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Endophytic fungal communities in the bark of canker-diseased *Toxicodendron vernicifluum*

Shuhei TAKEMOTO[a], *Hayato MASUYA*[b], *Masanobu TABATA*[a,*]

[a]*Department of Forest Microbiology, Forestry and Forest Products Research Institute, 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan*

[b]*Tohoku Research Center, Forestry and Forest Products Research Institute, 92-25 Nabeyashiki, Shimokuriyagawa, Morioka, Iwate 020-0123, Japan*

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ABSTRACT

Comparatively little is known about how tree diseases influence fungal communities. To examine this relationship, we studied the spatial and seasonal variations in the communities of endophytic and lesion-associated fungi in the bark of *Toxicodendron vernicifluum* trees with canker disease. The fungal communities included mainly *Lophiostoma* sp., *Phomopsis* spp., *Botryosphaeria dothidea* and *Ascochyta* sp. Two fungi, *Phomopsis* spp. and *B. dothidea*, were overrepresented in the lesions. Spatial and seasonal variations were significant in the relative abundance of dominant OTUs (operational taxonomic units) and in the overall community composition. Tissue specificity of the fungi was also indicated. Fungal OTUs specific to inner bark were richer in number than those specific to lesions. Sound trees had significantly more endophytic OTUs than expected, but the diseased trees did not. Therefore, we concluded that canker disease significantly affected the community composition and species richness of fungi in the bark. This is the first study indicating the influence of a disease on the community of bark endophytes.

Introduction

Toxicodendron vernicifluum is a deciduous forest tree belonging to the family Anacardiaceae. The tree is of economic and cultural importance, especially for the production of lacquer sap that is used in the manufacture of lacquer wares and repairing cultural artifacts (Miyamoto and Kakuda, 2008). Recently, outbreaks of canker disease in *T. vernicifluum* trees arose in Japan, and the cause of the disease is currently being studied (Tabata et al. unpublished). In parallel, we examined the distribution patterns of bark endophytes and lesion-associated fungi in *T. vernicifluum* trees. The entire fungal community in the bark may include the causal pathogens of the disease and other species that may interact with, or even repel or defeat, the pathogens. Prior to our study, only limited research on plant pathogens of *T. vernicifluum* has been conducted. Basic information on the distribution patterns of bark endophytes and lesion-associated fungi in *T. vernicifluum* trees had not been assembled.

Endophytes are fungi that inhabit plant tissues without any obvious negative effects on their hosts and are ubiquitous and very diverse (Petrini, 1991; Stone et al., 2000; Arnold, 2007). The distribution pattern of endophytes is influenced by various factors. Spatio-temporal variations in endophytic fungi

* *Corresponding author*. Tel.: +81 29 874 3720; fax: +81 29 829 8245.
E-mail address: butter@ffpri.affrc.go.jp (M. Tabata).

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are well-known in tree leaves (e.g., Petrini, 1991). Plant diseases also affect leaf endophytes (Osono, 2006; Douanla-Meli et al., 2013). In bark tissues, however, whether or not these factors affect the community of endophytic fungi is unknown.

At our research site, a disease of unknown etiology causes canker on the branch of *T. vernicifluum* (Tabata et al. unpublished). The disease can degrade the bark tissue and destroy the habitat of pre-existing bark endophytes. Additionally, the disease may affect the health status of trees, thereby possibly altering the community composition of bark endophytes. As for most *T. vernicifluum* plantations in Japan, our research site is located on a mountainside and is extensively managed. Pest and disease control measures are rarely practiced there, and the plantation is open to natural environments nearby. Therefore, the endophytic flora associated with the trees may reflect those in the surrounding forest, left undisturbed, except for the occurrence of the canker disease that arose recently. This situation was ideal for studying the influence of this canker disease on the community of bark endophytes.

In this study, we hypothesized that the canker disease of *T. vernicifluum* could affect the community of bark endophytes. To test the hypothesis, we compared fungal community composition and diversity indices between the intact inner bark and debarked canker lesions, and between sound and diseased trees. Similarly, the distribution patterns of some dominant fungal OTUs (operational taxonomic units) were analyzed. To evaluate the relative importance of the effect of the canker disease among other factors, we considered spatio-temporal variations in the statistical analyses, simultaneously.

