https://doi.org/10.17221/785/2018-PSE

Mycorrhizal alfalfa and surfactant affect the uptake and dissipation of phenanthrene in soil

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Citation: Wu N.Y., Li Y.Q., Zhou Q., Chai X.C. (2019): Mycorrhizal alfalfa and surfactant affect the uptake and dissipation of phenanthrene in soil. Plant, Soil and Environ., 65: 267–272.

Abstract: A greenhouse experiment was conducted to compare the effects of biosurfactant Rhamnopyranoside and chemical surfactant Triton X-100 on the uptake of phenanthrene by mycorrhizal alfalfa (*Medicago sativa* L.) and phenanthrene dissipation in soil. Mycorrhizal treatment led to a higher phenanthrene accumulation in alfalfa and enhanced phenanthrene dissipation from the treated soil compared with non-mycorrhizal control. Inoculation combined with Rhamnopyranoside application gave the highest phenanthrene removal rate of 85.50% from treated soil and highest phenanthrene accumulation in plants (2.92 μg in root and 1.58 μg in shoot per pot) among all treatments. Besides, such combination treatment resulted in the highest freeze-dried biomass of plant (4.13 g for root and 3.31 g for shoot per pot), suggesting low toxicity for this treatment. These observations indicate co-effects of Rhamnopyranoside and inoculation may have potential as a biotechnological approach for decontamination of soil contaminated with phenanthrene.

Keywords: phytoremediation; organic contaminant; *Glomus etunicatum*; phototoxicity; biodegradation; biosurfactant

Arbuscular mycorrhizal (AM) fungi are ubiquitous in terrestrial ecosystems (Smith and Read 1997), and it has been shown to contribute to plant survival (Verdin et al. 2006). Experimental evidence has been obtained for the impact of AM fungi on the dissipation of polycyclic aromatic hydrocarbons (PAHs) in soils (Joner and Leyval 2003, Gao et al. 2011). These indicate that AM fungi have potential as a bioremediation agent that should be considered in phytoremediation schemes.

PAHs are widely distributed hydrophobic organic contaminants and accumulate in soils that have been contaminated with crude oil, creosote, or coal tar. Among PAHs, phenanthrene is often studied by researchers because it contains basic carcinogenic and mutagenic molecular structure K-regions and bay regions. Moreover, the concentration of phenanthrene can be easily detected in the aqueous phase (Liu et al. 2019). Therefore, phenanthrene is selected as an optimum PAHs model for our study. PAHs in soils are contaminants of major concern, remediation of PAH-contaminated soils gains increasing interest. However, the rate and extent of PAH biodegradation in soils are restricted due to their strong adsorption on soils (McElroy et al. 1989). Therefore, consideration should be given to enhance their release from the soils to increase their bioavailability.

Application of surfactants has been proven to be a feasible alternative for improving the bioavailability of organic contaminants in soils (Cheng and Wong 2006). However, it has drawn attention to the potential toxicities caused by the introduction of chemical surfactants into soils (Zhu and Zhang 2008). Biosurfactants are biological origin compounds which have high surface-activity, which can affect the bioavailability of hydrophobic organic contaminants in soil (Wolf and Gan 2018). With the advantage of biocompatibility and good stability, the use of biosurfactants in soil remediation should be more acceptable than that of chemical surfactants. Nevertheless, application of

Supported by the Natural Science Foundation of Hebei Province, Grant No. B2017402026, and by the Science and Technology Research and Development Projects of Handan City, Project No. 1722201064-2.

https://doi.org/10.17221/785/2018-PSE

biosurfactants is limited because they are still not capable of competing economically with the chemical surfactants. Rhamnosurfactants are among the most effective biosurfactants known today (Maier and Soberón-Chávez 2000). The ease of synthesis (Barragan-Montero et al. 2005) and the lack of toxicity (Dewey 1986) make them good candidates for soil remediation. Thus Rhamnopyranoside was selected in this study. Growing research concerning biosurfactant-enhanced contaminant degradation is documented (De et al. 2015, Kim et al. 2015). We hypothesize that the effects of AM fungi on soil phytoremediation would be further enhanced by the application of a biosurfactant.

