Final report of the project[1]

Root colonizing dark septate endophytes – diversity and function

K109102; PI: Gábor M. Kovács

2013-09-01 – 2017-08-31

Background, state of the art at the start of the project, main aim

Fungi have a fundamental role in ecological processes as decomposers, mutualistic symbionts and parasites/pathogens. Endophytes, which consist of living organisms that colonize plant tissues during some period of their life cycle yet cause no symptoms of tissue damage to their hosts (Saikkonen et al. 1998; Schultz & Boyle 2005) are found in all biomes. Among these endophytes, fungi commonly play important roles in ecosystem functioning (Porras-Alfaro & Bayman 2011). Some fungal endophytic interactions have been widely studied due to the general interest in economically important hosts (e.g. tall fescue) or fungi (e.g. clavicipitaceous fungi) (Cheplick & Faeth 2009). Although there is an increasing interest in fungal endophytes, our knowledge was and still is biased toward grasses and their above-ground tissues.

Dark septate endophytes (DSE) are found worldwide and comprise a group of root-colonizing endophytic fungi that belong to a few orders of the phylum Ascomycota (Jumpponen & Trappe 1998). Although there has been a continual increase in interest in DSE fungi, our knowledge on the diversity of DSE fungi and their function in ecosystems is limited and not as well understood as that of the common root colonizer mycorrhizal fungi or the above mentioned grass endophytes. Meta-analyses of the functional studies show different effects of the interaction on the host plants (from negative via neutral to positive) but generally with a high variability, especially between studies (Mayerhofer et al. 2013; Newsham 2011). That time only a few studies focused on the possible functional variation, the majority of the results are about the PAC (*Phialocephala fortinii* s.l. - *Acephala applanata* species complex) group, common DSE of trees (Grünig et al. 2008; Queloz et al. 2011; Zaffarano et al. 2011; Reininger et al. 2012).

DSE fungi are frequently found in arid and semiarid grasslands, and these communities share some important global key-players (Knapp et al. 2012). The need for a better understanding of the role of DSE fungi in ecosystem functioning has been stressed by many authors (e.g. Mandyam & Jumpponen 2005; Mandyam et al. 2010; Porras-Alfaro & Bayman 2011).

In the project K109102 (2013.09–2017.08) we aimed to study the compositional and functional diversity of DSE fungi, especially focusing on some groups found to be common and frequent root colonizers in semiarid grassland communities.

Our main hypothesis was that compositionally diverse DSE fungi were functionally diverse as well.

[1] We highly appreciate the possibility to submit the final report of K109102 now. The reason for this extension is that the publication process of several interesting results had taken much more time as we planned (in some cases it took years); in the final report we aimed *to show our results* as *published* after peer review, to illustrate the success of the project not only by results/data gained.

Compositional diversity

During the four years of the study we followed two main strategies in the isolation, collection of new strains:

(i) *General characterization of DSE communities*. **(i.a)** We isolated fungi from the roots of *Festuca vaginata*, *Stipa borysthenica*, *Juniperus communis*, *Salix rosmarinifolia* and *Ailanthus altissima* at the (semi)arid sandy grasslands of the Kiskunság, Hungary, mainly from Fülöpháza. **(i.b)** We isolated root endophytes from *Stipa krylovii* from a natural steppe zone located in the Nalaikh district, Mongolia. Altogether these samplings resulted ~600 new isolates to our strain collection (~460 Hungarian and ~140 Mongolian)

(ii) *Fine scale isolations to obtain high number of conspecific isolates*. During these works we sampled individual tufts/plants either randomly or via transects at the grasslands near Fülöpháza. We made a molecular identification step (either diagnostic PCR /see below/, or sequenced the DNA barcode region of the isolates) as soon as it was possible and keept only the interesting taxa. During these isolations we gained 118 *Periconia macrospinosa*, 53 *Darksidea alpha* and 35 *Cadophora* sp. isolates. (nb: This *Cadophora* taxon represents a well-determined lineage; which might be conspecific with *Cadophora meredithiae* (Walsh et al. 2018) but its taxonomic position is still ambiguous; so here and below, *Cadophora* sp. means our distinct, well-characterized lineage.) We sampled the hosts *Festuca vaginata*, *Stipa borysthenica*, *Juniperus communis*, *Salix rosmarinifolia* and *Ailanthus altissima*.

Molecular taxonomy/phylogenetics

For the molecular taxonomic identification after DNA extraction (methods applied: CTAB method, Qiagen, EZNA) the DNA-barcode sequence of the fungi (Schoch et al. 2012) the nrDNA ITS region of the isolates studied during the four year of the project was amplified using general protocols of the fungal specific primer pairs (ITS1F-ITS4) and sequenced at a service company (AGOWA/LGC, Berlin). Altogether we gained ~600 ITS sequences during the project. When needed for detailed taxonomic/phylogenetic analyses we also amplified and sequenced the nrDNA 28S large subunit (LSU) region with the primers LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990), the nrDNA 18S small subunit (SSU) region with the primers NS1 and NS4 (White et al. 1990), the partial actin gene (ACT) using the primers ACT-512F (Carbone & Kohn 1999) and ACT-2Rd (Quaedvlieg et al. 2011), part of the translation elongation factor 1-α gene (TEF) using EF1-728F (Carbone & Kohn 1999) and EF-2Rd (Groenewald et al. 2013) primers, part of the calmodulin gene (CAL) using the primers CAL-228F (Carbone & Kohn 1999) and CAL-2Rd (Groenewald et al. 2013) and ß-tubulin gene (TUB) with the primers CYLTUB1F (Groenewald et al. 2013) and Bt-2b (Glass & Donaldson 1995). Altogether ~300 non-ITS sequence have been obtained. We also detected fungal associated bacterial communities in/on the hyphae of our DSE fungi (Simon et al. 2014)

ISSR

The study of Inter Simple Sequence Repeats (ISSR) profiles could help us to study the intraspecific genetic differences. For that we used the ISSR primers published previously by Lim et al. (2004) choosing six probes for the analyses. We involved the following taxa (with the number of isolates studied): *Periconia macrospinosa* (66), *Darksidea alpha* (31), *Flavomyces fulophazii* (13) and *Cadophora* sp. (35)

Taxon specific – PCR, SCAR

To carry out rapid identification of isolates we used species specific diagnostic PCRs developed for *Periconia macrospinosa*, *Darksidea alpha*, *Cadophora* sp.. In case of those taxa we did not have to do ITS sequence based identification. A qPCR-based identification was also used to study the presence of

hyphae *Cadophora* sp. in the soil (Yakti et al. 2018). The above mentioned isolate specific ISSR profiles helped us to develop isolate specific SCAR markers with which we could test the presence of certain isolates (Zajta 2014).

FISH

We previously developed and published an *in planta* visualization technique based on nrRNA fluorescent in situ hybridization (nrRNA FISH) (Vági et al. 2014). During the four year of the project we developed and designed several probes successfully used for identification of *Periconia macrospinosa* and *Cadophora* sp. (Vági et al. 2015; Zajta 2014) and helped us to screen experimental setups (Yakti et al. 2018, see below).

Results

ISSR

During the ISSR based screening of the genetic heterogeneity we successfully applied six probes (**Fig 1**). Based on the patterns we could analyse the intraspecific groups to screen genetic diversity (**Fig 2**) in *Periconia macrospinosa*, *Darksidea alpha*, *Cadophora* sp. and *Flavomyces fulophazii* species. Our previous findings showed there was no obvious specificity (e.g. host, microhabitat) of DSE fungi on species level (Knapp et al. 2012). Based on the ISSR results we could not see any specificity either. No hosts (e.g. *Festuca* vs *Stipa*) no collecting season and area, and no colony morphology patterns could be correlated with the ISSR based grouping. Based on the ISSR results we could demark "genotypes" what we could use in intraspecific functional diversity studied (Knapp et al. 2015a) (see below)

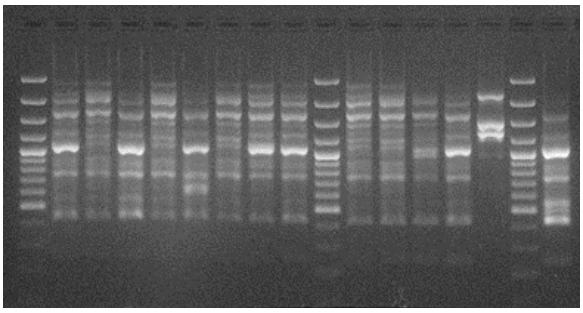

Fig. 1: ISSR profiles of 14 *Periconia macrospinosa* isolates using the ISSR primer $(AAG)_6$

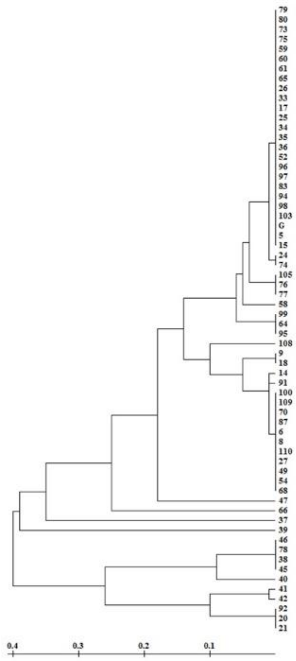

Fig. 2: Unweighted pair group method with arithmetic mean (UPGMA) tree of 66 *Periconia macrospinosa* isolates based on 6 ISSR profiles.