Materials and methods

Sample collection and fungal isolation

The study site was located in a *T. vernicifluum* plantation on a mountainside in Ninohe, Iwate Prefecture, northeastern Japan. The site was ca. 380 m above sea level, facing southwest with an average slope of 5°. Trees had 2 yr. regrowth after coppicing. About 60 % of the trees had the canker disease showing visible symptoms on the trunks.

We collected samples in early Jun. 2011 (early disease development) and late Jul. 2011 (most severe symptoms). At both times, we sampled three apparently diseased trees (“D”) and three apparently sound trees (“S”) arbitrarily (See Supplementary Fig 1), ca. 20 m apart from each other, throughout the research site. A vertical strip of outer bark was peeled off a 1- to 2-year-old main shoot with a surgical blade. Within each of six to ten 7 cm segments, seven fragments of sound tissue were punched out with a 4 mm cork borer from each of the peeled and unpeeled sides. Fragments from the peeled side contained only inner bark (“I”), and those from the unpeeled side contained whole bark (“W”). Additionally, up to seven tissue fragments with visible lesions (“L”) were punched out after the shoots were peeled.

The fragments were immersed in 70 % ethanol for 30 s and in sodium hypochlorite solution (1 % available chlorine) for 3 min, rinsed twice in sterile distilled water, and blotted dry on sterile filter paper for 15 min. Single fragments were then placed on the surface of 1 % malt extract agar (MEA) medium in culture plates. The plates were held at 15 °C in the dark and observed intermittently under a dissecting microscope. Fungal isolates were obtained from any distinguishable sector of colonies growing from the fragments and maintained on 1 % MEA medium in culture plates.

The isolates were grouped by gross morphology. Several representative isolates of each group and all unclassified isolates were used in the molecular analyses.

DNA isolation, sequencing and OTU identity

Fungal isolates were cultured on 1 % MEA plates for 2 weeks. DNA was directly extracted in sterilized distilled water from growing mycelial tips and amplified by PCR in a Gene Amp 9700 Thermal Cycler (Perkin Elmer, Waltham, MA, USA) in a 25 μl reaction mixture modified from the method of Suyama et al. (1996). Reactions contained 12 μl GoTaq Green Master Mix (Promega, Madison, WI, USA) and primers ITS3 (White et al., 1990) and NL4 (Kurtzman and Robnett, 1997). The amplified products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were sequenced using an ABI PRISM 3100 Genetic Analyzer (Perkin Elmer) with a Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit v. 3.1 and primers NL1 (Kurtzman and Robnett, 1997) and NL4 according to the manufacturer’s instructions.

The D1/D2 region of LSU rDNA (ca. 570 base pairs) was used for the phylogenetic analysis using MEGA v. 5 software (Tamura et al., 2011). The sequences were deposited in the DNA Data Bank of Japan under accession nos. AB808023–AB808471. Reference sequences were obtained by BLAST searches. Reference hits were scrutinized for reliability (e.g., sequences from the CBS collection were preferred). Phylogenetic positions were inferred in molecular phylogenetic trees constructed by the maximum likelihood method in the MEGA software. When sequences could not be identified to a particular taxonomic level, the lowest common affiliation of reliable reference sequences was taken. The cut-off for distinct species was set to 99 % (Klaubauf et al., 2010), unless BLAST results for two closely related sequences gave distinct hits to well characterized strains.

On the basis of the phylogenetic affinities, the above mentioned morphological groups were revised and recombined into OTUs that represented the taxonomic units implied by phylogenetic analysis, without overlap. The taxonomic affiliation of OTUs was confirmed as much as possible from the morphology of anamorphic states produced in culture and from teleomorphic states collected additionally at the same site whose isolates had D1/D2 sequences identical to those of the focal OTUs. The addition of autoclaved *T. vernicifluum* twigs to the medium and exposure of the plates to diffuse daylight by a window promoted spore formation.

Statistical analyses

All statistical analyses were performed with R software v. 2.15.1 (R Core Team 2012). Model selection was based on Akaike’s information criterion (AIC), if needed.

For all analyses, isolated fragments without fungi were first removed. Relative abundance was determined as the