Theses of doctoral dissertation

STUDY OF DARK SEPTATE ENDOPHYTIC (DSE) FUNGI ON SEMIARID SANDY AREAS OF THE GREAT HUNGARIAN PLAIN

Dánial G. Knapp

Doctorate School in Biology, Eötvös Loránd University
Head of the Doctorate School: Prof. Anna Erdei, DSc
Experimental Plant Biology PhD Program
Head of the Program: Prof. Zoltán Szigeti, DSc

Supervisor: Gábor M. Kovács, PhD, dr. habil., associate professor
Institute of Biology, Department of Plant Anatomy, Eötvös Loránd University

Institute of Biology, Department of Plant Anatomy, Eötvös Loránd University

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INTRODUCTION

The majority of terrestrial vascular plants live together with different non-pathogenic fungi. Among others, roots can be colonized by fungal endophytes, which colonize plant tissues during some period of their life cycle yet cause no symptoms of tissue damage to their hosts. Dark septate endophytes (DSE) comprise a form group of root-colonizing endophytic fungi that are septate and generally have melanized hyphae that colonize the epidermal and cortical cells and intercellular regions of roots.

DSE fungi occur in all main climate regions and major biome types colonizing roots of more than 600 species of more than 150 plant families. These fungal root endophytes are widespread and have a broad host range, however studies on their presence and diversity is sporadic. Although DSE fungi are relatively frequent in extreme environments with strong abiotic stress, such as arid and semiarid areas, our knowledge on their role in these ecosystems is far limited.

Several studies have gained information about the function of DSE and their relationship with their host plants, however general statements are still missing and it seems, this symbiosis highly depends on circumstances and experimental conditions. Positive, neutral and negative effects of DSEs on numerous features of plant performance could be detected in experiments established with different fungal and plant species.

As dark septate endophytes are grouped together based on their connection to roots and similar morphological characteristics, these fungi do not represent one monophyletic lineage or taxon. They are ascomycetes belonging to numerous orders of subphylum Pezizomycotina (e.g. Helotiales, Pleosporales, and Hypocreales). DSEs are anamorphic fungi; neither sexual state nor sexual morph nor ascospore production is known. Even conidiogenesis is infrequent and conidiogenous processes have only been induced in a minority of isolates after specific treatments.

Although the presence of pigmented and septate hyphae in plant roots has been known for more than a decade, our knowledge on DSE colonization of roots is still far limited. Only a couple of studies have focused on structural and ultrastructural examination of root colonizing endophytes, applying mainly light microscopy in these publications. The majority of studies used the DSE species Phialocephala fortinii sensu lato complex mostly associated with forests and woody plant species. Our information about the presence of DSE fungi
within roots can be obtained by culturing methods or molecular identification, and the knowledge about their exact position in the root and colonizational features is poor.

Although DSE fungi are present in all major ecosystems, abundant in certain nutrient-limited environments and certainly have an important role in plant survival in these areas, our knowledge on several of their fundamental features (e.g. their biology, ecological function, phylogenetic placement, community structure, root-colonization of DSE or their effect to the host plant) is far limited.

• Our objectives were to study the compositional diversity of DSE fungi colonizing the plants of semiarid sandy areas. To find the generalist, dominant and frequent members of the community indigenous and invasive plants were sampled. We aimed to test the area specificity and the seasonality and to establish artificial synthesis experiments to test whether an isolate could be considered a DSE fungus.

• Further aims were to investigate the genetic variability of conspecific isolates of dominant DSE clades grouped based on ITS (internal transcribed spacer) region of nrDNA, using the genomic fingerprinting technique ISSR (inter sample sequence repeat) that could show the slight differentiations.

• Our aims were to adapt, set and apply a fluorescent method for specific detection and visualization of fungal endophytes in the root that enables simultaneous specific labelling and localization of different fungi in the same root section.

• Further aims of the study were to gain information on ultrastructural features of colonization of a DSE fungi using transmission electronmicoscopy (TEM) that would get insights into the colonization and strategy of not one fungus but even of the DSE fungi generally.