Thus, the objectives of this study were to assess the influence of AM inoculation on phenanthrene uptake by alfalfa and the dissipation of phenanthrene in combination with surfactants. Surfactants of Rhamnopyranoside and Triton X-100 were compared aiming to evaluate the advantages of biosurfactant Rhamnopyranoside over the commonly used chemical surfactant of Triton X-100.

MATERIAL AND METHODS

Soil preparation and experimental design. A calcareous sandy soil initially free of phenanthrene contamination (the detection limit of the analytical method was 0.2 μg/kg) were collected from the 5–25 cm depth zone of an agricultural field in Beijing. Soil properties are described in details in Table 1. The soil was air-dried, ground and passed through a 2 mm nylon sieve. The growth medium (referred to as 'soil' below) was a 1:1 (w/w) mixture of sand (1–2 mm) and soil, which was sterilized by γ-radiation (10 kGy, 10 MeV γ rays) to inactivate indigenous AM fungi and received mineral nutrients at the rates of 30 mg P (KH_2PO_4), 60 mg N (NH_4NO_3), and 67 mg K (K_2SO_4)/kg soil. The soil was then artificially spiked with phenanthrene (Sigma Chemical Co., St. Louis, USA, purity of 96%) in acetone. When acetone was evaporated, the spiked soil was mixed with uncontaminated soil to produce the initial concentration of 5.0 mg/kg in soil, thoroughly mixed and preconditioned by inoculation for 4 weeks at room temperature in the dark. The experimental design consisted of six treatments: non-mycorrhizal control; mycorrhizal treatment; addition of Triton X-100; amendment with Rhamnopyranoside (kindly provided by Kebosi Bio technology Co. Ltd, Beijing, China), inoculum plus Triton X-100, inoculum plus Rhamnopyranoside. All treatments were set up in triplicate.

Inoculum and host plants. The AM fungus *Glomus etunicatum* (BGC USA01) isolated from non-contaminated soil was obtained from the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry. The inoculum contained spores, mycelium, sandy soil and root fragments with approximately 350 spores/100 g soil (dry weight soil basis, DW).

Alfalfa seeds (*Medicago sativa* L.) were purchased from the Chinese Academy of Agricultural Sciences, Beijing, China. They were surface sterilized in a 10% (v/v) solution of hydrogen peroxide for 10 min, rinsed with sterile distilled water and pre-germinated on moist filter paper overnight and ready for sowing.

Pot experiment. Each pot with a dimension of 10 × 10 cm (diameter × height) received 650 g of treated soil with the phenanthrene concentration of 2.15 mg/kg. Mycorrhizal treatments received 50 g inoculum. Non-mycorrhizal treatments received an equivalent amount of sterilized soil-sand mixture. Five uniform seedlings of alfalfa were planted in each pot immediately after germination. The upper 2–5 mm of each pot was covered with non-spiked sterilized soil to minimize the loss of phenanthrene by evaporation. All pots were lined with polyethylene to prevent cross-contamination. Rhamnopyranoside or Triton X-100 was watered into the soil to give a final concentration of 0.1% (w/w). The pots were positioned randomly and re-randomized every two days. The plants grew under a 25°C/20°C day/night temperature regime with a 14 h photo period at a

Table 1. Chemo-physical properties of experimental soil

Soil property		Value
pH (1:2.5 in water)		8.04
Soil organic matter (g/kg)		12.54
Proportion of	clay (0–5 μm) (v/v)	12.2%
	silt (5–50 μm) (v/v)	50.6%
	sand (50–2000 μm) (v/v)	37.2%
Available N (mg/kg)		6.39
Extractable (mg/kg)	P	10.6
	K	36.24
	As	0.29
	Cu	1.26
	Pb	1.58
	Zn	0.93

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light intensity of 250 μmol/m^2/s provided by supplementary illumination. Distilled water was added as required to maintain soil moisture content at 70% of field capacity by regular weighing. Nitrogen (as NH_4NO_3) was added to each pot 30 and 45 days after sowing to provide a total of 120 mg N per pot.

