A functional assessment of root endophytic fungal diversity and their context dependent effect on plants

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Summary

The fungal interaction with plants is a 400 million years old phenomenon, which presumably assisted in the plants' establishment on land. In a natural ecosystem, all plants—ranging from large trees to sea-grasses—are colonized by fungal endophytes, which can be detected inter- and intracellularly within the tissues of apparently healthy plants, without causing obvious negative effects on their host. These ubiquitous and diverse microorganisms are likely playing important roles in plant fitness and development. However, the knowledge on the ecological functions of fungal root endophytes is scarce. Among possible functions of endophytes, they are implicated in mutualisms with plants, which may increase plant resistance to biotic stressors like herbivores and pathogens, and/or to abiotic factors like soil salinity and drought. Also, endophytes are fascinating microorganisms in regard to their high potential to produce a great spectrum of secondary metabolites with expected ecological functions. However, evidences suggest that the interactions between host plants and endophytes are not static and endophytes express different symbiotic lifestyles ranging from mutualism to parasitism, which makes difficult to predict the ecological roles of these cryptic microorganisms.

To reveal the ecological function of fungal root endophytes, this doctoral thesis aims at assessing fungal root endophytes interactions with different plants and their effects on plant fitness, based on their phylogeny, traits, and competition potential in settings encompassing different abiotic contexts. To understand the cryptic implication of non-mycorrhizal endophytes in ecosystem processes, we isolated a diverse spectrum of fungal endophytes from roots of several plant species growing in different natural contexts and tested their effects on different model plants under axenic laboratory conditions. Additionally,

we aimed at investigating the effect of abiotic and biotic variables on the outcome of interactions between fungal root endophytes and plants.

In summary, the morphological and physiological traits of 128 fungal endophyte strains within ten fungal orders were studied and artificial experimental systems were used to reproduce their interactions with three plant species under laboratory conditions. Under defined axenic conditions, most endophytes behaved as weak parasites, but their performance varied across plant species and fungal taxa. The variation in the interactions was partly explained by convergent fungal traits that separate groups of endophytes with potentially different niche preferences. According to my findings, I predict that the functional complementarity of strains is essential in structuring natural root endophytic communities. Additionally, the responses of plant-endophyte interactions to different abiotic factors, namely nutrient availability, light intensity, and substrate's pH, indicate that the outcome of plant-fungus relationships may be robust to changes in the abiotic environment. The assessment of the responses of plant-endophyte interactions to biotic context, as combinations of selected dominant root fungal endophytes with different degrees of trait similarity and shared evolutionary history, indicates that frequently coexisting root-colonizing fungi may avoid competition in inter-specific interactions by occupying specific niches, and that their interactions likely define the structure of root-associated fungal communities and influence the microbiome impacts on plant fitness.

In conclusion, my findings suggest that dominant fungal lineages display different ecological preferences and complementary sets of functional traits, with different niche preferences within root tissues to avoid competition. Also, their diverse effects on plant fitness is likely host-isolate dependent and robust to changes in the abiotic environment when these encompass the tolerance range of either symbiont.

Zusammenfassung

Die Interaktion von Pilzen mit Pflanzen ist ein 400 Millionen Jahre altes Phänomen. Es wird davon ausgegangen, dass sie den Pflanzen ermöglicht hat, sich an Land zu etablieren. Fast alle Pflanzen eines natürlichen Ökosystems, von großen Bäumen bis hin zu Seegräsern, werden durch Pilz-Endophyten besiedelt. Diese Endophyten sind in sowohl inter- als auch intrazellulär in scheinbar gesunden Pflanzen zu finden, ohne ihre Wirte sichtbar negativ zu beeinflussen. Wenn es um die Rolle von Endophyten geht, werden diese normalerweise in clavicipitalean (gras-besiedelnd) und non-clavicipitalean (nicht gras-besiedelnd) gruppiert. Im Allgemeinen sind die gras-besiedelnden Endophyten besser untersucht als die nicht gras-besiedelnden. Basierend auf ihrer Funktion und ihrem Besiedlungsmuster werden die nicht gras-besiedelnden Endophyten in drei Gruppen eingeteilt. Die tatsächliche Rolle dieser Endophytengruppe ist jedoch nicht gut untersucht. Als mögliche Funktion wurde unter anderem eine mutualistische Lebensweise in Betracht gezogen, durch die die Wirtspflanze Resistenzen gegenüber biotischen Stressoren, wie Herbivoren und Pathogenen, sowie abiotischen Stressoren wie Salzen und Trockenheit erlangt. Endophyten zeichnen sich ebenso durch ihre außerordentliche Fähigkeit aus, eine große Bandbreite sekundärer Metabolite mit ökologischen Funktionen zu bilden. Bisherige Untersuchungsergebnisse weisen jedoch darauf hin, dass die Beziehung zwischen Wirtspflanze und Endophyt nicht statischer Natur ist. Vielmehr reichen die symbiotischen Lebensweisen von Parasitismus bis hin zu Mutualismus. Ob ein Endophyt asymptomatisch im Wirtsgewebe lebt oder Krankheitssymptome auslöst, hängt von der Anpassung des Endophyten an das jeweilige Wirtsorgan ab. Eine Rolle spielen dabei das Entwicklungsstadium beider Partner, die Virulenz des Endophyten, eventuelle Abwehrreaktionen des Wirtsorganismus und andere Umweltbedingungen. Dementsprechend bestimmt die antagonistische Balance zwischen Wirt und Endophyt, in Abhängigkeit ihres genetischen Hintergrundes, ihres

Entwicklungsstadiums und diverser Umweltfaktoren, sehr wahrscheinlich die Natur ihrer Beziehung. Dadurch sind die Ergebnisse der Wechselwirkungen zwischen Pflanze und Pilz-Endophyt unterschiedlicher Natur und kontextbasiert. Obwohl klar ist, dass diese vielfältigen und ubiquitären Mikroorganismen die Anpassung ihres Wirtes an ihre Umwelt beeinflussen können, sind die spezifischen Funktionen dieser Symbiosen und ihre Modulationen durch Umweltfaktoren in den meisten Fällen nicht bekannt.

