleaf weeds in the cultivation of a variety of crop plants, particularly vegetables and cereals. It is absorbed by plant roots and transported passively, via the xylem, to leaves where it inhibits photosynthesis by disrupting Photosystem II (USEPA 1984). Linuron is moderately persistent in soils. In aerobic conditions in the lab its half-life was 75 days, while under field conditions it was 230 days (Pest Management Regulatory Agency 2002). It is known to enter surface waters in agricultural runoff, and its residues have been detected in surface waters, drinking water and foodstuf [USEPA 1995; PMRA 2012].

Linuron is highly toxic to non-target aquatic organisms such as fish and shellfish, while in mammals it disrupts male reproductive function acting as an antiandrogen.

In this work we investigated the remediation potential of *T. assperellum* and *P. chrysosporium* to linuron in soil.

MATERIAL AND METHODS

Examinations of herbicide degradation in soil consisted of three separate experiments. Herbicide linuron was applied in each experiment. Solution was prepared by dissolving of commercial herbicide Afalon in tap water, in order to prepare three different final concentrations of linuron: 0.1 ppm, 0.45 ppm and 1 ppm, in three experimental conditions as it will be explained further below. The treatments in all experiments described below were split in two groups, one with and the other without herbicide applied in soil where plantlets of tomato or lettuce were transplanted. Both groups were split into the following subtreatments differing in the application of fungal suspensions: control, only *Trichoderma*, only *Phanerocheate* and both *Phanerocheate* and *Trichoderma* application.

Experimental conditions 1

Soil mixture was prepared (1:1:5 V ratio of soil:sand:peat) and half of it was sprayed with linuron solution to achieve final concentration in soil of 1 ppm. In the growth chamber experiment 635 g of soil mixture was weighted in transparent Plexiglas pots (depth of 24 cm), covered with aluminum foil. Plants were watered up to 75 % of maximum soil mixture water capacity. Lighting was provided with fluorescent bulbs in 14 h day / 10 h night light regime.

Two days after herbicide application, 5 ml of *P. chrysosporium* suspension (5.84×10^7 CFU/ml) was applied in each pot of *Phanerocheate* subtreatment group. One week after herbicide application, tomato plantlets were transplanted and 5 ml of *T. asperellum* suspension (32.5×10^6 CFU/ml) was applied to pots in only *Trichoderma* or *Phanerocheate* and *Trichoderma* subtreatment group. Non-destructive measurements of plants were done at two time points: two days after transplantation (I) and nine days after transplantation (II). Each treatment was done in three pots, with two plants per pot.

Experimental conditions 2

In order to be comparable with the first experimental setup, the final concentration of linuron in field experiments was calculated per soil weight also, assuming the area of experimental plot and soil depth of 15 cm. So, a half of the field plot was treated with linuron in final concentration of 0.45 ppm.

After seven days, 5 ml of both fungal suspensions were applied according to the described experimental design (*P. chrysosporium* suspension = 0,194 x 10^7 CFU/ml, *T. asperellum* suspension = 0,145 x 10^6 CFU/ml), followed by transplantation of tomato plantlets five days later. Non-destructive measurements of plants were done one month after fungal suspensions were added. Each treatment was done in plants with four pots (replicates).

Experimental conditions 3

A half of the field plot in this experiment was treated with herbicide linuron in the final concentration of 0.1 ppm in 15 cm soil layer, which is a recommended concentration in common agricultural practice. Both fungal suspensions were applied according to the described experimental design. Two days after herbicide application, 5 ml of the *P. chrysosporium* suspension ($1x10^7$CFU/ml) was applied to the future soil transplanting spot. One week after herbicide application, plantlets of lettuce were transplanted and 5 ml of the *T. asperellum* suspension ($1x10^6$CFU/ml) was applied. Non-destructive measurements of plants were done one month later.

The content of linuron in the soil was determined at two time points with two weeks interval (two and four weeks after transplantation). Each treatment was done in 10 plants (replicates).