Sample preparation. Alfalfa shoots and roots were harvested separately after growth for 60 days. Root fragments were collected by sieving the soil and adding them to the root samples. Roots were first carefully washed with tap water to remove any adhering soil particles. Then roots and shoots were rinsed thoroughly with distilled water, blotted dry and weighed. Soil samples were collected from each pot by gently crushing the soil and shaking the roots. A portion of fresh root subsample was taken from each treatment and cut into 1 cm long segments. They were cleared in 10% KOH for 10 min at 90°C in a water bath, rinsed in water, and then stained with 0.1% Trypan blue (0.4%) for 3–5 min at 90°C in a water bath. Mycorrhizal colonization was determined by the grid line to intersect method (Giovannetti and Mosse 1980). The soil samples and the remainder plant samples were freeze-dried, weighed, and stored at 4°C.

Phenanthrene analysis. 0.1 g of soil samples were submitted to Soxhlet extraction with dichloromethane and acetone (1:1, v/v) for 24 h and then cleaned with silica gel column with 5 mL elution of hexane and dichloromethane (1:1, v/v). The same Soxhlet extraction was conducted on 0.2 g of plant samples after they were chopped with pruning shears. Plant extracts were cleaned with Florisil columns with 5 mL elution of hexane and dichloromethane (1:1, v/v). All eluates were then evaporated, exchanged by methanol and analyzed with a reverse-phase high-performance liquid chromatography (C_{18} column, 4.6 mm × 25 cm, Agilent 1200, Agilent Technologies, Everett, USA) with a UV detector (Everett, USA). The mobile phase was methanol:water (90%:10%) with a flow rate of 1 mL/min. Recovery experiments were carried out by a selected blank sample spiked with phenanthrene. Recovery values ranged from 93.2% to 105.1% with a relative standard deviation < 1.7% (n = 5).

Desorption of phenanthrene from spiked soil. Exactly 1 g of phenanthrene-spiked soil (DW) was combined with 10 mL of Triton X-100 or Rhamnopyranoside solution with different initial concentrations (0.005, 0.01, 0.05, 0.1, 0.15, 0.5, 1 and 2% (w/v)). The mixtures were shaken on a reciprocating shaker at 25°C for 48 h. The supernatants were taken after centrifuging for 20 min at 4000 rpm. Finally, they were filtered through a 0.45 μm syringe filter and ready for phenanthrene analysis. All the treatments were set up in triplicate.

Data analysis. Data were subjected to two-way analysis of variance using the SPSS version 10.0 software package (SPSS Inc., Chicago, USA) to determine the significance of AM inoculation with or without the surfactant as sources of variation. Comparison of means was carried out by calculation of least significant differences (*LSD*) at the 5% level.

Table 2. Mean plant dry matter yield (freeze-dried basis) and mycorrhizal colonization (mean ± standard error, n = 3)

Treatment		Root weight	Shoot weight	Mycorrhizal colonization (%)
		(g/pot)		
Non-mycorrhizal control		0.68 ± 0.13^b	0.47 ± 0.04^c	0^d
Mycorrhizal control		4.10 ± 0.45^a	2.98 ± 0.24^b	0.30 ± 0.03^a
Triton X-100		0.42 ± 0.05^b	0.29 ± 0.03^c	0^d
Rhamnopyranoside		0.93 ± 0.14^b	0.49 ± 0.05^c	0^d
Mycorrhizal + Triton X-100		0.53 ± 0.12^b	0.28 ± 0.03^c	0.23 ± 0.03^b
Mycorrhizal + Rhamnopyranoside		4.13 ± 0.56^a	3.31 ± 0.22^a	0.11 ± 0.01^c
Significance of:	inoculation	***	**	***
	surfactant	ns	ns	***
	inoculation × surfactant	ns	ns	***

***P < 0.001; **P < 0.01; ns – not significant. Means within each column with the same letter are not significantly different at the 5% level

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Table 3. Total phenanthrene accumulation amount in plant parts per pot (μg per pot, freeze-dried basis) and bioconcentration factor (BCF) of phenanthrene in plant parts per pot (mean ± standard error, n = 3)