• As small numbers of DSE species have been described to date, our objectives were to carry out polyphasic taxonomy and describe unidentified DSE lineages revealed in our investigations if needed.
MATERIALS AND METHODS

Sampling, isolation and identification of endophytes, in vitro tests

The samples were collected from three sandy grasslands on the Great Hungarian Plain (Hungary); Bugac, Fülöpháza and Tatárszentgyörgy. Samples for investigation of structural diversity, season- and area-specificity of DSE were taken from each of the three areas, while targeted isolations were carried out using samples derived from only grasslands of Fülöpháza. The roots of eleven plant species were collected in the area, including three invasive and eight native species. They were chosen because they represent different life forms and they are characteristic components of these plant communities. To test season- and area-specificity, samples were collected from 10 marked indigenous juniper trees (Juniperus communis) from each of the three areas in three seasons (spring, summer and fall) for two years.

For isolation of endophytes, segments were cut from different regions of each root sample followed by surface sterilization, and then the root sections were placed on MMN media. Hyphae growing out of the root segments were isolated to separate plates and total DNA was extracted from different isolates from each root sample using the CTAB method. To identify the members of the DSE community ITS and LSU (28S RNA, large subunit) regions of nrDNA were amplified. In vitro tests were established with the representatives of each group obtained by the analyses of ITS sequences of the fungal isolates using leek (Allium porrum) to test whether an isolate could be categorized as a DSE fungus. We considered an isolate to be a DSE fungus if it colonized the root and formed microsclerotia and had no visible negative effect to the plant.

Molecular works

For taxonomic works of the 40 isolates of the three groups chosen (DSE-4, DSE-8A, DSE-7), DNA was isolated from all isolates using UltraCleanTM Microbial DNA Isolation Kit, amplified and sequenced using both forward and reverse primers for ITS, partial 18S nrRNA (SSU), 28S nrRNA (LSU), actin (ACT) and calmodulin (CAL) genes; for the DSE-7 isolates, partial transcription- elongation factor 1-α (TEF) and β-tubulin (TUB) gene sequences were also determined. Additionally, information was gained from indel regions of the ITS region as well using the software GapCoder.

Alignments of our sequences supplemented with sequences from GenBank of respective loci were made using the online version of MAFFT v. 6. The alignments were checked and
edited with MEGA v. 5. The best-fit nucleotide substitution model was selected using the program jModelTest considering the selection of the Akaike Information Criterion (AIC) and the model was implemented in the Bayesian analyses performed with MrBayes v. 3.1.2. Topological convergence was checked by AWTY. RAxML phylogenetic analyses were carried out with raxmlGUI v. 1.3 and ML bootstrap analysis with 1 000 replicates was used to test the support of the branches. The phylogenetic trees were visualised and edited by FigTree v. 1.4.0 and MEGA v. 5.

The primers (AAC)₆, (AAG)₆, (AGG)₆, (ATC)₆, (CCG)₆, (AC)₈ és (AG)₉ were used for ISSR-PCR. The bands of the ISSR profiles were analysed based on presence and absence in a fixed positions and were defined as binary data. The ISSR profile analyses were carried out based on these binary matrixes.

**Fluorescent in situ hybridization**

Oligonucleotid probes were designed for specific intraradical fluorescent labelling of isolates belonging to DSE-1 group, based on their LSU sequence. To test the specificity of the probes and for simultaneous labelling two different fungi, another probe was designed using LSU region of *Rhizophagus intraradices* (Glomeromycota). The 5’ end of the two oligonucleotids was labelled with fluorophores with different excitation/emission wavelengths. Root segments of maize inoculated by fungi in resynthesis systems were cut, and 90–120 μm thick sections of plant roots were prepared to keep the structures of colonizing fungi as intact as possible. These samples were incubated at 46°C in hybridization buffer containing probes (50 % dimetylformamide), then washed in hybridization buffer without probes to eliminate aspecifically binded labelled oligonucleotids. Root samples mounted in fluoroshield were analyzed using epifluorescence and confocal microscopy.

**Light and transmission electron microscopy**

For light microscopic surveys roots were decolorated in KOH solution (10%), stained using aniline blue, covered in PVLG (polyvinyl-lacto-glycerol) and were examined using Nomarski optics.

For ultrastructural investigations, root segments of leek colonized by *Cadophora* sp. (DSE-1 group) for 6 weeks were fixed with the surrounding media in 2.5% glutaraldehyde. The samples were postfixed in osmium-tetroxide and were embedded in Durcupan ACM. Half-thin sections were stained using neofuxin-crystal violet and were examined by light
microscopy. Ultra-thin sections were stained with uranyl-acetate and lead citrate, and were examined using transmission electron microscopy.