Fungal suspension

Strains *Trichoderma asperellum* SZMC 20866 and *Phanerochaete chrysosporium* 78 SZMC 20961 were from the Szeged Microbiological Collection (SZMC), Department of Microbiology. Fungal isolates were maintained on Potato Dextrose Agar (PDA) medium at 4 °C.

Prior to preparation of fungal suspensions, *T. asperellum* isolate was preincubated at 25 °C in the dark, and *P. chrysosporium* isolates were preincubated at 28 °C in the light. Suspensions were prepared as follows: pure culture of *T. asperellum* isolate was grabbed from a Petri dish, resuspended in 100 ml of tap water, and shaken for 2 h on 50 rpm. Pure culture of *P. chrysosporium* isolate was grabbed from 10 Petri dishes, resuspended in 100 ml of tap water, and shaken for 2 h on 50 rpm.

Determination of linuron in soil

Soil samples from pot experiments were taken in 3 replicates and in field experiment in 5–10 replicates. Linuron was determined by HPLC (Agilent 1220 Infinity LC). The column used was a stainless steel Phenomenex Synergy 2.5 µm Fusion RP 100 A (50 mm x 2.1 mm I.D.). The chromatographic conditions were as follows: eluent, methanol-water (65:35, v/v); flowrate, 0.4 ml/min; injection volume, 10 µl; wavelength, 254 nm. Column temperature was ambient.

To obtain a solution of extractable linuron, 0.5 g of soil was shaken with 5 ml of water for 24 h. The suspension was then centrifuged at 14,000 rpm for 30 min and the aqueous extract was separated [Sánchez-Martín *et al.*, 1996].

The response of the detector as referred to peak areas was linear in the range assayed (0.1–1.2 μg/ml) and least squares linear regression analysis of the data provided an excellent correlation (R^2=0.999). LOD was 0.03 μg/ml or 0.3 μg/g of soil.

Non-destructive measurements

Chlorophyll content of the leaves was measured nondestructively with SPAD 502 + chlorophyllmeter (Konika Minolta Sensing Inc, Japan). Soil water content and temperature were measured with WET sensor with HH2 (Delta T).

RESULTS AND DISSCUSSION

Experiment 1

Data on soil water content (WET= 30 ± 2%) and soil temperature (T= 25 ± 0.2 °C) measured around each tomato plant used for measurements, indicate that plant growth conditions were uniform and optimal. Chlorophyll content of the leaves (SPAD, rel. units) in plants that were treated with 1 ppm did not decrease in comparison with control plants measured two days after plant transplantation. However, chlorophyll content decreased in plants treated with herbicide nine days after transplantation regardless on the fungal treatment (Table 1). One week after the herbicide application the plants wilted. However, the content of linuron in the soil after plant harvest was lower in all fungal treatments (Table 2).

Table 1. *Parameters of chlorophyll content measured* in situ *(SPAD) as influenced by herbicide and different fungal treatments in growth chamber experiment. I – refers to the first measurement performed two days after transplantation; II – refers to the second measurement performed nine days after transplantation.*

Treatment	SPAD (rel.units) ±s.d.	Treatment	SPAD (rel.units) ±s.d.
Linuron	I 33.1±0.75 II 30.83±1.86	Control	I 33±0.46 II 37.93±0.64
Linuron and *Trichoderma suspension*	I 34.17±1.55 II 30.07±2.29	*Trichoderma* suspension	I 33±0.46 II 37.93±0.64
Linuron, *Phanerochaete* and *Trichoderma* suspension	I 33.63±0.57 II 29.83±1.8	*Phanerochaete* and *Trichoderma* suspension	I 33.2±0.31 II 39.07±2.21
Linuron and *Phanerochaete* suspension	I 32.9±0 II 29.2±1.27		

Table 2. *The percentage of linuron content in soil related to the value at the beginning of the experiment, as influenced by different fungal treatments in growth chamber experiment.*

Treatment	Linuron [%]
Linuron	90
Linuron and *Trichoderma* suspension	68
Linuron, *Phanerochaete* and *Trichoderma* suspension	74
Linuron and *Phanerochaete* suspension	75

Experiment 2

Data on soil water content (WET= 17.7 ± 2.3%) and soil temperature (T= 33.8 ± 1.0 °C) measured in the field around each tomato plant used for measurements, indicate that plant growth conditions were uniform and optimal.