Ziel dieser Doktorarbeit ist ein besseres Verständnis des Einflusses von Phylogenie und Eigenschaften der Pilz-Endophyten, sowie abiotischer Faktoren auf die Bildung von Wurzelpilzgemeinschaften und deren symbiotische Wechselwirkungen mit den Wirtspflanzen. Um den Einfluss nicht-mykorrhizierender Endophyten auf Vorgänge im Ökosystem zu entschlüsseln, haben wir eine Vielfalt von Pilz-Endophyten aus den Wurzeln diverser Pflanzen isoliert, die unter verschiedenen Bedingungen gewachsen sind (Glynou *et al*., 2016). Getestet wurden daraufhin deren Effekte auf verschiedene Modelpflanzen unter axenischen Laborbedingungen. Des Weiteren wurden die Effekte mehrerer biotischer und abiotischer Parameter auf die Symbiose zwischen Wurzelpilz-Endophyt und den Pflanzen untersucht. Wir haben uns in dieser Arbeit auf Wurzel-Endophyten konzentriert, weil ihre Besiedlung systemisch und wahrscheinlich in die Nährstoffaufnahme der Wirtspflanze involviert ist. Damit nehmen diese Endophyten höchstwahrscheinlich Einfluss auf die Entwicklung der Wirtspflanze. In diesem Umfang untersuchten wir eine große Auswahl von Pilz-Endophytenstämmen, die zu 60 operativen taxonomischen Einheiten (OTU) von zehn Pilzordnungen gehören und aus verschiedenen Pflanzen aus unterschiedlichen Regionen Europas isoliert wurden. Um den Einfluss auf die Entwicklung der Wirtspflanze zu untersuchen, wurden die Stämme getrennt voneinander in die Wurzeln drei verschiedener Pflanzen inokuliert. Zusätzlich wurden verschiedene Eigenschaften der Endophyten dokumentiert, die vermutlich die Beziehung zwischen Endophyt und Wirt beeinflussen. Auf unseren Screening-Ergebnissen basierend wurde eine Untergruppe von Stämmen selektiert, um den Einfluss verschiedener abiotischer Faktoren (pH, Lichtintensität, Nährstoffangebot)

auf die Wechselwirkungen mit verschiedenen Pflanzen zu untersuchen. Daraus resultierend wählten wir einige Stämme stellvertretend für die am häufigsten vorkommenden Gattungen (*Alternaria, Cadophora, Fusarium*) aus. Sowohl die Wechselwirkungen zwischen den dominantesten Wurzelbesiedlern unterschiedlichen Grades phylogenetischer Ähnlichkeit und anderen Eigenschaften wurden untersucht, als auch der Einfluss ihres gleichzeitigen Auftretens auf ihre Fähigkeit die Wurzel zu besiedeln und auf das Wachstum der Wirtspflanzen. Diese Doktorarbeit besteht aus sechs Kapiteln, die meine Forschungsaktivitäten beschreiben, um die Hauptziele zu erreichen. Diese Aktivitäten umfassen eine Arbeit mit vergleichsweise geringem Beitrag meinerseits zur Sammlung, Isolierung und Klassifizierung der Endophyten in Kapitel drei und wesentliche eigene Arbeiten im vierten bis sechsten Kapitel.

In Kapitel vier war es das Ziel die generellen Interaktionsmuster zwischen Wurzelpilz-Endophyten und ihren Wirtspflanzen zu verstehen (Kia *et al*., 2017). Durch die Nutzung einer großen Auswahl von wurzel-endophytischen Stämmen konnten in dieser Arbeit basierend auf Pilzeigenschaften und Wechselwirkungen mit Pflanzen funktionelle Gruppe definiert werden, um Gemeinschaftsbildung und symbiotische Assoziationsprozesse vorherzusagen. Dafür haben wir die Wechselwirkungsmuster von 128 Wurzel-Endophyten diverser Taxa und Ursprünge mit den Brassicaceen *Arabidopsis thaliana* und *Microthlaspi perfoliatum* und der Poacee *Hordeum vulgare* untersucht, um relevante ökologische Funktionen zu identifizieren. Für jede Kombination wurde das Pilzwachstum in den Wurzeln quantitativ und qualitativ evaluiert. Der Effekt der Besiedlung durch den Pilz auf das Gewicht der Pflanze sowie die Entwicklung von Symptomen wurden aufgezeichnet. Zusätzlich wurden die Enzymaktivitäten und morphologischen Eigenschaften (Vermehrung durch Sporen, Wachstumsrate, Melanisierung) verschiedener Endophyten als potentielle Einflüsse auf die Wechselwirkungen zwischen Wirt und Endophyt bestimmt. Alle untersuchten Endophyten waren fähig, die Wurzeln der drei Wirtspflanzenarten unter den gegebenen Versuchsbedingungen zu besiedeln. Die Mehrzahl hatte dabei keinen signifikanten Effekt

auf die Entwicklung der Pflanzen. *In vitro* Tests der Wechselwirkungen der Endophyten-Stämme mit den Pflanzen zeigten alles in allem einen negativen Effekt der Pilzbesiedlung auf das Pflanzenwachstum. Diese Effekte korrelierten teilweise mit der phylogenetischen Zugehörigkeit der Stämme, unterschieden sich aber auch in Abhängigkeit von der Pflanzen/Endophyt-Kombination. Die Differenzen konnten teilweise durch Pilzeigenschaften erklärt werden, die von mehreren verschiedenen Stämmen geteilt werden, wie z.B. Wachstumsraten oder Melanisierung. Die Herkunft der Stämme beeinflusste die Symbiosen ebenfalls. Endophyten, die aus *Microthlaspi* spp. Populationen isoliert worden waren, waren schädlicher für *M. erraticum* als Stämme aus anderen Quellen. Unsere Ergebnisse implizieren, dass die Verbindungen von Pflanzen und Endophyten lokalen Selektionsprozessen unterworfen sind. Dabei treten in unterschiedlichen Landschaften unterschiedliche Kombinationen von Symbionten bevorzugt auf. Es konnte ebenso gezeigt werden, dass unterschiedliche, häufig vorkommende Endophyten unterschiedliche Sets von Eigenschaften aufweisen die Wechselwirkungen beeinflussen können. Diese weisen auf eine funktionelle Komplementarität hin, die die Häufigkeit des gemeinsamen Vorkommens in natürlichen Gemeinschaften bestimmen könnte.

