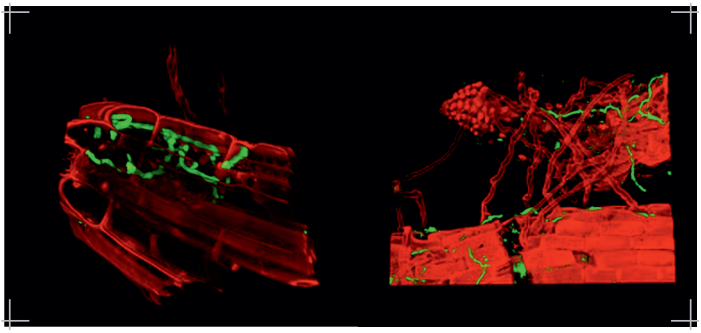
MEYSAM TAGHINASAB

# Function and diversity of root fungal endophytes in grassland under elevated temperature and $CO_2$ regimes



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# Function and diversity of root fungal endophytes in grassland under elevated temperature and $CO_2$ regimes

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**(Doktor der Agrarwissenschaften)**
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Contents

**List of abbreviations**

| | |
|---|---|
| $aCO_2$ | Ambient $CO_2$ |
| AMF | Arbuscular mycorrhizal fungi |
| ATP | Adenosine triphosphate |
| C | Ambient temperature |
| *Ca*Gc | *Candidatus* Glomeribacter gigasporarum |
| CM | Complex medium |
| CO | Control |
| CP | Mono-calcium phosphate |
| CTAB | Cetyl trimethylammonium bromide |
| cv. | Cultivar |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxy ribonucleic acid |
| DSE | Dark septate endophyte |
| $eCO_2$ | Elevated $CO_2$ |
| EDTA | Ethylenediamine tetra-acetic acid |
| FACE | Free Air $CO_2$ Enrichment |
| FISH | Fluorescence *in situ* hybridization |
| ITS | Internal transcribed spacer |
| $MgSO_4$ | Magnesium sulphate |
| MS | Murashige & Skoog |
| MYP | Malt & yeast extract, Peptone |
| OTUs | Operational taxonomic unit |
| P | Phosphorus |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PI | Propidium Iodide |
| $P_i$ | Inorganic orthophosphate |
| RA | Relative abundance |
| RNA | Ribonucleic acid |
| RP | Rock phosphate |
| rpm | Round per minute |
| RrF4 | *Rhizobiom radiobacter* F4 |
| *Sh*-P2A | *Stereum hirsutum* P2A |
| SSU | Small subunit rRNA |
| T | +2°C increased temperature |
| TEM | Transmission electron microscopy |
| TGW | Thousand-grain weight |
| *Tv*-WC16GW | *Trametes versicolor* WC16GW |
| WGA | Wheat germ agglutinin |

# 1 Introduction

## 1.1 What is endophytes

Understanding the mutualistic relationship of plant-fungal interactions in natural ecosystems has become a major research topic in plant science and agricultural food production (van der Putten et al. 2013). In this study we use the term endophyte to mean 'microorganism inhabiting the interior of plants irrespective of the function in association with the plant' (Brader et al. 2017). Endophytes constitute a major part of hidden fungal diversity inside of almost all plants (Peršoh 2013; Riess et al. 2014). Particularly, grasses have intimate symbiotic relationship with a diverse community of root endophytic fungi (Waller et al. 2005; Herrera et al. 2011; Khidir et al. 2010; Porras-Alfaro et al. 2014).

It is thought that virtually all land plants form symbioses with microorganisms. Plant-friendly-fungi, such as beneficial endophytes and mycorrhiza, contribute to plant biomass by influencing root development (Fidelibus et al. 2001; Franken 2012), extracting inorganic nutrients - primarily P - which are otherwise poorly accessible to plants (Raghothama1999; López-Arredondo et al. 2014), and protect them against pathogens and pests (Kogel et al. 2006; Pieterse et al. 2014; Shikano et al. 2017). In turn, plants supply symbiotic fungi with energy-rich carbohydrates produced by photosynthesis. Symbiosis enhances plant growth and health even under adverse nutrient-depleted soil conditions (Ngwene et al. 2016), stress (Porras-Alfaro and Bayman 2011) and pest infestation (Schouten 2016). Beneficial endophytes are microorganisms that live at the surface and/or within plant organs and colonize plant tissue without damage to their hosts (Hardoim et al. 2015). They have some practical and economical advantage over mycorrhiza fungi as they can be multiplied in axenic culture. Fungal endophytes also are capable of using recalcitrant substrates because of their wide-ranging enzymatic capabilities, and their ability to translocate nutrients through their hyphae (Mandyam and Jumpponen 2005). Controlled cultivation of plants in symbiosis with endophytic fungi might help in reducing the quantity of fertilizers and pesticides that need to be expended in sustainable agriculture.

### 1.1.2 Plant root endophyte diversity

Mutualistic fungi habitually residing in plant roots (Traveset and Richardson 2014) have commonly been associated with taxonomically and functionally different fungal

groups, including Glomeromycotan arbuscular mycorrhizal fungi (AMF, Peay 2016), ectomycorrhiza (Whitman et al. 1998), dark septate Ascomycota endophytes (Mandyam and Jumpponen 2005), and Sebacinalean Basidiomycota (Weiss et al. 2016). Most studies on beneficial root-colonizing fungi have been focused on AMF, because of their universality of the symbiotic partnership and ubiquity of their symbiotic associations (Treseder 2004), although non-AMF fungal symbiotic associations likewise modulate beneficial activities in their host plants (Franken 2012). Most beneficial non-AMF belong to Ascomycota, while beneficial Basidiomycota are rare (Rodriguez et al. 2009).

Root endophyte communities are commonly dominated by Ascomycota and less by Basidiomycota (Rodriguez et al. 2009; Toju et al. 2013; Khalmuratova et al. 2015). Apart from pathogenic ones, they have commonly been classified as either arbuscular mycorrhiza (AM, Glomeromycota), ectomycorrhiza (EM, Basidiomycota; Ascomycota), or non-mycorrhyal endophytes as dark septate endophytes (DSE, Ascomycota) and Sebaciniod endophyte (*Sebacinales*, Basidiomycota, e.g. *Piriformospora indica*, Weiss et al. 2016; Mandyam and Jumpponen 2005). Although, most studies on fungal root endophytes have focused on AM (Treseder 2004), both AM and non-AM fungal endophytes have ubiquitous symbiotic association with plants (Franken 2012). Indeed, non-AM fungi have the advantage of axenic propagation that place them as an ideal model of agri-horticulture application.

### 1.1.3 Plant microbiota

In this study we used the term microbiota to mean 'all microorganisms sharing a given environments' (Brader et al. 2017). During the past few years, analyses of the plant associated microbiota gave a new perspective in plant-microbe interaction. Amplicon sequencing of bacterial and fungal ribosomal genes and internal transcribed spacers and studies including the total genetic content of the root microbiota (microbiome studies) gave more detailed inside into the complex interaction of the plant microbiota with the plant host and their contribution to plants health (Bulgarelli et al. 2013). Several studies showed that the plant microbiota could be reshaped by environmental condition namely precipitation, temperature, and drought stress (Zimmerman et al. 2012; Fitzpatrick et al. 2018; Aydogan et al. 2018). However, to understand plant-

microbe interaction in detail the cultivation of key players of the plant associate microbiota for detailed plant-microbe interaction studies are required.

### 1.2 Plant-fungal endophyte interaction

Endophytic fungi are able to modulate plant growth and facilitate adaptation to environmental condition (Dovana et al. 2015). Particularly, DSE, common grasslands endophytes, may play an important role in ecophysiology of plants under environmental stress (Rodriguez et al. 2009). As an example, the DSE fungus *Curvularia* sp. provided thermal protection of its host plant at high temperature (Redman et al. 2002). Besides fungal endophytes have a significant contribution in biocontrol of plant pathogenic fungi (Andargie et al. 2017), insects (Greenfield et al. 2016), and nematodes (Bajaj et al. 2017). As an important case in point, *P. indica* as a model Basidiomycetes endophyte, has gained considerable interest (Weiss et al. 2016). *P. indica* (syn. *Serendipita indica*) is originally discovered in the Indian Thar desert (Verma et al.1998; Weiss et al. 2016). The fungus colonizes roots from a broad range of crop plants such as maize (Varma et al.1999), tobacco (Sherameti et al. 2005), barley (Achatz et al. 2010a, b) and wheat (Serfling et al. 2007), which leads to increasing biomass and grain yield.

Application of *P. indica* in agriculture has been well studied concerning plant growth promotion (Varma et al. 1999), facilitating mineral nutrient uptake (Achatz et al. 2010), inducing defence resistance against pathogens (Deshmukh and Kogel 2007; Rabiey et al. 2015; Serfling et al. 2007) and higher yield (Waller et al. 2005), thereby some plant-endophyte symbiotic mechanisms were identified (Deshmukh et al. 2006; Schäfer et al. 2009; Waller et al. 2008).

#### 1.2.1 Fungal endophytes and plant defence

Symbiotic microorganisms can directly mediate plant defence by producing special molecules that affect plant interaction with natural enemies (Fravel 1988). Interaction between plant immune system and endophyte is complicated. Endophytic fungi could reprogram plant gene expression, therefore induced systemic resistance (ISR) resulted. They could induced the different plant signalling pathway as salicylic acid

(SA), jasmonic acid (JA). At first step of interaction plant recognise endophytic fungus as biotrophic pathogens and activate the SA pathway (Pozo et al. 2015). In response the microorganisms produce some enzyme that suppress the SA pathway (Siciliano et al. 2007). Allowing to colonize host tissues and establish the symbiotic interaction (Jung et al. 2012). Particularly *P. indica* modulate gibberellin synthetic pathway to pass plant defence mechanisms (Schäfer et al. 2009). In addition, enhanced level of JA has been observed in symbiotic plants (Pieterse et al. 2012).

### 1.2.2 Fungal endophyte and global warming

Global warming is considered to increase the surface temperature in a range of 2.0 to 4.9°C by 2100 (Raftery et al. 2017). Increase in surface temperature could affect fungal community composition, hence plant-fungi interactions (Classen et al. 2015). Particularly, AM community composition in soil was significantly affected by high temperature but even so AM community composition of roots was not affected (Yang et al. 2013). Results from Free Air $CO_2$ Enrichment (FACE) experiments showed that elevated $CO_2$ ($eCO_2$), one of the main global warming factors, has minor effect on the fungal community composition, nevertheless, $eCO_2$ increases soil C availability, leads to increase fine-root production and root biomass increase (Norby and Zac 2011). $eCO_2$ is likely to affect mycorrhizal fungi communities to a greater extent than bacterial and saprotrophic fungal communities (Drigo et al. 2008), and likewise more than endophyte communities (Compant et al. 2010). Specifically, $eCO_2$ increased mycorrhizal density by 47% (Treseder 2004) and ectomycorrhizal density by 21% (Alberton et al. 2007).

## 1.3 Phosphorus a fundamental nutrient

Phosphorus (P), a crucial and often-limiting soil nutrient in nature, is confined to minerals, rocks and oceanic deposits (Ruttenberg 2003). Phosphate ions that are released into the soil after weathering are accessible to plants. Only inorganic orthophosphate ($P_i$, $PO_4^{3-}$), can be absorbed directly by the roots (Vazquez et al. 2000). Pi utilization creates a special zone in the rhizosphere, which is called Pi depletion zone, an area where active Pi acquisition takes place (Schachtman et al. 1998) but is not replaced at the same rate due to a low P diffusion rate. When plants

are unable to take up sufficient amounts of $P_i$, they undergo physiological and morphological changes, particularly root branching and lengthening of root hairs (Jiang et al. 2017; Raghothama 1999). P deficiency drives plants to devote more energy to reinforce $P_i$ acquisition, which leads to less energy being available for growth and reproduction and eventually resulting in less productivity (Mongon et al. 2017). Many biological molecules, particularly phospholipids, ATP, and nucleic acids contain P. They are present in many fundamental metabolic processes including carbon allocation, energy transfer, photosynthesis and respiration (Russell et al. 2016).

Total soil P content typically ranges from 100 to 2000 mg/kg of which only 10–15% is soluble P (Hinsinger 2001) and therefore sufficient P is often not available to the plant from the soil. Therefore, an adequate P application from an early stage of growth is essential for optimal crop production (Grant et al. 2001). However, the practice of supplying additional P has posed some environmental concerns such as under-ground water contamination, eutrophication of lakes and other water bodies. Moreover, a considerable reduction in the available phosphate resources is anticipated at the end of century (Akhtar et al. 2009), which in turn will mean that the cost of P will increase. In addition, the need to feed an increasing global population will lead to a higher demand for $P_i$ fertilizer and eventually will make producing crops more costly. Therefore, the development of new techniques to increase the efficiency of the supply and utilisation of inorganic $P_i$ for crop production is necessary. To manage $P_i$ depletion and overcome related problems, application of root colonizing endophytes that could multiply in axenic cultures for large-scale production has received particular attention. Fungal hyphae from such endophytes allow the plant to exploit a larger volume of soil (Schachtman et al.1998) and maximizing the total absorptive area (Johri et al. 2015). Moreover, endophytes acquire P from organic sources that are not available directly to plants (Behie et al.2013).

### 1.4 Endobacteria living in fungal hyphae

Interestingly, endophytic fungi such as some members of *Diversiporales* (Bianciotto et al. 2000) and *Sebacinales* (Sharma et al. 2008) often harbor endobacteria. Bacteria are key players in providing the largest reservoir of carbon, nitrogen, and phosphorus on the planet (Whitman et al. 1998). Despite their omnipresence, only approximately one percent of the existing bacteria are cultivable due to special nutrition requirement

or complex symbioses partnerships. The intimate relationship of beneficial fungi and endobacteria has attracted considerable attention (Salvioli et al. 2016; Glaeser et al. 2017). Phylogenetically diverse bacteria were detected in AMF including *Actinomycetales*, *Bacillales*, *Pseudomonadales*, *Burkholderiales*, *Rhizobiales*, and Mollicutes-related endobacteria (MRE) (Agnolucci et al. 2015). Particularly, MRE are widely distributed across Mucoromycota including *Glomerales*, *Endogonales* and *Mortierellomycotina* (Desirò et al. 2015; Bonfante and Desirò, 2017; Desirò et al. 2018). The photosynthetic and nitrogen-fixing cyanobacteria *Nostoc* spp. colonizes the fungal bladders of *Geosiphon pyriforme* (Schüßler et al. 1994; Gehrig et al. 1996), and Sebacinalean fungi are associated with Gram-stain positive bacteria of the genera *Paenibacillus* (Firmicutes) and *Rhodococcus* (Actinobacteria) and Gram-stain negative bacteria of the genera *Acinetobacter* (Gammaproteobacteria) and *Rhizobium*/*Agrobacterium* (Alphaproteobacteria) (Sharma et al. 2008, Glaeser et al. 2016; Guo et al., 2017). Besides endobacteria seem to be predominant in saprophytic, *Mortierella* (Uehling et al. 2017), and pathogenic, *Rhizopus* (Partida-Martinez et al. 2007), species of Mucoromycota.

### 1.4.1 Role of endobacteria in plant fungal interaction

Endobacteria improve the interplay between fungi and their host plants in diverse manners (Kobayashi and Crouch 2009). Curing the spores of *Gigaspora margarita* from the endobacterium *Candidatus* Glomeribacter gigasporarum (*Ca*Gc) strongly restrained fungal growth and altered hyphal wall and vacuole morphology (Lumini et al. 2007). Moreover, *Ca*Gc improves fungal fitness by priming mitochondrial metabolic pathways thereby increasing AMF success to face environmental stresses (Salvioli et al. 2016) as well as *Mycoavidus cysteinexigens* significantly altered fungal host metabolism (Uehling et al. 2017). Similarly, the Alphaproteobacterium *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*, "*Agrobacterium fabrum*") strain F4 (RrF4) isolated from the Sebacinalean basidiomycete *P. indica* improves plant growth and systemic resistance to fungal and bacterial pathogens (Sharma et al. 2008; Glaeser et al. 2016). Comparative studies of bacteria-depleted *P. indica* cultures and bacteria-rich subcultures that were freshly isolated from plant roots suggested that mycelia with high amounts of RrF4 cells were more efficient in supporting plant growth and resistance (Guo et al. 2017). An example for endobacteria supporting fungi in

pathogenic interactions is the toxin-producing *Burkholderia* endosymbiont of the *Mucorales* fungus *Rhizopus microsporus*, the causative agent of rice seedling blight (Partida-Martinez and Hertweck 2005), and the endobacterium *Enterobacter* sp. of *Rhizoctonia solani* AG 2-2IIIB, which increased fungal virulence in creeping bentgrass (Obasa et al. 2017). Besides, the nematode-biocontrol fungus *Esteya vermicola* is associated with an endobacterium identified as *Pseudomonas stutzeri* (Wang et al. 2017). Considering the high diversity of the so far known different fungus - endobacteria systems, it is plausible that high abundance of those symbioses may be exist.

### 1.5 Objectives

Here we investigated root endophytic fungi of the permanent grassland from the „Environmental Monitoring and Climate Change Impact Research Station Linden“ located near Giessen, Germany. This site is a semi-natural permanent grassland typical for German lowlands, hosting two long-term climate change experiments, a FACE experiment increasing the atmospheric $CO_2$ concentration during day light hours to a level of +20% relative to ambient concentration since 1988 and a warming experiment using IR lamps to increase soil surface temperature to +2°C relative to ambient from 2008-2014. Our aim was to isolate new fungal endophytes from the ecologically sustainable semi-natural grassland for introducing them for further research aiming sustainable agriculture with respect to future climate conditions. Therefore, it was aimed to isolate fungal root endophytes from plants exposed to long term $eCO_2$ (>14 years) and increased temperature (+2°C, 6 years). Illumina 18S - 28S rRNA gene internal transcribed spacer 2 (ITS2) amplicon sequencing was used to determine the relative abundance and specificity of the isolated fungi in the fungal microbiota present in plant roots grown under ambient and changed environmental conditions for selected samples.

In addition, we describe the isolation and characterization of two fungal strains identified as *Trametes versicolor* (*Tv*-WC16GW) and *Stereum hirsutum* (*Sh*-P2A), which represent two endophytic basidiomycete fungi isolated from the dicotyledonous *Galium album* and the monocotyledonous *Arrhenatherum elatius*, respectively, grown in a permanent grassland ecosystem. *T. versicolor* and *S. hirsutum* have been known

as white rot fungi, which decompose wood cell wall polymers, including lignin, in forest habitats (Shirkavand et al. 2017), and both fungi cause peach scaffold branch decay (Adaskaveg et al. 1993). Nevertheless, they also grow symptomless in roots (*S. hirsutum* F66) and aerial organs such as glumes (*S. hirsutum* F66, *T. versicolor* F72) of wheat (Comby et al. 2016). Moreover, bioactive compounds of *T. versicolor* strain C inhibited aflatoxin production by different *Aspergillus* species (Scarpari et al 2017) and *S. hirsutum* (Sh134-11) secondary metabolites showed antifungal activity against *Botrytis cinerea* (Aqueveque et al. 2017). These data together let us reason that both fungi – at least under yet unknown conditions - exhibit beneficial activities. Endobacteria have not been reported for those fungi. Inspired by the recent findings that beneficial fungi often contain endobacteria, hence to search for endobacteria in the fungal mycelia different detection and visualization methods including 16S rRNA gene amplification and sequencing, fluorescence in situ hybridization (FISH) with universal Bacteria 16S rRNA probes and transmission electron micrograph (TEM) were applied.

