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Letter to the Editor

Effects of Inoculum Density on Plant Growth and Hydrocarbon Degradation

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ABSTRACT

The combined use of plants and bacteria is a promising approach for the remediation of soil contaminated with organic pollutants. Different biotic and abiotic factors can affect the survival and activity of the applied bacteria and consequently plant growth and phytoremediation efficiency. The effect of inoculum density on the abundance and expression of alkane-degrading genes in the rhizosphere of plant vegetated in hydrocarbon-contaminated soil has been rarely observed. In this study, an alkane-degrading bacterium (*Pantoea* sp. strain BTRH79), at different inoculum densities (10^5 to 10^8 cells cm^{-3} soil), was inoculated to ryegrass (*Lolium perenne*) vegetated in diesel-contaminated soil to find the optimum inoculum density needed for its efficient colonization and hydrocarbon degradation activity. Bacterial inoculation improved plant growth and hydrocarbon degradation. Maximum plant growth and hydrocarbon degradation were observed with the inoculum having the highest cell density (10^8 cells cm^{-3} soil). Moreover, the inoculum with higher cell density exhibited more abundance and expression of alkane hydroxylase gene, *CYP153*. This study suggests that the inoculum density is one of the main factors that can affect bacterial colonization and activity during phytoremediation.

Key Words: alkane-degrading bacterium, gene abundance, gene expression, phytoremediation, plant-bacteria partnership

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INTRODUCTION

Phytoremediation, *i.e.*, use of plants, is an effective, economically attractive and environmental friendly approach for the remediation of soil polluted with petroleum hydrocarbons. However, hydrocarbons inhibit plant growth as well as microbial proliferation due to their toxicity and hydrophobic nature (Kirk *et al.*, 2005; Khan *et al.*, 2013). The inoculation of plants with hydrocarbon-degrading bacteria has been found to be very useful for the efficient remediation of soil polluted with hydrocarbons (Weyens *et al.*, 2009; Glick, 2010). In plant-bacteria partnership, plant provides nutrients for microbial colonization in the rhizosphere (Weyens *et al.*, 2009; Afzal *et al.*, 2014). Furthermore, plant releases easily degradable hydrocarbons in rhizosphere which stimulate the hydrocarbon-degrading genes of rhizobacteria (Xie *et al.*, 2012; Afzal *et al.*, 2013). In response, rhizobacteria facilitate plants to grow in the contaminated environment by reducing the toxicity of hydrocarbons through mineralizing these compounds into simple inorganic compounds and elements (Yousaf *et al.*, 2011). Moreover, rhizobacteria having the potential to consume 1-amino-cyclopropane carboxylic acid (ACC), immediate precursor of ethylene, improve plant health and development even in the hydrocarbon-contaminated soil (Glick, 2003).

There are a number of reports indicating the poor colonization and low activity of the inoculated bacteria in the rhizosphere of the inoculated plant (Gerhardt *et al.*, 2009). Among the biotic factors, the inoculum density can affect bacterial colonization and activity in the rhizosphere of a plant. However, the effect of inoculum density on the persistence and metabolic activity of the inoculated bacteria during the phytoremediation of organic pollutant-contaminated soil has been rarely studied. The current study was aimed to find optimum inoculum density of an alkane-degrading bacterium

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(*Pantoea* sp. strain BTRH79) needed for its efficient colonization and pollutant degradation activity in the rhizosphere of ryegrass planted in the diesel-contaminated soil.

MATERIALS AND METHODS

Experimental setup

An alkane-degrading bacterium, *Pantoea* sp. strain BTRH79, was used in the present study. This strain contained alkane hydroxylase *CYP153* gene and also exhibited ACC-deaminase activity (Yousaf *et al.*, 2010; Afzal *et al.*, 2011). The bacterial culture was prepared by growing *Pantoea* sp. strain BTRH79 in Luria-Bertani broth. Inoculum with various cell densities was prepared using turbidimetric method (Sutton, 2011). Each pot was inoculated with 100 mL of inoculum (10^5, 10^6, 10^7 and 10^8 cells cm^{-3} soil) immediately after seeds sowing.