Im fünften Kapitel wurde untersucht, ob die Variation verschiedener abiotischer Parameter den Einfluss von Wurzelpilz-Endophyten auf das Pflanzenwachstum bedingen könnte (Kia *at al*., 2018). Es wurde evaluiert wie genau die eingestellten Umweltparameter die pflanzliche Reaktion auf die Inokulation mit den Wurzelpilz-Endophyten vorhersagen konnten. Als ein hinweisendes Maß für die Wechselwirkungen wurden Änderungen in Auftreten und Stärke eines Effektes der Endophyten auf das Pflanzenwachstum gemessen. Im Speziellen wurde die Beantwortung folgender Fragen beabsichtigt: (1) Ist die Beziehung zwischen den Wirtspflanzen und den Wurzelpilz-Endophyten stabil bei Veränderung abiotischer Einflussgrößen? (2) Sind Beziehungen, die von abiotischen Faktoren abhängig sind, abhängig vom Endophytenstamm? (3) Sind die Beziehungen, die von abiotischen Faktoren abhängen, stabil zwischen den Wirtspflanzenspezies? Dazu nutzten wir eine Auswahl von

Pilz-Endophytenstämmen, die in Kia et al. (2017) untersucht wurden, um den Einfluss der abiotischen Umwelt auf deren Beziehung mit den Wirtspflanzen zu dokumentieren. Die Auswahl der Stämme basierte auf ihrer phylogenetischen Zugehörigkeit, ihrer ökologischen Ursprünge, ihrer beobachteten unterschiedlichen Eigenschaften und ihrem Einfluss auf das Pflanzenwachstum. *In vitro* Kokultivierungsassays wurden angewendet um den Einfluss einer Auswahl von Endophyten unterschiedlicher Herkunft auf das Wachstum von *Arabidopsis thaliana, Microthlaspi erraticum* und *Hordeum vulgare* in Abhängigkeit von Nährstoffgradienten, Lichtintensität und pH-Wert des Substrates zu untersuchen. Die meisten Pilze zeigten einen schwachen, negativen Einfluss auf das Pflanzenwachstum. Nur wenige hatten einen dauerhaft schädlichen Einfluss auf die unterschiedlichen Pflanzen unter den unterschiedlichen Bedingungen. Veränderungen der abiotischen Parameter hatten einen Effekt auf das Pflanzenwachstum, aber nur einen geringen Einfluss auf die Reaktion der Pflanzen auf die Inokulation mit den Wurzelpilzen. Von den untersuchten Parametern verursachte die Variation des Nährstoffangebotes die größten Unterschiede in der Beziehung zwischen Endophyt und Wirtspflanze. Diese Unterschiede waren jedoch schwach und stammspezifisch. Diese Ergebnisse implizieren, dass der Einfluss von Wurzelpilzendophyten auf das Pflanzenwachstum unempfindlich ist gegenüber Veränderungen abiotischer Parameter, wenn sich diese innerhalb der Toleranzgrenzen beider Symbionten bewegen.

In Kapitel sechs geht es um die Untersuchung des Einflusses von Interaktionen zwischen verschiedenen Endophytenstämmen verschiedener Pilzarten auf den Wirtspflanzenzustand. Im ersten Kapitel konnte gezeigt werden, das einzelne wurzelbesiedelnde Pilze unterschiedliche Einflüsse auf Pflanzen haben. Diese reichen von schädlich bis nützlich. Wie sich zwischenartliche Interaktionen letztendlich auf die Pflanzengesundheit auswirken, ist kaum bekannt. Deswegen untersuchten wir *in planta* Interaktionen zwischen dominant wurzelbesiedelnden Pilzen mit unterschiedlichem Maß an Übereinstimmung phylogenetischer und anderer Eigenschaften. Untersucht wurde der Einfluss ihres

gleichzeitigen Auftretens, auf ihre jeweilige Fähigkeit die Wurzeln zu besiedeln, und auf das Pflanzenwachstum. Dafür wurde eine *In vitro Assay* mit *Arabidopsis thaliana* als Wirtspflanze genutzt, um individuelle oder Artkombinationen von Pilzen zu ko-kultivieren. Die Wurzelbesiedlung durch die Pilze wurde mit Hilfe von real-time quantitative PCR (qPCR) überwacht. Der Einfluss auf die Wirtspflanze wurde durch die Messung der Pflanzenbiomasse quantifiziert. Die Wurzelbesiedlung unterschiedlicher Arten hatte unterschiedlichen Einfluss auf das Pflanzenwachstum. Dieser wurde durch die Präsenz anderer Pilzarten abgemildert. Die Masse der die Wurzeln besiedelnden Pilze verhielt sich bei Ko-Inokulation unterschiedlich. Die Konkurrenz zwischen den Arten mit ähnlichen funktionellen Eigenschaften war dabei am größten. Diese Ergebnisse implizieren, dass Wechselwirkungen zwischen wurzelbesiedelnden Pilzarten die Struktur wurzelassoziierter Pilzgemeinschaften maßgeblich mitbestimmen und dass das Myckbiom die Pflanzengesundheit beeinflusst.