In addition, we compare P. *indica's* activity to enhance wheat biomass and grain yield under different inorganic P sources with *T. versicolor*, a basidiomycete endophyte, we isolated from roots of *G. album* at Linden permanent grassland near Giessen under semi natural experimental conditions.

## 2 Material and Methods

### 2.1 Study site

Field samplings took place at the „Environmental Monitoring and Climate Change Impact Research Station Linden" near Giessen in the Giessen Free Air $CO_2$ Enrichment (GiFACE) facility and a warming experiment in permanent temperate grassland. The research station is located at 50°32'N and 8°41.3'E, 172 m above sea level. The mean annual temperature is 9.4°C with mean annual precipitation of 560 mm for the period 1995-2014.

The warming experiment started in 2008 and took place at a 100 $m^2$ site divided into 16 equally sized plots. Each plot was under the influence of one IR lamp elevating the temperature of both the plant and the soil surfaces (Jansen-Willems et al. 2016). The

plant roots were sampled in 2014 at 4 plots with +2 °C increased plant and soil surface temperature (T) plots and from four respective ambient temperature (C) plots.

The set-up and performance of the GiFACE system has been described in detail by Jäger et al. (2003) and Andresen et al. (2017). In brief, from May 1998 until present, atmospheric $CO_2$ concentrations were enriched by 20% above ambient, all-year-round during day light hours.

The $CO_2$ enrichment was applied in three circular plots, each 8 m in diameter (eCO2). Three equally sized control plots were maintained at ambient atmospheric $CO_2$ levels ($aCO_2$). The soil of the study site is classified as a Fluvic Gleysol (FAO classification) with a texture of sandy clay loam over a clay layer (Jäger et al. 2003). The experimental design was a randomized block design. A block consisted of two plots to which either ambient ($aCO_2$) or elevated ($eCO_2$) treatments were randomly assigned. A characteristic attribute of the study site is a soil moisture gradient, resulting from a gradual terrain slope (2-3°) and varying depths of a subsoil clay layer. Within each of the three blocks, soil moisture conditions were relatively homogeneous, small moisture differences between blocks may occur during summer, while in the rest of the year the water table is close to the soil surface. Volumetric soil water content of the 0-15 cm soil depth was measured daily with four permanently installed TDR probes (Imko, Germany, type P2G) per plot.

The vegetation is an Arrhenatheretum elatioris -Filipendula ulmaria subcommunity, dominated by *Arrhenatherum elatius*, *Galium album*, and *Geranium pratense*. At least 12 grass species, 15 non-leguminous herbs and up to 5 legumes with small biomass contributions (<5 %), are present within a single plot. The grassland has not been ploughed for at least 100 years, being managed as a hay meadow with two cuts per year, with granular mineral calcium-ammonium-nitrate fertilizer applied at the rate of 40 kg N $ha^{-1}$ $yr^{-1}$ in mid-April. Before 1996, fertilizer was applied at a rate of 50–100 kg N $ha^{-1}$ $yr^{-1}$ (Kammann et al. 2008) and details in (Andresen et al. under review). Meteorological data were available from to meteorological stations at the field site.

## 2. 2 Isolation of fungi and endobacteria

### 2.2.1 Fungal endophyte isolation

Endophytic fungi were isolated from root samples of two dominant plant species *A. elatius* (family: *Poaceae*) and *G. album* (family: *Rubiaceae*) which were collected in spring 2014.These samples were taken with a soil corer between 0.0 - 7.5 cm depths and placed on ice. At the laboratory, plant specific roots were carefully separated from soil and other roots of the dense grassland root mad. The roots were selected for analysis if they appeared healthy, exhibited no obvious lesions, and were connected to the green tissue of the respective plants. The samples were rinsed with tap water over a mesh screen. Parts of the roots were directly frozen in liquid nitrogen and stored at -80 °C before further molecular analysis. For the cultivation of fungi, once free of soil, the roots from each plant was cut into fragments then surface sterilized by 20 min incubation in 3% sodium hypochlorite solution followed by washing with distilled water one-time, incubating in 70% ethanol for 1 min and washing 3 times with sterile distilled water (Imani et al. 2011). Root segments were placed on MYP agar medium (per 1 L: 7 g malt extract, 0.5 g yeast extract, 1.5 g proteose peptone, and 13 g agar) cultured at 24 ˚C for two weeks. Each growing fungus was purified by transferring hyphal tips onto a fresh plate. Isolates were kept in solid complete medium (CM, Pham et al. 2004) and potato dextrose agar (PDA, per 1 L: potato extract 4 g, dextrose 20 g, agar 14 g), for laboratory use (sub-cultured every 3 months), and liquid medium at 4˚C for long term preservation (sub-cultured every 1.5 year).

### 2.2.2 Isolation of endobacteria

Mycelia of 14-day-old *T. versicolor* and *S. hirsutum* cultures were crushed in Gamborg B5 medium (Duchefa Biochemie, the Netherlands) supplemented with 0.45 M mannitol using a fine blender. Homogenate was filtered through a miracloth (22–25mm) filter and centrifuged at 100 g for 7 min. The supernatant was collected and subsequently centrifuged at 3200 g for 10 min. Therefore, the final pellet was suspended in LB medium (Per 1 L: D-Glucose 1g, Proteose peptone 7g, Soya peptone 8g, Sodium chloride 6g, Yeast extract 3g, Beef extract 3g) containing 0.8 % sucrose and 2.5% (v/v) fungal extract (25 g crushed mycelium suspended in 25 ml, filter sterilized). For anaerobic cultivation some LB medium tubes were covered with paraffin.

### 2.3. Microscopically analyses

#### 2.3.1 Light microscopy

Cultural characteristics such as colony appearances, hyphal shaped and pigments, presence of septa, conidiation, conidia shape and colour of some isolated endophytic fungi were observed by light microscopy using a Leica DM lL microscope (100 x magnification).

#### 2.3.2 Transmission electron microscopy (TEM) analysis

To detect the presence of bacteria in the mycelium of fungi by TEM, fungal mycelium was fixed in 2% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 and then embedded in gelatine (Fluka, Germany), post fixed in 1% osmium tetroxide washed and incubated in 1% aqueous uranylacetate (Polysciences) overnight at 4°C. Specimens were dehydrated in an ethanol series [30, 50, 70, 80, 90, 96, 100% (v/v), 20 min each] and embedded in Spurrs resin (Serva, Heidelberg, Germany). From the blocks cured by heat ultrathin sections were cut and finally contrasted in uranyl acetate and lead citrate. Ultrathin sections were inspected in the TEM (EM912a/b – ZEISS, Germany) at 120 kV under zero-loss conditions and images were recorded at slight under focus using a cooled 2k x 2k slow-scan ccd camera (SharpEye/ TRS, Germany) and the iTEM package (Olympus-SIS, Germany).

#### 2.3.3 Microscopically observation of the fungal root colonization

Hyphae in natural root segment, of *G. album* and *A. elatius* obtained from Giessen grassland, were stained with the chitin-specific binding wheat germ agglutinin (WGA) conjugated with Alexa Flour ® 448 (Thermo Fisher Scientific, US). Root material was fixed and stored in a solution containing 20% (v/v) chloroform 80% (v/v) ethanol and 0.15% (w/v) trichloroacetic acid for years. Before staining fixed roots were washed 3 times for 5 min with deionized water, then treated with 10% (w/v) potassium hydroxide (Kohler et al. 2015) by boiling for 30 sec, and then rinsed 3 × 10 min with phosphate buffered saline (PBS: KCl, 0.2 g; $KH_2PO_4$, 0.2 g; $Na_2HPO_4$, 1.15; $H_2O$, 1 l; pH 7.4). Then the root fragments were incubated in 5 ml PBS containing 10 µg WGA Alexa Flour 488 $ml^{-1}$ and 0.02% (v/v) Silwet L-77. During the incubation, the roots were stained with vacuum infiltration three times for one minute and kept in staining solution for 10 min. After washing with 1 x PBS buffer, the roots were incubated in 20 µM

propidium iodide solution (Sigma-Aldrich, US) in PBS for 10 min; then roots were analysed by confocal laser scanning microscope Leica TCS SP8.

### 2.4 molecular biology methods

#### 2.4 1 fungal genomic DNA extraction

For the phylogenetic identification of fungal isolates genomic DNA was extracted from pure cultures of fungal mycelia grown in liquid MYP medium for 2 weeks at 25°C and ground to a fine powder with liquid nitrogen using a mortar and pestle (sterilized for 3 h at 200˚C and subsequently one hour by UV-C exposure). For DNA extraction 400 mg of each sample was mixed with 700 µl of pre-heated (60°C) CTAB (cetyl trimethylammonium bromide) extraction buffer in the presence of β-mercapto-ethanol and was incubated at 60°C for 20 min. Sample was extracted with adding 700 µl of chloroform-isoamyl-alcohol (24:1, v/v) and centrifuged at 10000 rpm for 15 min at room temperature. Aqueous phase was transferred to a new tube and sample re-extracted by adding 600 µl CIA and repeating centrifuge. Aqueous phase was transferred to a new tube and DNA was precipitated by the addition of 500 µl ice-cold isopropanol. After incubation on ice for 20 min, precipitated DNA was collected by centrifugation at 15000 g for 10 min. pellet was washed with 500 µl ethanol (70%) for 10 min by shaking and centrifuge at 15000 g for 10 min again. DNA pellet was dried by removing supernatant and let ethanol to evaporate for 1 h. Pellet was dissolved with 30 µl TE buffer.

#### 2.4.2 DNA extraction from root samples for Illumina amplicon sequencing

For the cultivation-independent analysis of fungal root endophytes approximately 500 mg of frozen surface disinfected root samples of the same host plants of C and T plots that had been used freshly for culture-based approach, were used for DNA extraction with the Nucleospin® soil (Macherey Nagel) DNA extraction kit as described by the manufacturer. Finally, the DNA was eluted in 50 µL DNase and RNase free water, quantified spectrophotometrically using a Nano Drop and diluted to a DNA concentration of 5 ng $\mu L^{-1}$.

### 2.4.3 DNA extraction for chloroplast detection

The genomic DNA of *A. elatius* was extracted by NucleoSpin Plant II kit (Macherey-Nagel) and the *G. album* DNA by ZR Plant/Seed DNA MiniPrepTM kit (Zymo Research).

### 2.4.4 Polymerase chain reaction (PCR)

The conventional amplification was performed in a PCR Thermo cycler (Biometra, GmbH, Germany). GoTag®DNA polymerase, was used to amplify genes of interest from different DNA templates. The standard PCR mixture is given below. The elongation time normally depends on the size of the PCR product (1kb/min), while the annealing temperature is adjusted to the primers.

| GoTag DNA Polymerase | PCR mixture |
|---|---|
| 5×Buffer | 2 µL |
| dNTPs 2 mM | 0.5 µL |
| MgCl2 25 mM | 1.5 µL |
| Fwd primer 10 µM | 0.3-1.0 µL |
| Rev primer 10 µM | 0.3-1.0 µL |
| DNA Polymerase | 5 u/µL 0.25 µL |
| Template<br>dist. H2O | 1-3 µL<br>Up to 25 µL |

#### 2.4.4.1 Fungal DNA PCR

The 18S rRNA small subunit (SSU) region and the internal transcribed spacer (ITS) region (including the 5.8S gene) between the fungal 18S and 28S rRNA genes (Badotti et al.2017) were amplified by PCR using fungal universal primers NS1 (5´-GTAGTCATATGCTTGTCTC-3´) and NS5 (5'-AACTTAAAGGAATTGACGGAAG-3′) / ITS4 (5′-TCCTCCGCTTATTGATATGC-3′, White et al.1990). PCR products were purified using a PCR purification kit (Qiagen, DE) and sequenced with the Sanger sequencing method using primers NS1, NS5, and ITS4 by LGC Genomics (Berlin).

#### 2.4.4.2 Plant genomic DNA PCR for fungal endophyte detection

The ITS2 region of fungi was amplified with primers ITS7F (5′-GTGARTCATCGAATCTTTG-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) and was sequenced at IGC Genomics (Berlin, Germany) with the 300 bp paired-end read method using an Illumina MiSeq V3 sequencing method.

#### 2.4.4.3 Fungal genomic DNA PCR for detection of endobacteria

Amplification of the 16S rRNA gene was carried out using the bacterial universal primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′, Lane 1991). PCR amplification was performed in a total volume of 50 µl containing PCR Master Mix (Fermentas, Life Sciences, St Leon-Rot, Germany), 150 to 200 ng of DNA and 2 µM of each primer. After an initial denaturation step at 95°C for 5 min, 34 cycles with denaturation at 95°C for 1 min, primer annealing at 59°C for 1 min, elongation at 72°C for 1 min 45 s and a final extension at 72°C for 10 min were performed. PCR products were purified using a Gel Extraction Kit (Promega, Mannheim Germany) and sequenced.

#### 2.4.4.4 Plant genomic DNA PCR for plant-derived chloroplast detection

Chloroplast sequences (Chloroplast _2, Chloroplast_4) of the two plants species, *G. album* and *A. elatius,* were amplified with universal bacterial 16S rRNA gene targeting primer system (GC-339F / 907R, Muyzer et al. 1993). PCR fragments were separated by denaturing gradient gel electrophoresis (DGGE).

### 2.4.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a technique to separate DNA fragments with the same length but different G+C content and nucleotides according to the mobilities under the increasing gradient of formamide and urea based denaturing gel conditions. DGGE as community fingerprint pattern can be used to characterize microbial populations in the environment (Muyzer and Smalla 1998). The DNA used for DGGE analysis was extracted by NucleoSpin® Soil Kit (Macherey-Nagel, Germany) with adjusted protocol as described in 2.4.2. The bacterial 16S rRNA gene was used as target gene. PCR products 25 µl mix with 5 µl loading dye were loaded on a pre-warmed polyacrylamide gradient gel. Polyacrylamid gels containing formamide and urea (40-70%) were used for the DGGE analysis. The solution A, solution B and stacking gel were prepared from

0% and 80% stock solutions supplemented with TEMED and APS. Then solutions were poured in the front chamber through a pipe connected with a pump (Minipuls 2, Gilson. Inc). The casting was stopped before the gel reaching the comb. Stacking gel was pipetted on the top of the gel to generate slots on the DGGE gel. The comb was taken out after one h, the gel was stored over night at 4 ºC. The electrophoresis was performed in a tank containing 17 L pH 7.4 1×TAE buffer. The samples were loaded after the gradient gel was pre-warmed up to 60 ºC under 100 V. The electrophoresis was performed with the parameters 100 V, 60 ºC and 22 h. The bottom glass plate attached with gel was stained in ethidium bromide (30 µL ethidium bromide mixed with 500 mL pure water) for 20 min and washed in pure water for 10 min. The gel images were taken with imaging system software (Fluor-STM MultiImageer, BioRad) by exposure under UV 10-40 seconds.

| Loading dye | |
|---|---|
| Glycerol | 7 mL |
| Bromphenol blue | 250 µL |
| Xylene cyanol | 250 µL |
| DNase and RNase free water | 2.5 mL |

| 50×TAE buffer (pH 7.4) | |
|---|---|
| Tris base | 242 g |
| Glacial acetic acid | 57.1 mL |
| EDTA (0.5M, pH 8.0) | 100 mL |
| Pure water | Up to 1000 mL |

| Stock solution 80% (150mL) | |
|---|---|
| Urea | 50.4 g |
| Formamide | 48 mL |
| 50 TAE 40% | 3 mL |
| Acrylamid/bisacrylamid | 26.25 mL |

| Stock solution 0% (150 mL) | |
|---|---|
| 50 TAE | 3 mL |
| 40% Acrylamid/bisacrylamid | 26.25 mL |

| Stacking gel | |
|---|---|
| Stock solution 0% | 9 mL |
| Stock solution 80% | 0 |
| TEMED | 8 µL |
| APS (10%) | 100 µL |

### 2.5 Sequence analysis and alignment tools

#### 2.5.1 Molecular identification of endophytic fungi

Template DNA was sent to Sanger dideoxy sequencing separately with primers ITS4, NS5, and NS1. Sequenced template DNA's were manually corrected based on the electropherograms and merged in MEGA 6 (Tamura et al. 2011). The corrected 18S rRNA gene sequence including the ITS1-5.8S-ITS2 sequences were placed into

GenBank (accession numbers were mentioned in Table 1 and 2). A first phylogenetic identification of the isolates was performed by BLAST analysis of the sequences against the GenBank database. GenBank entries included into the analysis were checked for their reliability according to recognition to species level and publication status. Sequence with ≥80% query coverage and similarity ≥99%, was assigned to the same species (Raja et al.2017)

### 2.5.2 Phylogenetic identification of uncultured endofungal bacteria

Genomic DNA extracted from two-week-old mycelium with the CTAB method was used to detect endofungal bacteria. Sequences were assembled with MEGA6 and compered with the deposited 16S rRNA gene sequence in EzBioCloud database (Yoon et al. 2017). Reference phylogenetic analysis was performed in MEGA6. A maximum likelihood (ML) tree was constructed using the Kimura's 2-parameter distance model with uniform rates between sites to generate the similarity matrix. Support for specific nodes on ML tree was calculated with 1,000 replications (bootstrapping) to assess the relative stability of the branches.

### 2.5.3 Identification of plant-derived chloroplast

For sequence analysis, chloroplast bands were cut out from the DGGE gel and purified as described in 2.4.5. DNA was reamplified with primer system GC-339F (5´-CTCCTACGGGAGGCAGCAG-3´) and 907R (5´-CCGTCAATTCMTTTRAGTTT-3´) and controlled by DGGE. Sequencing was performed by LGC Genomics (Berlin, Germany) with the Sanger method using primer 341F (5´-CCTACGGGAGGCAGCAG-3´; Muyzer et al. 1993) sequences were corrected using Chromas and MEGA6 (Tamura et al. 2013) and identified in the 16S rRNA gene sequence database of EzBioCloud (Yoon et al. 2017).

### 2.5.4 Illumina sequences analyses

Sequences were demultiplexed using the Illumina's CASAVA data analysis software. After adaptor and primer clipping and discharge of sequences <100 bp forward and reverse reads were combined using BBMerge 34.48 (http://bbmap.sourceforge.net/) and subjected to further analysis. For quality control and taxonomic classification, sequences were imported into MGX (Jaenicke et al. 2018) and analysed using a data processing pipeline based on mother (Schloss et al., 2009) and the UNITE database;

for this, 100 bootstrap iterations and a cutoff of 70 was used. Absolute sequence numbers and relative abundances per taxonomic paths, were used for statistical analysis. Richness and diversity of the fungal endophyte community were calculated with the Chao 1, Shannon, evenness, and dominance indices (Harper, 1999) and rarefaction curve analysis (Krebs, 1989) considering the number of taxonomic paths and the number of individual reads per taxonomic path. Analysis was performed in PAST3 (Hammer et al. 2001).