Promoting sporulation

To induce sporulation for classical taxonomic investigations, isolates were incubated under different conditions and distinct treatments were applied. Isolates of the three chosen groups were cultured onto six different media. Isolates were also cultured onto autoclaved plant parts (barley shoots, pine needles, stinging nettle stems, rye grass roots and white elm stems), colonies were burned with a red-hot needle, exposed to near-ultraviolet light or incubated at different temperatures.

RESULTS AND DISCUSSION

The DSE community

Nearly 200 samples were collected from indigenous or invasive plant species of the three sampling areas to gain information about the composition of DSE community. Based on the ITS region, the 241 isolates were grouped into 41 groups. For the in vitro tests, 59 representative isolates of the 41 groups were chosen, and of the 41 groups of total isolates, 14 groups were considered DSE. These groups contained 142 of the 241 isolates. Henceforth, these groups will be referred to as DSE groups 1–14. The fourteen groups were nested into the orders Pleosporales (7 groups), Helotiales (2 groups), Hypocreales (2 groups), Eurotiales (1 group), Xylariales (1 group) and Glomerellales (1 group) based on their ITS and LSU sequences.

All but one of the isolates originated from junipers belonged to DSE-1, DSE-2 and DSE-3 groups and none of these three groups contained an isolate from only one area or one season. Accordingly, DSE groups showed no specificity to area or season. The main DSE groups containing high numbers of isolates were generalist and colonized both native and invasive species. The generalist and dominant DSE groups (e.g. DSE-1, DSE-3, and DSE-7) of the areas were identified.

The majority of GenBank hits similar to ITS and LSU regions of our isolates origined from semiarid areas. The core members of the DSE community of the region were the same with those found in semiarid grasslands of North America. Taking into account a previous hypothesis about the common root colonizers of those grasslands and our results reported
here, we hypothesize that plants of (semi)arid grasslands share common dominant members of the DSE fungal community on a global scale.

*Inter sample sequence repeat analysis*

For two DSE groups (DSE-1 and DSE-7) out of the three (DSE-1, DSE-3 and DSE-7) chosen for ISSR, further strains were isolated. Thirteen isolates of DSE-1 and nineteen for DSE-7 group were gained using a group-specific PCR enabling fast and accurate targeted sampling. No correlation was found between the finer grouping of isolates based on the ISSR profiles and collection date (season), neither collection area and host plant. Nevertheless, high variability of conspecific isolates was found using the ISSR analysis.

With a few exceptions, the vast majority of isolates of each three DSE group had unique ISSR profile, which enables strain-specific identification using this method.

**In planta FISH**

A RNA FISH protocol was adapted and used successfully for specific labelling and visualization of DSE-1 isolates within the plant root. Structures formed by the *Cadophora* sp. within the root could be specifically labelled and differentiation of two root colonizing species (*Cadophora* sp. and *Rhizophagus irregularis*) was successful due to the simultaneous specific labelling.

The first successful adaptation of a method based on fluorescence *in situ* hybridisation, that allows for simultaneous specific visualization of different DSE fungi within the same plant root even in field collected roots was demonstrated here.

**Ultrastructural investigations**

Examining root sections of leek colonized by a *Cadophora* sp. (DSE-1) isolate resulted observations on hyphae colonizing necrotic and living plant cells too. The structures in degraded plant cells were similar to those described in previous studies on ultrastructural characterization of DSE colonization.

In case of hyphae running in living plant cells, both the fungal and plant cells were surrounded by their intact cell membranes. Loose matrix materials were found between the fungal cell wall and plant cell membrane. Invaginated membrane parts/vesicules could be regularly detected on cell membranes of both the DSE fungus and the plant cell indicating significant endo- and/or exocytotic processes.
This was the first observation of DSE hyphae in functioning plant cells and the first description of a perifungal membrane in case of DSE colonization, which is known to be specific to biotrophic interactions.

**Taxonomy**

Based on the results of taxonomic work on the three chosen unidentitfied DSE groups, each group belonged to the order Pleosporales (Dothideomycetes). The group DSE-4 represented a basal lineage in *Morosphaeriaceae*, while isolates of group DSE-8A formed a well-supported incertae sedis clade together with *Massarina igniaria*, *Noosia bankssiae* and *Periconia macrospinosa* (DSE-8B) related to the family *Massarinaceae* in the suborder *Massarineae*. Representative isolates of group DSE-7 formed a well-supported clade in the family *Lentitheciaceae*.