Zusammenfassend ist zu sagen, dass der verwendete Experimentaufbau adäquat war, um die in dieser Arbeit aufgestellten Fragen zu Wechselwirkungen zwischen Pflanzen und einer großen Bandbreite an endophytischen Pilzarten zu untersuchen. Unter den gewählten Bedingungen verhielten sich die untersuchten Endophyten alle schwach parasitär. Unterschiede ergaben sich aber bei Verwendung verschiedener Arten von Wirtspflanzen und Endophyten. Die verschiedenen Endophytenarten haben unterschiedliche Strategien für die Symbiose mit Pflanzen entwickelt. Dadurch dass sie aber variable Verbindungen eingingen, ist davon auszugehen, dass sie grundsätzlich Gegenstand lokaler Selektionsprozesse waren. Ein Teil der Unterschiede in den Wechselwirkungen kann mit konvergenten Eigenschaften der Pilzarten erklärt werden, die Klassen von Endophyten mit eventuell unterschiedlichen Nischen abgrenzen. Die funktionellen Übereinstimmungen von Arten, die zu unterschiedlichen Gruppen gehören, kann durch die natürliche Struktur ihrer endophytischen Gemeinschaften vorhergesagt werden. Die Einteilung endophytischer Diversität in potentielle funktionelle Gruppen kann behilflich bei zukünftigen Untersuchungen

über deren Rolle in Ökosystemen sein. Die Untersuchungen des Einflusses von Nährstoffangebot, Lichtintensität und Substrat-pH auf die Endophyt-Pflanzen-Beziehung implizieren, dass die Art der Wechselwirkungen zwischen Pilz und Pflanze abiotischen Faktoren im Feld gegenüber relativ unempfindlich sind. Bei den hier untersuchten abiotischen Parametern handelt es sich in natura sehr wahrscheinlich nicht um entscheidende Einflussgrößen auf die Pflanzen-Endophyt-Beziehung. Eine Ausweitung der Untersuchungen auf extremere und länger einwirkende Bedingungen ist notwendig, um die Rolle abiotischer Faktoren in der Pflanzen-Endophyt-Beziehung abschließend zu klären. Die Untersuchungen der Reaktion der Pflanzen-Endophyt-Beziehung auf biotische Faktoren, wie die Kombination mehrerer Endophyten mit verschiedenen Graden der Ähnlichkeit und geteilter Evolution implizieren, dass die Konkurrenz zwischen verschiedenen wurzelbesiedelnden Endophyten, die häufig in natürlich vorkommenden Wurzeln dominieren und koexistieren, ein entscheidender Faktor in Hinblick auf die Struktur wurzelassoziierter Pilzgemeinschaften sind. Des Weiteren können sie einen Einfluss auf den Einfluss des Mikrobioms auf die Pflanzengesundheit haben. Weitere Untersuchungen zu zeitlichen Mustern von Besiedlungspräferenzen und der unterschiedlichen Nutzung von Wurzelteilen und/oder Nährstoffen sind notwendig, um den Stellenwert von Mikroben-Mikroben-Interaktionen in der Struktur des pflanzlichen Mikrobioms und dessen Funktion zu verstehen.

1. Introduction

1.1. Definition of fungal endophytes and brief research history

All plants in every ecosystem host a broad spectrum of microorganisms within their tissues, inter- and intracellularly (Bloemberg & Carvajal 2006; Schulz & Boyle 2006; Partida-Martínez & Heil 2011). This community of microorganisms can be composed of bacteria, fungi, archaea, algae, amoeba and protozoa (Sieber 2002; Malcolm *et al.* 2013). In this regard, terrestrial plants' phyllosphere and rhizosphere serve as a special niche for specific microorganisms (Sieber 2002; Berendsen *et al.* 2012; Peñuelas & Terradas 2014) and these "microorganisms located within apparently healthy, functional plant tissues at the moment of sampling" are acknowledged as endophytes (Sieber 2002). Although endophytes feed from their host plant, they do not cause an obvious harm to the host and often their interaction with the plant is symptomless (Schulz & Boyle 2006). However, in some cases endophytes can become pathogens during host senescence and therefore can be categorized as latent pathogens (Fisher & Petrini 1992; Delaye *et al.* 2013). Some endophytes are obligatory symbionts and cannot live independently of a living host, such as the arbuscular mycorrhizal fungi (Marschner & Dell 1994). On the other hand, some endophytes can be temporary plant colonizers with different lifestyles, like saprotrophs (Guerreiro *et al.* 2018). Various studies indicate that endophytes may switch their lifestyles from mutualistic to parasitic depending on various environmental factors (Saikkonen *et al.* 1998; Schulz & Boyle 2006; Mandyam & Jumpponen 2015).

At the beginning of the 19th century, microorganisms living within plant tissues were described by Heinrich Friedrich Link, who used the term "*Entophytae*" (Hardoim *et al.* 2015).

Later, in 1866 Heinrich Anton de Bary coined the term “endophyte”, which has been defined as “all organisms that invade and reside within host plant tissue or cells” (Sieber 2002; Hardoim *et al.* 2015). In 1991, the term is used by Orlando Petrini to define endophytes as “all organisms that for some time in their life inhabit plant organs without causing apparent harm to their host” (Petrini 1991; Sieber 2002).

Most commonly studied endophytes are bacteria and fungi and we have only started to understand the consequences of their interactions on host plant fitness (Sturz & Nowak 2000; Bonito *et al.* 2014; van der Heijden *et al.* 2016). Among fungi, mycorrhizal symbionts are extensively studied, yet the knowledge about non-mycorrhizal root fungal endophytes and their ecological functions is rather neglected. Given the importance of fungi in ecosystem processes like litter decomposition, nutrient cycling and carbon sequestration (Strickland & Rousk 2010), as well as on plant community dynamics (Bever *et al.* 2010), this doctoral thesis attempts to assess the functional diversity and possible roles of diverse fungal endophytes on plant fitness. Also, due to the importance of systemic colonization of plant roots by endophytes and the expected involvement of these symbionts in the host’s nutrient uptake—and consequently in plant development—the focus of this thesis is on fungal root endophytes.