Formal taxonomy

Novel pleosporalean taxa[2]

In our previous study (Knapp et al. 2012) based on an *in vitro* resynthesis assay, isolates of 14 lineages were considered as DSE fungi, several groups of which could not be identified. Three of these groups (DSE-4, DSE-8 and DSE-7 *sensu* Knapp et al. 2012) clustered in the Pleosporales. In case of DSE-4, no

[2] Knapp, D. G., Kovács, G. M., Zajta, E., Groenewald, J. Z., Crous, P. W. 2015. Dark septate endophytic pleosporalean genera from semiarid areas. Persoonia 35: 87–100. (Q1(D1), IF: 5.725)

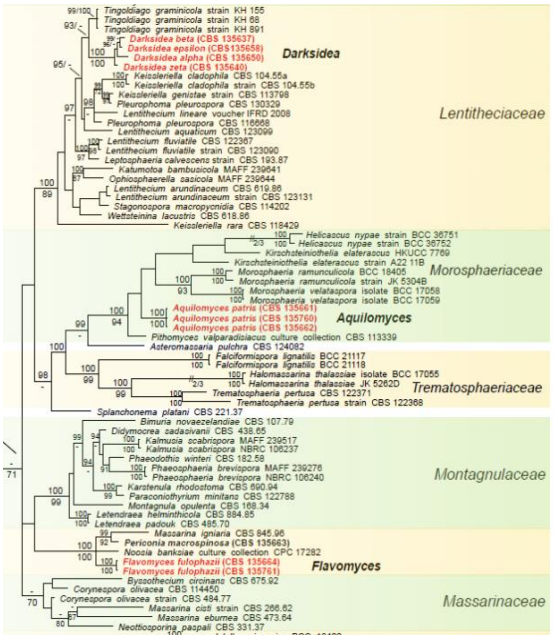

Fig. 3: A partial phylogenetic tree of representatives from Dothydeomycetes and from the DSE genera *Darksidea*, *Aquilomyces* and *Flavomyces* (red). The 50 % majority rule consensus phylogram inferred from Bayesian analysis of three loci (LSU, SSU, TEF) (Knapp et al. 2015b).

similar sequences (either cultured or uncultured fungi) were found in public databases. Although group DSE-7 was found to be the third most frequent DSE clade (Knapp et al. 2012), and similar findings were obtained in other studies (Porras-Alfaro et al. 2008; Herrera et al. 2010; Khidir et al. 2010), the identity and phylogenetic placement of this taxon and other DSE fungi in the Pleosporales remained unclear. During the general and targeted isolations, several root endophytes which represented those formally not described lineages were collected. Based on those isolates and our previous collections we carried out a detailed, polyphasic taxonomic study and based on the results we formally described three new genera and eight new species of those genera. The *Darksidea* genus groups into Lentitheciaceae, *Aquilomyces* to Morosphaeriaceae and *Flavomyces* was incertae sedis (**Fig 3**).

Aquilomyces patris D.G. Knapp, Kovács, J.Z. Groenew. & Crous, gen. & sp. nov.
MycoBank MB810756 (genus); MB810757 (species)

Flavomyces fulophazii D.G. Knapp, Kovács, J.Z. Groenew. & Crous, gen. & sp. nov.
MycoBank MB810758 (genus); MB810759 (species)

Darksidea D.G. Knapp, Kovács, J.Z. Groenew. & Crous, gen. nov.
MycoBank MB810760
Darksidea alpha D.G. Knapp, Kovács, J.Z. Groenew. & Crous, sp. nov. MycoBank MB810761
Darksidea beta D.G. Knapp, Kovács, J.Z. Groenew. & Crous, sp. nov. MycoBank MB810762
Darksidea gamma D.G. Knapp, Kovács, J.Z. Groenew. & Crous, sp. nov. MycoBank MB810763
Darksidea delta D.G. Knapp, Kovács, J.Z. Groenew. & Crous, sp. nov. MycoBank MB810764
Darksidea epsilon D.G. Knapp, Kovács, J.Z. Groenew. & Crous, sp. nov. MycoBank MB810765
Darksidea zeta D.G. Knapp, Kovács, J.Z. Groenew. & Crous, sp. nov. MycoBank MB810766

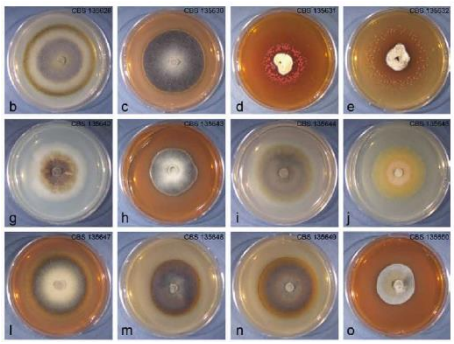

Fig. 4: Conspecific isolates of *Darksidea alpha* grown on MMN medium at identical conditions in the same age. (Knapp et al. 2015b)

We detected a high intraspecific variation of the growing characteristics of the isolates (**Fig 4**), because of that the *Darksidea* species could only be separated based on DNA sequence motifs. During the morphological investigations, although the trials to induce sporulation were not consistent and reproducible, we could detect ascomata, and in several cases asci and ascospores formed by *Darksidea* isolates (**Fig 5**). Although asexual sporulation is also rarely observed among many of the DSE fungi (Jumpponen & Trappe 1998), it could be induced in culture (e.g. Sieber & Grünig 2006), e.g. extreme long incubation times at low temperatures (Wang & Wilcox 1985;

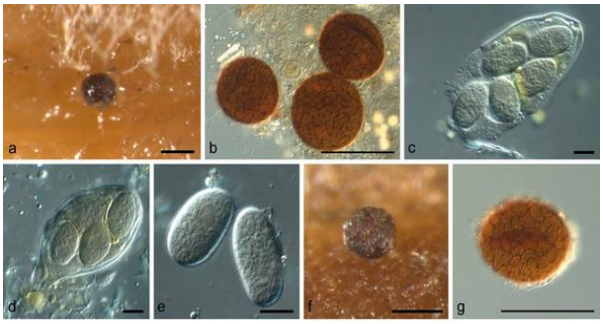

Fig. 5: Ascomata, asci and ascospores of *Darksidea* spp. **a–e**: *Darksidea zeta*. **a, b**: ascomata; **c, d**: asci; **e**: ascospores. **f–g**: *Darksidea alpha*. Scale bars: a, b = 200 µm, c,d,e = 10 µm, f, g = 180 µm. (Knapp et al. 2015b)

Grünig et al. 2009). Previous studies hypothesised that the sexual stage of some DSE and/or cryptic sexual reproduction exists and/or existed (e.g. Grünig et al. 2004; Zaffarano et al. 2011). Although sterile ascocarpium-like structures with no ascospores were observed in studies investigating other DSE species in *Acephala* sp. (UAMH 6816, Currah et al. 1993), to our best knowledge, our study was the *first in which sexual morphs formed by DSE fungi were observed*. This demonstrated capability for ascospore production in DSE fungi might help us to better understand the widespread and common occurrence of these root colonizing fungi.