An agricultural loamy soil was spiked with diesel (10 g kg^{-1} soil) and transferred in the pots (1.5 kg pot^{-1}). Surface-sterilized ryegrass seeds were sown in the pots (100 seeds pot^{-1}). Seedlings were counted after one week of seed germination and the poor-growing ones were removed to 75 seedlings pot^{-1}. Plants were grown in the greenhouse at 25 ± 2 °C for 90 d. Moreover, the plants were irrigated with tap water when the soil seemed to be drying.

Parameter determination

After 90 d of seed germination plants were cut and growth parameters (shoot length, shoot and root dry weight) were determined. The bulk and rhizospheric soil samples were collected and preserved at −80 °C for further examination.

The population of inoculated bacterium, *Pantoea* sp. strain BTRH79, in the rhizospheric soil was enumerated by plate count method. DNA from rhizosphere was extracted by using FastDNA Spin Kit for soil (Qbiogene, USA), whereas RNA was isolated with the FastRNA Pro Soil-Direct Kit (MP Biomedicals, USA). Reverse transcription was performed with 10–20 ng RNA, the specific primer P450-for and SuperScript II Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions. Abundance and expression of *CYP153* gene were quantified by real-time polymerase chain reaction (PCR) by using an iCycler IQ (Bio-Rad, USA) as described earlier (Andria *et al.*, 2009; Afzal *et al.*, 2011). Besides melting curve analysis, PCR products were examined on 2% agarose gels. No primer-dimers were detected. The *CYP153* gene copy numbers were quantified relative to a standard curve of positive control.

The residual hydrocarbon concentration in the soil was estimated by using fourier transform-infrared spectroscopy as explained previously (Yousaf *et al.*, 2010).

Ames test was executed using *Salmonella typhimurium* TA98 and TA100 strains to determine the genotoxicity of soil samples as described previously (Tara *et al.*, 2014). The data was presented as mutagenicity ratio (MR), *i.e.*, the ratio between induced revertants and spontaneous revertants. The value of MR $\geq$ 2 is the indication of significant mutagenicity.

Statistical analysis

The Microsoft Excel and SPSS software packages were used for the statistical analyses. The results were interpreted by analysis of variance and their mean values ± standard deviations were compared to determine whether the means differ significantly by Duncan's multiple range test (Duncan *et al.*, 1983). A Pearson correlation was used to determine the correlation coefficient between gene expression and gene abundance, gene abundance and hydrocarbon degradation, and gene abundance and toxicity reduction.

RESULTS AND DISCUSSION

Effect of inoculum density on plant growth

In this study, lower values of seed germination, shoot length and biomass of the plants were obtained in the diesel-contaminated soil compared to those of the plants grown in the soil without diesel (Table I). This reduction in growth and development of ryegrass in the diesel-contaminated soil can be attributed to the toxicity and hydrophobicity of diesel hydrocarbons (Hou *et al.*, 1999; Babu *et al.*, 2001; Kirk *et al.*, 2005). However, the toxic effects of diesel hydrocarbons on plant growth were reduced by inoculating plants with *Pantoea* sp. strain BTRH79. It is well established that the phytotoxicity of pollutants can be reduced by applying the bacteria having pollutant degradation potential (Kim *et al.*, 2006; Afzal *et al.*, 2012). Moreover, plant growth can be improved due to the ACC-deaminase activity of rhizobacteria during phytoremediation of a contaminated soil (Arshad *et al.*, 2007; Glick and Stearns, 2011).

The maximum shoot length and biomass were exhibited by the plants inoculated with the highest number (10^8 cells cm^{-3} soil) of bacterial cells. This indicated that the inoculum with higher number of bacterial cells was more efficient in improving plant growth

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