1.2. Groups of fungal endophytes

According to fossil records, the association of plants with fungi is a longstanding relationship (Redecker *et al.* 2000; Heckman *et al.* 2001). Fungal endophytes encompass a great diversity of species, which associate with plant roots and aerial parts in all terrestrial ecosystems (Arnold 2007; Rodriguez *et al.* 2009; Glynou *et al.* 2017). Excluding well-studied mycorrhizal fungi, endophytes are grouped in clavicipitalean (e.g. some grass-inhabiting) and non-clavicipitalean (Schulz & Boyle 2005; Rodriguez *et al.* 2009) and, usually, the clavicipitalean grass-inhabiting endophytes are better studied than others. In a study by

Rodriguez and colleagues (2009), authors attempted a classification of endophytes based on their function and colonization in four classes. The clavicipitalean endophytes were grouped as class 1 endophytes and non-clavicipitalean endophytes were grouped within three classes. While class 2 endophytes supposedly have broad host range and extensive colonization of different plant organs, class 3 and 4 endophytes occurrence are restricted to only shoots and roots, respectively. Class 4 endophytes mainly comprise root specialized dark septate endophytes. Yet, the actual ecological diversity and function of non-clavicipitalean endophytes awaits further studies.

It is likely that different compartments of a plant harbor specific endophytic communities, which have developed efficient features to colonize and persist in different settings of above and below ground plant tissues (Bloemberg & Carvajal 2006; Hardoim *et al.* 2015). For example, the foliar endophytes should have characteristics to stand against UV radiation, dehydration and lack of nutrition (Arnold 2007). On the other hand, root endophytes should adapt to the moist and dark rhizospheric environment (Juniper 1991). Therefore, the phyllospheric and rhizospheric fungi most likely have distinct traits to persist in these environments (Arnold 2007; Hoffman & Arnold 2008).

1.2.1. Above ground fungal endophytes

Foliar fungal endophytes are mainly ascomycetous fungi that live asymptomatically within the photosynthetic tissues of plants (Arnold 2007). These endophytes primarily comprise species in the classes Eurotiomycetes, Dothideomycetes, Leotiomycetes, Pezizomycetes and Sordariomycetes (Arnold 2007; Higgins *et al.* 2007; Rodriguez *et al.* 2009). This group commonly occurs in aerial tissues of plants in almost every ecosystem from the Arctic to the tropics (Arnold 2007). The above ground colonization of endophytes occurs inter/intracellularly and localized at the tissue level (Boyle *et al.* 2001; Schulz & Boyle 2005). Foliar endophytes are more specialized than root endophytes, owing to distinct morphology

of plant areal parts among plant species and due to lack of survival or nutritional reserve structures (Sieber 2002). The reproduction and persistence over time of the phyllospheric endophytes are relatively shorter than root endophytes (Sieber 2002). The ecological roles of this group of endophytes and their effects on hosts are not yet well known, nevertheless, conference to hosts of increased resistance toward diseases and herbivory are among potential functions of this group (Clay 1988, 1991; Arnold *et al.* 2003). For instance, anamorphic *Neotyphodium* spp. (teleomorph: *Epichloë* spp.) are broadly studied grass-associated endophytes that are vertically transmitted with plant seeds (Schardl *et al.* 2004), which are known for their function of herbivore deterrence due to alkaloid production and also for assisting Gramineae plants to stand against insects and pathogens (Faeth & Fagan 2002; Malinowski & Belesky 2006).

1.2.2. Below ground fungal endophytes

Fungal root endophytes colonize plants inter- and/or intracellularly via the rhizoplane (Schulz 2006). These endophytes are very diverse and mainly comprise species in the classes Dothideomycetes, Eurotiomycetes, Leotiomycetes, Pezizomycetes, Sordariomycetes and Taphrinomycetes. However, these endophytes represent a large group of fungi that have not yet been well defined taxonomically and ecologically (Rodriguez *et al.* 2009; Andrade-Linares & Franken 2013). The colonization of roots by non-mycorrhizal root endophytes differs among host species, due to hosts' structural differences and/or source-sink relationships between host and fungi. Either the host provides photosynthetic sources to fungi, or under specific circumstances lipid stored in fungal hyphae may provide energy source for the host (Schulz & Boyle 2006). Nonetheless, the root colonization is extensive from epidermal to cortical cells and in some cases it is accompanied by the formation of special structures like microsclerotia (Andrade-Linares & Franken 2013). In general, the non-pathogenic root colonizers do not infect the vascular cylinder (Abdellatif *et al.* 2009), which is a common infection pattern for necrotrophic fungi. Non-clavicipitalean endophytes are

usually horizontally transmitted (transmission among different individuals of same species rather than mother-offspring transmission) and the attraction of fungi to plant roots by root exudates mainly remains to be explored (Schulz & Boyle 2006; Rodriguez *et al.* 2009; Andrade-Linares & Franken 2013). Root-secreted hormones like jasmonic acid, strigolactone and ethylen are probably involved in this attraction (Khatabi *et al.* 2012; Nagata *et al.* 2016; Rozpądek *et al.* 2018). The specific penetration site into the host is not clear, but the general assumption is that endophytes get inside plants through cracks caused by emergent lateral roots or via wounds caused by pests (Mercado-Blanco 2015).

Well-known examples of root specialized non-mycorrhizal endophytes among Ascomycota are species within the genera *Cadophora, Chloridium*, *Exophiala, Leptodontidium*, *Phialocephala* and *Phialophora*, which are root specialized dark-septate endophytes (DSE), owing their name to the special morphology with dark, septated hyphae and often sterile mycelium (Sieber 2002; Schulz & Boyle 2006; Andrade-Linares & Franken 2013). DSE have broad host ranges across different ecosystems and are likely involved in plant community dynamics (Mandyam & Jumpponen 2005). Nutrient acquisition by hydrolytic enzyme activity and herbivory inhibition via secondary metabolites produced by fungi are among suggested functions for DSE, but empirical studies to unravel the ecological roles of this group are mostly lacking (Mandyam & Jumpponen 2005; Rodriguez *et al.* 2009).

A well-studied basidiomycete root endophyte is *Serendipita indica* (formerly *Piriformospora indica*). The root colonization of barley plants by this fungus increases with root maturation and the establishment of mutualistic interaction between *S. indica* and barley plant requires host cell death (Deshmukh *et al.* 2006). This fungus likely plays a role as plant growth promoter and contributes to host tolerance against abiotic stresses (Waller *et al.* 2005; Deshmukh *et al.* 2006; Hilbert *et al.* 2012; Varma *et al.* 2012; Banhara *et al.* 2015). However, a recent study shows that other species in the Serendipitaceae family also have the potential to promote plant growth under poor soil fertility conditions (Venneman *et al.* 2017), which merits further considerations.