Our isolates were found to represent three novel genera with eight novel species within the suborder *Massarineae* in the order Pleosporales. Isolates of group DSE-7 represented a novel genus (*Darksidea gen. nov.*) with six species (*D. alpha*, *D. beta*, *D. gamma*, *D. delta*, *D. epsilon* and *D. zeta spec. nov.*), while the groups DSE-4 (*Aquilomyces patris gen. & spec. nov.*) and DSE-8A (*Flavomyces fulophazii gen. & spec. nov.*) represented two monotypic genera.

**Spore production**

Although numerous media and culture conditions were set and tested to induce sporulation, sporocarp-like structures could be observed only on one occasion. These structures were observed on the surface of stinging nettle stem kept on SNA at room temperature 3–4 wk after inoculation with *Darksidea* (DSE-7) species. Except for one structure, these were 180–250 µm sized dark ascomata containing asci with 4–6 ascospores. Although, a *D. gamma* isolate seemed to produce similar sized, shaped and coloured structures, these were sterile pycnidial-like sporocarps with a central ostiole.

To the best of our knowledge, we could induce DSE to form their sexual morphs for the first time, where asci and ascospores could be detected in the sporocarps. Because *Darksidea* species (e.g. *D. alpha*) seem to be common in arid and semiarid regions of different continents and we have no information about sexual spore production of other DSE species, they can be used in experiments aimed at broadening our understanding of the broad distribution, propagation and function of DSE fungi in arid and semiarid environments.
SUMMARY

• More than 200 samples were taken from native and invasive plants of the three sites. According to the analyses of 296 ITS sequences obtained from 241 isolates, isolates clustered into 41 groups and found that 14 of these were DSE.

• The generalist and dominant DSE groups were identified, neither area specificity nor seasonality were found. According to the significant similarities with root associated fungal communities of semiarid regions of North America we may extend this statement by hypothesizing that plants of semiarid grasslands share common dominant DSE fungal community.

• Isolates of assigned DSE groups could not be differentiated based on sampling area, sampling date or host plant using ISSR, however high variability were found among the isolates that enables specific identification of conspecific strains.

• An rRNA fluorescent in situ hybridization (FISH) method was adapted for specific labeling of fungal endophytes in root for the first time enabling simultaneous specific detection of DSE fungi colonizing the same root.

• In this study, we reported DSE hyphae in living plant cell for the first time and cellular changes using ultrastructural examination. A loose matrix material was found between the fungal and plant cell and we observed for the first time a perifungal membrane that is known to be specific to biotrophic interactions.

• Based on the results of taxonomic work of three unidentified DSE groups, isolates were found to represent three novel genera with eight novel species (Aquilomyces patris, Flavomyces fulophazii, Darksidea alpha, D. beta, D. gamma, D. delta, D. epsilon and D. zeta) within the suborder Massarineae in the order Pleosporales.

• In our study, we managed for the first time to induce DSE to form their sexual morphs, where asci and ascospores could be detected in the sporocarps.
LIST OF PUBLICATIONS

Publications directly related to the PhD thesis:

Articles published in peer reviewed international journals:


Articles published in peer reviewed Hungarian journals:


Conferences – proceedings:


Knapp DG, Kovács GM. 2012. Inter sample sequence repeat analysis of dark septate endophytes of semiarid sandy grasslands. Annual Meeting of the Hungarian Society for Microbiology, Keszthely, Hungary. [oral lecture, best lecturer award]

Knapp DG, Pintye A, Kovács GM. 2011. Study of dark septate endophytic fungi colonizing invasive and indigenous plants on semiarid sandy areas. XVI. Congress of European Mycologists (CEM), Halkidiki, Greece. [oral lecture]

Knapp D, Kovács GM. 2009. Study of root colonizing dark septate endophytic fungi of invasive and indigenous plants of semiarid sandy areas. Second Central European Forum for Microbiology (CEFORM), Keszthely, Hungary. [poster, best poster award]


Publications not directly related to the PhD thesis:

Articles published in peer reviewed international journals:


Conferences – proceedings:

