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Se enhanced phytoremediation of diesel in soil by *Trifolium repens*

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ABSTRACT

A pot-culture experiment was conducted to assess the effects of selenium (Se) (0.5 mg kg^{-1}) on *Trifolium repens* exposed to various levels of diesel (0, 15, 20, 25 g kg^{-1}) for 30 days and 60 days. Exposure to diesel for 60 day led to concentration-dependent decreases in root morphogenesis, chlorophyll content and CAT activity, and to dose-dependent increases in MDA content and SOD activity. The residual diesel concentration in soil increased and the removal efficiency decreased with soil diesel concentration. The chlorophyll content and residual diesel concentration after were slightly higher at 30 days than at 60days. Application of Se to soil increased *Trifolium repens* tolerance to diesel and significantly increased the phytoremediation effect at 60 days, with a removal rate of 36 ± 8%, compared to 28 ± 7% in the control. These results contribute to the ongoing effort to develop an effective phytoremediation system for soils highly contaminated by diesel.

1. Introduction

Diesel is a petroleum-derived fuel containing polyromantic hydrocarbons(PAHs), and diesel is a source of PAHs to the environment (Ruma et al., 2007). Anthropogenic activities such a soil extraction and processing and accidental leakage during transportation results in hydrocarbon pollution (Souza and Vessoni-Penna, 2014; Sajna et al., 2015; Saeki et al., 2009)that threatens environmental and public health due to its toxicity carcinogenicity and mutagenicity (Kang et al., 2017; Janbandhu and Fulekar, 2011). Thus, cost-effective, environment-friendly and sustainable remediation strategies to remove hydrocarbon contaminants from soils are needed.

Phytoremediation is an in-situ, nondestructive, and cost effective technique to remove hydrocarbon pollutants (Reynoso-Cuevas et al., 2008). However, most phytoremediation studies have focused on the interaction between plants and soil bacteria at low organic pollutant concentrations (Liu et al., 2013a, 2013b; Li et al., 2013; Sun et al., 2011). Peng et al. (2009) found that *Mirabilis jalapa* effectively promoted the degradation of hydrocarbons at soil concentration slower than 10,000 mg kg^{-1}. The microorganisms play a vital role in phytoremediation of organic pollution, but microorganisms do not adapt to petroleum-contaminated soil when the concentration reaches 20,000 mg kg^{-1} (Liu et al., 2013a, 2013b). Therefore, the challenge of developing phytoremediation systems able to tolerate and degrade highly contaminated soils remains.

Many studies have been conducted to investigate means of enhancing phytoremediation (Kuppusamy et al., 2016; Chirakkara et al., 2015; Li et al., 2012), but few have focused on Se-assisted phytoremediation of organic pollutants in soils.

Low concentrations of Selenium (Se) have been reported to stimulate plant growth, increase chlorophyll content and increase crop resistance to oxidative stress (Barrientos et al., 2012; Hawrylak-Nowak, 2009; Djanaguiranman et al., 2005). A better understanding of the underlying mechanisms could lead to improvements in the use of Se-assisted phytoremediation (Seppänen et al., 2003; Lyons et al., 2009). Hartikainen et al. (2000) studied the effect of Se on ryegrass (*Lolium perenne* L.) and found that Se was harmful at soil concentrations above 10 mg kg^{-1}, but beneficial at a concentration of 0.1 mg kg^{-1}. However, there are few reports on the use of Se to enhance phytoremediation of soils highly contaminated by diesel.

The objective of this investigation was to assess the effects of Se on phytoremediation of high levels of diesel in soil by *Trifolium repens* (white clover). According to previous reports, *Trifolium repens*, has good removal ability in phytoremediation of soils contaminated by organics (Wang and Oyaizu, 2011, 2009). This study contributes to the development of a phytoremediation system appropriate for soils highly contaminated by diesel.

2. Materials and methods

2.1. Experimental design

Soil samples collected at a depth of 20–60 cm from the botanic garden on the campus of Three Gorges University, without detectable

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diesel, was used in this study. The soil was treated with both Se (0.5 mg kg^{-1}) and diesel (0–25 g kg^{-1}). The diesel purchased at a Sinopec petrol station was mixed with soil samples. Volatile components were evaporated with constant stirring in a fume hood for 12 h. The resulting diesel-spiked soil was then mixed, again stirring constantly, with appropriate amounts of diesel-free soil to give uniform soil mixtures with initial concentrations of 0, 15, 20 and 25 g kg^{-1}. No Se was added to soil used as the Se control and soil with added Se was prepared according to Lavu et al. (2013). Briefly, sodium selenite, Na_2SeO_3, (Sigma, St. Louis, MO, USA) was dissolved in water and then added to soil to give a Se concentration of 0.5 mg kg^{-1}. The contaminated soil was sieved (2 mm), placed in pots (6 L pots with 3000 g dry soil per pot) and left in the air for 7 days to permit volatilization. The soil was stirred every day to allow the diesel constituents reach equilibrium with the soil particles. The pots were watered every 3 days with tap water to maintain ~ 70% saturation.

Trifolium repens was the plant selected for this study. Plants of similar biomass were collected from gardens on the Three Gorges University campus and maintained for 4 days in uncontaminated soil before transplanting to test pots.

A randomized block design (4 diesel treatments × 2 Se treatments × 1 plant) was employed and treatments were run in triplicate: (1) control (CK), plant grown in untreated soil; (2) no plant with diesel only, Se only, diesel and Se; and (3) plant, soil with diesel at four concentrations, with and without Se (36 pots total).

2.2. Determination of physical and chemical properties of soil

The basic physical and chemical properties of the soil are provided in Table 1. Total phosphorus (TP), total nitrogen (TN), the pH, organic matter (OM), and the texture of soil samples were determined using, respectively, alkali fusion with Mo-Sb spectrophotometric detection (HJ632-2011), the Kjeldahl method (HJ717-2014), a glass electrode (NY/T1377-2007), the potassium dichromate method (NY/T1121.6-2006) and hydrometer method (FHZDZTR0008). The Se content in soil was determined according to method of De et al. (2014) using inductively coupled plasma mass spectrometry (ICP-MS, Thermo Fisher Scientific, USA)

2.3. Analysis of Se in plant

For the analysis of Se, *Trifolium repens* were divided into two parts (shoot and root) and placed in a drying oven at 60 °C for 24 h before being ground to a fine powder with mortar and pestle. The powdered sample (0.5 g) was then digested in a digestion vessel with an acid mixture (1 mL HF, 4 mL HNO_3), heated at 180 °C for 12 h in a thermostatic oven and allowed to cool to room temperature. The digested samples were placed on a hot plate at 100 °C, evaporated to 2 mL and mixed with 1 mL of 30% H_2O_2. The samples were diluted to 10 mL with ultrapure water, allowed to settle overnight and analyzed by ICP-MS (Huang et al., 2017).

2.4. Analysis of plant biomass and root morphology

Shoots and roots were separated, rinsed with deionized water and weighed at harvest. *Trifolium repens* biomass was determined by measuring the fresh weight of the root and shoot of 4 plants randomly selected from each pot. The averaged value for each treatment was expressed as the percentage of relative fresh weight with respect to the plants grown without Se. Mass was measured using an analytical balance (+ 0.1 mg).

Root morphology, including root length, surface area and number of nodes were measured and analyzed using LA-S Root Analysis software (WSeen, China).

Table 1
Physical and chemical properties of test soil.

Texture (%)			Se (mg kg^{-1})	OM (g kg^{-1})	pH	TN	TP
Sand	Silt	Clay					
31	61	8	0.03	2.89	7.4	306	415.1

2.5. Chlorophyll parameters

Chlorophyll parameters were recorded with a SPAD-502 m (Minolta Camera Co., Osaka, Japan), according to the procedures of Nauš et al. (2010) and Uchino et al. (2013), for 30 days and 60 days. After cleaning dust from the leaf surface, SPAD readings at 10–20 points (according to leaf size) were recorded for each leaf and the mean value was reported. Chlorophyll meter readings of the top four leaves in each pot were averaged to minimize the influence of leaf position and sampling location on chlorophyll readings.

2.6. Assays of antioxidant enzymes and malondialdehyde

Leaf tissue (0.5 g) was manually homogenized in a tissue grinder with 2 mL of pre-chilled 0.1 mol L^{-1}phosphate buffer (pH = 7.4). The buffer was prepared by mixing 0.1 mol L^{-1} solutions of K_2HPO_4 (80.2 mL) and KH_2PO_4 (19.8 mL) to give a final pH of 7.4. The homogenates were then centrifuged at 4 °C for 10 min at 3500 RPM. The catalase (CAT) and superoxide dismutase (SOD) activities, total protein and malondialdehyde (MDA) concentrations of *Trifolium repens* leaves were determined by using SOD, CAT, protein and MDA assay kits (Jiancheng, Ltd, Nanjing, China). Readings were taken on a Spectra Max® Paradigm® Multi-Mode Detection Platform using the manufacturer's protocols and assays were carried out in triplicate. One unit of CAT activity was defined as the quantity of H_2O_2decomposed by CAT per second, per gram of protein, under assay conditions (U g^{-1} protein). One unit of SOD activity was defined as the amount of enzyme per mg of protein required to inhibit the rate of xanthenes reduction by 50% under assay conditions (Umg^{-1} protein). Lipid peroxidation was assayed by determining an end product, MDA, using the 2-thiobarbituric acid (TBA) method. Absorbance was measured at 532 nm, using a molar extinction coefficient of 0.155 mM cm^{-1}, and MDA was expressed as nmol g^{-1} fresh weight according to Wu et al. (2016).

2.7. Determination of diesel degradation

Residual diesel in soils for 30 and 60 days was determined using gravimetric analysis (Escalante-Espinosa et al., 2005). Approximately 5 g of soil sample was ultrasonically extracted with 15 mL of chloroform for 15 min. The sample was centrifuged for 15 min at 4000 RPM and the supernatant was transferred to a flask and dried to a constant weight. The solution was filtered and collected. The process was repeated twice. The combined filtrate was dried at 45 °C by rotary evaporators and then evaluated using a gravimetric method. The removal ratio is defined as (Liu et al., 2013a, 2013b):

$$\text{Removal}(\%) = \frac{(diesel\ concentration)_{control} - (diesel\ consentration)_{experimental}}{(diesel\ concentration)_{control}} \times 100$$

2.8. Statistical analyses

Each treatment was run in triplicate and results were reported as mean ± SD. The effect of diesel, selenium and the interaction effect were measured by two-way analysis of variance using SPSS version 17.0. When the differences among diesel concentrations were considered significant at $p < 0.05$, Tukey's range test was performed to