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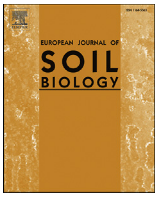

Original article

Endophytic fungal diversity and space-time dynamics in sugar beet

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ABSTRACT

We studied species richness, composition and distribution of sugar beet endophytic fungi with location, plant species and plant growth period using the methods of PCR-based 454 pyrosequencing and terminal-restriction fragment length polymorphism. In total 4345 fungal nuclear ribosomal internal transcribed spacer region sequences (rDNA ITS) obtained from all sugar beet grown in two locations (Shihezi and Changji) on the north slope of Tianshan Mountain, China. The most abundant fungal groups in all sugar beet were Basidiomycota (97.26%). The other dominant phyla were from Ascomycota (0.61%) and Glomeromycota (2.13%). There was a marked difference in diversity of endophytic fungi in sugar beet for different growth stages. The greatest number of Operational T-RFLP Units was detected during sucrose accumulation (84) and tuber growth (74). Endophytic fungal diversity was reduced during seedling growth (18 OTUs) and rosette formation (58 OTUs). One OTU was common to all four periods of growth. There were more OTUs in Shihezi than in Changji. The dynamics of endophytic fungal communities were influenced by plant genotype and plant growth stage. Results indicated that the three factors were significantly related ($\alpha = 0.05$) to the distribution of endophytic fungi, with host species the most important, followed by sampling dates and sampling locations. To our knowledge, this study is the first application of 454 pyrosequencing and T-RFLP to characterize and compare multiple sugar beet samples.

1. Introduction

Endophytic fungi are defined as those fungus that are cultivable and non-cultivable, inhabit the interior of plant tissues and organs without causing harm but can be of two types: I) those that do not produce external structures to the plant and II) those that produce external structures, like Mycorrhizae [1]. Endophytic fungi are common in plants [2–4], and have been found to be ubiquitous within all examined plants [5–7]. Endophytic fungi have been reported from different plants such as marine algae [8], mosses and fern [9], coniferous tree [10], tropical palms and monocots [11] and broad leaf trees [12]. In addition, endophytic fungi have been isolated from different plant tissues, including flowers, seeds, roots, stems and leaves [13,14]. Previous studies have found that some endophytic fungi have roles within the plant in relation to growth [15], enhanced stress resistance [16], degradation of pollutants [5], and the production of bioactive substances in the host [17]. Little is known about symptomless endophytic symbionts of other cultivated plants.

The presence of endophytic fungus within healthy sugar beet (*Beta vulgaris* L.) leaves and roots have been demonstrated [18,19] (Larran et al., 2000; Shi et al., 2009). The endophytic fungi could promote sugar beet growth and photosynthesis and increase sugar content, due to increased chlorophyll, leading to a consequent increased carbohydrate synthesis [20]. These endosymbionts enhance plant nutrient absorption, leading to improved vegetative growth of the host plant. Little is known about the rich endophytic fungal communities that exist in sugar beet on the north slope of Tianshan mountain, China.

Most information about endophytic fungal diversity has been obtained using culture-dependent approaches. Due to the limitations of traditional isolation techniques, it is highly probable that some or even numerous endophytic fungi are never isolated. This is because some endophytic fungi cannot grow or grow slowly and are outcompeted by fast-growing species on the artificial media. To overcome the potential technical bias, molecular techniques have been employed in the detection of endophytic fungi directly within

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the host tissues.

These methodological constraints have seriously limited our knowledge of endophytic fungus. More recently, molecular approaches for the isolation and characterization of fungal endophytes and plant-associated fungus and communities have been reviewed by Sun et al. (2012) [21,22]. Microbial communities inhabiting stems, roots and tubers of various varieties of plants were analyzed by 16S rRNA gene-based techniques such as terminal restriction fragment length polymorphism analysis (T-RFLP), denaturing gradient gel electrophoresis (DGGE) as well as 18S rRNA gene cloning and sequencing [23–26].

18S ribosomal RNA gene (rRNA) and ITS sequence-based methods can provide additional information for analyzing the community composition of endophytic fungi. This approach has been successfully used for fungal community analysis in a great variety of environments, including soil ecosystems [27], marine [28], rhizosphere [29], food [30], and human intestine [31] to overcome the limitations of culture-dependent approaches. These molecular biology methods lack sufficient sequences to capture comprehensive and systematic information on diverse microbial communities. A new highly parallel sequencing technique 454 GS-FLX pyrosequencing, can generate 400,000 sequences (100–400 bp in length), compared with the 192 sequences (700 bp) produced by one run of Sanger sequencing [32]. Pyrosequencing can provide significant insight into themicrobial community structures in bioelectrochemical systems.

T-RFLP is a culture-independent method to effectively assess the diversity of a microbial community and is one of the most commonly used molecular methods for community structure determination [33–35]. T-RFLP can compare multiple microbial communities rapidly and accurately.