### 2.4 Plant material and growth condition

Wheat (*Triticum aestivum*) cv. Bobwhite and *Arabidopsis thaliana* (ecotype Colombia-0, N10192) were used in this research.

#### 2.4.1 Germination of seeds

Kernels of wheat cv. Bobwhite were sterilized with 3% (v/v) NaOCl for 1 h and 70% (v/v) ethanol for 1 min, then rinsed with sterilized water 3 times for 5 min and germinated for 4 days on wet-sterilized filter paper in glass jars at 23°C and under 8 h light.

*A. thaliana* seeds were sterilized with 70 % ethanol for 90 s and 0.5 % sodium hypochlorite plus 0.01% Tween 20 for 5 min. After four washing steps, first with pH 3 water then in distilled water, seeds were sown on circle petri dishes filled with ½ MS medium (Per 1 L: MS 2.20 g, Sucrose 5 g and pH 5.8) plus 0.35% (w/v) gelrite (Duchefa) for germination.

#### 2.4.2 Root inoculation

The fungal strains *P. indica* DSM 11827 was freshly re-isolated from barley roots (Guo et al. 2017), *Tv*-WC16GW, and *Sh*-P2A were grown in MYP broth for four weeks at 21˚C in the dark. Roots of 4 days wheat seedlings were inoculated in a solution of sheared mycelium that was prepared by shaking 15 g of mycelium in 15 ml sterile purified water for 3 × 15s. Inoculated seedlings were transferred to pots containing heat sterilized (121°C for 1 min at 15 psi) Linden grassland subsoil. An experiment was arranged in a randomized design with four replicate per treatment for a total 16

pots (3 plants in each pot). Plants were grown for four weeks in a growth chamber under 60% relative humidity and a photoperiod of 16 h.

*Arabidopsis* seedlings, 10 days after germination, were dip inoculated in *Sh*-P2A crushed mycelium (1 g $ml^{-1}$) for 1.5 h. Subsequently, seedlings were transfer to square agar plates (10 ×10 mm) containing ½ MS medium with 0.5% (w/v) sucrose plus vitamins and stratified at 4 °C for 48 h. Afterwards, plants were grown under photoperiod of 16 h light/8 h darkness with a light intensity of 183 µM $m^{-2}$ $s^{-2}$, and a temperature of 22°C. Plates were placed at an angel of 75° to allow root growth along the agar surface and to proper aerial growth of the hypocotyls.

### 2.4.3 Mitscherlich pots experiments growth condition

An experiment in Mitscherlich pots was arranged in a completely randomized design with four replicates per treatment [endophyte (2) x P treatment (2)] for a total of 80 pots. Pots were prepared by combining 6 kg of subsoil (0.5–1.0 m depth), from a Luvisol derived from loess (44.92% sand, 34.71% silt, 20.38% clay, pH in 0.01 M $CaCl_2$ 6.4, 0.17% total C, 0.03% total N and 6.3 mg CAL-P $kg^{-1}$ soil), taken from Linden, Giessen, Germany, with the inorganic P sources and basic fertilizer nutrients. P treatment was applied by adding 100 mg P $kg^{-1}$ soil of the inorganic P forms: mono-calcium phosphate [Ca $(H_2PO_4)_2$, CP]. Soils receiving no additional P served as a control (CO treatment). Additional macro- and micronutrients were supplied per kg of soil as follows: 1 g N ($NH_4NO_3$); 3.6 g K ($K_2SO_4$ & KCl); 0.16 g Mg ($MgSO_4$); 3 mg B ($H_3BO_3$); 30 mg Cu ($CuSO_4$); 120 mg Mn ($MnSO_4$); 0.7 mg Mo [$(NH_4)_6Mo_7O_{24}$] and 0.65 mg Zn ($ZnSO_4$).

### 2.4.4 Plant development and yield parameter analysis

During growth, the number of tillers per plant and the number of ears per plant were determined 5 and 9 weeks, respectively, after planting. Plants were harvested 11 weeks after planting, when the development of the ears was complete, and their colour changed to yellow. Afterwards, grain (dry weight) and straw (fresh weight) were measured by weighting plant samples. Moreover, 1000 grain weight and bran (fresh weight) were measured only for *T. versicolor*-colonized plants.

#### 2.4.4.1 Phosphorus concentration analysis

The total concentration of P in the wheat biomass (straw and grain) was determined by a dry ashing method. Dry plant samples (0.8 g) were ashed in a porcelain crucible in a muffle furnace at 550 °C overnight. The ash was moistened with 2 ml of demineralized water. The P concentration was measured as an NH4-molybdovanado-complex at 450 nm after adding 2.5 mL of 5 M HNO3, boiling for 1 min and filtering (Gerike and Kurmies1952). The P contents of wheat straw and wheat grain were calculated by multiplying the P concentration (mg/g of plant biomass) with the grain (or straw) yield weight (g/plant).

## 2.5 In situ detection of bacteria and fungi

### 2.5.1 Wheat germ agglutinin (WGA) staining

WGA is a specific plant lectin that strongly binds to chitin (Meyberg 1988). Roots of wheat plants (0.1 g) were fixed for 24 h in a solution containing 20% (v/v) chloroform, 80% (v/v) ethanol, and 0.15% (w/v) trichloroacetic acid. Before staining, fixed roots were washed 3-5 min with distilled water, cleared with 10% (w/v) potassium hydroxide by boiling for 30 seconds (Kohler et al. 2015), and rinsed 3 times for 5 min with phosphate buffered saline (PBS; KCl 0.2 g, $KH_2PO_4$ 0.2 g, $Na_2HPO_4$ 1.15 g, $H_2O$ 1 liter; pH 7.4). Roots were then incubated in 5 ml PBS containing 10 µg $ml^{-1}$ WGA-Alexa Flour 488 (Thermo Fisher, Ohio, USA) and 0.02% (v/v) silwet L-77 (Momentive, New York, USA). The roots were stained with vacuum infiltration three times for one minute and kept in staining solution for an additional 10 min. After washing with 1x PBS buffer, roots were analyzed by fluorescence microscopy.

### 2.5.2 Detection of endobacteria by fluorescence in situ hybridization (FISH)

FISH was performed as described by Manz et al. (1992). To detect *Bacteria* in general, a universal EUB 338 probe mixture, which consists of probes (EUB 338: 5'-GCT GCC TCC CGT AGG AGT-3', EUB 338-II: 5'- GCA GCC ACC CGT AGG TGT -3' and EUB 338-III: 5'- GCT GCC ACC CGT AGG TGT -3', Amann et al. 1990; Daims et al. 1999) was applied and analyzed by fluorescence microscopy. Probes were labelled by fluorescein dye.

Two to four-week-old pure fungal cultures were fixed with 4% paraformaldehyde solution. One volume of samples was mixed with 3 volumes of fixation solution and

incubated at 4°C for 16 h. Fixed samples were washed with PBS (16,000 g, 2 min, 4°C) to remove residual fixation solution and finally re-suspended in a 1:1 mixture of 1x PBS and 99.8% ethanol. Samples were then stored at -20°C before further processing. Formaldehyde solution fixed fungal samples were spread on a gelatin-coated glass slides (Paul Marienfeld, Germany) and dried for 10 min at 37°C to improve the attachment of the samples. A modified permeabilization protocol was used to enhance penetration of the probes into the cells. Samples were treated by applying 15 µl of chitinase (1 mg $ml^{-1}$), 10 µl of lysozyme solution (10 mg $ml^{-1}$), 10 µl proteinase K (10 µg $ml^{-1}$) for 20 min at 37°C and 0.01 M HCl for 10 min at 20°C, respectively. After each permeabilization treatment slides were dipped into ice-cold 1x PBS and air dried. Prior to the hybridization fixed fungal samples were dehydrated by dipping the slides into an ethanol series (50, 80 and 96% ethanol, 3 min each) and air dried. Each sample was hybridized with 10 µl of hybridization buffer containing 900 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.01% SDS, a formamide concentration of 35% and probes in a ratio of 10:1 (v/v) for 90 min at 46°C. For hybridization, the slides were incubated with pre-warmed washing buffer containing 20 mM Tris-HCl, 0.01% SDS, 5M EDTA (pH 8:0), NaCl, and 35% formamide concentration for 15 min at 48°C. In the following step, the slides were washed with autoclaved pure water and dried in the dark. Samples were counterstained with DAPI (2.5 µg $ml^{-1}$) for 10 min and dried again in the dark at room temperature. Slides were embedded with antifading reagent (Citiflour, London, UK) and sealed with a cover glass for fluorescence microscopy. Slides were analyzed with a fluorescence microscope (Leica Microsystems DFC3000G, Wetzlar, Germany) with a magnification of 1,000-fold.

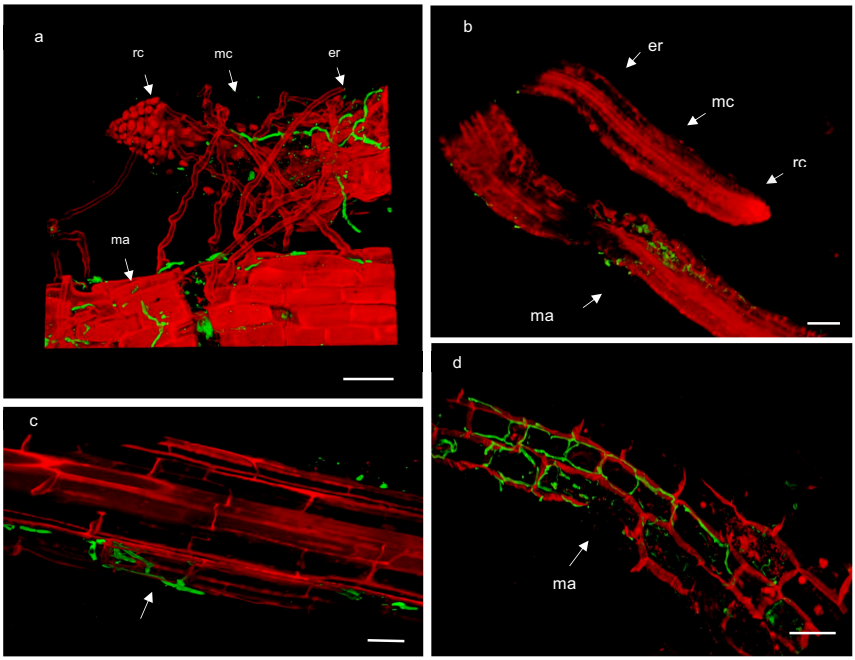
**2.6 Statistical analysis**

First data were checked for normal distribution, then one-way ANOVA was performed using IBM SPSS version 19. The significance of the treatment effects was judged by the magnitude of the F-value ($P \leq 0.05$). Then Fishers protected LSD was performed for separation of means.

## 3 Results

### 3.1 Colonization pattern of endophytic fungi on natural roots

Randomly selected roots of *G. album* and *A. elatius* were stained with wheat germ agglutinin (WGA) to visualize fungal hyphae and with propidium iodide for root cell detection. The root cap zones of both plants and the meristem and elongation zone of *G. album* were free of hyphae (Fig 1 a and b). However, several fungal hyphae were detected at the root surface of the elongation and maturation zone of *A. elatius* (Fig. 1 a). In both plants, fungi colonized the root surface and had penetrated into the epidermal cell layer with inter/intra cellular hyphae; within the maturation zone fungi colonized sub-epidermal and outer cortical layers (Fig 1 c and d). Intracellular hyphae branched and proliferated within root cells and passed from cell to cell (Fig 1 e and f). Root cortex colonization was not observed. Putative endophytic fungi also produced spores at the surface of root (Fig 1 g).



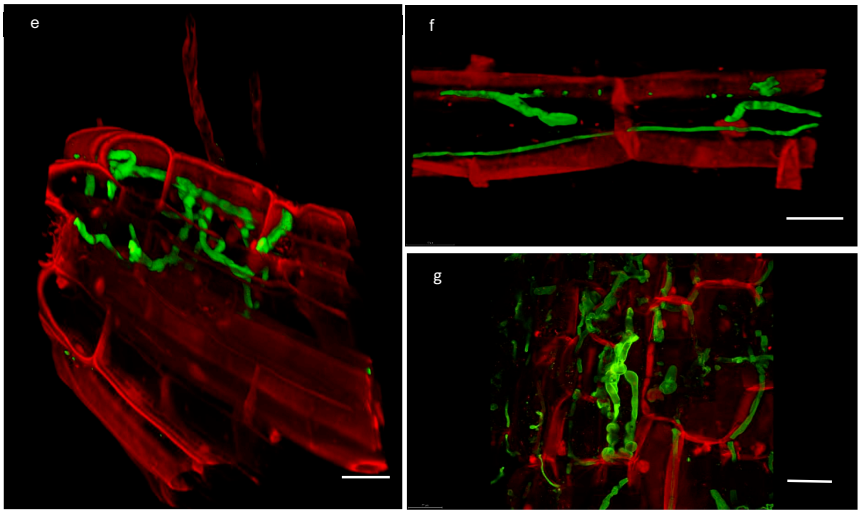
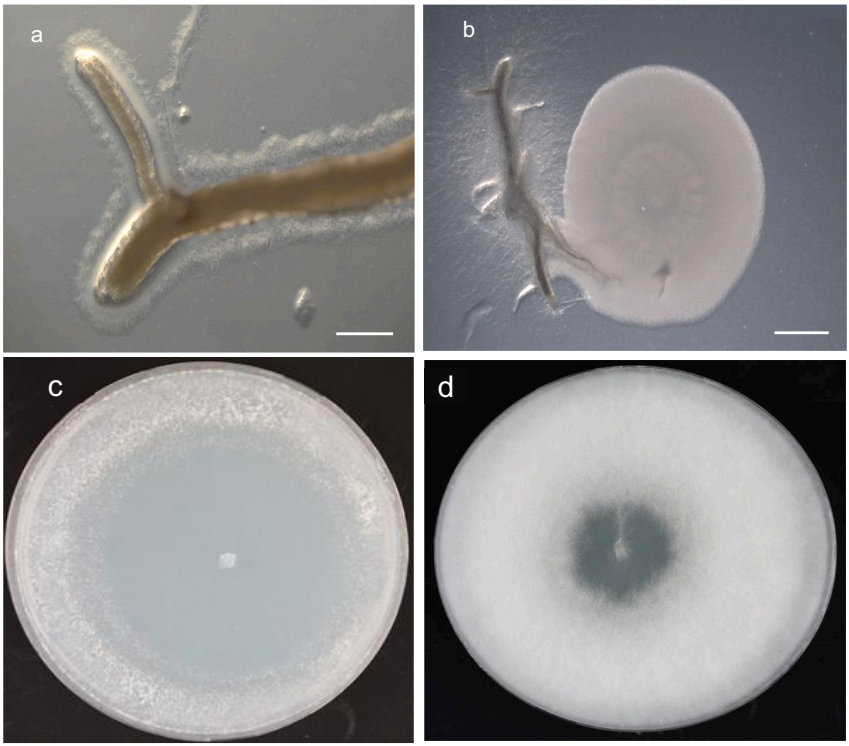



Fig. 1 Fungal colonization of randomly selected natural roots of *Galium album* and *Arrenatherum elatius* of Giessen grassland using WGA staining and propidium iodide. **a** Root cap zone (rc) of *A. elatius*; and **b** root cap zone, meristem zone (mc), and elongation zone (el) of *G. album* are free of hyphae, except maturation zone (ma). c *A. elatius*, d *G. album,* root surface hyphae, after penetration, colonizing sub-epidermal layer. **e** *A. elatius* **f.** *G. album*, Intracellular hyphae branching and proliferation within sub-epidermal space then passed from one sub-epidermal cell to another. **g** spore production at the surface of *G. album* root. Scale bar, a, d 100 µm; e 50 µm; f 10 µm; b, c, g 20 µm. (Images were provided with cooperation of Dr. Jens Steinbrenner)

### 3.2 Fungal endophytes isolated from plants at Giessen grassland

For the isolation of endophytic fungi, 150 surface sterilized pieces of fine surface disinfected root segments of *G. album* and *A. elatius* collected from controls, e$CO_2$ and T plots were incubated on MYP agar. mycelium of the fungi grew out from surface-sterilized root pieces kept on MYP agar plates after two-week of incubation at 25ºC (Fig. 1a, b). Finally, 15 fungi were distinguished and incubated in CM and 10% PDA

media at 25°C. Most of the fungal isolates did not produce spores on the culture media. Fungal strains *Tv*-WC16GW and *Sh*-P2A were isolated from the surface-sterilized roots of *G. album* and *A. elatius*, respectively. Axenic colonies of *Tv*-WC16GW on 10% PDA had white to pale cream aerial mycelium with velvety texture (Fig. 1c). Colonies of *Sh*-P2A grew slowly on 10% PDA at 25°C. Aerial mycelium was sparse, appressed, and cottony to woolly (Fig. 1d). Other two spore producing fungal isolates were obtained which were further studied in detail with respect to their morphology. Isolate WC10G1 formed white colonies on CM (Fig. 2 e) with branched septated mycelium that produced conical shaped in connection site (Fig. 2 f). The fungus produced chlamydospores with melanised single cell that release from chain shaped conidiophores (Fig. 2 g). Isolate WC18G1 had a septated and branched mycelium. It produces single cell spores with ellipsoid to egg shaped and brown colour. Oil droplets were visible inside the spore plasma (Fig. 2 h).



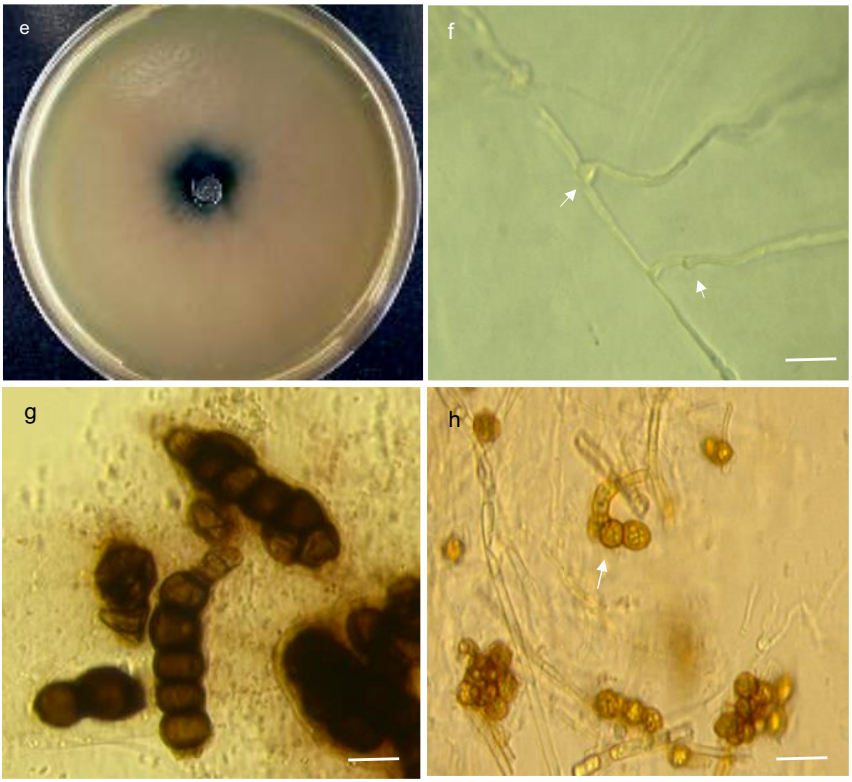



Fig. 2 Morphology of fungal strains *Tv*-WC16GW (*Trametes versicolor*), *Sh*-P2A (*Stereum hirsutum*), WC10G1 (*Periconia macrospinosa*) and WC18G1 (*Paraphaeosphaeria neglecta*), (a) *Tv*-WC16GW mycelium isolated from surface-disinfected roots of *Galium album* two weeks after cultivation on MYP medium. (b) Colony of Sh-P2A two weeks after the incubation of surface disinfected roots of *Arrhenatherum elatius* on MYP medium. (c) Whitish colony of Tv-WC16GW on PDA medium (d) Cottony-like colony of Sh-P2A on PDA medium. (e) WC10G1, four-week pale white colony on CM medium, with dark spores in the centre. (f) WC10G1, septate and branched mycelium with 3×23 µm in diameter and conical shape in the crotch or connect (arrowhead). (g) WC10G1, chlamydospores, dark brown, and round, 10 µm in diameter. (h) WC18G1, septate mycelium in PDA medium, each cell 2×7 µm in diameter, spores egg shaped with oil droplet inside the cell (arrowhead) spore length is seven µm. Scale bar, a, b, 1mm, f, g, h 0.01 mm.