Parameters of plant vitality and chlorophyll content in leaves indicate that the application of *P. chrysosporium* fungal strain, alone or in combination with *T. asperellum* fungal strain significantly reduced negative effect of 0.45 ppm linuron treatment of tomato plants (Table 3).

Table 3. *Parameters of chlorophyll content measured* in situ *(SPAD) as influenced by herbicide and different fungal treatments in field conditions.*

Treatment	SPAD (rel.units) ±s.d.	Treatment	SPAD (rel.units) ±s.d.
Linuron	48±12	Control	106±5
Linuron and *Trichoderma* suspension	54±13	*Trichoderma* suspension	106±8
Linuron, *Phanerochaete* and *Trichoderma* suspension	84±14	*Phanerochaete* and *Trichoderma* suspension	104±10
Linuron and *Phanerochaete* suspension	79±17		

The content of linuron in the soil after plant harvest was the lowest after the application of both fungal suspensions (Table 4).

Table 4. *The percentage of linuron content in soil related to the value at the beginning of the experiment, as influenced by different fungal treatments in field conditions*

Treatment	Linuron %
Linuron	62.5
Linuron and *Trichoderma* suspension	70
Linuron, *Phanerochaete* and *Trichoderma* suspension	46.5
Linuron and *Phanerochaete* suspension	63

Experiment 3

Data on soil water content (WET= 23 ± 2%) and soil temperature (T= 17 ± 1 ^{o}C) measured in the field around each lettuce plant used for measurements, indicate that plant growth conditions were uniform and optimal. Chlorophyll content of the leaves (SPAD, rel. units) treated with 0.1 ppm linuron did not differ significantly from untreated plants. Basically, there was no difference among the treatments (Table 5). The decrease of linuron content in the soil was the highest when both fungi were applied at both measurement time points (Table 6).

Table 5. *Parameters of chlorophyll content measured* in situ *(SPAD) as influenced by different fungal treatments in greenhouse conditions in lettuce plants. The concentration of applied Linuron was 0.1 ppm.*

Treatment	SPAD (rel.units) ±s.d.	Treatment	SPAD (rel.units) ±s.d.
Linuron	19.9 ± 2.2	Control	19.3 ± 2.1
Linuron and *Trichoderma* suspension	19.7 ± 2.3	*Trichoderm*a suspension	22.4 ± 1
Linuron, *Phanerochaete* and *Trichoderma* suspension	20 ± 2.5	*Phanerochaete* and *Trichoderma* suspension	19.9 ± 3.4
Linuron and *Phanerochaete* suspension	19.7 ± 3.1		

Table 6. *The percentage of linuron content in soil related to the value at the beginning of the experiment, as influenced by different fungal treatments in field conditions*

Treatment	Linuron % 2 weeks after plant transplantation	Linuron % 4 weeks after plant transplantation
Linuron	83	42
Linuron and *Trichoderma* suspension	69	59
Linuron, *Phanerochaete* and *Trichoderma* suspension	45	0
Linuron and *Phanerochaete* suspension	87	27

Investigations of the possibilities of pesticide microbial degradation are of great importance in the field of environmental protection. Species belonging to *Phanerochaete* and *Trichoderma* genus are known for their application in biotechnology, due to their production of lignin peroxidase, manganese peroxidase enzymes [Vágvölgyi *et al*., 2014], as well as laccases [Da Silva Coelho-Moreira *et al*., 2013; Körmöczi *et al*., 2013]. Vágvölgyi *et al*. [2014] showed that *P. chrysosporium* strains exert good degradation potential of herbicides, parabens and phenol derivatives. Also, it is well known that *P. chrysosporium* possesses a great ability to degrade isoproturon, atrazine, propanil, bentazon, and diuron [Da Silva Coelho-Moreira *et al*., 2013].

