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Influence of nitrogen fertilization on diazotrophic communities in the rhizosphere of the Jerusalem artichoke (*Helianthus tuberosus* L.)

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Abstract

Diazotrophs in the soil may be influenced by plant factors as well as nitrogen (N) fertilization. In this study, we investigated potential diazotrophic communities in the rhizosphere of the Jerusalem artichoke (*Helianthus tuberosus* L.) supplied with differing amounts of N. The community structure of N_2-fixing bacteria was profiled using the length heterogeneity polymerase chain reaction (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP) based on a variation in the *nifH* gene. Higher numbers of diazotrophs were detected by T-RFLP compared to LH-PCR. The lowest number of N_2-fixing bacteria was observed in the rhizosphere soil with high N fertilization. T-RFLP was a better method than LH-PCR for profiling microbial diversity of diazotrophs using multidimensional scaling (MDS) and analysis of similarity (ANOSIM) of fingerprints as well as diversity measures. The supply of N fertilizer appeared to negatively influence the abundance of diazotrophs in the rhizophere of the Jerusalem artichoke.
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Keywords: Jerusalem artichoke; Diazotrophs; Bacterial community structure; Rhizosphere; LH-PCR; T-RFLP

1. Introduction

Biological nitrogen fixation (BNF) is an important process for making nitrogen from air available to living organisms in both terrestrial and aquatic habitats. BNF was shown to be responsible for 65% of total nitrogen input into the biosphere, whereas only 25% of the total nitrogen fixation per year was due to industrial processes (Newton, 2002). In addition to legume–rhizobia symbiosis, free-living soil microorganisms (rhizosphere diazotrophs) are important contributors to N_2 fixation (Cleveland et al., 1999; Kanungo et al., 1997; Roper et al., 2006). In rhizosphere soils and in the rhizoplane and endorhizosphere, non-symbiotic diazotrophs have been shown to be the dominant N_2-fixing microbes (Lovell et al., 2000; Zehr et al., 2003), including α-, β-, γ-, and δ-Proteobacteria, Firmicutes and Archaea. Most of these bacteria have yet to be cultured under laboratory conditions (Coelho et al., 2008, 2009; Poly et al., 2001b; Wartiainen et al., 2008).

Cultivation-independent studies using molecular-based tools (Piceno and Lovell, 2000; Poly et al., 2001a; Wartiainen et al., 2008; Zhang et al., 2006) have already shown the great diversity of *nifH* genes (encoding the iron protein subunit of nitrogenase) in natural environments. The *nifH* gene is highly conserved among both cultured and unculturable microorganisms isolated from multiple environments (Demba et al., 2004; Deslippe and Egger, 2006; Tan et al., 2003; Zehr et al., 2003). The close phylogenetic correlation between *nifH* and the 16S rRNA gene fragment (Raymond et al., 2004; Young, 1996; Zehr et al., 2003) makes *nifH* an ideal gene marker for investigation of potential N_2-fixing organisms in natural environments. It is recognized that the diversity of *nifH* is linked to functioning of soil ecosystems (Hsu and Buckley, 2008), making it useful for studying the functionality of diazotrophs in soils.

In recent years, polymerase chain reaction (PCR)-based technology has been widely used to profile diversity and the

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community structure of soil bacteria (Kirk et al., 2004; Thies, 2007). The length heterogeneity polymerase chain reaction (LH-PCR) (Rappé et al., 1997; Suzuki et al., 1997) and terminal restriction fragment length polymorphism (T-RFLP) (Clement et al., 1998; Liu et al., 1997), two "fingerprinting" techniques, analyze differences in lengths of gene fragments (usually rDNA genes) due to insertions, deletions and point mutations affecting restriction–recognition sites, and estimate the relative abundance of each fragment. A DNA-based assessment of community structure targeting functional genes can be linked to a potential ecosystem function (Kennedy and Egger, 2010).

The Jerusalem artichoke (*Helianthus tuberosus* L.) has recently attracted great interest due to its high economic value as a good source of fructose and inulin (Baldini et al., 2004; Saengthongpinit and Sajjaanantakul, 2005). The Jerusalem artichoke is a salt-tolerant and drought-tolerant species and can be cultivated with a relatively small amount of N fertilization (90 kg/ha, 2 g/plant) (Zhao et al., 2008), suggesting that diazotrophs can contribute a notable proportion of nitrogen to the rhizosphere soil (Wartiainen et al., 2008).