### 3.3 Molecular identification of isolated fungal endophytes

The comparison of the fifteen fungal isolates based on their 18S rRNA gene and the ITS sequences in GenBank using BLAST, showed that 13 isolates were assigned to Ascomycota (*Pleosporales*, *Eurotiales*, *Helotiales*, and *Hypocreales*), and two isolates to Basidiomycota (*Polyporales* and *Russulales*) (Table 1 and 2). All isolates were assigned here to the genus as lowest taxonomic level based on a high sequence similarity to the next related strains (>99%). *Pleosporales* was the only common order (Table 1 and 2) that was represented by seven isolates and the most common taxon by far was *Periconia macrospinosa* Tode: Fr. (isolates F2F, PMT2, F1FH & WC10G1) and *Drechslera nobleae*. (Isolates F1F, WC10A1B and WC10A1W, Table 1).

The 18S rRNA gene sequence of isolate WC16G (GenBank KU981161) was 100% sequence identical to the 18S rRNA gene sequence of *Cadophora orchidicola* strain UAMH 8152 (GenBank DQ521603), an endophyte of the herbaceous plants in the forest of western Canada (Narisawa et al. 2007). Moreover, isolate WC16G shared 99% ITS sequence similarity with *C. orchidicola* strain P35.1 (GenBank KX611558), isolated as endophyte from hairy roots of ericaceous plant, *Gaultheria pumila* living in high altitude in Argentina (Bruzone et al. 2017).

The 18S rRNA gene sequences of four isolates, F2F (GenBank KU886525), PMT2 (GenBank KU954527), F1FH (GenBank KU954528), and WC10G1 (GenBank KU981158) shared 99-100% identity with the 18S rRNA gene sequence of *P. macrospinosa* strain CBS135663 (GenBank KP184080), an endophyte of *Festuca vaginata*, native grass species in semiarid grassland near Fülöfaza, Hungary (Knapp et al. 2015). While, the ITS sequences of the isolates F2F, F1FH, and WC10G1 were 99% identical to the ITS sequences of *P. macrospinosa* strain REF145 and R28 (GenBank JN859365 and KP183999) isolated of *Festuca vaginata* roots (Knapp et al. 2012), the ITS sequence of isolate PMT2 was 99% similar to *P. macrospinosa* strain R28 (GenBank KX169243) that was isolated as endophyte of corn in Iran.

The 18S rRNA gene sequences of isolates WC18G1 (GenBank KU981162) and WC16G1 (GenBank KU981160) were 99% and 100% similar to the 18S rRNA gene sequence of *Paraphaeosphaeria* sp. strain E5-3C (GenBank AB665311), which was isolated as endophyte of *Ulmus davidiana* var. *japonica* (Tomita 2003). The ITS

sequences of those strains shared 99% sequence identity with the ITS sequence of *Paraphaeosphaeria neglecta* strain CBS683.83 (GenBank JX496107) obtained from seeds of *Quercus robur* (Verkley et al. 2014).

Based on the 18S rRNA gene sequence comparison isolates F1F (GenBank KU862636), WC10A1W (GenBank KU981164), and WC10A1B (GenBank KU981163) shared 99% sequence similarity with *Drechslera nobleae* DAOM229296 (GenBank JN940959). The respective ITS sequences showed 100% identity to the ITS sequence of another *Drechslera* sp., strain BAFC3419 (GenBank FJ868975) an endophyte isolated from the grass *Lolium multiforum* (Scervino et al. 2009).

Isolate WC10AB (GenBank KU981165) shared 99% 18S rRNA gene sequence similarity with *Fusarium oxysporum* YW1 (GenBank KX262999) an endophytic fungus obtained from Iris tectorum (Qijia et al. 2016) and 99% ITS sequence similarity to *F. oxysporum* strain 5 (GenBank KU872777) an endophyte from roots of date palm *Phoenix dactylifera* (Mefteh et al. 2017).

The ITS sequence of isolate WC16GW (GenBank KX218391) was 99% similar to the ITS sequence of *Trametes versicolor* strain 5H1P0P52 (GenBank KP6989193) an endophyte in the leaf of *Huperzia serrata*, a Chinese medical plant (Gan et al. 2017) and 18S RNA gene sequence similar of 99% similar to another *T. versicolor* strain, HG9 (GenBank KM222266), that was collected from deep-subsea floor sediment near New Zealand island (Rédou et al. 2015).

Table 1. Phylogenetic assignment of fungal isolates according to 18S rRNA gene and ITS BLAST analysis in GenBank (NCBI)

| Fungal isolate | Host | NCBI closest match to 18S rRNA | NCBI closest match to ITS | Order | Genbank accession of fungi from this study (18S rRNA gene + ITS) |
|---|---|---|---|---|---|
| F1F | *G. album* | 99% *Drechslera nobleae* DAOM 229296 (JN940959) | 100% *Drechslera* sp. BAFC_3419 (FJ868975) | *Pleosporales* | KU862636 |
| F2F | *A. elatius* | 99 % *Periconia macrospinosa* CBS 135663 (KP184080) | 100% *Periconia macrospinosa REF145* (*JN859365*) | *Pleosporales* | KU886525 |
| PMT2 | *A. elatius* | 100% *Periconia macrospinosa* CBS 135663 (KP184080) | 99% *Periconia macrospinosa* R28 (KX169243) | *Pleosporales* | KU954527 |
| F1FH | *A. elatius* | 99% *Periconia macrospinosa* CBS 135663 (KP184080) | 99% *Periconia macrospinosa* strain CBS 135663 (KP183999) | *Pleosporales* | KU954528 |
| WC16G | *G. album* | 99% *Cadophora orchidicola* UAMH 8152 (DQ521603) | 99% *Cadophora orchidicola* P35.1 (KX611558) | *Helotiales* | KU981161 |
| WC10A1W | *A. elatius* | 99% *Drechslera nobleae* DAOM 229296 (JN940959) | 99% *Drechslera* sp. BAFC 3419 (FJ868975) | *Pleosporales* | KU981164 |
| WC18G1 | *G. album* | 99% *Paraphaeosphaeria* sp. E5-3C (AB665311) | 100% *Paraphaeosphaeria neglecta* CBS_683.83 (JX496107) | *Pleosporales* | KU981162 |
| WC10A1B | *A. elatius* | 99% *Drechslera nobleae* DAOM 229296 (JN940959) | 100% *Drechslera* sp. BAFC_3419 (FJ868975) | *Pleosporales* | KU981163 |
| WC16G1 | *G. album* | 99% *Paraphaeosphaeria* sp. E5-3C (AB665311) | 100% *Paraphaeosphaeria neglecta* CBS_683.83 (JX496107) | *Pleosporales* | KU981160 |

| | | | | | |
|---|---|---|---|---|---|
| WC10AB | *A. elatius* | 99% *Fusarium oxysporum* YW1 (KX262999) | 99% *Fusarium oxysporum* 5 (KU872777) | *Hypocreales* | KU981165 |
| WC10G1 | *G. album* | *99 % Periconia macrospinosa* CBS 135663 (KP184080) | 99% *Periconia macrospinosa* REF145 (JN859365) | *Pleosporales* | KU981158 |
| WC16GW | *G. album* | 99% *Trametes versicolor* 5H1-P0-P5-2 (KM222266) | 99% *Trametes versicolor* HG9 (KU517157) | *Polyporales* | KX218391 |

The ITS sequence of isolate WT14G (GenBank MH156142), obtained from a T plot, was 99% similar to the ITS sequence of a *Penicillium* sp., strain Y12EG-2010 (GenBank HM161749), which was isolated from rhizosphere of *Nicotiana tabacum* (Galiana et al. 2011) and its 18S rRNA gene sequence was 99% similar to the 18S rRNA gene sequence of *P. olsonii* strain PenI (GenBank FJ717701) isolated from the atmosphere of Porto city (Oliveira et al. 2010). Regarding two isolates from $eCO_2$ plot, the ITS sequence of isolate $P_1A_2$ (GenBank KP863919) was 99% similar to *Nectria* sp. strain aurim 671 (GenBank DQ069039) isolated from mycorrhizal roots of conifer seedling in Lithuania (Menkis et al. 2005) and 18S rRNA gene sequence was 99% sequence identical to *N. lugdunensis* strain CBE98 (GenBank AY204604) collected as a freshwater fungi in an alpine stream in Germany (Baschien et al. 2008). The 18S RNA gene sequence of isolate $P_2A$ (GenBank KX838369) was 100% sequence identical to *Stereum hirsutum* strain FPL8805 (GenBank SHU59095), the strain stored in USDA forest products laboratory (Hibbett 1996) and the ITS sequence of it was 100% sequence identical to another *S. hirsutum* strain, P24.7 (GenBank KX611567) isolated from surface sterilized hairy roots of *Gaultheria pumila* (Ericaceae, Bruzone et al. 2017). *S. hirsutum* is morphologically very close to *S. rugosum* with slight difference in the spore diameters.

Table. 2 Phylogenetic assignment of fungal isolate of *Galium album* and *Arrhenatherum elatius* that were exposed to elevated $CO_2$ or +2°C temperature under GiFACE conditions according to 18S rRNA gene and ITS BLAST analysis in GenBank (NCBI)

| Fungal isolate | Host Plant | NCBI closest match to 18S rRNA | NCBI closest match to ITS | Order | Genbank accession of fungi from this study (SSU) |
|---|---|---|---|---|---|
| WT14G | *G. album* | *Penicillium olsonii* PenI (FJ717701) | *Penicillium* sp. Y12_EG-2010 (HM161749) | *Eurotiales* | MH156142 |
| $P_1A_2$ | *A. elatius* | *Nectria lugdunensis* CBE98 (AY204604) | *Nectria* sp. aurim 671 (DQ069039) | *Hypocreales* | KP863919 |
| $P_2A$ | *A. elatius* | *Stereum hirsutum* FPL8805 (U59095) | *Stereum hirsutum* P24.7 (KX611567) | *Russulales* | KX838369 |

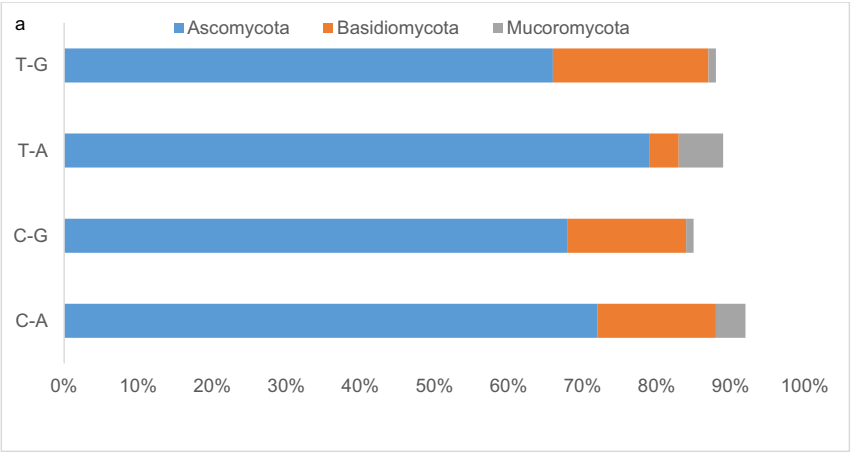
### 3.4 Diversity of endophytic fungi community of the warming experiment

With the intent to get a general view on the root fungal endophyte communities associated with *G. album* and *A. elatius* and tracking the relative abundance of taxa which represented the 15 isolates which were obtained by culture-dependent approach, Illumina ITS amplicon sequencing was applied. Sixteen root samples of *G. album* and *A. elatius* had been collected separately from the C and T plots. Segments of the same root samples that had been applied for cultivation were used for DNA extraction. DNA samples were pooled according to each host plant (*A. elatius* and *G. album*) and treatment-plot (+2°C temperature and ambient temperature). Four pooled samples were used for the fungal ITS amplicon amplification, one C and T sample for both plant species, *A. elatus* and *G. album*. In total 198,442 paired end sequences were obtained of the four samples. In summary 434 operational taxonomic unites (OTUs) were recognized and classified as taxonomic rank from phylum to species.

#### 3.4.1 Phylogenetic composition of the fungal root microbiota

The phylogenetic composition of the fungal microbiota was studied in more detail focused on OTUs which occurred with a relative abundance of 1% or higher in at least one of the C or T plots samples. The relative abundance (RA) is presented in terms of

percentage in total sequences in each sequenced root sample. In summary OTUs were assigned to three different phyla and 17 orders (Fig. 3 a and b). At the level of phyla Ascomycota (RAs 66-79%), Basidiomycota (RA 4-21%), and Mucoromycota (RA 1-6 %) were detected (Fig. 3 a). In the order ranking, Ascomycota, *Helotiales* (RA 17-61%), *Pleosporales* (RA 3-21%), *Chaetothyriales* (2-12%), *Sordariales* (1-10%) and *Hypocreales* (RA 1-7%) with, Basidiomycota, *Agaricales* (RA 2-12%) were as common taxon between both host plants grown in C and T plots samples (Fig. 3 a b). *Helotiales* (Ascomycota) was the most dominant order in all samples and included some well-known DSE. *Mortierellales* (RA 3- 5%) was the only order of Mucoromycota that was detected in both, C and T plots samples of *A. elatius*.



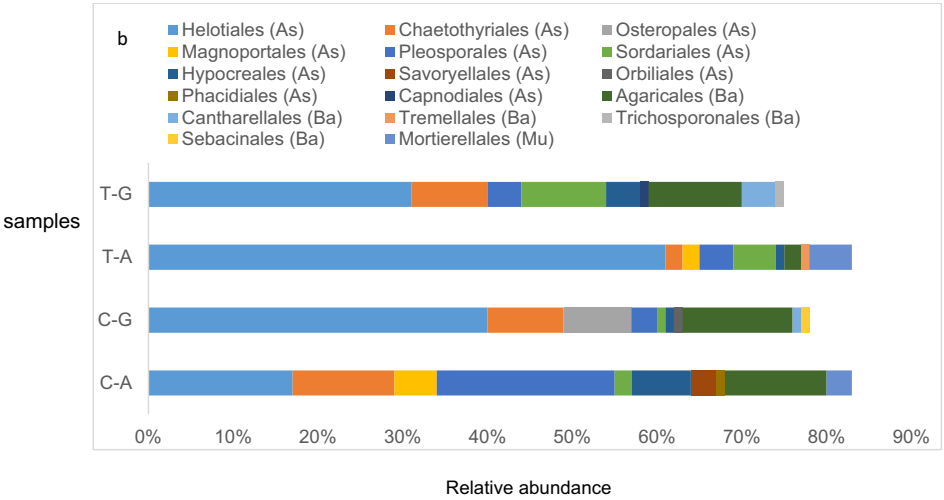



Fig. 3 Relative abundance (RA) of sequences in each sample that were attributed to different OTUs in control (C) and warming (T) plots. a Ascomycota (RA ≤ 75%), Basidiomycota (RA ≤ 20%), and Mucoromycota (RA ≤ 3.5). The RA is presented in terms of percentage in total sequences in each sample. b the most dominant Basidiomycota order were respectively *Agaricales*, *Cantharellales*, *Tremellales*, *Trichosporonales* and *Sebacinales*. Ascomycota dominant order were *Helotiales*, *Pleosporales* and *Chaetothyriales*, respectively. A: *Arrhenatherum elatius*. G: *Galium album.* As: Ascomycota. Ba: Basidiomycota. Mu: Mucoromycota. Data are shown for OTUs with ≥ 1% contribution.

### 3.4.1 Alpha diversity of the fungal root microbiota

The alpha (within-sample) diversity of the fungal root microbiota of *A. elatius* and *G. album* plants grown within C and T plots was estimated based on the total number of OTUs by calculating the Chao 1, Simpson's evenness, Simpson' dominance and Shannon indices. The Chao 1 index reflects the species richness of the fungal root microbiota, the evenness and dominance the relative abundance of OTUs within each root sample, while the Shannon index reflects the species diversity considering both, richness and evenness parameters.

Based on the Chao 1 index, the richness of the fungal endophyte communities was in the same range (287-300 OTUs) for *A. elatius* roots derived from C and T plots. For *G. album* the richness was lower in roots derived from T plots (187 OTUs) compared to roots obtained from control plants (275 OTUs, Fig. 4 a). The evenness of the communities strongly varied among the samples with different trends among C and T derived host plants (evenness index, Fig. 4 b). A high abundance of individual OTUs as indicated by high dominance value was only obtained for root samples of *A. elatius* derived from T plots (dominance index, Fig. 4 c). Vice versa the overall diversity of that root sample was lower indicated by a lower Shannon index value, while all other investigated roots samples contained fungal root microbiota of a similar higher diversity (Fig. 4 d). The difference among fungal richness for C and T samples for both host plants were also illustrated by difference in the dynamics of rarefaction curves. Rarefaction curves of T samples of *G. album* had lower slopes than the curves of the other samples indicating the least fungal species richness (Fig. 4 e). Because only pooled root samples were studied, a statistic-based correlation of the environmental treatments and the fungal root microbiota diversity cannot be given.