Treatment	Total phenanthrene		BCF	
	root	shoot	root	shoot
Non-mycorrhizal control	1.02 ± 0.12^{c}	0.53 ± 0.03^{c}	0.70 ± 0.05^{b}	0.53 ± 0.02^{d}
Mycorrhizal control	2.77 ± 0.24^{a}	1.22 ± 0.56^{a}	0.31 ± 0.08^{c}	0.19 ± 0.10^{e}
Triton X-100	1.61 ± 0.07^{b}	0.82 ± 0.07^{a}	1.78 ± 0.25^{a}	1.29 ± 0.07^{a}
Rhamnopyranoside	1.46 ± 0.16^{b}	0.67 ± 0.04^{b}	0.73 ± 0.03^{b}	0.63 ± 0.02^{c}
Mycorrhizal + Triton X-100	1.85 ± 0.36^{b}	0.49 ± 0.03^{c}	1.62 ± 0.03^{a}	0.81 ± 0.10^{b}
Mycorrhizal + Rhamnopyranoside	2.92 ± 0.14^{a}	1.58 ± 0.08^{a}	0.33 ± 0.03^{c}	0.22 ± 0.01^{e}
Significance of:				
Inoculation	***	ns	ns	ns
Surfactant	ns	ns	ns	ns
Inoculation × surfactant	ns	ns	ns	ns

***P < 0.001; ns – not significant. Means within each column with the same letter are not significantly different at the 5% level

RESULTS AND DISCUSSION

Root colonization and plant biomass. From Table 2, the presence of surfactant decreased mycorrhizal colonization (P < 0.001). The shoot and root weights increased for mycorrhizal treatment (P < 0.01), but they were not influenced by the addition of the surfactants. In comparison shoot and root, weights were higher in the presence of Rhamnopyranoside than Triton X-100 irrespective of mycorrhizal status, indicating lower toxicity for biosurfactant Rhamnopyranoside addition.

Phenanthrene accumulation in alfalfa. As shown in Table 3, phenanthrene accumulations in both roots and shoots were increased by mycorrhizal colonization. Addition of surfactants led to an increase in phenanthrene uptake in both shoots and roots compared to non-mycorrhizal control. But anyway, the co-effects of Rhamnopyranoside and mycorrhizal colonization obtained the highest phenanthrene accumulation among all treatments.

To better understand the accumulation potential of phenanthrene in alfalfa from soil under different treatments, the bioconcentration factor (BCF) values were calculated based on phenanthrene concentration in bulk soil (Table 3). Surfactant amendment alone increased BCF values compared with non-mycorrhizal treatment. Rhamnopyranoside plus mycorrhizal colonization gave the lowest BCF with only one exception of mycorrhizal control, indicating a lower accumulation capacity for phenanthrene in alfalfa under this treatment. It might be due to a limited desorption capacity of phenanthrene from the soil. Nevertheless, the combined treatment obtained the highest biomass. Thus the highest phenanthrene accumulation was obtained.

The phenanthrene removal rate in soils. Mycorrhizal treatment led to a significant increase in phenanthrene dissipation in the soil (Figure 1). Without AM colonization, the Triton X-100 addition resulted in a higher phenanthrene removal

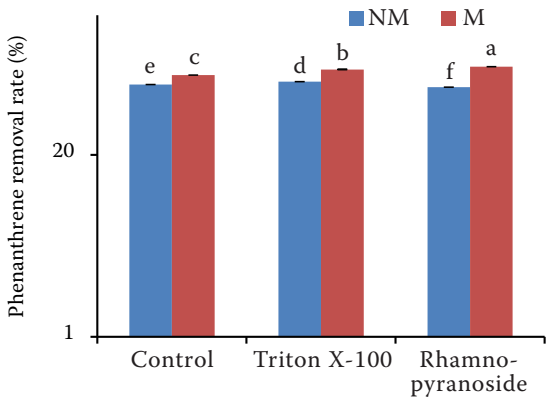

Figure 1. The phenanthrene removal rate in the soil for each treatment. Data are means of three replicates. Means with the same letter are not significantly different at the 5% level. NM – non-mycorrhizal control; M – mycorrhizal control

