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Plant tolerance to diesel minimizes its impact on soil microbial characteristics during rhizoremediation of diesel-contaminated soils

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ABSTRACT

Soil contamination due to petroleum-derived products is an important environmental problem. We assessed the impacts of diesel oil on plants (*Trifolium repens* and *Lolium perenne*) and soil microbial community characteristics within the context of the rhizoremediation of contaminated soils. For this purpose, a diesel fuel spill on a grassland soil was simulated under pot conditions at a dose of 12,000 mg diesel kg^{-1} DW soil. Thirty days after diesel addition, *T. repens* (white clover) and *L. perenne* (perennial ryegrass) were sown in the pots and grown under greenhouse conditions (temperature 25/18 °C day/night, relative humidity 60/80% day/night and a photosynthetic photon flux density of 400 μmol photon m^{-2} s^{-1}) for 5 months. A parallel set of unplanted pots was also included. Concentrations of n-alkanes in soil were determined as an indicator of diesel degradation. Seedling germination, plant growth, maximal photochemical efficiency of photosystem II (F_v/F_m), pigment composition and lipophylic antioxidant content were determined to assess the impacts of diesel on the studied plants. Soil microbial community characteristics, such as enzyme and community-level physiological profiles, were also determined and used to calculate the soil quality index (SQI). The presence of plants had a stimulatory effect on soil microbial activity. *L. perenne* was far more tolerant to diesel contamination than *T. repens*. Diesel contamination affected soil microbial characteristics, although its impact was less pronounced in the rhizosphere of *L. perenne*. Rhizoremediation with *T. repens* and *L. perenne* resulted in a similar reduction of total n-alkanes concentration. However, values of the soil microbial parameters and the SQI showed that the more tolerant species (*L. perenne*) was able to better maintain its rhizosphere characteristics when growing in diesel-contaminated soil, suggesting a better soil health. We concluded that plant tolerance is of crucial importance for the recovery of soil health during rhizoremediation of contaminated soils.

1. Introduction

Soil contamination with diesel fuel, an environmental problem of great concern, is for the most part caused by leakage of underground storage tanks and accidental spills during transportation. The use of plants and their associated microorganisms to remediate hydrocarbon-contaminated soils has gained increasing acceptance as a viable cleanup technology (van der Lelie et al., 2001; Arthur et al., 2005). This remediation technology, termed rhizoremediation, has been defined as "the acceleration of organic contaminant breakdown in soil as a consequence of the enhanced biodegradative activity of rhizosphere microorganisms" (Shaw and Burns, 2005). Indeed, plant roots can provide a beneficial, stimulating habitat for hydrocarbon-degrading microorganisms (Gaskin and Bentham, 2010). Within the phytoremediation field, rhizoremediation has been suggested as the primary mechanism responsible for hydrocarbon degradation in soil (Hutchinson et al., 2003; Yateem et al., 2007).

During remediation processes, apart from monitoring the removal of contaminants, it is most important to also assess the recovery of soil health, defined as "the capacity of soil to perform its functions" (Karlen et al., 2003). Traditionally, emphasis has been placed on physical and chemical soil properties as indicators of soil health but, in the last years, biological properties are becoming increasingly used due to their sensitivity and capacity to provide information that integrates many environmental factors (Mijangos et al., 2006). Soil properties that provide information on the biomass, activity and diversity of soil microbial communities (e.g., microbial biomass C, respiration, enzyme activities, structural and functional community profiling, etc.) have proved to have great potential for the assessment of the effectiveness of phytoremediation processes (Hernández-Allica et al., 2006; Epelde et al., 2009a, b).

Different authors (Palmroth et al., 2002; Kaimi et al., 2006; Tang et al., 2010) have investigated the capacity of legumes (such as *Trifolium repens*) and grass species (such as *Lolium perenne*) to enhance rhizodegradation of

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petroleum-derived compounds. However, to our knowledge, no study has been carried out to determine which plant species (*T. repens* versus *L. perenne*) has more potential for the recovery of soil microbial community characteristics (and, concomitantly, soil health) after a diesel oil spill. On the other hand, diesel phytotoxicity has been extensively studied (Adam and Duncan, 2002; Wyszkowski and Wyszkowska, 2005), but there is a paucity of studies on the possible implications of plant tolerance to diesel for the successful rhizoremediation of diesel-contaminated soils.

The objective of this work was to study the impacts of diesel fuel on plants (a legume and a grass species, i.e. white clover — *Trifolium repens* and perennial ryegrass — *Lolium perenne*, respectively) and soil microbial community characteristics within the context of the rhizoremediation of contaminated soils. To this aim, a short-term microcosm rhizoremediation experiment with *T. repens* and *L. perenne* was carried out with soil artificially contaminated with diesel fuel, in which we simultaneously determined plant physiological status, diesel degradation rate, and soil health as reflected by the values of soil microbial characteristics. A parallel set of unplanted pots was also included in the experiment. We hypothesized that, after a diesel oil spill, the more tolerant plant species is able to better maintain its rhizosphere soil microbial characteristics, thereby minimizing the impacts of diesel contamination on soil health.

2. Materials and methods

2.1. Soil characterization

Soil was collected from the top layer (0–30 cm) of a natural grassland located in Derio (43°17′ N; 2°52′ W, northern Spain), with no previous history of hydrocarbon contamination. After collection, the soil was sieved to <4 mm, air-dried at 30 °C for 48 h, and subjected to physicochemical characterization according to standard methods (MAPA, 1994). The soil was a clay loam, with a pH of 5.2, an organic matter (OM) content of 4.12%, a total nitrogen (N) content of 0.23%, a C/N ratio of 10.4, a phosphorus (P) content of 26.4 mg kg^{-1} and an electrical conductivity of 0.08 dS m^{-1}. Subsequently, the soil was artificially contaminated with commercial diesel fuel (12,000 mg kg^{-1} DW soil) purchased from a petrol station following ISO Norm CD 15952 (2006).