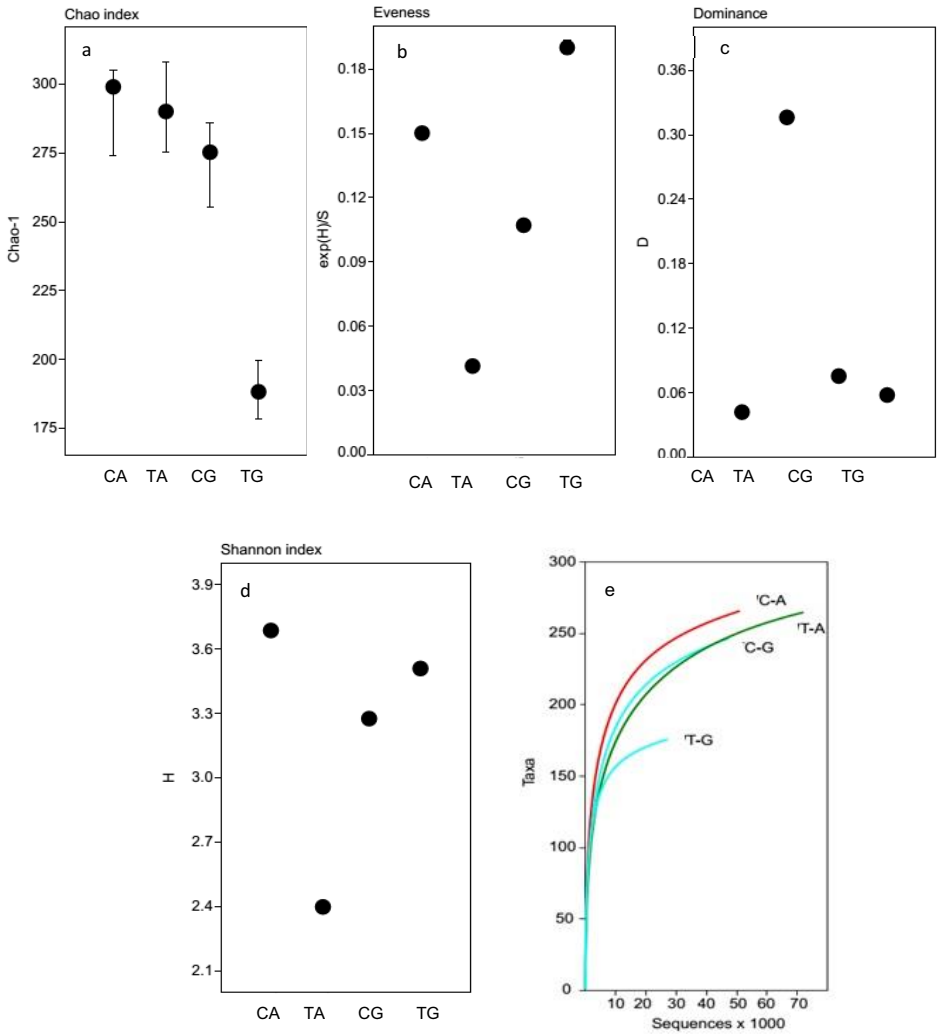



Fig. 4 Alpha diversity indices of fungal communities, a Chao 1, b evenness, c dominance, d Shannon index, and e rarefaction curves for the endophytic community of *G. album* and *A. elatius* roots from control (C) and warming (T) plots, were analysed based on Illumine ITS2 amplicon sequencing of fungal endophyte communities from both host plant in C and T plots.(Images provided by Dr. Stefanie Gläser)

### 3.4.2 Main shared fungal endophytes between the two host plants considering cultivation dependent and independent data

There was no Basidiomycetes or Ascomycetes preference between both host plants among isolated fungal endophytes. *Pleosporales* was the only common order between the two host plants and *Periconia macrospinosa* and *Drechslera* spp. was the most common genus among them which was furthermore represented by seven isolates (Table 1 and 2). Under $eCO_2$ conditions, both isolates cultured from monocotyledon while the only isolate of T plots was cultured from dicotyledonous plant (Table 2). Besides, in culture dependent approach, there was not any shared species among isolated endophytes of $eCO_2$ and T plots (Table 2). Regarding Illumina ITS2 amplicon sequencing output, among the 48 total OTUs (RA ≥ 1%), only 9 OTUs were shared between the two host plants, including *Glarea* sp., *Cadophora orchidicola* and *Mycena* sp., respectively (Fig. 5). Furthermore, among the fungal endophytes that were isolated from one or both plants in the culture-dependent approach, four OTUs including *C. orchidicola*, *P. macrospinosa*, *Drechslera* sp. and *Fusarium oxysporum* were identified. In contrast, 31 OTUs only became evident in one host plant. Accordingly, 18 OTUs were traced in *A. elatius*, such as *Lachnum* sp., *Delitschia* sp., *Slopeiomyces cylindrosporus*, *Myrothecium verrucaria* and *Periconia macrospinosa* (Fig. 5). In addition, 24 OTUs were detected only in *G. album*, such as *Tetracladium maxilliforme*, *Mycena citrinomarginata*, and *Cryptodiscus rhopaloides*, respectively, with the highest abundance (Fig. 5). Besides, isolates assigned as *Serendipita herbamans* and *Trichoderma hamatum*, were merely detected in *G. album*.

### 3.4.3 OTUs specifically detected in C compared to T plots

According to Illumina ITS2 amplicon sequencing 10 OTUs were detected only in roots of the T plot samples if OTUs with a relative abundance higher than 1% were considered, among those: *Schizothecium glutinans* with a RA of 1% and 10% in *A. elatius* and *G. album*, respectively. *Volucrispora graminea* (RA 3 %) and *Cladophialophora* sp. (RA 2 %) both detected in *G. album* roots had the highest relative abundance (Fig. 5). Moreover, *Penicillium olsoni* (RA ≤ 0.05 % in *G. album*) had been isolated in culture-dependent approach. In contrast, 14 fungal OTUs merely were detected from C plots samples (Fig. 5).

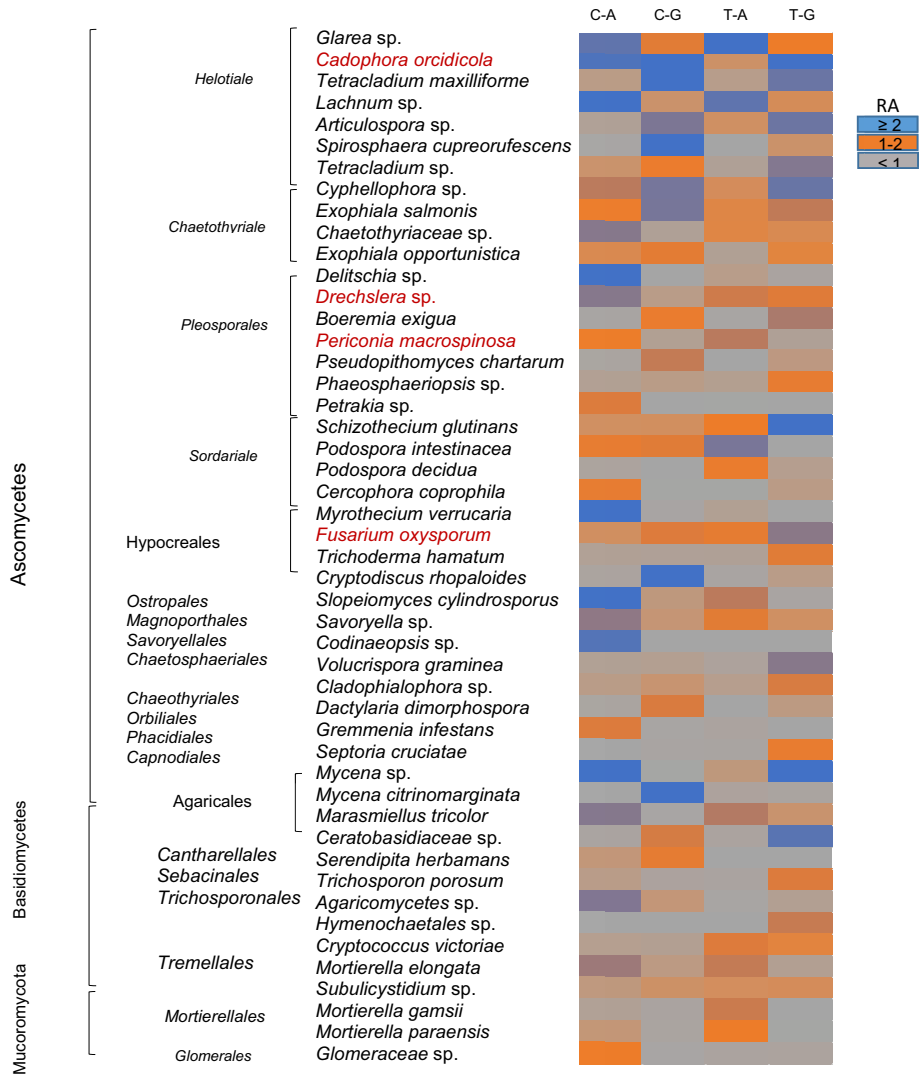

C-A
C-G
T-A
T-G
RA
≥ 2
1-2
< 1
Ascomycetes
Basidiomycetes
Mucoromycota
Helotiale
Chaetothyriale
Pleosporales
Sordariale
Hypocreales
Ostropales
Magnoporthales
Savoryellales
Chaetosphaeriales
Chaeothyriales
Orbiliales
Phacidiales
Capnodiales
Agaricales
Cantharellales
Sebacinales
Trichosporonales
Tremellales
Mortierellales
Glomerales
Glarea sp.
Cadophora orcidicola
Tetracladium maxilliforme
Lachnum sp.
Articulospora sp.
Spirosphaera cupreorufescens
Tetracladium sp.
Cyphellophora sp.
Exophiala salmonis
Chaetothyriaceae sp.
Exophiala opportunistica
Delitschia sp.
Drechslera sp.
Boeremia exigua
Periconia macrospinosa
Pseudopithomyces chartarum
Phaeosphaeriopsis sp.
Petrakia sp.
Schizothecium glutinans
Podospora intestinacea
Podospora decidua
Cercophora coprophila
Myrothecium verrucaria
Fusarium oxysporum
Trichoderma hamatum
Cryptodiscus rhopaloides
Slopeiomyces cylindrosporus
Savoryella sp.
Codinaeopsis sp.
Volucrispora graminea
Cladophialophora sp.
Dactylaria dimorphospora
Gremmenia infestans
Septoria cruciatae
Mycena sp.
Mycena citrinomarginata
Marasmiellus tricolor
Ceratobasidiaceae sp.
Serendipita herbamans
Trichosporon porosum
Agaricomycetes sp.
Hymenochaetales sp.
Cryptococcus victoriae
Mortierella elongata
Subulicystidium sp.
Mortierella gamsii
Mortierella paraensis
Glomeraceae sp.

Fig 5 Relative abundance (RA) patterns of fungal taxa associated with roots of *Galium album* (G) and *Arrhenatherum elatius* (A) in control (C) and warming (T) plots. Colour scale represent the RA of fungal species-level OTUs across all samples. Analysis were performed at the level of taxonomic paths withPAST3 software. Red-colour specified species isolated in culture-dependent approach. Data are shown for OTUs with ≥ 1% RA contribution.

### 3.5 Biological activity assay

#### 3.5.1 Arabidopsis endophytic colonization by *Stereum hirsutum*

The fungal isolate *Sh*-$P_2$A, was the only fungal isolate obtained from a $eCO_2$ plot. It was selected to further investigation aimed at endophytic lifestyle development in Arabidopsis. To pursue this aim, roots dip inoculated Arabidopsis seedlings were placed on ½ MS medium. Consequently, external mycelium emerged from primary roots 14 day after inoculation (Fig. 6 a and b). No diseases symptoms were observed in Arabidopsis.

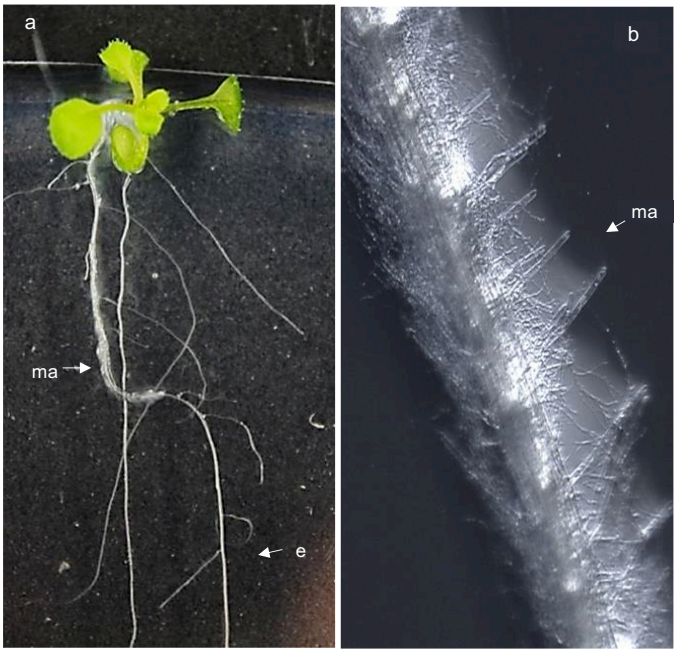
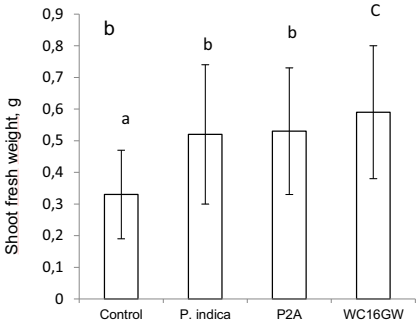


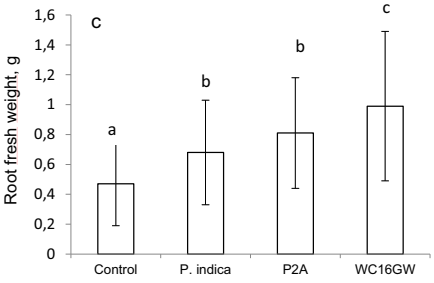
Fig. 6 Endophytic and host specificity assessment of a *Sh*-$P_2$A obtained from *A. elatius* from the $eCO_2$ plot. Endophytic lifestyle was checked in *Arabidopsis*. (a) *Arabidopsis* root colonized by *Sh*-P2A on ½ MS medium shows hyphal colonization in the primary root. (b) Mycelium attachment to hairy root of *Arabidopsis*.

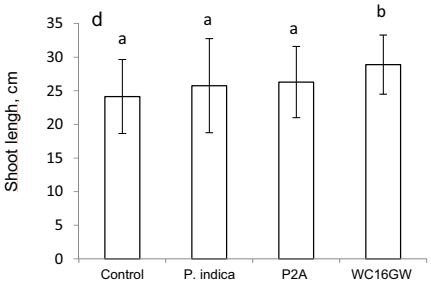
### 3.5.2 *Trametes versicolor* and *Stereum hirsutum* show beneficial activity in wheat

To assess the growth promoting activity of *Tv*-WC16GW and *Sh*-P2A, three-day-old wheat seedlings were dip-inoculated in crushed mycelia from four-week-old liquid cultures. The root endophyte *P. indica* DSM 11827 was used as reference for strong biological activity (Waller et al., 2005). After four weeks, plants inoculated with *Tv*-WC16GW and *Sh*-$P_2$A showed an overall increase in biomass (Fig. 7 a). Root and shoot fresh weights were elevated compared to mock-inoculated plants (Fig. 7 b,c). Notably, the increase in fresh weights was comparable to that induced by *P. indica*, suggesting that *Tv*-WC16GW and *Sh*-$P_2$A had a strong beneficial activity. Increase in shoot / root fresh weights was strongest in *Tv*-WC16GW-colonized plants, which also showed a significant increase in shoot length ($p$ = 0.05; Fig. 3 d). Visual analysis of the roots confirmed an increase in biomass and a strong induction of the formation of secondary roots upon fungal colonization (Fig 7 e - h).


a
Control
P. indica
P2A
WC16GW


Shoot fresh weight, g
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
a
b
b
b
C
Control
P. indica
P2A
WC16GW


Root fresh weight, g
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
c
a
b
b
c
Control
P. indica
P2A
WC16GW


Shoot lengh, cm
35
30
25
20
15
10
5
0
d
a
a
a
b
Control
P. indica
P2A
WC16GW


e


f

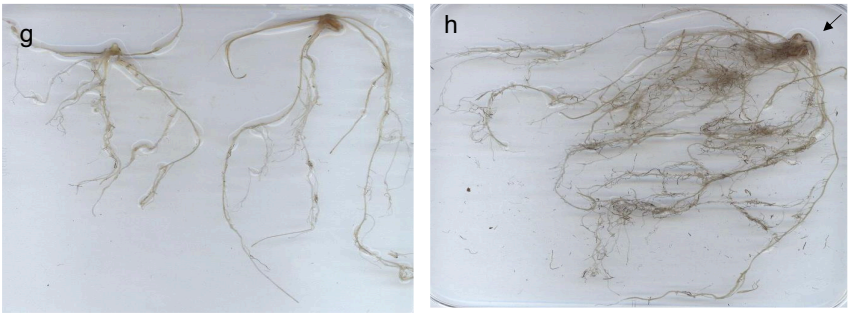



Fig 7 Wheat plant responses to *Tv*-WC16GW, *Sh*-P2A, and *P. indica* (positive control). Four-day-old wheat seedlings were dip-inoculated with approximately 1g/ml of crushed mycelium for 3 h and subsequently transferred to heat sterilized soil of Linden grassland, Giessen. (a) Wheat plants 4 weeks after inoculation; (B) shoot fresh weights, (C) root fresh weights and (D) shoot lengths; Mean values and standard deviation of 12 plants were shown. Randomly selected roots are shown from control plant (E), *P. indica* (F), *Sh*-P2A (G), and *Tv*-WC16GW (H); one root (arrowhead). Statistical analysis was performed in SPSS using One-Way ANOVA; different letters at top of the bars indicate statistically significant differences ($P = 0.05$).

### 3.6 Fungal colonization pattern in wheat roots

To trace the endophytic growth of the fungal strains in wheat, root colonization patterns were analyzed by staining with the chitin-specific dye Wheat Germ Agglutinin (WGA)-Alexa Fluor 488. The visualization of hyphae by fluorescent microscopy revealed that both fungal strains endophytically colonize wheat roots. Heavy fungal colonization of the root cap and meristem zone with an extracellular and intracellular network of *Tv*-WC16GW hyphae is visible (Fig.8 a). Moreover, the root elongation and maturation zone were colonized with extra- and intraradical hyphae (Fig.8 b), while non-inoculated roots were free of fungal hyphae (Fig. 8 c). *Sh*-P2A colonized predominantly the meristematic zones (Fig. 8 d), and only few hyphae were detected in the elongation zones (Fig. 8 e). Notably and in contrast to *P. indica,* neither *Tv*-WC16GW nor *Sh*-P2A showed sporulation on or within roots (Fig. 8 f). Thus, the colonization pattern of *T. versicolor* WC16GW virtually resembled the pattern described for *P. indica* (Waller et al. 2005; Deshmukh et al.2006; Jacobs et al. 2011).

