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Are endophytic fungi from *Rhododendron tomentosum* preadapted for its essential oil?

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ABSTRACT

Endophytic fungi have to cope with various secondary metabolites produced by their host plant. *Rhododendron tomentosum* is an evergreen shrub with a high content of essential oil showing antimicrobial effects. The first aim of our study was to reveal the diversity of culturable endophytic fungi colonizing leaves of *R. tomentosum*. We isolated and identified 30 species, mostly ubiquitous species colonizing various plants as endophytes and later turning into saprotrophs. The second aim was to explore whether strains obtained from *R. tomentosum* (RT-strain) are better adapted to growth in environments with essential oils, compared to strains of the same species obtained from different substrates. In five of the six species pairs tested, the growth of RT-strains was more inhibited by three components of the essential oil added to the medium, compared to the corresponding strains. Only the RT-strain of *Desmazierella acicola* was less susceptible compared to its corresponding strain. Our results show that the essential oil in leaves of *R. tomentosum* do not represent a selective barrier that can be crossed only by preadapted strains.

1. Introduction

In the last decades, endophytic fungi have received substantial attention due to their enormous diversity and potential use in biotechnologies (Wang and Dai, 2011). Less effort has been put into exploring the adaptations and factors affecting the presence of particular fungal species within the tissues of certain plant species, especially those that are well-known known producers of allelopathic secondary metabolites. Endophytes are supposed to preadapt to specific conditions in plant tissues. Ganley and Newcombe (2006) found that the only non-rhytismataceous endophytes isolated from leaves and seeds of *Pinus ponderosa* Douglas ex C.Lawson were able to grow on a nitrogen-limited medium, suggesting their ability to grow in the nutrient-free apoplast. A series of experiments with endophytic fungi isolated from *Sequoia sempervirens* (D. Don) Endl. and other conifers found endophytic fungi to be more sensitive to higher doses of different phenotypes of essential oils obtained from *S. sempervirens* compared to pathogens of the same tree species. However, most sensitive was a fungus obtained from *Pseudotsuga menziesii* (Mirb.) Franco, i.e. a tree species with a different composition of essential oils (Espinosa-García and Langenheim, 1991; Espinosa-García et al., 1993). Jurc et al. (1999) found that terpenes from *Pinus nigra* var. *austriaca* (Hoess) Aschers. & Graebn. stimulated the growth of the latent pathogen *Cenangium ferruginosum* Fr. isolated from the same tree species.

Rhododendron tomentosum Harmaja (= *Ledum palustre* L., Ericaceae) is an evergreen shrub colonizing acid soils, distributed in Central and Northern Europe, North America and Asia. A high content of secondary metabolites, especially essential oil with antibacterial and antifungal effects, is present in tissues of *R. tomentosum* (Dampc and Luckiewicz, 2013). The most frequent chemical compounds in the essential oil are ledol, palustrol, *p*-cymene, γ-terpineol and myrcene, but the exact content and composition of essential oil differs depending on the locality, age of the plant tissue or growth phase (Butkiene and Mockute, 2011; Gretšušnikova et al., 2010; Raal et al., 2014). Endophytic fungi in *R. tomentosum* have been hitherto overlooked compared to other, more frequently studied members of the Ericaceae (Okane et al.,

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1998; Petrini, 1984; Petrini et al., 1982). Recently, Tejesvi et al. (2011) provided the first insight into the diversity of endophytes in leaves of *R. tomentosum* in Estonia. They identified most of the 18 species obtained by culturing as members of the Sordariomycetes (Hypocreales, Sordariales and inc. sed.) and Eurotiomycetes (Eurotiales). Twenty-four per cent of strains showed antibacterial or antioxidant activities.

The primary aim of our study was to identify the most frequent foliar endophytes in leaves of *R. tomentosum* in the Czech Republic. Secondly, we aimed to find out whether selected essential oils from *R. tomentosum* would have a different effect on strains isolated from leaves of *R. tomentosum* compared to corresponding strains of identical fungal species isolated from other substrates. Essential oil is usually deposited in the intercellular space or under the leaf cuticle (Fahn, 1988; Gersbach, 2002); the endophyte should therefore interact with the essential oil at least while infecting the plant. Endophyte transmission is usually realized horizontally, throughout the cuticle (Arnold and Herre, 2003). We hypothesized that strains from *R. tomentosum* would grow better in the presence of essential oil to the corresponding strains.