In this study, 12 sugar beet samples (Table 1) were collected from 2 fields in Changji and Shihezi in Xinjiang, China. Pyrosequencing using the ITS rRNA gene as the biomarker was conducted to examine the fungal diversity of those sugar beet samples, to get a broader overview of the diversity and space-time dynamics of community structure of endophytic fungus throughout the development period of sugar beet.

2. Materials and methods

2.1. Plant sampling and plant surface sterilization

Sixty sugar beets (Table 1) were collected from one field per site on the north slope of Tianshan Mountain, in two locations (Changji and Shihezi), Xinjiang China, during the growing season of May–October 2011, and transported to the laboratory at 4 °C. Each sugar beet was washed with tap water to remove attached clay and ultrasonic wash for 5min in 124 mmol l^{-1} Na_2HPO_4. Plant surface sterilization was conducted as previously described [19]. Sterility checks were carried out for each sample to monitor the effectiveness of the disinfestation procedures. Microorganisms in the final rinse were examined by using culture-dependent and culture-independent molecular approaches based on 18S rRNA gene analysis.

2.2. DNA extraction and PCR amplification of the ITS rRNA gene

About 1.2 g of the surface-sterilized sugar beet plants was frozen with liquid nitrogen and ground to a fine powder. Then, DNA extraction was conducted as previously described [36]. The primers ITS1 (5′-FAM-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were chosen to amplify the internal transcribed spacer (ITS) region. The 50 μl of PCR reaction mixture contained 100 ng of DNA extract, 1 × Taq reaction buffer, 20 pmol of each primer, 200 μM of each dNTP and 1.5 U of Taq DNA polymerase (Sangong Biotech). After initial denaturation at 95 °C for 4 min, each thermal cycling consisted of denaturation at 94 °C for 1 min, annealing at 56 °C for 60 s and elongation at 72 °C for 1.5 min. At the end of 30 cycles, the final extension step was at 72 °C for 7 min.

2.3. T-RFLP complemented with pyrosequencing

To select the endonuclease with the highest power to resolve endophytic fungal communities, we used the following three restriction enzymes individually: *Bst*NI, *Mae*II and *Bfm*I (Fermentas) to digest aliquots (≤1 μl) of purified rDNA ITS PCR products at their optimal temperature for 6 h according to the manufacturer's protocol. Restriction digestion reactions were incubated at 37 °C for 6 h, followed by 20 min at 80 °C to denature the enzyme (*Bst*NI and *Bfm*I) and at 65 °C for 6 h, followed by 20 min at 80 °C to denature the enzyme (*Mae*II). Of the restricted PCR product, 2 μl was mixed with 0.75 μl of size standard LIZ1200 (ABI, Foster City, CA) and 7.25 μl of Hi-Di formamide (ABI). DNA fragments were scanned on an ABI 3730 automated DNA sequencer at Oklahoma State University's Recombinant DNA/Protein Core Facility. The T-RFLP data profiles were obtained and analyzed using Gene-Mapper Software version 4.0 (ABI). Fluorescently labeled T-RFs were detected with an ABI 373A automated DNA sequencer (PE Applied Biosystems Inc.) operated in the GeneScan mode. Lengths of labeled fragments were determined by comparison with the internal standard using the GeneScan 2.5 software package (PE Applied Biosystems Inc.).

Amplicon libraries were performed using a combination of tagged primers designed for the variable ITS-1 region, as recommended for the tag-encoded 454 GS-FLX amplicon pyrosequencing method [37]. A region 650 bp (ITS1/ITS4) in the nuclear ribosomal

Table 1
Sugar beet Variety, locations, elevations, pH and soil types.

Samples	Number of samples	Variety	Soil classification	Soil pH	Latitude	Longitude	Elevation (m)	The growth period	Location
1	5	Xintian 18	desert grey soil	7.72	44.338694 N	86.059056 E	422	seedling growth	Shihezi
2	5	Beta 580	desert grey soil	7.53	44.012222 N	87.361167 E	553	seedling growth	Changji
3	5	Beta 580	desert grey soil	7.53	44.012222 N	87.361167 E	553	seedling growth	Changji
4	5	Xintian 18	desert grey soil	7.72	44.338694 N	86.059056 E	422	rossette formation	Shihezi
5	5	Beta 580	desert grey soil	7.53	44.012222 N	87.361167 E	553	rossette formation	Changji
6	5	Beta 580	desert grey soil	7.53	44.012222 N	87.361167 E	553	rossette formation	Changji
7	5	Xintian 18	desert grey soil	7.72	44.338694 N	86.059056 E	422	tuber growth	Shihezi
8	5	Beta 580	desert grey soil	7.83	44.012222 N	87.361167 E	553	tuber growth	Changji
9	5	Beta 580	desert grey soil	7.83	44.012222 N	87.361167 E	553	tuber growth	Changji
10	5	Xintian 18	desert grey soil	7.61	44.338694 N	86.059056 E	422	sucrose accumulation	Shihezi
11	5	Beta 580	desert grey soil	7.83	44.012222 N	87.361167 E	553	sucrose accumulation	Changji
12	5	Beta 580	desert grey soil	7.83	44.012222 N	87.361167 E	553	sucrose accumulation	Changji