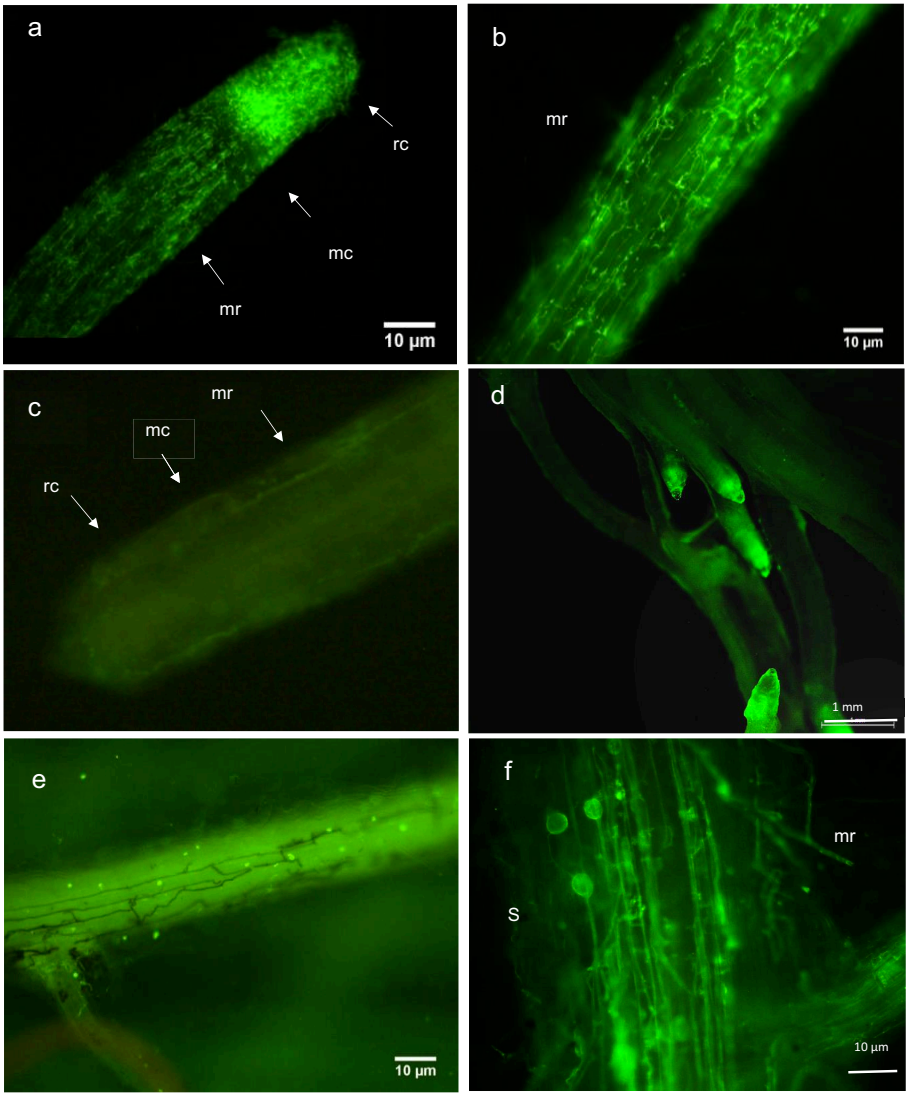

a
rc
mc
mr
10 µm
b
mr
10 µm
c
mr
mc
rc
d
1 mm
e
10 µm
f
mr
S
10 µm

Fig 8 Colonization of wheat roots with *Tv*-WC16GW and *Sh*-P2A. Fungal hyphae were stained with WGA-conjugated fluorophores in roots at 30 days after inoculation and visualized by fluorescent microscopy. Root colonization pattern of *Tv*-WC16GW (a) Heavy colonization of root cap zone (rc) and meristematic zone (me) (b) elongation zone (ma) and its sub-epidermal layer colonization (arrowhead). (c) mock-inoculated plant (negative control). (d-e) Root colonization pattern of *Sh*-P2A (d) root cap and meristematic zone (e) elongation zone. (f) *P. indica* sporulation within root tissue (positive control), spore (s).

### 3.6 endobacterial detection and identification

#### 3.6.1 *T. versicolor* WC16GW and *S. hirsutum* P2A host endobacteria

FISH analysis with the universal *Bacteria* 16S rRNA targeting probe indicated the presence of bacterial cells in axenically grown mycelia of the two fungi. Bacterial colonization has been seen in some hyphae of both fungi (Fig. 9 a and b). To confirm the endohyphal localization of bacterial cells, hyphae of strain *Tv*-WC16GW were further investigated by TEM. Hyphal cytoplasm contained spherical shaped with variable size and partially empty bacteria-harboring vacuoles (BHVs) with an electron-dense contents (Fig. 10 a). One fungal cell with size of 1.5 × 10 µm contained approximately 20 BHVs (Fig. 10 a). BHVs were 200 to 800 nm in diameter (Fig. 10 b). BHVs were separated from the fungal cytoplasm by a single membrane, indicating the fungal-derived membrane (Fig. 10 b).

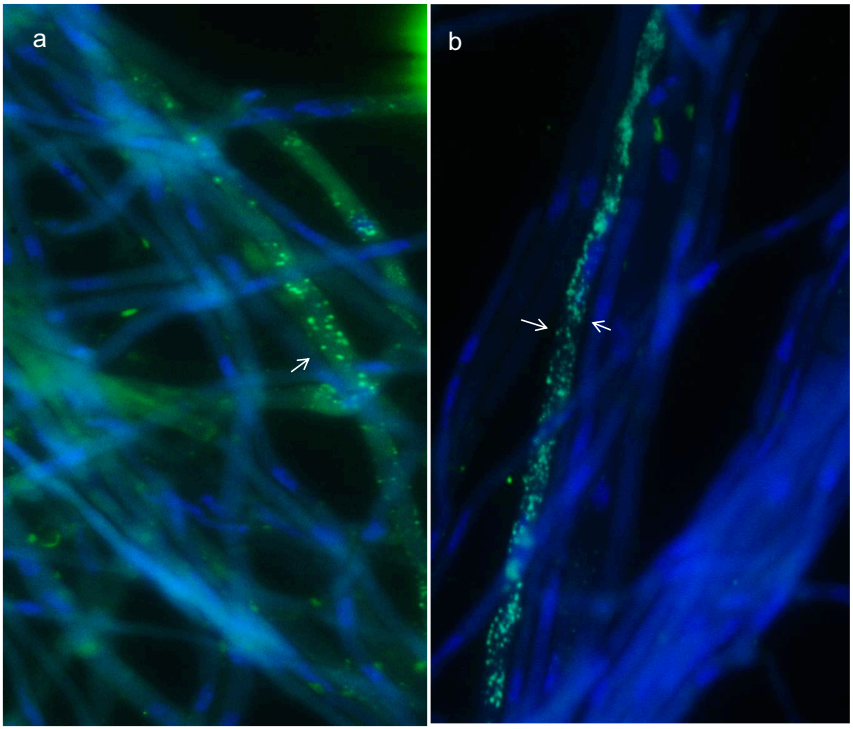



Fig 9 Detection of endo-fungal bacteria by FISH, mycelium of *Tv*-WC16GW (a) and *Sh*-P2A (b) fungal strains. Overlay images of the bacterial detected by FISH using the universal bacterial 16S rRNA probe EUB338 (green) and DAPI counterstaining (blue signal). White arrows point on endobacteria (green labeled). Scale bars, 3 μm. (Images were provided by Hossein Haghighi)

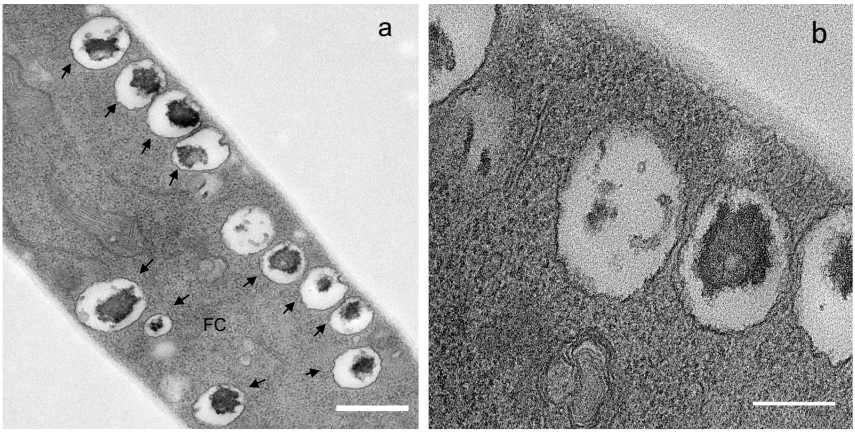



Fig 10 TEM micrographs of bacteria-harbouring vacuoles (BHVs) inside fungal hyphae of isolate Tv-WC16GW. (A) Numerous BHVs (arrowhead) are embedded in the fungal cytoplasm (FC), (B) single membrane structure around BHVs. Scale bars: (A) 500 nm (B) 200 nm. (Images were provided by Dr. Martin Hardt)

### 3.6.2 Phylogenetic identification of endobacteria

The detected endobacteria were phylogenetically identified by 16S rRNA gene sequence analysis. Nearly full-length 16S rRNA gene sequences were PCR-amplified from total DNA extracted from pure fungal biomass of *Ts*-WC16GW and *Sh*-P2A because bacteria could not be isolated. 16S rRNA similarity and phylogenetic analysis showed that the 16S rRNA gene sequence of both endobacteria (Genbank accession numbers KY321464 and MG954110) made monophyletic group with an uncultured bacterial symbiont of *Heliothrips haemorrhoidalis* (GenBank accession number KM582851; Nguyen et al. 2015 ) and choloroplast and plastid sequences derived from the mono-dicotyledonous plants besides they showed the highest sequence similarity together (Fig 11). To exclude the possibility that the sequences were impurities in fungal DNA extracts originating from chloroplast/plastid 16S genes of *A. elatius* or *G. album*, chloroplast sequences of the plants were analyzed in parallel. We found that the sequences from fungal DNA extracts were only 97% and 98% similar to chloroplast DNA sequences of *A. elatius* (GenBank accession number MG976759) and *G. album*

(GenBank accession number MG976759), respectively (Fig. 11). Consistent with this, we could not detect red-auto fluorescent organelles in the FISH analysis, further confirming the absence of chloroplasts and phototrophic bacteria in the fungal samples.

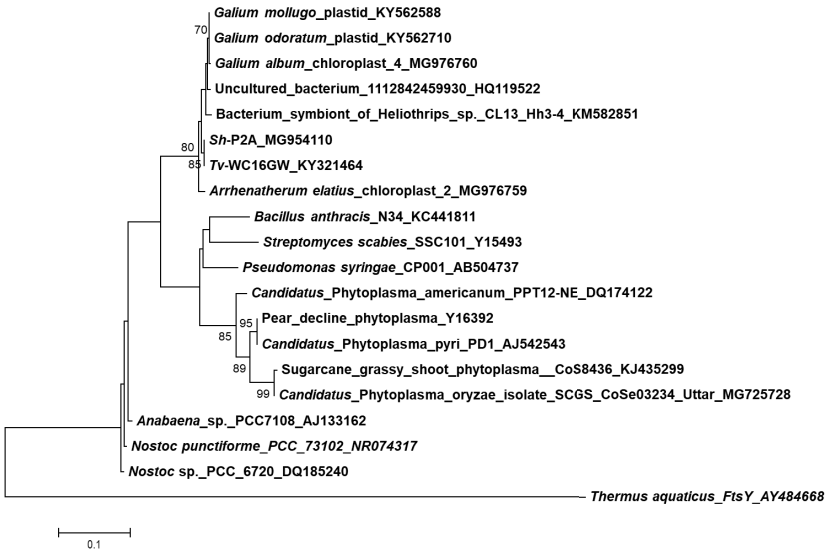



Fig 11 Phylogenetic placement of the uncultured endobacteria of fungal strains *Tv*-WC16GW and *Sh*-P2A. Maximum-likelihood analysis inferred from 16S rRNA gene sequence obtained from fungal genomic DNA extracts. 16S rRNA gene sequence of closest related sequences phylogenetic placement of *Tv*-WC16GW and *Sh*-P2A among members of the phylum bacteria, which were already known as fungal endobacteria. Reference sequences were retrieved from GenBank after NCBI BLAST search. Sequences analysis was performed in MEGA 6 using Clustal W for the alignment. The maximum-likelihood tree was calculated based on the distance matrix generated with the Kimura-2 model and based on 1,000 replicates (bootstrap analysis). Respective values (>70%) are given at branch nodes. The branch comprising sequence of the species *Thermus aquaticus* was used as an outgroup. Bar 0.01 and 0.05 substitutions per sequence position. (Chloroplast/plastid 16S genes of *A. elatius* or *G. album*, chloroplast sequences were provided by Olga Budich)

### 3.6.3 Isolation of endobacteria

No cultivable bacteria yielded on fungal extract-LB medium of both fungi in both aerobic and anaerobic conditions.

## 3.7 outdoor experiment and analysis

### 3.7.1 Effects of *T. versicolor* and *P. indica* on yield parameters under different phosphate regimes

Application of P in the form of $Ca(H_2PO_4)_2$ significantly increased the grain yield compared with the treatment without additional P (Fig.12). Plants inoculation with *T. versicolor* showed increased numbers of ears; and higher numbers of tillers per plant in the CO treatment. The grain yield was increased by 37% (CO treatment), 8.5% (CP treatment) and straw yield by 27% (CO treatment) (Table3; Fig.12). Plants inoculation with *P. indica* showed increased numbers of ears in the CP treatment (Table4; Fig.1). The grain yield was increased by 10% (CP treatment) and straw yield by 22% (CO treatment). Thus, both fungi showed increases in many of the parameters measured in P-deprived condition and grain yield improvement in both P-deprived and P-rich conditions. The increase in grain yield correlated with an increase in ear numbers except the *P. Indica* variant in P deprived conditions. In plants inoculated with *T. versicolor*, an increase in tiller number, ear number, straw yield and barn yield follows the same pattern as grain yield (Table3). Nevertheless, there was no correlation between thousand-grain weight (TGW) and grain yield increase in *T. versicolor*-colonized plants in both CO and CP treatment (Table3), suggesting that TGW was more or less constant and the effect of increasing P access had more of an effect on the earlier growth stages and yield component formation.

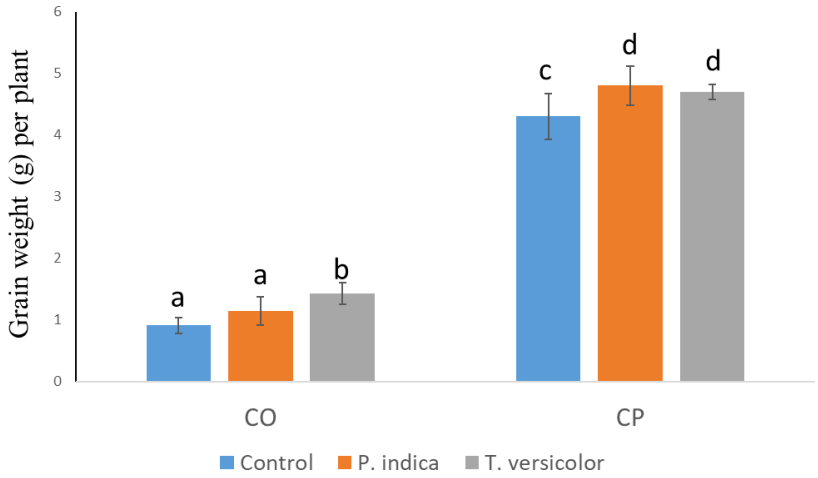



Fig. 12 Effect of *Piriformospora indica* DSM 11827 and *Trametes versicolor* WC16GW on wheat grain yield (weight per plant) under Ca $(H_2PO_4)_2$ (CP) treatment and control (CO). Values given are means of four pots (with 7-8 plants per pot). Statistical analysis was performed in SPSS using One-Way ANOVA. Means with different letters are significantly different ($p \leq 0.001$). Error bars represent ± the standard deviation of the mean. Different letters indicate significant difference between the treatments.

**Table 3**. Effect of *T. versicolor* on growth and yield parameters in wheat at different proportion of phosphate (P). Values given are means of four pots (with eight plants per pot). *Statistically significant differences between colonized plants and control.

| | ***T. versicolor*** | **Tillers plant** | **Ears / plant** | **Straw yield** g / plant | **Grain yield** g / plant | **Barn yield** g / plant | **TGW** g |
|---|---|---|---|---|---|---|---|
| P-deprived (CO) | - | 3.8 ± 0.26 | 2.2 ± 0.12 | 2.4 ± 0.15 | 0.9 ± 0.13 | 0.75 ± 0.09 | 4 ± 0.34 |
| | + | 4.8 ±0.47* | 2.6 ± 0.28* | 3.3 ± 0.38* | 1.43 ± 0.18* | 0.93 ± 0.08* | 4.1 ± 0.12 |
| P-rich (CP) | - | 5.8 ± 0,71 | 3.2 ± 0.42 | 6.04 ± 0.83 | 4.3 ± 0.37 | 1.44 ± 0.11 | 3.79 ± 0.21 |
| | + | 6.8 ± 0.5 | 4 ± 0.17* | 6.01 ± 0.6 | 4.7 ± 0.12* | 1.68 ± 0.08* | 3.88 ±0.26 |

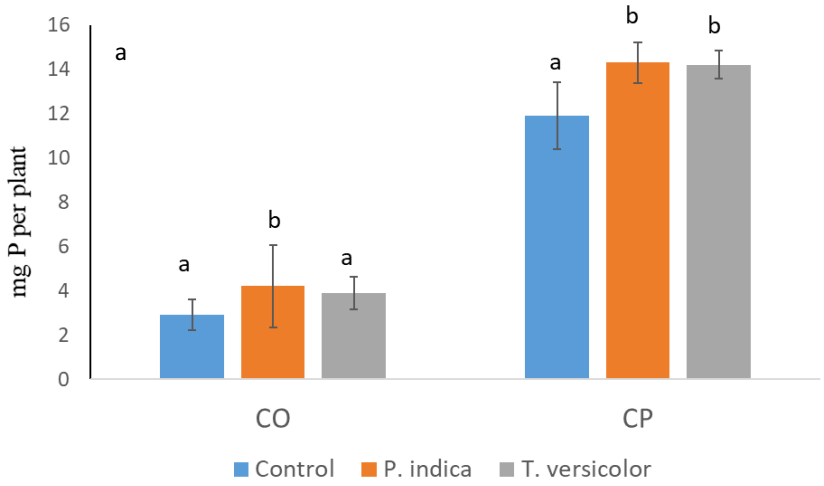
Table 4. Effect of *P. indica* on growth and yield parameters in wheat at different proportion of phosphate (P). Values given are means of four pots (with eight plants per pot). *Statistically significant differences between colonized plants and control.

| | ***P. indica*** | **Ears / plant** | **Straw yield** g / plant | **Grain yield** g / plant | **Grain [P]** mg / plant | **Straw [P]** mg / plant |
|---|---|---|---|---|---|---|
| P-deprived (CO) | - | 2.2 ± 0.12 | 2.4 ± 0.15 | 0.9 ± 0.13 | 2.90 ± 0,68 | 1.4 ± 0.14 |
| | + | 2.2 ± 0.1 | 3.08 ± 0.51* | 1.14 ± 0.23 | 4.2 ± 1,8* | 2.1 ± 0.31* |
| P-rich (CP) | - | 3.2 ± 0.42 | 6.04 ± 0.83 | 4.3 ± 0.37 | 11.9 ± 1.52 | 3.6 ± 0.96 |
| | + | 3.8 ± 0.32* | 5.5 ± 0.56 | 4.8 ± 0.32* | 14.2 ± 0.63* | 4 ± 0.31 |

### 3.7.2 Wheat P content under different soil phosphate treatment and endophyte inoculation

To evaluate the effect of two different levels of soil phosphate during wheat-fungi interaction on plant growth parameters, the total phosphate content of the straw and the grain was determined separately. *P. indica* improved the P content of the grain significantly by 30% (CO treatment) and by 16% (CP treatment), while *T. versicolor*

increased the grain P content by 16% (CP treatment) (Fig. 13 a). Regarding the P content of straw, *P. indica* improved the P content by 33% in the CO treatment and, in contrast, *T. versicolor* by 35% in the CP treatment (Fig.13 b).*P. indica* enhanced the P content significantly, which correlated both with improved vegetative growth in P-deprived conditions and improved grain yield in P-rich conditions, suggesting Pi uptake by *P. indica* could be a main factor that enhances plant growth and yield. However, *T. versicolor* in P rich conditions increased both the straw and grain P content significantly and likewise the grain yield. In contrast, in P deprived conditions both straw and grain P content did not change significantly in *T. versicolor* inoculated plants, despite a significant increase in vegetative growth and grain yield



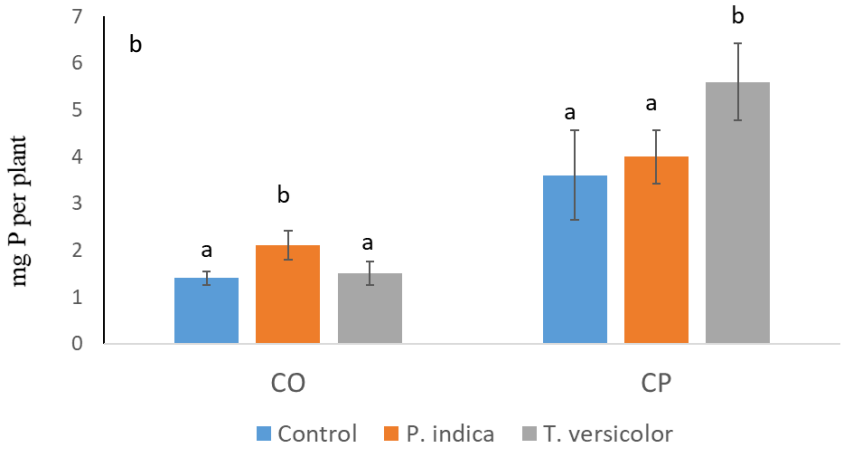



Fig. 13 Effect of *Piriformospora indica* DSM 11827 and *Trametes versicolor* WC16GW on wheat P content (mg/plant). (a) Grain P content and (b) straw P content. CO: control, CP: Ca $(H_2PO_4)_2$. P content separately measured for grain and straw. Statistical analysis was performed in SPSS using One-Way ANOVA. Error bars denote the ± S.D. from the mean of 28-32 plants in four Mitscherlich pots. Means with different letters are significantly difference ($p \leq 0.05$).