Novel helotialean taxa[3]

In our previous project two unidentified helotialean phylotypes were detected (Knapp et al. 2012) (**Fig 6**). One lineage, referred to as the "DSE-2" group isolated from various host plants was considered as DSE based on *in vitro* re-synthesis tests (Knapp et al. 2012). The other unidentified helotialean root-associated fungus was represented by only one isolate, REF050, classified in the DSE-5 group. This fungus was not considered a DSE based on previous criteria (Knapp et al. 2012). At the time of that study, no nrDNA ITS1-5.8S-ITS2 (ITS barcode) or nrDNA 28S (28S) sequences were found in public databases with high BLAST similarity to those from REF050. In contrast, sequences of the DSE-2 group were highly similar or identical to sequences of fungi mainly originating from soil and roots of a variety of plants from different continents (Knapp et al. 2012). Later, Glynou et al. (2016) isolated endophytes from roots of *Microthlaspi* spp. from a wide range of localities in Europe and Turkey, and found some isolates with ITS sequences highly similar to those of DSE-2. These isolates were classified within the operational taxonomic unit OTU037, which was sixth in frequency out of the 44 helotialean OTUs obtained in that study (Glynou et al. 2016). Lacercat-Didier et al. (2016) isolated endophytes from ectomycorrhizal poplar roots collected from a metal-contaminated site in France, some of which also showed high sequence similarities to both the DSE-2 clade and the DSE-5 lineage. All those isolates were able to colonize roots of hybrid *Populus* in re-synthesis experiments. The clade including these and other sequences was described as a "new endophytic putative species belonging to the Helotiales" (Lacercat-Didier et al. 2016). Further strains with high ITS sequence similarity to the DSE-2 clade were isolated from two additional sources: the inner tissue of a truffle ascoma collected from our sampling area and

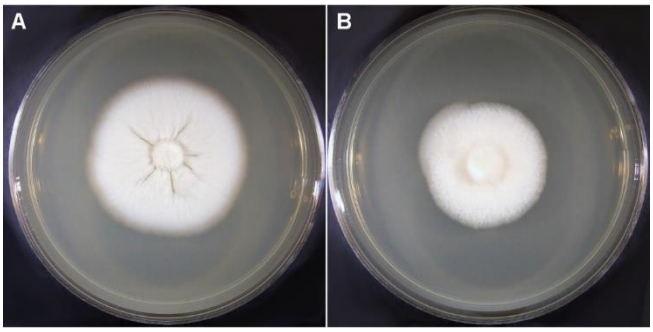

Fig. 6: Colonies of *Polyphilus* species on MMN. **A**. *Polyphilus sieberi* (DSM 106515). **B**. *Polyphilus frankenii* (DSM 106520). (Ashrafi et al. 2018)

[3] Ashrafi, S., Knapp, D. G., Blaudez D., Chalot, M., Maciá-Vicente, J. G., Zagyva, I., Dababat, A. A, Maier, W., Kovács, G.M. 2018. Inhabiting plant roots, nematodes and truffles — *Polyphilus*, a new helotialean genus with two globally distributed species. *Mycologia* 110: 286–299 (Q1, IF: 2.861)

eggs of the cereal cyst nematode (CCN) *Heterodera filipjevi* originating from the Central Anatolian Plateau of Turkey. We collected all those isolates to assess the taxonomic novelty of the strains and to determine their phylogenetic relationships. To accomplish this, within an international cooperation, we carried out a polyphasic taxonomic study using multilocus phylogenetic analyses. Based on that we described a new genus with two representative species:

Polyphilus D.G. Knapp, Ashrafi, W. Maier & Kovács, gen. nov.
MycoBank MB823756
Polyphilus sieberi Ashrafi, D.G. Knapp, W. Maier & Kovács, sp. nov. MycoBank MB823757
Polyphilus frankenii D.G. Knapp, Ashrafi, W. Maier & Kovács, sp. nov. MycoBank MB823758

Mongolian isolates[4]

We studied the DSEs community of a common dominant gramineous plant species, *Stipa krylovii* in a semiarid grassland of Mongolia. Root samples were collected in a natural steppe zone located in the Nalaikh district, ~38.6 km from the capital city of Ulaanbaatar, Mongolia. Sampling of *S. krylovii* roots was carried out in October 2016. Root samples were collected from 20 tussocks of *S. krylovii* with ~1 m distance from one another along a transect. For molecular identification of the isolates, the internal transcribed spacer (ITS) region of the nrDNA was sequenced for all the isolates investigated; furthermore, the partial translation elongation factor 1-α (TEF) gene, and large subunit (LSU) and small subunit (SSU) of rDNA were also amplified and sequenced in case of representative isolates. *In vitro* tests were used to examine the rough symbiotic nature of the fungi, and root colonization was visualized. A majority of the 135 isolates studied in detail was found to belong to several orders of Ascomycota (110 isolates) and some to Basidiomycota (25 isolates), the isolates represented 34 clades from which 30 comprised ascomycetous and 4 comprised basidiomycetous fungi. A significant number of the isolates represented presumably novel taxa, and dominant similarities of the lineages have been found with relatively frequent and known grass root endophytes of semiarid areas in other geographic regions. These endophytes included *Periconia macrospinosa*, *Microdochium bolley* and *Darksidea*, the genus of which comprised one fourth of the isolates. We found numerous lineages, which have been detected not only from Asian steppe ecosystems, but also from prairies in North America and sandy grasslands in Europe. Therefore, our results strengthen the hypothesized worldwide presence of a common and dominant core group of a DSE community in arid and semiarid grasslands.

NGS Metabarcode

In the original research plan we aimed to carry out next generation sequencing (NGS) based metabarcoding of the root associated fungal community. This method has been going through a rapid and significant change in the last 10 years; mostly used platforms and biochemistries (e.g. 454 Roche vs. Illumina) has changed. We took soil samples from an LTER (ExDrain) at Fülöpháza grassland in 2014 and used these samples to establish the protocol we aimed to use. We used and compared the MoBio PowerSoil DNA Isolation Kit with 0.25 g soil in 3 replicates and MoBio PowerMax Soil DNA Isolation Kit with 5 g soil. We first used F-ARISA (Fungal Automatic rRNA Intergenic Spacer Analysis) method to screen and compare the communities; we found that there might be differences according to the starting soil amounts and the 5 g soil results better representatively of the communities. Within the frame of the project here we took 12 samples and run an NGS metabarcode (ITS1F-ITS4 primers)

[4] Knapp, D. G., Imrefi, I., Boldpurev, E., Csíkos, S., Akhmetova, G., Berek-Nagy, P. J., Otgonsuren, B., Kovács, G. M. 2019. Root colonizing endophytic fungi of the dominant grass *Stipa krylovii* from a Mongolian steppe grassland. *Frontiers in Microbiology* 10: 2565 (Q1, IF: 4.259)

community analyses on Illumina MiSeq platform. There were 3000-38,000 fungal ITS2 sequences/samples, the OTU number varied between 60 and 310. Majority of the fungal taxa belonged to Dikarya. Within the Ascomycetes the well-known DSE fungi of the grasslands were detected as: *Polyphilus sieberi*, *Acrocalymma vagum*, *Cadophora* sp., *Curvularia inequalis*, *Periconia macrospinosa*, *Darksidea* spp. (Vajna et al. 2018). Because of the above mentioned changes of the methods which significantly influenced how and what kind of data could be published in highly ranked journals, we decided to continue the sampling after the project; following the manipulations at the ExDrain LTER. We took the last samples in 2019 and will do a total analyses of changes/characteristics of fungal community based on four sampling in a 6-year range.

Aims in the original plan with no sound results

We aimed to establish an online *DSE database*; we set an international group with the most known experts of DSE fungi we also set the main points of the database; unfortunately the IT-part of project failed, although there were promising progress at the beginning. We also aimed to screen DSEs on root level microscale – although SCARs and DSE taxon-specific FISH were designed (see above) we could not apply those on root level and this question was not checked during the four year of the project. We could only screen root-level results when the pathogen-DSE interaction was screened (see below).

Functional diversity

Functional anatomy

Originally we hypothesized that the potential functional differences of DSE fungi could be revealed with comparative functional anatomy. For that purpose we set different experiments during the project. First we inoculated leek (*Allium porrum*) a common host plant in DSE inoculation experiments with *Periconia macrospinosa* and *Cadophora* sp. We got very low colonization rates and gained only few good TEM view – in which we could reveal perifungal intact plant membranes within the colonized plant cells showing biotrophic interaction (**Fig 7**). We tried to use plant calli to screen differences in colonization by different DSE but after trials we stopped those experiments. We applied micropropagated *Cerasus* and *Sambucus* plantlets inoculated with *Periconia macrospinosa*, *Darksidea alpha*, *Flavomyces fulophazii* and *Cadophora* sp. DSE fungi. Although the experiments were repeated several times we could only sporadically detect colonization during the microscopic analyses and those information did not make possible presenting solid, sound results (**Fig 8**).