Perhaps the best studied fungal root endophytes are arbuscular mycorrhizal (AM) fungi, which have developed unique structures to penetrate and establish within roots of vascular plants (Hoeksema *et al.* 2010). These obligate symbionts form the most abundant mutualistic symbioses known in nature, involved in the assistance of plants in the uptake of nutrients like nitrogen and phosphorus in exchange for photosynthetic carbon (Marschner & Dell 1994; George *et al.* 1995). In contrast to AM fungi, non-mycorrhizal root endophytes are often generalist and their colonization pattern and ecological role is not well understood. However, recent evidence indicates that these fungi also have the potential to translocate nutrients to host similarly to AM fungi (Behie *et al.* 2012; Hiruma *et al.* 2016; Almario *et al.* 2017).

1.3. Diversity of fungal root endophytes

Plants are not standalone entities, because they harbor a rich and diverse group of microorganisms (Partida-Martínez & Heil 2011; Vandenkoornhuyse *et al.* 2015). After several decades of studies on the diversity of endophytes, the recent application of molecular approaches has shed light on the large diversity of fungi contributing to the plant microbiota (Vandenkoornhuyse *et al.* 2002, 2015). Before the advent of molecular approaches, the diversity of root endophytes of herbaceous plants was roughly estimated to be around 20 fungal species (Sieber 2002), but a study on the diversity of root fungal endophytes of the grass *Arrhenatherum elatius* by sequencing of the small subunit of the ribosomal rDNA (SSU) reported 49 different phylotypes in a single plant (Vandenkoornhuyse *et al.* 2002). Other investigations such as those on root associated fungal endophytes of the herb *Bistorta vivipara* by sequencing of the internal transcribed spacer (ITS) region show a large diversity, of up to 41 fungal operational taxonomic units (OTUs) per root system (Blaalid *et al.* 2012). It is clear that the interior of plant roots harbors a complex assembly of fungi that interact with each other as a community and with the host. Therefore, the

ecological outcomes of these complicated interactions are not easy to predict and remain poorly understood.

Non-mycorrhizal fungal root endophytes mainly belong to the phyla Ascomycota, Basidiomycota, or Mucoromycota (Sieber 2002; Vandenkoornhuyse *et al.* 2002; Rodriguez *et al.* 2009; Spatafora *et al.* 2016). Among ascomycetes, the orders Pleosporales, Hypocreales and Helotiales are often reported as dominant, widespread, and generalist endophytes (Vandenkoornhuyse *et al.* 2002; Hoffman & Arnold 2008; Abdellatif *et al.* 2009; Glynou *et al.* 2016). Apart from these dominant orders, Sordariales, Xylariales, Chaetothyriales, and Eurotiales are also among the ascomycetous root endophytes (Porras-Alfaro *et al.* 2008; Chen *et al.* 2015a). With a lower incidence, some orders within Basidiomycota like the Sebacinales, Agaricales, Atheliales, Auriculariales, Cantharellales, Hymenochaetales, Polyporales, Russulales, Septobasidiales, or Tremellales, are also reported as root endophytes colonizing various plants in almost all terrestrial habitats (Jumpponen & Trappe 1998; Porras-Alfaro *et al.* 2008; Weiß *et al.* 2011; Knapp *et al.* 2012; Martin *et al.* 2015). The occurrence of root endophytes among the Mucoromycota has been recently reported, like species of Mortierellales as endophytes inducing host stress tolerance (Uehling *et al.* 2017; Wani *et al.* 2017).

Fungal root endophytes are abundant in the rhizosphere across different bioclimatic zones (Timling *et al.* 2014) and seem to have efficient dispersal abilities so that they are found across broad geographic areas without showing a weak host specificity (Jumpponen & Trappe 1998; Queloz *et al.* 2011; Knapp *et al.* 2012; Timling *et al.* 2014; Glynou *et al.* 2016, 2017). However, several environmental abiotic factors like bioclimatic conditions (e.g. altitudinal and latitudinal associated temperature and precipitation gradients) and soil type and structure likely affect root endophytes communities (Blaalid *et al.* 2014; Geml *et al.* 2014; Glynou *et al.* 2016). For instance, a study assessing the fungal diversity in a neotropical forest shows that root endophytic fungi mainly within the Helotiales are mostly represented in high-elevated montane cloud forests, and in general the fungal community

structure is affected by the soil pH and the nitrogen (N), phosphorus (P) and organic matter contents (Geml *et al.* 2014). Similarly, a study on the diversity and distribution of fungal communities in the arctic shows that root endophytes, among other soil fungi, are effected by soil pH and climate, which are correlated with altitude (Timling *et al.* 2014). Likewise, Glynou and colleagues reported that among the root endophytes of *Microthlaspi* spp. plants sampled across Europe, Helotiales fungi (e.g. *Cadophora* spp.) occurrence is correlated with soil pH and magnesium content (Glynou *et al.* 2016). Among other bioclimatic factors, CO_2, elevation, temperature, humidity and sunlight hours are other important factors affecting richness of root fungal endophytes and their colonization capability (Lingfei *et al.* 2005; Brosi *et al.* 2011; Geml *et al.* 2015). While these abiotic factors have clear influence in determining the structure and composition of endophytic communities, their effect on plant-endophyte interaction is barely known.

It is likely that abiotic environmental factors, like soil properties and bioclimatic conditions, are more important than biotic factors, like host species identity, in determining fungal root endophytes' distribution and diversity (David *et al.* 2016; Glynou *et al.* 2016). The host specify of some DSE species, like *Phialocephala fortinii* s. l.—*Acephala applanata* species complex (PAC) argued before (Sieber 2002), however, later studies showed that the PAC is also not affected by host tree species nor by climatic factors, which suggest that these fungi are also generalists and do not have a biogeographic preferences (Queloz *et al.* 2011; Walker *et al.* 2011). Similarly, studies on herbaceous plant root endophytes from different ecosystems show that host species is not a filtering biotic factor for endophytic community distribution, but abiotic factors like soil characteristics of sampling sites are likely playing roles in below ground community structure and richness (Botnen *et al.* 2014; David *et al.* 2016; Glynou *et al.* 2016). Still, the role of host identity and fungal phylogeny in plant-endophyte interactions deserves further studies.