## 4 Discussion

### 4.1 Fungal endophytes obtained in culture-dependent approach

Culture-dependent approach was performed for isolation of endophytic fungi of two dominant plants of the permanent grassland at three different conditions, +2°C surface temperature, $eCo_2$ and ambient environmental conditions. Accordingly, *P. macrospinosa* was the prevalent species that could be isolated from both host plants. It is described as frequent endophytic fungus of temperate to tropical grassland (Luo et al. 2014), which forms typical DSE morphology (Knapp et al. 2012), especially in tallgrass prairie (Knapp et al. 2012; Luo et al. 2014; Mandyam et al. 2010). Accordingly, *Periconia* spp. normally produces melanised intracellular microsclerotia in the cortex of plant roots (Mandyam et al. 2010). Our study confirms the presence of *P. macrospinosa* as a common septate endophyte in temperate grassland. It is considered as a symbiotic fungus but can be a wheat pathogen under experimental conditions (Carter et al. 1999). *P. macrospinosa* could produce chlorine-containing compound but its biological effect have not been determined (Giles and Turner 1969). Besides *Periconia* sp. isolated from *Taxus cuspidata* produced antibacterial compounds (Kim et al. 2004). In addition, we isolated *D. nobleae* from both host plants as a DSE fungus. Exudates of *Drechslera* sp. could stimulate hyphal growth and branching in arbuscular mycorrhizal fungus *Gigospora rosea* symbiont of the grass *Lolium multiflorum* (Scervino et al. 2009).

Here, we use the term endophyte to mean 'microorganism inhabiting the interior of plants irrespective of the function in association with the plant' (Brader et al. 2017). It has been suggested that strains of each fungal species could express different lifestyles, either pathogenic or mutualistic depending on environmental condition or the host plant (Redman et al. 2001). For instance, some strains of *F. oxysporum*, a well-known plant pathogenic fungus, have been characterised as biocontrol agents (Fravel et al. 2003). In this study, *F. oxysporum* and *P. neglecta* were isolated as non-DSE endophytes. *F. oxysporum* is a common endophyte of grasses (Herrera et al. 2010) and *Parasphaerosphaeria* sp. was identified in halophylic plants (Khalmuratova et al. 2015) and forage grass (Herrera et al. 2010). Its anamorphic form, *Paraconiothyrium variabile*, when in association with leaves of *Cephalotaxus harringtonia*, had a profound impact on the physiology and defence reactions of the host plant by releasing a glycosylated flavonoid (Tian et al. 2014).

### 4.2 Diversity of endophytic fungi in warming experiment

To track the diversity of endophytic fungi in two dominant plants, a grass and a herb, of the permanent grassland at two different conditions, +2°C surface temperature and ambient environmental conditions, we performed Illumina sequencing of fungal ITS2 amplicons of surface washed root samples obtained from +2°C temperature and ambient temperature conditions and isolated fungal endophytes for further plant-microbe interaction studies under climate change conditions.

In total 48 OTUs (RA ≥ 1%) were identified, of which 45 were represent by a single species-level OTUs that display large part (93%) of total species richness (Fig 4). The richness of root associated fungi obtained in this study is in accordance with the Alpha diversity studies of other plant species (Glynou et al. 2017; Novotná et al. 2018).

The most prevalent OTUs in this study represented Ascomycota as endorsed in earlier studies (Rodriguez et al. 2009; Toju et al. 2013). Next abundant were Basidiomycota and Mucoromycota. Hence, 11 order within the Ascomycota were detected. The dominant orders, in average of four samples, were the orders *Helotiales* (RA 17% - 61%), *Pleosporales* (RA 3% - 21%), and *Chaetothyriales* (RA 2% - 12%) (Fig 3 b). In line with current study, *Helotiales* are normally dominant in fungal endophytes culture-independent approaches, particularly, single plant species such as oak (Toju et al. 2013), ecosystems such as California semiarid shrub-land (Lipson et al. 2014), and vascular plants in high arctic zone (Zhang and Yao 2015). Moreover, endophytic fungi diversity is strongly related to soil characters. A case in point is *Helotiales*, often associated with acidic soil (Glynou et al. 2016). In this experiment, the top soil in Giessen grassland was slightly acidic (pH 5.3-5.8) and evidently the predominant OTUs of fungal-endophytes community composition were assigned to the *Helotiales*.

Most of the dominant OTUs in this study were assigned to the *Pleosporales* which was also obtained in a study of a semiarid grassland in Hungary (Knapp et al. 2015), of seeds of a medical plant (Shen et al. 2014), and *Microthlaspi* plants collected from six European countries and Turkey (Glynou et al. 2016). Rodriguez et al. (2009) divided plants fungal endophytes in four groups, accordingly DSE were grouped in class 4. They are often Ascomycota (*Helotiales* or *Pleosporales*) and predominant in different ecosystems (Lipson et al. 2014; Newsham 2011). Approximately 33 species of DSE

have been described in literatures (Knapp et al. 2015) that have been hosted by around 600 plants (Jumpponen and Trappe 1998). Data from meta-analysis of 18 peer reviewed papers about DSE showed species of *Helotiales*, *Chaetothyriales,* and *Pleosporales*, which had been used as DSE type species, could increase shoot and root biomass (Newsham 2011).

In this study, *Glarea* sp. (RA 1-55%) and *C. orchidicola* (RA 0.5-15%) were the dominant species OTUs. *Glarea* sp., formerly *Zalerion* sp. (Bills et al. 1999), produce a secondary metabolite pneumocandin $B_0$, essential molecule for the antifungal drug caspofungin synthesis (Peláez et al. 2011) and is known as endophyte of Chinese medical herbs (Miller et al. 2012). *Cadophora* spp. includes different saprophytic and plant pathogenic species (Harrington et al. 2003). *C. orchidicola* is reported as an endophytic species with a wide host range (Rodriguez et al. 2009). Fungi of this species were able to transfer nitrogen and uptake phosphorus to provide nutrients to host plants (Diene et al. 2010). Both, *Glarea* spp. and *Cadophora* spp. are known as DSE. In other host plants, the most dominating OTUs were *Penicillium* spp. (Huang et al. 2014) and *Trichoderma* spp. (Novotná et al. 2018).

### 4.2.1 Alpha diversity of fungal endophyte communities

Illumina ITS2 amplicon sequencing showed different impact of moderate surface warming on alpha diversity of fungal endophyte communities of both host plants. In *A. elatius,* species richness in C and T samples was high, species abundance was higher in T compared to C, but species diversity was lower in T samples. In *G. album* species diversity and abundance were similarly high but species richness was decreased compare to C samples. In both host plants the composition of fungal endophyte was not equally distributed in C and T samples. Increasing nearly +2°C of both soil and air temperature during the whole growing season of balsam poplar trees (*Populus balsamifera*) decreased the diversity of foliar fungal endophytic communities (Bálint et al. 2015).

Fungal community composition of both host plants in the T plots samples versus C plots samples showed about 50% difference in terms of the most abundant species (Fig. 5). Change in temperature could influence not only the microbial community (Mayor et al. 2017), but also its function (Rubenstein et al. 2017). Eventually, it could

affect composition and function of the ecosystem (Classen et al. 2015). In this study 45 OTUs of non-pathogenic root-associates contrast with three OTUs of potential plant pathogenic fungi. Collectively, *Septoria cruciatae*, *Fusarium oxysporum* and *Drechslera* sp. in *G. album*, and *F. oxsporum* in *A. elatius* showed higher RA at T plots.

**4.3 high-through output sequencing versus culture-dependent approach**

New molecular technique such as high-through output sequencing facilitates mass identification of microorganisms. Accordingly, this study confirmed the principle that short-read sequencing can be useful tools to complex microbiota characterization by characterizing 48 OTUs versus 15 isolated species in a culture-dependent approach, corroborating cultivation strategies are unable to detect a large fungal richness. It seems culture-dependent approaches still needs more attention since some species do not grow well on standard agar media (Andrade-Linares and Franken 2013) or grow slowly (Narisawa et al. 2007). In line with our results, *Pleosporales* is the most dominant isolated fungi in culture-dependent approaches (Knapp et al. 2015; Shen et al. 2014) indicating its members could grow well on standard agar media. Whereas *Helotiales* is dominant in culture-independent studies (Lipson et al. 2014; Toju et al. 2013) and likewise in this study. Furthermore, isolation of endophytic fungi facilitates their further individual research and practical application.

**4.4 Basidiomycota in culture dependent/independent approach**

Basidiomycota as endophyte mostly constitute smaller proportion of species community than Ascomycota, notwithstanding they play more prominent role in symbiosis with plants as mycorrhiza. According to Illumina amplicon sequencing, only 16.5% of the detected OTUs were assigned to the Basidiomycota (Fig. 4a). In Basidiomycota totally five orders were classified including *Agaricales* (RA 2% - 13%), *Cantharellales* (RA 0% - 4%), and *Sebacinales* (RA 0% - 1%) (Fig 3 b). Similarly, fungi of the order *Agaricales* represented the predominant endophytes in common forage grass, *Bouteloua gracilis*, in Mexico (Herrera et al. 2010). Nevertheless, in culture-dependent approaches two isolates, which were assigned to *Russulales* and *Polyporales,* were isolated.

The most dominating Basidiomycota OTUs represented a *Mycena* sp. (RA 0-11 %). Fungi of that genus are well known as mycorrhizal fungi, which can improve seedlings

growth in the orchidaceous plant *Dendrobium officinale* in both natural (Zhang et al. 2012) and artificial cultivated assays (Chen et al. 2016). In addition, both genera *T. versicolor* and *S. hirsutum,* to which the Basidiomycota isolates obtained from Giessen grassland were known as famous saprophytes related to wood decay process (Hiscox et al. 2015; Ortiz et al. 2013). As endophytes, both, *T. versicolor* and *S. hirsutum,* were recognized in wheat (Comby et al. 2016) and *Hevea* sp. (Martin et al. 2015). Moreover, *T. versicolor* was isolated as endophyte of marine algae (Flewelling et al. 2013) and saprophyte in agricultural soil (Thorn et al. 1996). Both *T. versicolor* and *S. hirsutum* likely have two ecological type species as either saprophyte or endophyte that phylogenetically branched from same ancestors (Kohler et al. 2015).

The strain *Tv*-WC16GW showed high ITS and 18S rRNA sequences similarity respectively to *T. versicolor* strain HG9 that isolated as a wood white rot fungus from common hazel, *Corylus avellana*, in Czech Republic and the strain 5H1-P0-P5-2 that was isolated from a deep-subsea floor sediment near New Zealand ( Tomovský et al. 2006; Rédou et al. 2015). The *Sh*-P2A strain, which was isolated from a $eCO_2$ plot, shared high ITS and 18S rRNA sequences identity respectively to *S. hirsutum* strain P24.7 , was isolated from surface sterilized hair roots of *Gaultheria pumila* (*Ericaceae*) and strain FPL8805 , which is stored in USDA forest products laboratory (Hibbett, 1996; Bruzone et al. 2017). Interestingly, both strains isolated here showed plant growth promotion activity similar to *P. indica*. *Sh*-P2A also exhibited endophytic development in dicotyledonous *Arabidopsis* (Fig. 6).

Both fungi have been known as saprotrophs and the white rot wood decomposer fungi (Shirkavand et al. 2017) widely spread across forest ecosystems such as European beech trees *Fagus sylvatica* (Hiscox et al. 2015) and white-rotted wood of a temperate rainforest in Chiole National Park in Chile (Ortiz et al. 2013). They were known as secondary pathogens of plants and mostly associated with decline of peach trees (Adaskaveg et al. 1993; Chen et al. 2015), apple trees dieback (Doepel 1962), and decay of camphor tree *Ocotea usambarensis* (Nsolomo et al. 2000).
Notwithstanding that *Tv*-WC16GW and *Sh*-P2A were originally isolated from two different non-crop plants of Giessen grassland, they efficiently colonized roots of wheat, a monocotyledons crop plant. Similar to the well-characterized endophyte *P. indica* both fungi promoted root and shoot growth (Fig. 7 a-d). This finding also suggests that both fungi do not have a pronounced host specificity. Secondary

metabolites of *T. versicolor* have been shown to inhibit production of aflatoxin B1 by *Aspergillus flavus* in maize seeds (Scarpari et al. 2014). however, as an endophyte of tall fescue *Festuca arundinacea*, it could not reduce the damaging consequences of a root feeding insect, the European chafer (*Rhizotrogus majalis*; Gan et al. 2017).

### 4.5 Both *T. versicolor* and *S. hirsutum* harbour same Endobacteria

The two fungal species of different basidiomycetes orders - *Polyporales* and *Russulales* – were accompanied by 16S rRNA gene sequence contained identical endobacteria (Fig 11) raising the possibility that they form tripartite complex symbioses with host plants as have been shown in the Sebacinalean symbiosis (Sharma et al. 2008; Guo et al. 2017), although in the previously identified fungal strains assigned to the species *T. versicolor* and *S. hirsutum* endofungal bacteria have not been described. There are two groups of endohyphal bacteria, obligate (e.g. *Mollicutes*, *Ca*Gg and *M. cysteinexigens*) and facultative free-living e.g. *Burkholderiaceae* and *Rhizobiaceae* symbionts associated with endophytic fungi (Glaeser et al. 2016; Partida-Martinez 2017; Bonfante and Desirò, 2017). Electron microscopy (Bonfante and Anca 2009), FISH (Bertaux et al. 2005; Sharma et al. 2018; Guo et al. 2017), and PCR amplification of endobacterial genes (Sharma et al. 2008; Desiro et al. 2015, Guo et al. 2017) accomplished detection and characterization of endobacteria. Consistent with previous reports, *Tv*-WC16GW and *Sh*-P2A, contained only a single type of endobacteria whose 16s rRNA derived phylogeny revealed a monophyletic clade together with uncultured bacterial symbionts of *Heliothrips haemorrhoidalis* (Nguyen et al. 2015) and chloroplasts, supporting evolutionary relationships among fungal endobacteria and plastids/chloroplasts (Fig 11). Besides, culturing free-living bacteria was not successful possibly due to the fact that they are obligate symbionts or cultivation conditions are just unknown.

Phylogenetic analysis on 16S rRNA gene sequence similarities of the endobacteria following by the exclusion of eukaryotic organelles showed that *Tv*-WC16GW and *Sh*-P2A contain endobacteria showed high 16S rRNA gene sequence similarity to uncultured bacterial symbionts of thrips. Thrips could transmit a particular mollicute (Powell et al. 2015), which are well known as vector of Tospovirus (Riley et al. 2011). The endobacteria of both fungal strains were identical based on the 16S rRNA gene sequences (Fig 11). Likewise, endobacterial strains identified as *Luteibacter* sp.

(*Gammaproteobacteria*) were isolated from two different endophytic ascomycetes, *Microdiplodia* sp. (Dothideomycetes), and *Pestalotiopsis* sp. (Sordariomycetes). Strains of those genera successfully cross-inoculated between both fungi (Arendt et al. 2016). Furthermore, four isolates of the fungus *Mortierella elongata* possess identical endobacteria (Sato et al. 2010). Thus, harboring of same endobacteria by different endophytic fungal species may be attributed to either metabolic dependence to the host fungus (Naito et al., 2015) or horizontal transmission among fungal species in the same ecological niche (Toomer et al. 2015). Particularly, *Candidatus* Moeniiplasma glomeromycotorum (*Mollicutes*) horizontally transmitted among distinct *Glomeromycotina* host lineage (Naito et al. 2017). Therefore, it is possible that the endobacteria detected in this study have been transmitted between different fungal species in the Linden grassland.

**4 5.1 Morphology of endobacteria**

TEM microscopy revealed presence of BHVs in the hyphae of *Tv*-WC16GW isolate (Fig 10 a, b). Vacuoles are one of the prevalent organelles in the hyphae compartment of filamentous fungi. They have spherical or tubular shapes with variable size and mostly appear empty in electron microscope (Weber, 2002; Richards et al. 2010). Interacellular bacteria prefer to localize in vacuolar niches to provide maximal protection (Kumar and Valdivia 2009). Mammalian bacterial pathogens exploit the endocytose machinery of host cells for intravacuole residence. The property of bacteria-harboring vacuoles differs among bacteria (Cossart and Helenius, 2014). There are different bacteria-containing vacuoles types particularly *Salmonella*- and *Chlamydia* type (Yu et al. 2010; Bastidas et al. 2013). Moreover, an obligate endosymbiont bacterium of arthropods employs host cells vacuoles as well (Mediannikov et al. 2010). Complete absence of peptidoglycan structure was reported in some obligate intracellular bacteria (Otten et al. 2017).