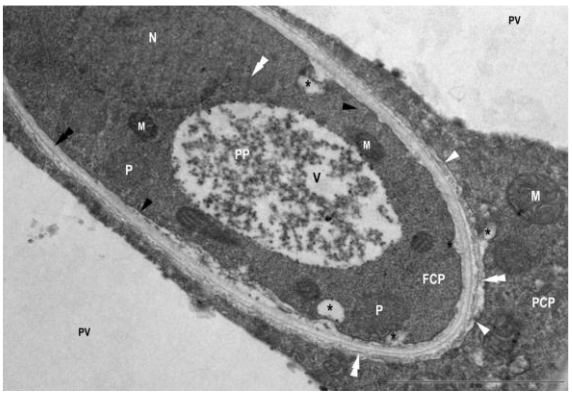

Fig. 7: Micrograph (TEM) showing *Allium porrum* root cell colonized by *Cadophora* sp. (DSE1049). An intracellular hypha. FCP: fungal cytoplasm, M: mitochondrion, N: fungal nucleus, P: peroxisome, PCP: plant cytoplasm, PP: polyphosphate granules, PV: plant vacuole, V: fungal vacuole, *: vesicles, black arrowhead: fungal plasma membrane, black double arrowhead: fungal cell wall, white arrowhead: plant plasma membrane, double whit arrowhead: matrix material. Scalebar = 500 nm

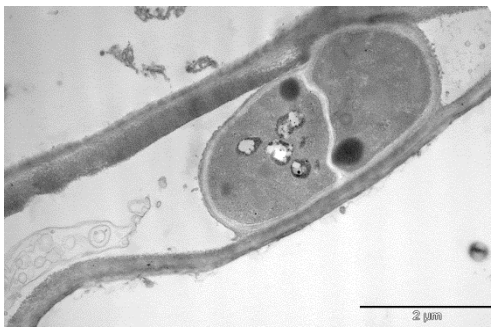

Fig. 8: Intracellular hypha of *Darksidea alpha* in root of micropropagated *Sambucus nigra*. Scale bar = 2 µm.

Interspecific enzymatic differences[5]

We aimed to test if functional diversity exists among DSE fungi representing different lineages of root endophytic fungal community of our sampling site at the semiarid sandy grasslands. For this we studied representatives of fifteen different DSE fungal taxa/lineages we detected at our sites. To address the question and to gain general information on function of DSEs, we adopted api-ZYM and BioLog FF assays to study those non-sporulating filamentous fungi and characterized the metabolic activity of the different DSE species. The api-ZYM assay, originally designed for fingerprinting of bacteria, is a simple, rapid, semiquantitative test of 19 constitutively expressed of lipid, protein and carbohydrate degrading enzymes of non-pre-treated organisms (e.g. Humble et al. 1977). It has been used to characterize both communities (e.g. Bending et al. 2002) and species or serotypes (e.g. Ahmed et al. 2013) of bacteria. Before us, only few studies examined fungal enzyme activities with this test (e.g. Bidochka et al. 1999; Tekere et al. 2001). The other assay for physiological identification, BioLog FF MicroPlate was designed to identify and/or characterize sporulating fungi and yeasts. MicroPlates supplied with 95 different carbon sources enable fingerprinting of metabolic activity of fungi based on the degree of substrate degradation. BioLog FF test has been used for e.g. characterization of closely related fungal species (e.g. Singh 2009) and was successfully applied to distinguish different genera and species, e.g. in the case of the well-known root colonizing *Oidiodendron* species (Rice & Currah 2005). We successfully adopted both systems to screen DSEs (**Fig 9**). Although there were striking differences of among the species, all of the substrates tested were utilized by the DSEs. When endophytes characteristic to grasses and non-grass host plants were separately considered we found that the whole substrate repertoire was used by both groups (**Fig 10**). This might illustrate the complementary functional diversity of the communities root endophytic plant associated fungi. The broad spectra of substrates utilized by these root endophytes illustrate the functional importance of their diversity, which can play role not only in nutrient mobilization and uptake of plants from with nutrient poor soils, but in general plant performance and ecosystem functioning.

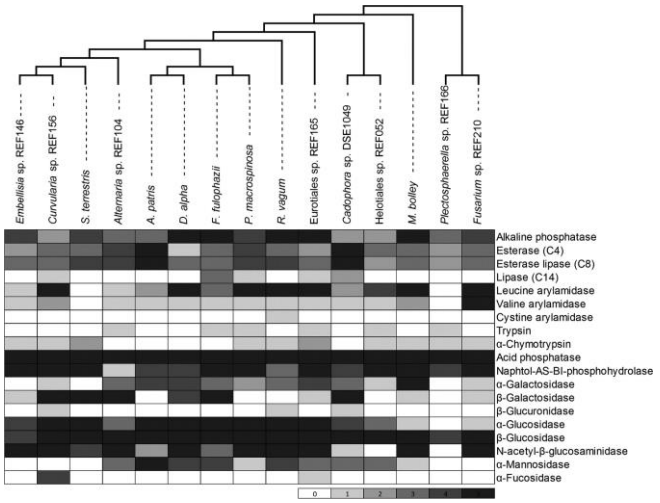

Fig. 9: Constitutive enzyme production of the 15 DSE species determined with the api-ZYM tests. Cells are shaded according to the intensity of hydrolyzation of the 19 substrates of the test from zero (null reaction) to five (strongest reaction). The maximum likelihood (ML) tree was gained from the analysis of a combined dataset of ITS and LSU sequences. (Knapp & Kovács 2016)

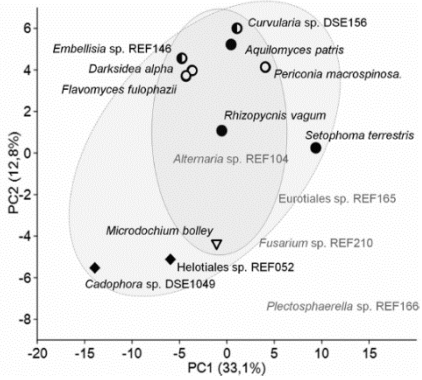

Fig. 10: PCA of categorized OD values (0–5) derived from BioLog FF system of the 15 DSE species. PC1 accounts for 33.1% and PC2 for 12.8% of the variation. DSE species of grasses are marked by empty symbols and circled by solid line, DSEs of non-grass hosts are marked by black symbols and circled by dashed line, half black symbols indicates DSEs of mixed hosts, while symbols for species cannot clearly assigned to host types are not shown. (Knapp & Kovács 2016)

[5] Knapp, D. G., Kovács, G. M. 2016. Interspecific metabolic diversity of root colonizing endophytic fungi revealed by enzyme activity tests. *FEMS Microbiology Ecology* 92: fiw190. (Q1, IF: 3.720)

Plant/nutrient focused functional studies[6]

We set several experiments to screen functional differences in nutrient uptake of plants inoculated with different DSEs. Unfortunately several trials did not resulted sound data: e.g. N and P content of plants varied a lot and in other cases the experimental setups failed. However, we could establish a complex experiment using *Periconia macrospinosa* and *Cadophora* sp. isolated from our semiarid grasslands. Phosphate uptake plays a crucial role in non-pathogenic root-fungus interaction (Yakti et al. 2019[7]), and both phosphate and nitrate form could influence the effect of DSE on plants (see e.g. metaanalyses of Newsham 2011).

We established a study to examine the effect of DSEs on the nutrition and growth of tomato (*Solanum lycopersicum*); the plant had a well-established experimental setup and know-how so we hoped that less technical problems could affect the experiments. We used the two different DSE species *Periconia macrospinosa* and *Cadophora* sp. and screened effects when different forms of N and P (organic and inorganic) was applied. We also set an *in vitro* bioassay with tomato plantlets to screen the compatibility of these fungi with the plant. Pot-culture experiments with and without compartments were conducted to study the effects of these DSEs on the growth and nutrient uptake of tomato plants grown with organic and inorganic N and P sources. We found that *Periconia macrospinosa*, but not *Cadophora* sp., increased the root and shoot biomass of tomato plants when organic nutrient resources were present, and both DSEs promoted shoot growth when cultivated with inorganic fertilizers (**Fig 11**). Analysis of N and P concentrations indicated that the growth response of tomato with inorganic fertilisation was not based on DSE-improved plant nutrition. However, *Periconia macrospinosa* improved N uptake from organic sources. Presence of hyphae could be proven using species specific FISH probes hyphal colonization was not strong and not important from the point of view of the effects, which is similar to previous findings (Newsham 2011). We could prove that there are functional differences between the two DSE fungi used in the experiments. We could also conclude that the positive effects of DSEs could be due to nutrient mobilization rather than to hyphal transport to the plant.