CONCLUSION

According to our results, the applied fungal strains significantly reduced toxic effect of 0.45 ppm linuron in plants, which was not the case for 1 ppm linuron. Both fungi, applied separately or in combination, were effective in decreasing the linuron content in the soil. However, in field conditions the combination of both fungi was the most effective. Investigations should be continued in order to determine which metabolites are produced due to microbial degradation of linuron as they could be more toxic than herbicide itself.

ACKNOWLEDGMENT

The research is co-financed by the European Union through the Hungary-Serbia IPA Cross-border Co-operation Programme (PHANETRI, HUSRB/1002/214/068) and by the Ministry of Education and Science of the Republic of Serbia (Project No. III43010).

REFERENCES

Beškoski V, Gojgić-Cvijović G, Milić, J, Ilić, M, Miletić S, Jovančićević B, Vrvić M (2011): Bioremediation of soil polluted with crude oil and its derivatives: microorganisms, degradation pathways, technologies. *Chem. Ind.* 66: 275–289.

Da Silva Coelho-Moreira J, Bracht A, Da Silva de Souza AC, Ferreira Oliveira R, De Sá-Nakanishi AB, Giatti Marques de Souza C, Peralta RM (2013): Degradation of Diuron by *Phanerochaete chrysosporium*: Role of Ligninolytic Enzymes and Cytochrome P450. *BioMed. Research International*. Article ID 251354.

Harman GE, Lorito M, Lynch JM (2004a): Uses of *Trichoderma* spp. to Alleviate or Remediate Soil and Water Pollution. *Adv. Appl. Microbiol.* 56: 313–330.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004b): *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2: 43–56.

Jovicic Petrovic J, Danilovic G, Curcic N, Milinkovic M, Stosic N, Pankovic D, Raicevic V (2014): Copper tolerance Of *Trichoderma* species. *Arch. Biol. Sci.* 66: 137–142.

Körmöczi P, Manczinger L, Sajben-Nagy E, Vágvölgyi C, Danilović G, Panković D, Jovanović L, Pucarević M, Kredics L (2013a): Screening of *Trichoderma* strains isolated from rhizosphere samples for laccase production. *Rev. Agric. Rural Develop.* 2: 325–330.

Körmöczi P, Danilović G, Manczinger L, Jovanović L, Panković D, Vágvölgyi C, Kredics L (2013b): Species composition of *Trichoderma* isolates from the rhizosphere of vegetables grown in Hungarian soils. *Fresen Environ. Bull.* 22: 1736–1741.

Kredics L, Hatvani L, Naeimi S, Körmöczi P, Manczinger L, Vágvölgyi C, Druzhinina I (2014): *Biodiversity of the genus Hypocrea/Trichoderma in different habitats, in Biotechnology and Biology of Trichoderma*, ed. by Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy M, Elsevier Science BV, Amsterdam, 3–24.

Pest Management Regulatory Agency (2012). Proposed Re-evaluation Decision PRVD2012-02, Linuron. Tecnical report prepared for Pes tManagement Regulatory Agency, Health Canada: Ottawa, Ontario, Canada.

Ray DK, Mueller ND, West PC, Foley JA (2013): *Yield Trends Are Insufficient to Double Global Crop Production by 2050*. <DOI:10.1371/journal.pone.0066428>.

Review report for the active substance linuron: EC, Health&Consumer Protection Directorate--General Directorate E – Food Safety: plant health, animal health and welfare, international questions E1 – Plant health, Linuron, 7595/VI/97-final, 2 December 2002.