1.4. Ecological importance of fungal root endophytes

Since 1904, when Lorenz Hiltner defined the term “rhizosphere” and suggested the importance of root-inhabiting microbes for plant fitness (Hartmann *et al.* 2008), the plant root microbiome as microbial assembly associated with plant root compartments either on surfaces or within tissues have been increasingly recognized by biologists, due to the large diversity and prevalence in all existing ecosystems and plants (Hirsch & Mauchline 2012; Berg *et al.* 2014a, b).

Plant roots harbor and interact with a broad range of microbes, which are likely involved in plant fitness via expanding plant metabolites and promoting plant germination and growth, mainly due to production of phytohormones (e.g. IAA, cytokines, gibberellins), vitamins, amino acids, and volatiles (Hilbert *et al.* 2012; Vos *et al.* 2013). Also, root endophytes may enhance plant resistance against disease and stress (Yuan *et al.* 2010; Berendsen *et al.* 2012; Kusari *et al.* 2012; Berg *et al.* 2014a, 2016) via the production of antibiotics, salicylic acid, jasmonic acid, siderophores, volatiles, and lipopolysaccharides, which are plant priming factors that enhance plant resistance toward pathogens, insect pests and herbivores by increasing their competitiveness (Zhang *et al.* 2006; Khan *et al.* 2014; Pieterse *et al.* 2014). In addition, fungal endophytes are known for their capacity to protect plants via producing compounds like alkaloids, terpenoids, lignins, phenolics and defense enzymes such as peroxidases and chitinases, which inhibit the growth of plant pathogens and herbivores (Zhang *et al.* 2006; Porras-Alfaro & Bayman 2011; Khan *et al.* 2014). Moreover, endophytes are known to show a beneficial effect on their hosts when abiotic stressors are present, including drought, high salinity, high temperature, heavy metal presence, or low pH (Schulz & Boyle 2006; Zhang *et al.* 2006). Drought tolerance mechanisms induced by endophytes comprise several strategies, including osmotic adjustment/protection, water-use efficiency, production of reactive oxygen species (ROS) as a signaling molecule for inducing an early stress response in plants and lipid accumulation within hyphae, which serves as a carbon source reserve for host (Schulz & Boyle 2006;

Singh *et al.* 2011; Hardoim *et al.* 2015). The response to salt stress encompasses an array of changes in plants like increases in lipid peroxidation and desaturation, reduction of oleic acid and changes in the fatty acid composition of the plant and increases in antioxidant enzymes in roots (Waller *et al.* 2005; Singh *et al.* 2011).

Most plants establish close associations with mycorrhizal fungi for scavenging soil nutrients, but some plant families like the Brassicaceae lost the ability to establish these symbioses over evolution (Heijden *et al.* 2017), which could entail that they utilize alternative strategies for nutrient acquisition from rhizosphere. For instance, studies on root-associated fungal endophytes of the Brassicaceae species *Arabis alpina* and *Arabidopsis thaliana* show the involvement of non-mycorrhizal endophytic fungal species in the uptake of P and the growth of the plant under P deficiency conditions (Hiruma *et al.* 2016; Almario *et al.* 2017). Also, groups of DSE show the capacity to solubilize inorganic P and mineralizing organic forms of it, independently of the presence of AM fungi and the host species and of increasing the available P in soil (Newsham 2011; Della Monica *et al.* 2015). In general, there are various strategies involved in P acquisition by the endophytic microbiome, including solubilization and mineralization. For instance, organic and inorganic acids, like hydrogen chloride or nitric acid, oxalate, or acid phosphatases are produced by fungi and are involved in decreases of soil pH and facilitating the solubilization or mobilization of P (Plassard *et al.* 2011; Richardson & Simpson 2011; Khan *et al.* 2014).

Hydrolytic enzymes, like extracellular proteinases and chitinases, are also involved in the mobilization of nitrogen from organic compounds, which are important in the release of low-molecular-mass compounds to be adopted by the native flora (Chalot & Brun 1998). An interesting example of nitrogen-scavenging endophytic fungi is *Metarhizium* spp., which is a common soil inhabiting entomopathogen. A study indicates that this fungus can infect and kill soil insects and transfer the insect-derived nitrogen to plants via fungal mycelia and endophytic association (Behie *et al.* 2012).

The mentioned nutrition uptake abilities are described for some non-mycorrhizal fungal root endophytes, including species from genera such as *Chaetomium, Cladorrhinum, Colletotrichum*, *Cryptosporiopsis, Fusarium, Heteroconium, Oidiodendron, Phialocephala, Prifomospora*, and *Stagonospora* (Schulz 2006; Schulz & Boyle 2006; Hiruma *et al.* 2016; Almario *et al.* 2017). These fungi are mostly reported to improve their hosts' growth via nutrient supply and synthesis of plant hormones and by obtaining nutrients saprotrophytically from soil via ligninolytic enzymatic activities (Schulz 2006). Therefore, plant root associated microbes may play important roles in plants' phenotypic plasticity and evolution through modulation of plant development and defense responses (Goh *et al.* 2013) and, consequently, by regulating the effect of the plant community on terrestrial ecosystems' productivity (Kent & Triplett 2002; Van Der Heijden *et al.* 2008; Harris 2009; Lugtenberg & Kamilova 2009; Partida-Martínez & Heil 2011; Bakker *et al.* 2014; Berg *et al.* 2014b).