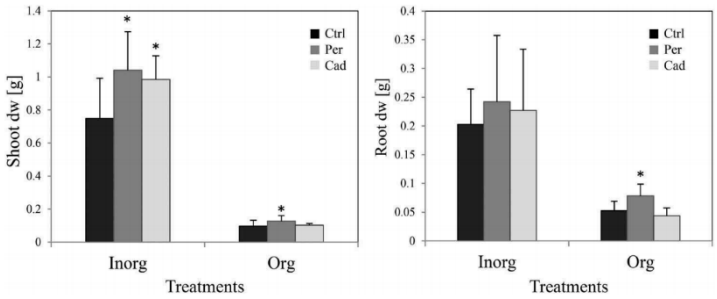

Fig. 11: Effect of DSEs on tomato vegetative growth in a pot-experiment. Tomato plants were non-inoculated (Ctrl), inoculated with *Periconia macrospinosa* (Per) or *Cadophora* sp. (Cad); grown in organically fertilised (Org) or inorganically fertilised (Inorg) soils. The dry weight of roots and shoots was measured. (Yakti et al. 2018)

Interaction of DSE and pathogen fungi[8]

Beside influencing the nutrient uptake of the plant DSEs could affect the interaction of the plants with pathogenic fungi and this could be an important functional effect of those root colonizers. To test this possible hypothesis we applied our *Cadophora* sp. against the soilborne pathogens *Rhizoctonia solani*, *Pythium aphanidermatum* and *Verticillium dahliae*. To investigate their interactions, we conducted *in*

[6] Yakti, W., Kovács, G. M., Vági, P., Franken, P. 2018. Impact of dark septate endophytes on tomato growth and nutrient uptake. *Plant Ecology and Diversity* 11: 637-648. (Q2, IF 1.73)

[7] Yakti, W., Andrade-Linares, D. R., Ngwene, B., Bitterlich, M., Kovács, G. M., Franken, F. 2019. Phosphate nutrition in root-fungus interactions. In: Hodkinson, T. H., Doohan, F. M., Saunders, M. J., Murphy, B. R. (eds) Endophytes for a Growing World. Cambridge University Press pp: 120-142.

[8] Yakti, W., Kovács, G. M., Franken, P. 2019. Differential interaction of the dark septate endophyte *Cadophora* sp. and fungal pathogens *in vitro* and *in planta*. *FEMS Microbiology Ecology* 95: fiz164 (Q1, IF 4.098)

vitro assays followed by greenhouse experiments in which the above mentioned tomato experimental setups were used. RNA level of certain tomato pathogenesis-related genes and of *Cadophora* sp. genes with possible antifungal function was analysed. The DSE had a negative effect on the growth of the fungal pathogens *in vitro* and vice versa; a negative impact of the pathogens on the growth of the DSE was also detected. In roots, however, this antagonism could not be observed. Beside other results, our data indicate that plants could have an effect on the interaction between different fungi and, therefore, *in vitro* detected antagonism can not necessarily be extrapolated for *in planta* competitions.

Genomic analyses[9]

We aimed to screen genetic/genomic background of different strategies related to potential functions of DSEs. First we screened gene expressions of different genes which could be related to symbiotic/saprobic nature of fungi. After several optimization tests of different steps of the methods, based on literature survey we designed qPCR probes for around 30 different genes, e.g. invertases and phosphate transporters. We worked both on *Cadophora* sp. and *Periconia macrospinosa* and different strains of *Periconia* and we screened free living and symbiotic fungi, too (Németh et al. 2016). The promising pilot results showed obvious functional differences not only between the species but among the different isolates of *Periconia macrospinosa* (**Fig 12**). Although we could not extend our works using RNA-sequencing based comparative analyses (a nowadays must for proper comparison), we could do a comparative genomic analyses of DSE fungi after gaining the possibility of full genome sequencing of two of our DSEs: *Periconia macrospinosa* and *Cadophora* sp. within the 1000 Fungal Genome Program of JGI DOE. Beside the preparation of proper DNA and RNA samples and genome sequencing we carried out a detailed comparative genomic analyses to reveal evolutional and ecological features of DSEs. For the comparative analyses, beside our two new genomes, another 32 ascomycete genomes of different lifestyles were involved into the analyses. *Cadophora* sp. and *Periconia macrospinosa* have

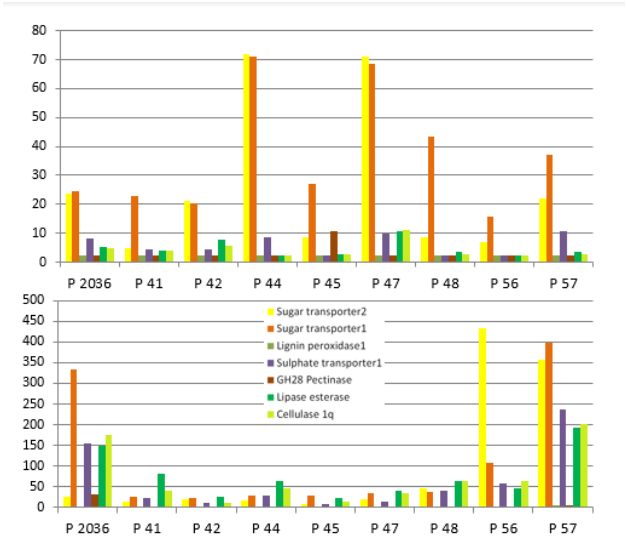

Fig. 12: Relative gene expression levels of nine *Periconia macrospinosa* isolates in barley roots (above) and on MS medium with cellophane (below). X axis: *P. macrospinosa* isolates, Y axis: gene expression R values.

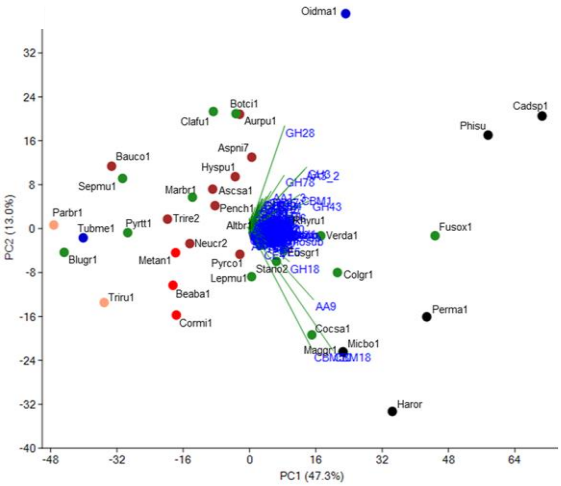

Fig. 13: PCA of carbohydrate active enzymes (CAZymes) of *Cadophora* sp., *Periconia macrospinosa*, and 35 other ascomycetes including three further DSE species. PCA based on CAZyme copy numbers. Different fungal lifestyles are labelled in red (ap/e; animal pathogens/endophytes), brown (sap; saprotrophs), green (plp; plant pathogens), black (dse, dark septate endophytes), blue (myc; mycorrhizal fungi), or pink (ap; animal pathogen). (Knapp et al. 2018)

[9] Knapp, D. G., Németh, J. B., Barry, K., Hainaut, M., Henrissat, B., Johnson, J., Kuo, A., Ping Lim, J. H., Lipzen, A., Nolan, M., Ohm, R. A., Tamás, L., Grigoriev, I. V., Spatafora, J. W., Nagy, L. G., Kovács, G. M. 2018. Comparative genomics provides insights into the lifestyle and reveals functional heterogeneity of dark septate endophytic fungi. *Scientific Reports* 8: 6321 (Q1(D1), IF 4.011)

genomes of 70.46 Mb and 54.99 Mb with 22,766 and 18,750 gene models, respectively. These are relatively high predicted gene model numbers. The majority of DSE-specific protein clusters lack functional annotation with no similarity to characterized proteins, implying that they have evolved unique genetic innovations. Both DSE possess an expanded number of carbohydrate active enzymes (CAZymes), including plant cell wall degrading enzymes (PCWDEs). Those were similar in three other DSE fungi (*Harpophora oryzae*, *Phialocephala subalpine*, *Microdochium bolleyi*) published previously (Xu eta al. 2014; David et al. 2016; Schlegel et al. 2016), and contributed a signal for the separation of root endophytes in principal component analyses of CAZymes, indicating shared genomic traits of DSE fungi (**Fig 13**). Number of secreted proteases and lipases, aquaporins, and genes linked to melanin synthesis were also relatively high in our fungi. In spite of certain similarities between our two DSE, we observed low levels of convergence in their gene family evolution and high number of specific gene duplication events at the terminal branches of our DSEs (**Fig 14**). These results, similarly to our other findings (enzyme studies, pot experiments, intraspecific analyses etc) strengthen further our hypothesis that the mobilization, saprobic capacity of DSEs could play a crucial role in their functions in ecosystems. Our results suggested that, despite originating from the same habitat, these two fungi evolved along different evolutionary trajectories and display considerable functional differences within the endophytic lifestyle.