Sánchez-Martín MJ, Delgado-Pascual R, Iglesias-Jiménez E, Sánchez-Camazano M (1996): Determination of linuron in aqueous soil extracts by high-performance liquid chromatography, *J. Chromatogr.* 754(1–2): 295–299.

USEPA (U.S. Environmental Protection Agency). 1984. Pesticide fact sheet: Linuron. USEPA, Office of Pesticide Program, Washington DC.

USEPA (United States Environmental Protection Agency) (1995): R.E.D. Facts Linuron; Technical report no. EPA-738-F-95-003; prepared for Special Review and Reregistration Division (7508W), Office of Pesticides Programs, United States Environmental Protection Agency: Washington, DC.

Vágvölgyi C, Vörös M, Bóka B, Pucarević M, Jovanović L, Panković D, Manczinger L (2014): Isolation and characterization of *Phanerochaete* strains suitable for bioremediation purposes. *Rev. Agr. Rural Deveopl*. 2: 347–350

Vidali M (2001): Bioremediation. An overview. *Pure Appl. Chem.* 73: 1163–1172.

Verma JP, Jaiswal DK, Sagar R (2014): Pesticide relevance and their microbial degradation: a state of art. *Rev. Environ. Sci. Biotechnol.,* 13: 429–466.

Woo SL, Ruocco M, Vinale F, Nigro M, Marra R, Lombardi N, Pascale A, Lanzuise S, Manganiello G, Lorito M (2014): *Trichoderma*-based products and their widespread use in agriculture. *The Open Mycol. J.* 8 (Suppl-1, M4): 71–126.

ПРИМЕНА ДВА РАЗЛИЧИТА СОЈА ГЉИВИЦА У ДЕГРАДАЦИЈИ ЛИНУРОНА У ЗЕМЉИШТУ

Гордана М. ДАНИЛОВИЋ[1], Наташа Ж. ЋУРЧИЋ[1], Мира М. ПУЦАРЕВИЋ[1], Љубинко Б. ЈОВАНОВИЋ[2], Чаба ВАГВЕЛЂИ[3], Ласло КРЕДИЧ[3], Дејана М. ПАНКОВИЋ[1]

[1]Факултет заштите животе средине, Универзитет Едуконс, 21208 Сремска Каменица, Србија
[2]Факултет еколошке пољопривреде, Универзитет Едуконс, 21208 Сремска Каменица, Србија
[3]Департман за микробиологију, Факултет природних наука и информатике, Универзитет у Сегедину, Сегедин, Мађарска

РЕЗИМЕ: Да би се испитао потенцијал за деградацију хербицида, два соја гљивица су примењена на земљиште загађено линуроном у оквиру три експериментална система. У првом експерименту са судовима земљиште је загађено линуроном у коначној концентрацији од 1 ppm. Суспензије спора *Phanerocheate*

chrysosporium и *Trichoderma asperellum* су примењене појединачно или у комбинацији. Након расађивања на биљкама парадајза је мерен садржај хлорофила два пута у току вегетације. У другом експерименталном систему примењена је нижа концентрација линурона, у коначној концентрацији од 0,45 ppm. У трећем експерименту примењена је коначна концентрација линурона од 0,1 ppm на експерименталној парцели у пољу. Након расађивања на биљкама салате је мерен садржај хлорофила као показатељ стања стреса. Концентрација линурона у земљишту је одређивана HPLC методом. Примењени сојеви гљивица су значајно смањили токсичне ефекте 0,45 ppm линурона на биљке, што није био случај при концентрацији од 1 ppm линурона. Оба соја гљивица, примењена појединачно или у комбинацији, била су ефикасна у смањивању садржаја линурона у земљишту. Међутим, у условима огледа у пољу најефикаснија је била примена комбинације оба соја.

КЉУЧНЕ РЕЧИ: *Trichoderma asperellum*, *Phanerochaete chrysosporium*, биоремедијација, хербицид, линурон