TEM microscopy did not indicate any bacterial cell with plasma membrane or cell wall (Fig 10 a, b). Peptidoglycan cell wall is essential for cell shape determination and protection against osmotic stress (Desmarais et al. 2013). Lack of a cell wall is the feature of either *Mollicutes* or L-form bacteria among prokaryotes (Tully et al. 1993; Errington 2013). As a consequence of the lack of a cell wall, bacterial cells adapt mostly to coccoid morphology (Strahl and Errington 2017). Nevertheless, *Glomus versiforme,* Mollicute-related endobacteria of AMF, has an electron-dense layer

outside of plasma membrane resembling a cell wall (Naito et al. 2017). Host cytoplasm serves as an osmoprotective substrate for endobacteria (Cayley and Record 2003), hence saving energy with host-dependency.

### 4.5.2. Isolation of endobacteria was unsuccessful

Unfortunately, in our study the isolation of endobacteria was unsuccessful. This can be an evidence for strict dependence of the bacterium to its host fungus, e.g. for nutritional supply (Ghignone et al. 2012). The in situ detection of endobacteria by FISH showed a presence of the large number of endobacteria in some hyphae of both fungi (Fig 11 a, b). TEM images have verified FISH analysis by showing that only some hyphae possess numerous bacteria-harboring vacuoles in the cytoplasm. A large number of endobacteria in single hypha was observed in other studies as well (Obasa et al. 2017; Uehling et al. 2017). Moreover, two different endobacteria, *Ca*Gc and coccoid MRE, coexisted in a single cell (Desirò et al. 2014). The role of the novel endobacteria in the new fungal isolates must be further studied, as plant-associated fungi - endobacteria interaction can be rather complex among three-level inter- kingdom domain (Guo et al. 2017; Bonfante and Desiro, 2017).

## 4.6 Both *T. versicolor* and *P. indica improved wheat* yield parameters

We have confirmed the hypothesis that the potential of fungal endophytes to support the uptake of P by plants is dependent on both P form and the endophyte species (Ding et al. 2016). In particular, the yield parameters improved under mono-calcium phosphate (CP) but not under rock phosphate in the inoculated wheat plants (data not shown). Both fungal endophytes, *T. versicolor* and *P. indica*, enhance the grain P content under monocalcium phosphate conditions (Tables 3 and 4). Evidently, under this condition, both fungi improve wheat grain yield which also correlated with increased numbers of ear per plant. Achatz et al. (2010a) showed that increased numbers of ears in *P. indica*-colonized plants is due to increased number of tillers. We noticed the trend of increased number of tillers and ears resulting in higher grain yield in *T. versicolor*-colonized plants under mono-calcium phosphate condition. Indeed, increased number of tillers could be a first indication of a colonization by an endophyte. Endophytic development at an early plant growth stage could be dependent on both host genotype (Cheplick 2008) and nutrient supply. Namely, P

decreases the number of tillers by diminishing the emergence of leaves on the main stems (Rodríguez et al.1999) that could be remedied by fungi endophytes.

We show here that *T. versicolor* WC16GW can induce a considerable increase in grain yield (37%) and straw yield (8.5%). We also saw a slight, though under the given experimental conditions not significant, increase in P content under P deprived condition. Therefore, it cannot be ruled out that *T. versicolor* also exploits additional mechanisms to improve grain and straw yield in P-deprived condition. Instead, the fungus improved both grain yield and grain P content in P-rich condition. It remains to be established whether the growth promoting activity of *T. versicolor* is a result of an effect on plant growth hormones as it was demonstrated for *P. indica*-colonized plants (Schäfer and Kogel 2009).

Plants have developed various mechanisms to encounter P deficient conditions (Shen et al.2011), such as the exudation of special proteins which solubilize phytate, the main organic P form in the soil (Gerke 2015), the production of $P_i$ transporter proteins (Raghothama 1999), or acidic phosphatase (Mehra et al.2017). Interestingly the same mechanisms have been found in *P. indica* for P acquisition and transportation to the plant. P transporter encoding fungal (PT)-genes (PiPT1–4, Pedersen et al. 2013), genes encoding acidic phosphatase (PiPA1 and PiPA2) and phytases ($P_i$PHY1 and $P_i$PHY2) have been recognized in the *P. indica* genome (Ngwene et al. 2016). High P concentration (500 mg P $kg^{-1}$ soil) restrained RNA accumulation of all these genes, except PiPT4, under in vitro condition (Ngwene et al. 2016). Furthermore, in *P. indica* interaction with maize seedlings, PiPT1 gene was active only in P-deprived conditions (Kumar et al. 2011; Yadav et al. 2010). In our study, *P. indica* increased wheat P content not only in P-deprived, but also in P-rich conditions [100 mg P $kg^{-1}$, $Ca(H_2PO_4)_2$]. Hence, it remains unresolved whether an unknown P transporter becomes active under P-rich conditions or, alternatively, known genes are active also in P-rich conditions.

We confirmed that *P. indica*-mediated wheat yield increases were associated with improvement of P uptake. Consistent with our data, increased P uptake was also observed in maize (Yadav et al.2010; Kumar et al. 2011) and Arabidopsis (Shahollari et al. 2005) colonized with *P. indica*. Arbuscular mycorrhizal (AM) fungi establish widespread obligate symbioses with the majority of terrestrial plants (Corradi and Bonfante2012). Their major advantage in symbiosis is acquiring P for host plants;

nevertheless, their usefulness in high-input agriculture is ambiguous (Grace et al. 2009; Veiga et al. 2011; Kim et al. 2017). As AM fungi cannot be cultured axenically, large-scale production of inoculum is dependent on the appropriate host, hence making the mass inoculum production costly (Ijdo et al. 2011). Besides, high soil P concentration mostly reduced AM fungi colonization (Mäder et al. 2000; Breuillin et al. 2010; Balzergue et al. 2013; Liu et al. 2016). High level of residual P on much agricultural land due to the cumulative input of P fertilizer, namely in the period of 1965–2007 in Europe (1.115 kg $he^{-1}$, Sattari et al. 2012) suggests endophytes will be functional in future agricultural systems as they are able to increase P uptake into the plant at high soil P levels. It seems that the basidiomycetes species used in this study behaved in a similar manner.

### 4.7 Conclusions

Most endophytes of this experimental approach belong to class 2 endophytes defined by Rodriguez et al. (2009), which could be transmitted via seed coat from maternal plants to offspring (Redman et al. 2002). Accordingly, 48 OTUs with RAa ≥1% were detected in either a mono- or dicotyledonous plant in the GiFACE ecosystem (Fig. 5). Only nine OTUs were discovered in both host plants (Fig. 5). Such a considerable difference in fungal communities (85%) suggests each plant has its unique fungal species and many endophytes exhibit host plant specificity, which is in line with other studies (Wearn et al. 2012; Kembel and Mueller, 2014). Some species were ubiquities and common among both co-occurring host plants, suggesting the possibility of a horizontal transmission between them (Rodriguez et al. 2008). According to our data, plants in grassland ecosystem have a hidden variety of fungal endophytes that may have an influence on crops production. However, more research is required to gain a full picture on the communities. Especially the endophytes obtained from grasslands exposed to climate change conditions are promising candidates for detailed studies of niche adaptation and plant-microbe interactions under changing environmental conditions excepted in the next years

My study discovered two phylogenetically different plant growth promoting endophytic fungi isolated from two plant species within the same grassland. Both endophytic fungi contained identical endobacteria as determined by 16S rRNA sequence comparison. The bacteria were also found to be closely related to uncultured bacterial symbionts

of *Heliothrips haemorrhoidalis* and chloroplast sequences. The colonization of *Tv*-WC16GW and *Sh*-$P_2$A mycelia by endobacteria was confirmed by microscopy. Whether the endobacteria have a functional role in the mutualistic plant-fungus interaction needs to be elucidated in further studies. It is interesting that phylogenetically identical, and potentially obligate endobacteria seem to have different host fungi. As both fungi were isolated from the same grassland, an inter-fungal transfer is a possible transmission pathway. Open questions concern the mechanisms of bacterial transfer, the mode of exclusion of other bacteria from fungal mycelia, and strategies of extra-fungal survival. So far, the cultivation of the endobacteria was not successful, thus requiring further studies on the endobacteria during their endofungal life style. Metagenome sequence will give more insights in the fungal - bacterial relationships.

In addition, I show here the beneficial activity of *T. versicolor*, a fungal endophyte isolated from the root of *G. album* at Linden grassland, for the first time under experimental condition. I show its ability to induce growth promotion in wheat and likewise increases grain yield. Moreover, we show its ability to increase P uptake in P-rich conditions. These results further confirm that P acquisition and transportation is a key factor in plant-endophytic fungi interaction and has a strong influence on plant yield improvement. My data show that additional experiments under more practical field conditions by using winter wheat are required as the growing time of 11 weeks in our experiments is short compared to the practical situation.

# 5 Summary/ Zusammenfassung

## 5.1 Summary

I surveyed the diversity and the composition of endophytic fungal communities in roots of two predominant plant species, the monocotyledonous *Arrhenatherum elatius* and the dicotyledonous *Galium album* in the permanent grassland of the „Environmental Monitoring and Climate Change Impact Research Station Linden" near Giessen, Germany. Samples were taken from a 1998 started Free Air $CO_2$ Enrichment (FACE) system (+20 % relative to ambient) and a 2008 started warming experiment using IR-lamps to increase soil surface temperature to +2°C above ambient temperature. We used both, cultivation independent high-throughput Illumina 18S - 28S rRNA gene internal transcribed spacer 2 (ITS2) amplicon sequencing of the fungal endophyte

communities, and a cultivation-dependent approach to determine respective fungal endophytes. The most dominating taxa among fungal communities were assigned as *Helotiales,* Ascomycota (e.g. *Cadophora orchidicola*), *Pleosporales,* Ascomycota (e.g. *Periconia macrospinosa*) and *Agaricales,* Basidiomycota (e.g. *Mycena* spp.). Fifteen fungal isolates were isolated from surface disinfected roots tissue of the two plants grown under elevated $CO_2$, +2°C temperature, or ambient condition. Phylogenetic identification showed that 13 isolates belonging to Ascomycota (mostly *Pleosporales*), and two to Basidiomycota (*Polyporales* and *Russulales*). The most common isolated endophytic fungi within the two host plants were closest related to *Periconia macrospinosa* (4/15 sequences; 99-100% 18S rRNA gene sequence similarity). According to the Illumina ITS2 amplicon sequencing results, most of the endophytic fungi with a relative abundance of more than 1 % were host specific. Fungal isolates obtained from plants adapted to climate change conditions are promising candidate for future studies of climate change effects.

During the investigation of endophytic fungal communities of *A. elatius* and *G. album*, two endophytic fungi were isolated from surface-disinfected root fragments belongs to Basidiomycota. The fungal strains were identified as Agaricomycetes and assigned to the species *Trametes versicolor* (*Polyporales*) and *Stereum hirsutum* (*Russulales*), respectively, with 100% 18S rRNA gene and internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, and ITS2 sequence identity to described members of these species. Upon wheat inoculation, both fungi colonized roots and showed strong plant growth promoting activities, suggesting beneficial life styles. Fluorescence in situ hybridization (FISH) using an universal *Bacteria* 16S rRNA targeting probe mixture (EUB 338 I-III) and transmission electron microscopy (TEM) showed that both fungi contained endobacteria. While these bacteria proved to be so far non-culturable, they were identified by sequence analysis of 16S rRNA genes amplified from fungal genomic DNA. Unexpectedly, the bacteria from both fungi contained identical 16S rRNA gene sequences with highest sequence similarity to 16S rRNA gene sequences of the bacterial symbiont of the insect *Heliothrips haemorrhoidalis*, and 16S rRNA gene sequences of chloroplasts, different from chloroplast sequences of the host plants. Since there was neither evidence for contamination of mycelia by chloroplasts nor for non-green plastids of plants in TEM and chlorophyll-specific autofluorescence of suspicious spherical bacterium-like organisms (BLOs) by epifluorescence microscopy,

the data suggest the possibility that phylogenetically similar or even identical endophytic bacteria of a yet to be not further characterized bacterial lineage were found in different fungal endophytes of different plant species in a grassland ecosystem.

Soil phosphorus (P) deficiency occurs in many developing and transition countries. One method of resolving soil P deficiency is a strong application of mineral and organic fertilizers in order to saturate the P binding capacity of soil. Another promising method is the implementation of crop-endophyte symbioses in combination with the application of smaller amount of P fertilizer. This study examined the effect of the fungal endophytes *T. versicolor* and *P. indica* in P-deprived and P-rich conditions on P uptake and yield in wheat (*Triticum aestivum* L., cv. Bobwhite).

Three-day-old wheat seedlings were dip-inoculated with mycelia of (a) *T. versicolor* WC16GW axenically isolated from *G. album*, a dicotyledonous plant obtained from grassland in Linden near Giessen, Germany, and (b) axenic cultures of *P. indica* DSM 11827 freshly re-isolated from surface-sterilized barley roots. Seedlings were subsequently grown in 6 l Mitscherlich pots (eight seedlings per pot) in soil containing mono-calcium phosphate [CP, Ca $(H_2PO_4)_2$] with 100 mg P $kg^{-1}$ soil and control (CO) with 6.3 mg CAL-P $kg^{-1}$ soil P in an open-air pot experiment station for three months. Colonization of wheat roots by *T. versicolor* and *P. indica* increased plant biomass, yield and P content. *T. versicolor*-colonized plants exhibited a significant increase in grain yield of 37% (CO treatment) and 8.5% (CP treatment), as well as straw yield of 27% (CO treatment) as compared to non-colonized plants. *P. indica*-colonized plants showed a significant increase in grain yield 10% under high P (CP treatment) and straw yield of 22% (CO treatment). Moreover, *P. indica* improved grain P content by 30% (CO treatment), 16% (CP treatment) and straw P content by 33% (CO treatment), while *T. versicolor* increased grain P content by 16% (CP treatment) and straw by 35% (CP treatment). In Conclusions Both *T. versicolor* and *P. indica* improved wheat P uptake in both P-deprived and P-rich condition. *T. versicolor* supported a high grain yield under the CO and CP treatments, suggesting this fungus has a promising potential for P management in cereal crops.

## 5.2 Zusammenfassung

Ich habe die Vielfalt und Zusammensetzung endophytischer Pilze in Wurzeln von zwei vorherrschenden Pflanzenarten, dem monokotylen *Arrhenatherum elatius* und dem dikotylen *Galium album*, in der „Umweltbeobachtungs- und Klimafolgenforschungsstation Linden“ in Gießen, Deutschland untersucht.

Es wurden Proben von einem 1998 gestarteten Freiluft $CO_2$ Anreicherungsexperiment (Free Air $CO_2$ Enrichment (FACE)) und von einem 2008 gestarteten Erwärmungsexperiment, in welchem die Bodentemperatur auf 2°C über der Umgebungstemperatur erwärmt wird, genommen.

Wir haben sowohl kultivierungsunabhängige 18S – 28S rRNA Gen „internal transcribed spacer 2“ (ITS2) Amplikons der endophyten Gemeinschaft mit Hilfe der Illumina-Technik sequenziert, als auch einen kultivierungsabhängigen Ansatz verwendet, ,um die entsprechenden pilzlichen Endophyten zu bestimmen. Die vorherrschenden Taxa unter den pilzlichen Gemeinschaften wurden als Heliotiales (Ascomycota, z.B. *Cadophora orchidicola*), Pleosporales (Ascomycota, z.B. *Periconia macrospinosa*) und Agaricales (Basidiomycota, z.B. *Mycena* spp.) bestimmt.

Es wurden 15 verschiedene pilzliche Isolate aus oberflächendesinfiziertem Wurzelgewebe aus den zwei Pflanzenspezies, die unter erhöhten $CO_2$-Konzentrationen, erhöhter Temperatur wuchsen, gewonnen. Die phylogenetische Analyse hat gezeigt, dass 13 dieser Isolate zu den Ascomycota (hauptsächlich *Pleosporales*), und zwei zu den Basidiomycota (*Polyporales* und *Russulales*) gehören, enger Verwandter von *Periconia macrospinosa* (4/15 Sequenzen; 99-100% 18S rRNA Gen Übereinstimmung).

Nach den Ergebnissen der Illumina ITS2 Amplikon Sequenzierungen waren die meisten endophytischen Pilze mit einer Häufigkeit von über einem Prozent wirtsspezifisch.

Während der Untersuchung der endophyten pilzlichen Gemeinschaften in *Arrhenatherum elatius* und *Galium album* wurden zwei Pilze aus oberflächensterilisierten Wurzelfragmenten isoliert, die zu den Basidiomyceten gehören.

Diese Pilzstämme wurden als Agaricomycetes identifiziert und den Spezies *Trametes versicolor* (Polyporales) und *Stereum hirsutum* (Russulales) aufgrund der 100%-igen

Übereinstimmung der 18S rRNA Gene, der internal transcribed spacer 1 (ITS1), 5.8S ribosomale RNA Gene und der ITS2 Sequenzen mit beschriebenen Isolaten der entsprechenden Spezies zugewiesen.

Nach der Inokulation von Weizen, konnten beide Pilze die Wurzel besiedeln und zeigten starke Förderung des Pflanzenwachstums, was eine mutualistische Lebensweise indiziert.

Außerdem konnte, mittels Fluoreszenz in situ Hybridisation (FISH) mit einer Sondenmischung, welche bakterielle 16S rRNA targetiert (EUB 338 I-III) und Elektronen Transmissionsmikroskopie gezeigt werden, dass beide Pilze Endobakterien enthalten.

Weiterhin konnte ich Effekt der pilzlichen Endophyten *T. versicolor* und *P. indica,* unter P-mangel und P-reichen Bedingungen, auf die P-Aufnahme und Ertrag in Weizen (*Triticum aestivum* L., cv. Bobwhite) zeigen. Die Besiedelung von Weizenwurzeln mit *T. versicolor* und *P. indica* steigerte die Biomasse, den Ertrag und den P-gehalt. *T. versicolor* besiedelte Pflanzen zeigten eine signifikante Steigerung des Kornertrags von 37% (gegenüber Kontrollbehandlung) und 8.5% (P-Düngung), als auch des Strohertrags von 27% (Kontrollbehandlung) im Vergleich mit nicht besiedelten Pflanzen. *P. indica* besiedelte Pflanzen zeigten eine signifikante Steigerung des Kornertrags von 10% bei P-reichen Bedingungen (P-Düngung) und eine Steigerung des Strohertrags von 22% (Kontrollbehandlung). Des weiteren verbesserte *P. indica* den Korn-P-Gehalt um 30% (Kontrollbehandlung) und um 16% (P-Düngung) und den Stroh-P-Gehalt um 33%, während *T. versicolor* den Korn-P-Gehalt um 16% (P-Düngung) und den Stroh-P-Gehalt um 35% steigerte.

Zusammenfassend verbesserten *T. versicolor* und *P. indica* die P-Aufnahme unter P-reichen und P-armen Bedingungen. *T. versicolor* unterstützte eine Kornerstragssteigerung während gedüngten und ungedüngten Behandlungen, was dafür spricht, dass der Pilz ein vielversprechendes Potential für das P-Management in Getreiden hat

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## 7 Erklärung

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Gießen,

Meysam Taghinasab

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