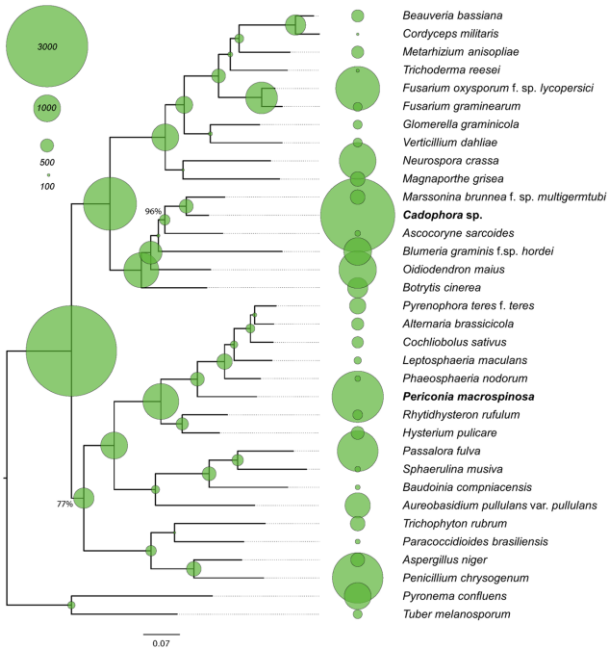

Fig. 14: Genome-wide reconstruction of gene duplication histories of *Cadophora* sp., *Periconia macrospinosa* and another 32 ascomycetes. Green circles indicate observed (on terminal branches) and reconstructed (on internal nodes) gene copy numbers. The two DSEs are marked in bold. (Knapp et al. 2018)

New experimental setup and fluorescence based biomass estimation to screen interspecific differences[10]

To test further the functional differences just like to test importance of the degrading/saprobic capacity of DSEs we decided to establish a new experimental setup coupled with a fluorescence based biomass estimation method. In the new micro-scale experimental setup we applied substrates made of different organs of different plant species; so we could use different plant substrates as growing media for fungi. Namely: grounded powder of roots and shoots of barley, cabbage and ribwort were set in wells of a 4 x 6 microwell plate (**Fig 15**). We involved five DSE taxa into the first experiments (*Periconia macrospinosa* and *Darksidea alpha*, *Setophoma terrestris*,

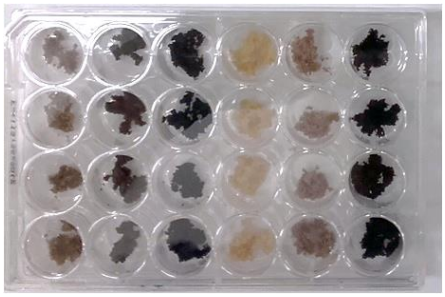

Fig. 15: Powdered shoot and root as media prepared from three plant species in a microwell system before fungal inoculation.

[10] The manuscript: Németh, J. B., Knapp, D. G., Kósa, A., Hegedűs, P. Á., Herczeg, G., Vági, P., Kovács, G. M. Novel micro-scale experimental system coupled with a WGA titration-based fluorescence biomass estimation of fungi reveal species-specific utilization of plant substrates by root endophytes. Is now under revision after two supporting reviews and an editorial „encouraged resubmission" decision at the *Methods in Ecology and Evolution* (Q1(D1), IF 7.099)

Cadophora sp., *Polyphilus sieberi*). We modified, adopted, and tested reliable and simple WGA titration-based method (Ayliffe et al. 2013) for estimation of *total* fungal biomass within the substrates using fluorescence labelled lectin (WGA-AlexaFluor488). When finished the calibration experiments, we found that the relationship between fluorescence intensity and fungal dry weight was strong and linear, but differed between fungi tested (**Fig 16**). The GLM revealed that the fluorescence intensity – fungal dry weight relationship is strongly species-specific (species: F3,55 = 3.65, P = 0.02; partial η2 = 0.17; dry weight: F1,55 = 549.38, P < 0.001; partial η2 = 0.91; species × dry weight: F3,55 = 53.84, P < 0.001; partial η2 = 0.75). The linear regressions revealed that fungal dry weight accounted for more than 80% of variation in fluorescence intensity in both species, but the highly divergent slopes depicted almost five-fold difference between species (*Periconia macrospinosa*: R2 = 0.88; B = 159.71; Standard Error of B [SE] = 15.68; t = 10.18; P < 0.001; *Cadophora* sp.: R2 = 0.83; B = 507.71; SE = 63.87; t = 7.95; P < 0.001; *Polyphilus sieberi*: R2 = 0.96; B = 781.21; SE = 41.48; t = 18.83; P < 0.001; *Darksidea alpha*: R2 = 0.96; B = 701.53; SE = 40.00; t = 17.54; P < 0.001). The fungal dry weight – fluorescence intensity relationship was almost identical between *Polyphilus sieberi* and *Darksidea alpha*, but the other two species differed considerably (**Fig 16**). On the other hand, it was impossible to measure the biomass of *Setophoma terrestris* using this technique. The effect of the plant organ (i.e. root vs. shoot) used as substrate on fungal growth depended on plant species and also varied between fungal species. Comparison of growth profiles of the examined DSE fungi showed striking interspecific differences in growing capacity on different plant substrates. Beside others, these might be due to differences in metabolic, cell-wall and tissue composition of the different plant species and their different organs, the amount of easily utilizable sources and different enzyme spectra of the fungi. The novel microscale experimental system is useful to screen the utilization of different substrates which can help to understand ecological roles and or functions of fungi. Our fungal biomass estimation method that can be applied in various fields. As the estimation is based on fungal cell wall, it measures total cumulative biomass produced in a certain environment.

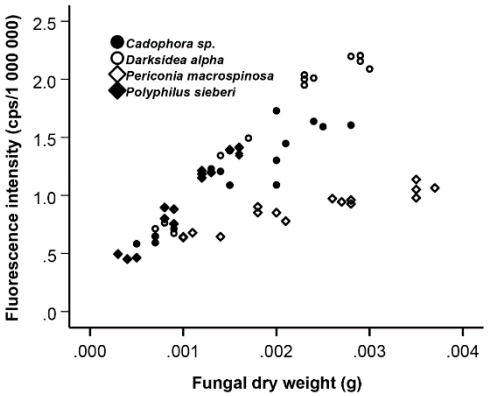

Fig. 16: Species-specific linear fluorescence intensity – fungal dry weight relationships revealed by GLM based on dry hyphal weight (g) and average fluorescence (counts per second - cps) of hyphal suspensions of *Periconia macrospinosa*, *Cadophora* sp., *Polyphilus sieberi* and *Darksidea alpha*.

Intraspecific functional diversity

When testing the intraspecific functional heterogeneity, we hypothesized that there were functional differences between genetically different isolates of the same species. Based our previous findings and the results of the ISSR analyses (see above) we used strains isolated from two grasses (*Stipa borysthenica* and *Festuca vaginata*) of the same locality at the same time. The isolates spent the same time *in vitro* and had the same passage numbers. We chose 8-8 genetically different isolates of three pleosporalean fungi: *Darksidea alpha*, *Periconia macrospinosa* and *Flavomyces fulophazii*. We set different experiments to screen functional diversity. First we compared “saprobic” vs “symbiotic” behaviour, when screened biomass growth on medium (with no monosaccharide) and effect on inoculated *Zea mays* plants, respectively. Interestingly when repeatability (here heritability, too) were analysed in case of *Periconia macrospinosa* we had strong heritability of the effect on *Zea* shoot growth and saprobic feature, in case of *Darksidea alpha* we obtained strong heritability of the effect on *Zea* root growth and saprobic feature, while there was no significant heritability of any feature of *Flavomyces fulophazii* isolates. We applied api-ZYM and biolog-FF enzyme tests for all the 8-8 isolates

of the three DSE taxa gained which showed intraspecific enzymatic differences, too. We applied above mentioned qPCR assays as well (**Fig 12.**) for *Periconia macrospinosa* isolates. For the 8 *Periconia macrospinosa* isolates we also applied the above mentioned novel, microwell based saprobic tests which gave highly significant results: strong repeatability on growing features (except on cabbage shoot) and there was a genetically highly determined strategy of *Periconia macrospinosa* isolates according to the different plant species as substrate (Knapp et al. 2015a).