In this study, LH-PCR was used together with T-RFLP to characterize the structure of diazotrophic communities in the rhizosphere of the Jerusalem artichoke, followed by sequencing and phylogenetic analysis. The objective of this study was to detect potential changes in the structure and composition of the diazotroph community between the rhizosphere and bulk soil as influenced by different amounts of N fertilization.

2. Materials and methods

2.1. Field characteristics and soil sampling

The field experiment was carried out at Laizhou, Shandong, China, located at latitude 37°38′N and longitude 119°38′E. *H. tuberosus* was planted on April 20, 2010 and harvested after 8 months. Some of the physical and chemical characteristics of the soil are given in Table 1. There was no cultivation in the sampling area before the experiment was set up. Plot size was 5 m in length and 4 m in width. The experimental design consisted of two nitrogen (N) application levels: 1.2 g/plant and 8.4 g/plant, denoted as NL and NH, respectively. Plant row spacing was 60 cm and plant spacing was 50 cm. The urea fertilizer containing 46% N was spot-applied 15–25 cm away from the roots when *H. tuberosus* was planted. The treatment with no fertilizer application and no plants growing was considered the control (CK). The soil adhering to the roots (=rhizosphere soil) was removed by shaking and collected in sterile tubes. Bulk soil was sampled from the CK. Samples were kept at −70 °C until DNA extraction. Three replications of soil samples for each treatment were evaluated.

2.2. DNA extraction from soils

DNA was extracted from the rhizosphere soil [high (NH) and low (NL) fertilizer treatments] and bulk soil samples (CK) using the soil DNA isolation kit (OMEGA, GA, USA). Extracted DNA was visualized on 1.0% (w/v) agarose gels to assess integrity and then stored at 4 °C prior to PCR amplification.

2.3. PCR amplification of the nifH gene for length polymorphism

The *nifH* gene was amplified from DNA extracted from soil samples using semi-nested PCR. The primary PCR reaction was carried out with PolF and PolR and the second amplification with PolFL (labeled with 6-FAM) and AQE (Table 2) (Coelho et al., 2009; Poly et al., 2001a). Each 50 μl reaction cocktail contained 1× PCR buffer, 3 mM $MgCl_2$, 2.5 units of high-fidelity Taq DNA polymerase, 0.2 mM deoxyribonucleoside triphosphate (dNTP), each primer at 1 μM and a 200 ng DNA template. For the second PCR, 1 μl of the first PCR product was used as a template. Thermocycler conditions were the same for both reactions, except for the annealing temperature: initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturing, annealing and extension at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1.5 min. The final extension required 10 min at 72 °C. The annealing temperature in the second PCR was 48 °C. The PCR products were analyzed by 1% (w/v) agarose gel electrophoresis followed by staining with ethidium bromide to verify amplification of a single product of the expected size, and were stored at −20 °C after gel purification until analysis of LH-PCR and T-RFLP.

2.4. LH-PCR and T-RFLP

The PCR products were used directly for LH-PCR. To obtain profiles of T-RFLP, the PCR products were digested with two endonucleases (*Nde* II and *Mnl* I) according to the manufacturer's instructions. The restriction enzymes were chosen to maximize T-RFLP richness as reported by Poly et al. (2001a). The digested products were precipitated using 100 μl of cold ethanol/sodium acetate solution (95% v/v ethanol, 5% v/v 3 M sodium acetate, pH 4.6). The suspension was briefly

Table 1
Physical and chemical characteristics of soil in the experimental field.

Depth, cm	pH	Salt content, g kg^{-1}	Organic matter, g kg^{-1}	Sand (2–0.02 mm), g kg^{-1}	Silt (0.02–0.002 mm), g kg^{-1}	Clay (<0.002 mm), g kg^{-1}	Bulk density, g cm^{-3}
0–20	7.55	0.379	10.52	817.0	98.4	84.6	1.27
20–40	7.50	0.401	4.42	826.3	80.7	93.0	1.58
40–60	8.17	0.480	2.84	826.1	80.5	93.4	1.50
60–80	7.71	0.647	–	859.9	69.1	71.0	1.48
80–100	7.94	0.731	–	855.6	70.3	74.1	1.50

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