https://doi.org/10.17221/785/2018-PSE

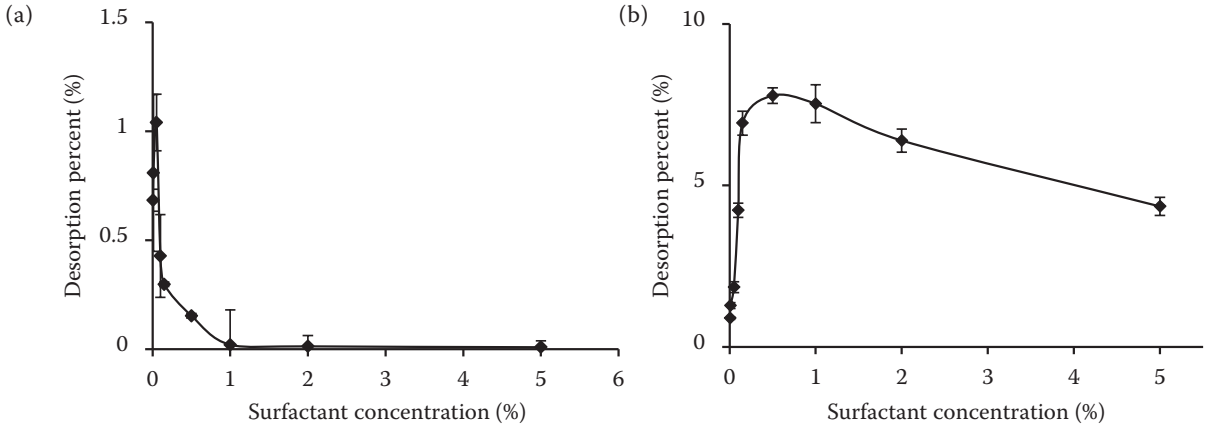

Figure 2. Effect of surfactant (a) Rhamnopyranoside and (b) Triton X-100 concentration on phenanthrene desorption from the spiked soil. Data are means of three replicates

rate in the soil than Rhamnopyranoside addition. However, the combination of AM inoculation and Rhamnopyranoside led to the highest removal rate in the soil, about 85.50%.

As shown in Figure 1, AM inoculation plus Triton X-100 had positive effects on the dissipation of phenanthrene. The ability of surfactants to increase the apparent aqueous solubility of hydrophobic organic chemicals has been well documented (Li et al. 2015). However, the inhibitory effects of non-ionic surfactants on bacterial growth has been observed (Chen et al. 2000), even under the critical micelle concentration. It is expected that the surfactants may have phytotoxicity on plant growth, thus affect phenanthrene biodegradation in soils. Biosurfactant was reported to facilitate the transfer of contaminants from soil particles to the cell membrane (Schippers et al. 2000), therefore enhancing bacterial degradation. As the results, the effects of AM on phenanthrene dissipation was significantly enhanced by biosurfactant Rhamnopyranoside addition ($P < 0.05$).

Desorption of phenanthrene from the spiked soil. To compare the transfer capacity of phenanthrene with different surfactants addition, desorption of phenanthrene from the spiked soil in the presence of Triton X-100 and Rhamnopyranoside were investigated.

Surfactant addition significantly enhanced desorption of phenanthrene from the soil (Figure 2). Phenanthrene desorption reached the highest at the concentration of 0.5% Triton X-100 or of 0.05% Rhamnopyranoside. Phenanthrene desorption is higher for Triton X-100 compared to Rhamnopyranoside, which should be a benefit for the bioavailability of phenanthrene in soil. The result can be explained for higher BCF of Triton X-100 treatment irrespective of mycorrhizal treatment. Nevertheless, the inhibitory effects of Triton X-100 on bacterial growth cause inhibitory effects on plant growth, thus affect phenanthrene accumulation in plants. Lower toxicity of Rhamnopyranoside compared to Triton X-100 lead to higher biomass for Rhamnopyranoside addition irrespective of inoculation treatment. AM inoculation plus Rhamnopyranoside witnessed the highest phenanthrene accumulation in plants among all treatments. Besides, this combined treatment gave the highest phenanthrene removal rate compared to other treatments. These evidence suggest that the combination of AM inoculation and Rhamnopyranoside is promising for soil remediation of phenanthrene. Additionally, the advantage of Rhamnopyranoside over Triton X-100 for plant growth and phenanthrene dissipation was manifested.

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Received on December 4, 2018
Accepted on May 6, 2019
Published online on May 9, 2019