Conclusions and outlook

During the four year of the project we could gain solid data to answer our original hypothesis: the root colonizing DSE fungi are genetically diverse, there is a plethora of unknown diversity, either on species and higher level or on intraspecific level. This ***"taxon" diversity of DSE fungi represents obvious functional diversity as well*** when ***both inter- and intraspecific*** functions/strategies are screened. The successful four year of the project resulted several new questions and directions. The international interest in the results is well illustrated by one of our taxonomic paper (Knapp et al. 2015, see above) which gained international reputation/citation made possible to apply for "KH" research support.

Publications

As we aimed to use new methods we gave a conservative plan for publication plan in the original plan. Nevertheless, one important experience of the project was the extreme long time of publication of the results especially of the experimental works. Although we could well demonstrate and present our results on different conferences, and e.g. our presentation were chosen for "highlight talk" on the 8th International Congress of Mycorrhizae (2015, Flagstaff, Arizona, USA) the peer reviewed publications processes were long – and in one case it has still been running and "blocks" the finalization our last paper.

In all publication which were based on, or partly based on the results shown above the support of 'K109102' was added, and will be added. In this report we have listed the publications, the peer reviewed papers (and one chapter) is given in footnotes at the exact parts, too.

Altogether ***7 papers have been published from the works reported above; 6 of them were published in "SCImago" Q1 journals (2 out of them are D1) (sum WoS IF: 26.404)***. We also published a peer-reviewed book chapter on nutrient uptake functions of root colonizers where the support was given.

Book chapter

▪ Yakti, W., Andrade-Linares, D. R., Ngwene, B., Bitterlich, M., Kovács, G. M., Franken, F. 2019. Phosphate nutrition in root-fungus interactions. In: Hodkinson, T. H., Doohan, F. M., Saunders, M. J., Murphy, B. R. (eds) Endophytes for a Growing World. Cambridge University Press pp: 120-142.

Papers

▪ Ashrafi, S.*, Knapp, D. G.*, Blaudez D., Chalot, M., Maciá-Vicente, J. G., Zagyva, I., Dababat, A. A, Maier, W., Kovács, G.M. 2018. Inhabiting plant roots, nematodes and truffles — *Polyphilus*, a new helotialean genus with two globally distributed species. *Mycologia* 110: 286–299 (Q1, IF: 2.861) (* equally contributed)

▪ Knapp, D. G., Kovács, G. M., Zajta, E., Groenewald, J. Z., Crous, P. W. 2015. Dark septate endophytic pleosporalean genera from semiarid areas. *Persoonia* 35: 87–100. (Q1 (D1); IF: 5,725)

▪ Knapp, D. G., Kovács, G. M. 2016. Interspecific metabolic diversity of root colonizing endophytic fungi revealed by enzyme activity tests. *FEMS Microbiology Ecology* 92: fiw190. (Q1, IF: 3.720)

▪ Knapp, D. G., Németh, J. B., Barry, K., Hainaut, M., Henrissat, B., Johnson, J., Kuo, A., Ping Lim, J. H., Lipzen, A., Nolan, M., Ohm, R. A., Tamás, L., Grigoriev, I. V., Spatafora, J. W., Nagy, L. G., Kovács, G. M. 2018. Comparative genomics provides insights into the lifestyle and reveals functional heterogeneity of dark septate endophytic fungi. *Scientific Reports* 8: 6321 (Q1 (D1), IF 4.011)

▪ Knapp, D. G., Imrefi, I., Boldpurev, E., Csíkos, S., Akhmetova, G., Berek-Nagy, P. J., Otgonsuren, B., Kovács, G. M. 2019. Root colonizing endophytic fungi of the dominant grass *Stipa krylovii* from a Mongolian steppe grassland. *Frontiers in Microbiology* 10: 2565 (Q1, IF: 4.259)

▪ Yakti, W., Kovács, G. M., Vági, P., Franken, P. 2018. Impact of dark septate endophytes on tomato growth and nutrient uptake. *Plant Ecology and Diversity* 11: 637-648. (Q2, IF 1.73)

▪ Yakti, W., Kovács, G. M., Franken, P. 2019. Differential interaction of the dark septate endophyte *Cadophora* sp. and fungal pathogens *in vitro* and *in planta*. *FEMS Microbiology Ecology* 95: fiz164 (Q1, IF 4.098)

Paper in the publication process

Németh, J. B., Knapp, D. G., Kósa, A., Hegedűs, P. Á., Herczeg, G., Vági, P., Kovács, G. M. Novel micro-scale experimental system coupled with a WGA titration-based fluorescence biomass estimation of fungi reveal species-specific utilization of plant substrates by root endophytes. Is now under revision after two supporting reviews and an editorial „encouraged resubmission" decision at the *Methods in Ecology and Evolution* (Q1 (D1), IF 7.099).

Manuscript in preparation

Only when the above mentioned methodical paper is accepted will we be able to submit our results on intraspecific functional diversity – as we applied the microwell based experiments in the study (see above). This will be our last paper where the K109102 will be shown as support.

Literature cited

Ahmed L, Jensen PR, Freel KC et al. 2013. *Salinispora pacifica* sp. nov., an actinomycete from marine sediments. Anton Leeuw 103: 1069–78.

Ashrafi S, Knapp DG, Blaudez D, Chalot M, Maciá-Vicente JG, Zagyva I, Dababat AA, Maier W, Kovács GM. 2018. Inhabiting plant roots, nematodes and truffles — *Polyphilus*, a new helotialean genus with two globally distributed species. Mycologia 110: 286–299.

Ayliffe M1, Periyannan SK, Feechan A, Dry I, Schumann U, Wang MB, Pryor A, Lagudah E. 2013. A simple method for comparing fungal biomass in infected plant tissues. Mol Plant Microbe Interact 26: 658–667.

Bending GD, Turner MK, Jones JE. 2002. Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities. Soil Biol Biochem 34: 1073–82.

Bidochka MJ, Burke S, Ng L. 1999. Extracellular hydrolytic enzymes in the fungal genus *Verticillium*: adaptations for pathogenesis. Can J Microbiol 45: 856–64.

Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553–556.

Cheplick GP, Faeth SH. 2009. Ecology and Evolution of the Grass Endophyte Symbiosis. New York, Oxford.

Currah RS, Tsuneda A, Murakami S. 1993. Morphology and ecology of *Phialocephala fortinii* in roots of *Rhododendron brachycarpum*. Can J Bot 71: 1071–1078.

David AS, Haridas S, LaButti K, Lim J, Lipzen A, Wang M, Barry K, Grigoriev IV, Spatafora JW, May G. 2016. Draft genome sequence of *Microdochium bolleyi*, a dark septate fungal endophyte of beach grass. Genome Announc 4: e00270–16.

Glass NL, Donaldson G. 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous ascomycetes. Applied Environ Microbiol 61: 1323–1330.

Glynou K, Ali T, Buch A-K, Haghi Kia S, Ploch S, Xia X, Çelik A, Thines M, Maciá-Vicente JG. 2016. The local environment determines the assembly of root endophytic fungi at a continental scale. Environ Microbiol 18: 2418–2434.

Groenewald JZ, Nakashima C, Nishikawa J, Shin H-D, Park J-H, Jama AN, Groenewald M, Braun U, Crous PW. 2013. Species concepts in *Cercospora*: spotting the weeds among the roses. Stud Mycol 75: 115–170.

Grünig CR, McDonald BA, Sieber TN, et al. 2004. Evidence for subdivision of the root-endophyte *Phialocephala fortinii* into cryptic species and recombination within species. Fungal Genet Biol 41: 676–687.

Grünig CR, Queloz V, Sieber TN, Holdenrieder O. 2008. Dark septate endophytes (DSE) of the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex in tree roots: classification, population biology, and ecology. Can J Bot 86: 1355–1369.

Grünig CR, Queloz V, Duò A, Sieber TN. 2009. Phylogeny of *Phaeomollisia piceae* gen. sp. nov.: a dark, septate, conifer-needle endophyte and its relationships to *Phialocephala* and *Acephala*. Mycol Res 113: 207–221.

Herrera J, Khidir HH, Eudy DM, Porras-Alfaro A, Natvig DO, Sinsabaugh RL. 2010. Shifting fungal endophyte communities colonize *Bouteloua gracilis*: effect of host tissue and geographical distribution. Mycologia 102: 1012–1026.

Humble MW, King A, Phillips I. 1977. API ZYM: a simple rapid system for the detection of bacterial enzymes. J Clin Pathol 30: 275–7.

Jumpponen A, Trappe JM. 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytol 140: 295–310.