2.2. Experimental design

One month after diesel contamination, non-contaminated (control) and diesel-contaminated soils were placed in 24 pots (2.5 kg DW soil pot^{-1}): a third of the pots were sown with *Trifolium repens* L. (300 seeds pot^{-1}), another third with *Lolium perenne* L. (200 seeds pot^{-1}), and the remaining third was left as unplanted control. The six treatments (no diesel, unplanted; no diesel, *T. repens* planted; no diesel, *L. perenne* planted; diesel-contaminated, unplanted; diesel-contaminated, *T. repens* planted; diesel-contaminated, *L. perenne* planted) were conducted in quadruplicate (four replicates per treatment per harvest; see below). Pots were placed in a greenhouse under the following conditions: temperature 25/18°C day/night, relative humidity 60/80% day/night and a photosynthetic photon flux density of 400 µmol photon m^{-2} s^{-1} by supplementing natural illumination with cold white lamps (Philips SON-T AGRO 400, Belgium). Throughout the experimental period, both planted and unplanted pots were bottom watered as required to maintain a 1-cm depth in the pot plates. After seed emergence, seedling number per pot was set at 100 seedlings pot^{-1} by manually removing extra seedlings with their roots.

Two and five months after sowing, all the plants in the experimental plots were harvested (4 pots per treatment and harvest) and manually separated into shoots and roots. After recording the total fresh weight of all plants in each pot, shoots and roots were carefully rinsed in tap water, soaked twice in deionized water, oven dried at 70 °C for 48 h, and finally their dry weights were recorded. In those pots intended for the second harvest, 2 months after sowing, the aerial part of *L. perenne* plants growing in non-contaminated and diesel-contaminated soil, as well as that of *T. repens* plants growing in non-contaminated soil, was trimmed to the soil surface, due to the elevated biomass present in the pots, and left to regrow for another 3 months. At both harvest times, soil was collected from the pots for the determination of n-alkanes. For the determination of microbial properties, soil was collected from the pots at the end of the experiment (i.e., at second plant harvest).

2.3. Plant physiological parameters

Prior to harvest, maximal photochemical efficiency of PSII (F_v/F_m) was determined in leaves of *T. repens* and *L. perenne* using a portable modulated fluorimeter (OS 5-FL, Optisciences, Tyngsboro, USA). Initial (F_0) and maximal (F_m) fluorescence were measured with a saturating pulse of 0.8 s. Then, leaf tissue (approximately 10 mg FW) was collected and kept in the dark for 12 h at room temperature (20–22 °C) to reduce the effects of diurnal variations in pigments and provide comparable conditions, "artificial predawn conditions", as described in García-Plazaola et al. (2000) and Tausz et al. (2003). Subsequently, dark adapted tissue was frozen in liquid N and stored at −80 °C until analysis. Photosynthetic pigments (chlorophylls and carotenoids) and lipophilic antioxidants (α-tocopherol) were determined according to García-Plazaola and Becerril (2001).

2.4. Soil microbial properties

β-glucosidase (EC 3.2.1.21), arylsulfatase (EC 3.1.6.1) and alkaline and acid phosphatase (EC 3.1.3.1 and EC 3.1.3.2) activities were determined according to Dick et al. (1996) and Taylor et al. (2002), as described in Epelde et al. (2008). Urease (EC 3.5.1.5) activity was determined according to Kandeler and Gerber (1988), as described in Rodríguez-Loinaz et al. (2008). A geometric mean was calculated to obtain an integrative view of the effect of treatments on total soil enzyme activity.

Community-level physiological profiles (CLPPs) with Biolog EcoPlates™ were determined as described in Epelde et al. (2009b). Average well color development (AWCD) was calculated after 44 h of incubation, which corresponded to the time of maximal microbial growth rate in the Biolog EcoPlates™. The number of utilized substrates (i.e., NUS = number of substrates with an absorbance value >0.25), equivalent to species richness, *S*, was calculated at 44 h incubation time. Shannon's diversity index ($H' = -\sum p_i \log_2 p_i$), where p_i is the ratio of the absorbance of a particular well to the sum of absorbances of all wells, was also calculated at 44 h, considering absorbance values at each well as equivalent to species abundance.

2.5. Aliphatic hydrocarbons

Prior to the study, n-alkanes present in the diesel fuel purchased from the petrol station were determined by GC–MS (gas chromatography–mass spectrometry). The chromatographic profile showed that the diesel fuel contained the following n-alkanes: decane (n-C10), dodecane (n-C12), tetradecane (n-C14), hexadecane (n-C16), octadecane (n-C18), nonadecane (n-C19), eicosane (n-C20), docosane (n-C22), tetracosane (n-C24); hexacosane (n-C26), and triacontane (n-C30). The concentrations of these aliphatic hydrocarbons in soil were determined throughout the experiment as an indicator of diesel degradation. Standard solutions were prepared using an aliphatic hydrocarbon mixture (INC ChemService, West Chester, PA; purity >99%). In order to check the recovery of n-alkanes from soil, samples (5 g) of non-contaminated soil were spiked with 5 mg kg^{-1} of the aliphatic hydrocarbon standard mixture, and extracted and analyzed as described below. Under our