2. Material and methods

2.1. Sites, sampling and isolation of fungi

Shoots of *R. tomentosum* were collected in October and November 2012 at seven localities in the Czech Republic. Except for one locality, the Botanical Garden of the Faculty of Science of Charles University in Prague, all localities included natural stands of *R. tomentosum* in open bog areas or pine forests (dominated by *Pinus sylvestris* L., for locality details see Suppl. Table). At each locality, five shoots, each from a different plant, were cut, stored in a plastic bag and processed in the laboratory within 48 h. In the laboratory, two leaves from each shoot were randomly selected, giving a total of 80 leaves for fungal isolations. Each leaf was sterilized in 96% ethanol for 10 s, in 0.5% sodium hypochlorite for 20 s and in 95% ethanol for 10 s, and washed in sterile distilled water (Fisher et al., 1984; Okane et al., 1998). Then, each leaf was cut into five pieces (approx. 1 cm wide) and placed on a Petri dish with 2% wort agar (2WA) prepared from brewery wort (Staropramen Brewery, Prague, Czech Republic). The final sucrose content was adjusted to 2% w/v, and the suspension was supplied with 18 g l^{-1} agar (Fassatiová, 1986). Petri dishes were incubated in the dark at approx. 25 °C. Emerging colonies were grouped into morphotypes according to their phenotypic characteristics (e.g. growth rate, colour and structure of colonies, sporulation) as they appeared in the primary isolation plates. The frequency of occurrence of each morphotype (the number of pieces from which the morphotype was growing) was recorded. Representative strains of each morphotype were deposited in the Culture Collection of Fungi (CCF, Charles University, Faculty of Science, Prague, Czech Republic).

2.2. DNA extraction, PCR and sequencing

At least one isolate from each morphotype was used for DNA extraction and sequence analysis. Genomic DNA was isolated from 7 to 14-day-old cultures using the Zymo Research.

Fungal/Bacterial DNA Extraction Kit (Zymo Research, Orange, USA). In the last step 30 μl of elution buffer preheated to 65 °C was used for DNA elution. Nuclear rDNA containing internal transcribed spacers (ITS1 and ITS2), 5.8S and the D1/D2 domains of the 28S region was amplified with the primer sets ITS1F/NL4 or ITS1/NL4 (O'Donnell, 1993; White et al., 1990) in all strains under study. PCR purification was carried out using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Bade City, Taiwan). Both PCR fragments were sequenced in the Sequencing Laboratory of the Faculty of Science, Charles University (Prague, Czech Republic). Consensus sequences were constructed in Geneious 6.1.5 software (Biomatters, Auckland, New Zealand).

2.3. Morphotype identification

Sporulating colonies were identified down to the genus or species level based on phenotypic characteristics. Slides were mounted in Melzer's reagent and examined in phase and differential interference contrast (BX-51, Olympus, Tokyo, Japan). Phenotypic and molecular data were taken into consideration in the final identification of taxa. To identify sterile morphotypes, their sequences were aligned and morphotypes with highly similar sequences (99–100% similarity) were joined together. Representative sequences were then compared with those in the GenBank database using the BLASTn algorithm (Altschul et al., 1990). Only a 99–100% match with reliable sources (ex-type sequences, sequences from taxonomic studies) was accepted as proof of identification.

2.4. Sample preparation

Chemical compounds from leaves were obtained by solid phase micro extraction (SPME). Absorption of volatile compounds was carried out using a fused silica fibre coated with a layer of polydimethylsiloxane/carboxene/divinylbenzene (PDMS/CX/DVB). One gramme of a pulverized sample was weighed and promptly transferred into a 25 mL headspace vial. The vial was heated at 40 °C, and the fibre was exposed to the headspace of the vial for 30 min. The fibre was then inserted into the GC system injector port for 3 min in order to achieve thermal desorption.

2.5. Gas chromatography – mass spectrometry

GC-MS analyses were performed using a GC7860/5742C MSD instrument (Agilent technologies, Santa Clara, USA) equipped with an Rxi-624-MS fused silica column (20 m × 0.18 mm ID × 1 μm film thickness, Agilent technologies, Santa Clara, USA). Helium (purity 99.9992%, Air product, Prague, Czech Republic) was used as the carrier gas with a flow rate of 35 cm s^{-1}. The analysis was carried out in the following conditions: injector and detector temperatures of 250 °C and 280 °C, respectively. The oven temperature was held at 35 °C for 2 min following injection and raised to 130 °C at 5 °C min^{-1} and then to 230 °C at 15 °C min^{-1}. Finally, the temperature was held isothermally for 5 min. All the injections were performed in splittles mode. The mass spectrometer was operated with electron impact ionization (70 eV), and the mass range was set from 30 to 450 Da in full scan acquisition mode. Components were identified by comparison of their mass spectra with those of a commercial library (Wiley library, Mass Spectral Database, Hewlett-Packard, Vienna, Austria).

2.6. Strains used in the growth tests

Strains of endophytes isolated from leaves of *R. tomentosum* (further referred to as RT-strains) were selected according to their frequency of isolation and availability of strains of the same species isolated from a different substrate (further referred to as corresponding strains). All strains belonged to ascomycete species, which corresponds with the overall prevalence of ascomycetes among plant endophytes (Rodriguez et al., 2009). The corresponding strains were purchased from the CCF and the Centraalbureau voor Schimmelcultures (CBS-KNAW, Fungal Biodiversity Centre, Utrecht, Netherlands, Table 1). Their species