Khidir HH, Eudy DM, Porras-Alfaro A, Herrera J, Natvig DO, Sinsabaugh RL. 2010. A general suite of fungal endophytes dominate the roots of two dominant grasses in a semiarid grassland. J Arid Environ 74: 35–42.

Knapp DG, Pintye A, Kovács GM. 2012. The dark side is not fastidious – dark septate endophytic fungi of native and invasive plants of semiarid sandy areas. PLoS ONE 7: e32570.

Knapp DG, Berek-Nagy PJ, Imrefi I, Herczeg G, Kovács GM. 2015a. Intraspecific functional diversity of dark septate endophytic fungi of semiarid sandy grasslands. Rhizosphere4 Congress. Maastricht, the Netherlands. [conference presentation]

Knapp DG, Kovács GM, Zajta E, Groenewald JZ, Crous PW. 2015b. Dark septate endophytic pleosporalean genera from semiarid areas. Persoonia 35: 87–100.

Knapp DG, Kovács GM. 2016. Interspecific metabolic diversity of root colonizing endophytic fungi revealed by enzyme activity tests. FEMS Microbiol Ecol 92: fiw190.

Knapp DG, Németh JB, Barry K, Hainaut M, Henrissat B, Johnson J, Kuo A, Ping Lim JH, Lipzen A, Nolan M, Ohm RA, Tamás L, Grigoriev IV, Spatafora JW, Nagy LG, Kovács GM. 2018. Comparative genomics provides insights into the lifestyle and reveals functional heterogeneity of dark septate endophytic fungi. Sci Rep 8: 6321.

Lacercat-Didier L, Berthelot C, Foulon J, Errard A, Martino E, Chalot M, Blaudez D. 2016. New mutualistic fungal endophytes isolated from poplar roots display high metal tolerance. Mycorrhiza 26:1–15.

Lim S, Notley-McRobb L, Lim M, Carter DA. 2004. A comparison of the nature and abundance of microsatellites in 14 fungal genomes. Fungal Genet Biol 41: 1025–1036.

Mandyam K, Jumpponen A. 2005. Seeking the elusive function of root-colonising dark septate endophytic fungi. Stud Mycol 53: 173–189.

Mandyam K, Loughin T, Jumpponen A. 2010. Isolation and morphological and metabolic characterization of common endophytes in annually burned tallgrass prairie. Mycologia 102: 813–821.

Mayerhofer MS, Kernaghan G, Harper KA. 2013. The effects of fungal root endophytes on plant growth: a meta-analysis. Mycorrhiza 23: 119–128.

Németh JB, Németh MZ, Hegedüs PÁ, Tamás L, Knapp DG, Kovács GM. 2016. Intraspecific differences in expression of genes related to symbiotic and saprobic lifestyle of a dark septate endophytic fungus. 13th European Conference on Fungal Genetics (ECFG). Paris, France. [conference presentation]

Newsham KK. 2011. A meta-analysis of plant responses to dark septate root endophytes. New Phytol 190: 783–793.

Porras-Alfaro A, Bayman P. 2011. Hidden fungi, emergent properties: Endophytes and microbiomes. Ann Rev Phytopath 49: 291–315.

Porras-Alfaro A, Herrera J, Sinsabaugh RL, Odenbach KJ, Lowrey T, Natvig DO. 2008. Novel root fungal consortium associated with a dominant desert grass. Appl Environ Microbiol 74: 1308–1315.

Quaedvlieg W, Kema GHJ, Groenewald JZ, Verkley GJM, Seifbarghi S, et al. 2011. *Zymoseptoria* gen. nov.: a new genus to accommodate Septoria-like species occurring on graminicolous hosts. Persoonia 26: 57–69.

Queloz V, Sieber TN, Holdenrieder O, McDonald BA, Grünig CR. 2011. No biogeographical pattern for a root-associated fungal species complex. Global Ecol Biogeog 20: 16–169.

Rehner SA, Samuels GJ. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. Mycol Res 98: 625–634.

Reininger V, Grünig CR, Sieber TN. 2012. Host species and strain combination determine growth reduction of spruce and birch seedlings colonized by rootassociated dark septate endophytes. Environ Microbiol 14: 1064–1076.

Rice AV, Currah RS. 2005. Profiles from Biolog FF plates and morphological characteristics support the recognition of *Oidiodendron fimicola* sp. nov. Stud Mycol 53: 75–82.

Saikkonen K, Faeth SH, Helander M, Sullivan TJ. 1998. Fungal endophytes: A continuum of interactions with host plants. Ann Rev Ecol Evol Syst 29: 319–343.

Schlegel M, Münsterkötter M, Güldener U, Bruggmann R, Duò A, Hainaut M, Henrissat B, Sieber CMK, Hoffmeister D, Grünig CR. 2016. Globally distributed root endophyte *Phialocephala subalpina* links pathogenic and saprophytic lifestyles. BMC Genomics 17: 1015.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, and Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Nat Acad Sci USA 109: 6241–6245.

Schulz B, Boyle C. 2005. The endophytic continuum. Mycol Res 109: 661–686.

Sieber TN, Grünig CR. 2006. Biodiversity of fungal root-endophyte communities and populations in particular of the dark septate endophyte Phialocephala fortinii. In: Schulz B, Boyle C, Sieber TN. (eds) Microbial Root Endophytes. Springer, Berlin 107–132.

Simon J; Knapp DG; Vági P; Lukács AF; Németh JB; Borsodi AK; Kovács GM. 2014. Félszáraz területen élő gyökér endofiton gombák szimbiotikus baktériumainak kimutatása és azonosítása., A Magyar Mikrobiológiai Társaság 2014. évi Nagygyűlése, Keszthely Abstracts [conference presentation]

Singh MP. 2009. Application of BioLog FF MicroPlate for substrate utilization and metabolite profiling of closely related fungi. J Microbiol Meth 77: 102–108.

Tekere M, Mswaka AY, Zvauya R et al. 2001. Growth, dye degradation and ligninolytic activity studies on Zimbabwean white rot fungi. Enzyme Microb Tech 28: 420–426.

Vajna B, Kröel-Dulay Gy, Kovács GM. 2018. Gombaközösségek diverzitásának tanulmányozása kiskunsági (fél)száraz homokgyepeken egy hosszútávú ökológiai kutatás keretében: a DNS-izolálás optimalizálása egy kisléptékű NGS elemzés segítségével. A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése, Eger. [conference presentation]

Vági P, Knapp DG, Kósa A, Seress D, Horváth A, Kovács GM. 2014. Simultaneous specific *in planta* visualization of root-colonizing fungi using fluorescence in situ hybridization (FISH). Mycorrhiza 24: 259–66.

Vági P, Knapp DG, Kósa A, Horváth ÁN, Zajta E, Kovács GM. 2015. *In planta* visualization of root colonizing fungi using fluorescence *in situ* hybridization (FISH)., WG3 meeting, COST Action FA1103: Endophytes in Biotechnology and Agriculture. 12-14 October 2015, Budapest. Abstracts [conference presentation]

Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. J Bacteriol 172: 4238–4246.

Walsh E, Duan W, Mehdi M, Naphri K, Khiste S, Scalera A, Zhang N. 2018. *Cadophora meredithiae* and *C. interclivum*, new species from roots of sedge and spruce in a western Canada subalpine forest. Mycologia 110: 201–214.

Wang CJK, Wilcox HE. 1985. New species of ectendomycorrhizal and pseudomycorrhizal fungi: *Phialophora finlandia*, *Chloridium paucisporum*, and *Phialocephala fortinii*. Mycologia 77: 951–958.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ. (eds) PCR Protocols: A guide to methods and applications, Academic Press Inc., New York 315–322.

Xu XH, Su ZZ, Wang C, Kubicek CP, Feng XX, Mao LJ, Wang JY, Chen C, Lin FC, Zhang CL. 2014. The rice endophyte *Harpophora oryzae* genome reveals evolution from a pathogen to a mutualistic endophyte. Sci Rep 4: 5783.

Yakti W, Kovács GM, Vági P, Franken P 2018. Impact of dark septate endophytes on tomato growth and nutrient uptake. Plant Ecol Diversity 11: 637–648.

Zaffarano PL, Queloz V, Duò A, Grünig CR. 2011. Sex in the PAC: A hidden affair in dark septate endophytes? BMC Evol Biol 11: 282.

Zajta E. 2014. Csoport- és törzsspecifikus FISH próbák és PCR markerek fejlesztése sötét szeptált endofitonok kimutatására. Eötvös Loránd Tudományegyetem, Növényszervezettani Tanszék, Budapest. [MSc Thesis]