1.5. *Recognized and presumed functions of fungal root endophytes*

Fungi-plant interactions are dated back to the Ordovician time (400–460 million years ago), when supposedly beneficial interactions assisted in the terrestrial establishment of vascular plants (Redecker *et al.* 2000). Accordingly, in a stable mutualistic association between partners, the fungus potentially helps the plant to scavenge nutrients form the soil and improves plants stress tolerance in exchange for photosynthetic carbohydrates. Contrarily, instable symbiotic interactions may result in weakened plant (Schulz 2006; Zeilinger *et al.* 2016). Therefore, plant fitness is affected by the interactions of the plant with its endophytic and also epiphytic microbiome, in a form of assembly of different species in an ecological unit known as the "holobiont" (Vandenkoornhuyse *et al.* 2015).

Endophytic fungi can comprise some species shared with the rhizospheric and epiphytic assemblages of the plant microbiome, hence commonly known epiphytic saprobes like *Alternaria alternata* can also be recognized as endophytes. The rhizospheric habitat is a

very dynamic environment, highly affected by plant root exudates and properties of the soil. Endophytes, unlike rhizospheric fungi, are established in the interior of plant tissues, so they may encounter less environmental stresses and competition (Yugan *et al.* 2010). In some cases, the colonization of plant tissues by fungal endophytes may develop further than normal and break the balance of a mutual interaction with the host. Thereby some fungi may become pathogens. However, unlike necrotrophic pathogens which extensively colonize the plant's vascular system and have detrimental effects on the host, endophytes usually have a weak parasitic interaction and do not cause an obvious harm on the host (Faeth & Fagan 2002; Brundrett 2004; Stone *et al.* 2004; Kia *et al.* 2017). Even though endophytes are commonly known as microorganisms that colonize healthy plant tissues without immediate and obvious symptoms, this definition includes the entire spectrum of interactions from parasitism to mutualism and, strictly speaking, endophytes encompass all types of microbes living inside of plants (Stone *et al.* 2004; Schulz & Boyle 2005; Partida-Martínez & Heil 2011). Indeed, many of the commonly reported endophytic fungi are known as pathogens, which may occur in both healthy and diseased plant tissues (Schulz & Boyle 2005). The differences between endophytes and latent pathogens are slight and are reflected in the duration of the dormant phase and the degree of damage caused to the host (Faeth & Fagan 2002). Additionally, many commensal saprobic fungi can show symptomless colonization of hosts (Redman *et al.* 2001; Stergiopoulos & Gordon 2014; Guerreiro *et al.* 2018). Therefore, fungi known as endophytes likely display a modest and sustained period of colonization, "waiting" for plant senescence and physiological changes to turn into a latent pathogen or a saprobe (Fisher & Petrini 1992; Stone *et al.* 2004; Zeilinger *et al.* 2016). The symptomless colonization of plant tissues by weak parasites may emphasize the heterogenic endophytic association and evolutionary continuum between latent pathogens and symptomless endophytes (Saikkonen *et al.* 1998). Recent comparative genomic analyses of 163 fungal strains revealed multiple switches between endophytic and necrotrophic lifestyles, often involving the expansion or contraction of gene families, which are coding plant cell-wall degradation enzymes (Delaye *et al.* 2013). Likely, the development of a repertoire of cell-

wall degrading enzymes makes endophytes less dependent on host's carbon sources, while it can imply several lifestyles with broad enzymatic activities which enable them to utilize different carbon sources in the absence of the host plant (Wang & Qiu 2006; Parrent *et al.* 2009; Delaye *et al.* 2013; Almario *et al.* 2017; Knapp *et al.* 2018).

Although plants developed resistance mechanisms against opportunistic fungi, endophytes coevolved with their hosts and somehow adapted to them (Conrath *et al.* 2002; Stergiopoulos & Gordon 2014). Therefore, endophytes could have overcome host defense systems during evolution, by specific host recognition, adhesion and spore germination, and also by structural diversification to avoid plant recognition and defense (Stone et al. 1994). The frequent occurrence of endophytic fungi suggests that they developed effective systems to overcome the host barriers and that plants benefit from these microbes as "genome extensions" to increase their adaptation capacity (Vandenkoornhuyse *et al.* 2015). However, the ecological functions of endophytes as a complex phenomenon requires more systemic *in planta* testing and genomic investigations. In addition, the cross-talk between above and below ground tissues can effect this interaction and in some cases an external factor like herbivores may be involved in this cross-talk (Turner *et al.* 2013), which merits further consideration.

The high diversity of endophytes and the variation in their interaction with hosts across environmental conditions may have ecological implications for the local assembly of natural communities and might be a consequence of adaptations to local conditions (Thompson 2005). Recent studies have shown that some endophytes have a high degree of flexibility to colonize genetically distant plants and show different lifestyles (Rai & Agarkar 2014; Selosse *et al.* 2018). For instance, the ability of *Colletotrichum* spp., *Fusarium* spp. and *Curvularia* spp. to switch lifestyles from pathogenic to mutualistic between different host plants under different abiotic conditions is recognized, which can lead to a conference of stress (salt and heat) tolerance to the host (Freeman *et al.* 2001; Redman *et al.* 2005; Bacon & Yates 2006; Rodriguez *et al.* 2008; Hiruma *et al.* 2016; Lofgren *et al.* 2018). This

phenomenon has been termed “habitat-adapted symbiosis”, in which both plant and endophyte survive varying environmental conditions by fungal endophytes providing mechanisms to the plant to adapt and withstand extreme abiotic conditions and the plant shelters the fungus from stressful conditions (Redman *et al.* 2005; Yuan *et al.* 2010).

As earlier mentioned, the outcome of the interaction between plants and endophytes often varies along a parasitism–mutualism continuum. This variation mostly occurs among environmental conditions gradients as abiotic and biotic contexts (Bronstein 1994; Piculell *et al.* 2008; Chamberlain *et al.* 2014). According to a meta-analysis, the symbiotic function of endophytes seems to depend on different factors, comprising the identity of the host plant and fungi, soil fertility and the biotic complexity of the soil (Davitt *et al.* 2010; Rousk *et al.* 2010; Mayerhofer *et al.* 2012; Mandyam & Jumpponen 2015). Some studies have argued that plant–endophyte interactions likely exhibit some level of host specificity with particular species combinations, resulting either in a positive or a negative effect on plant fitness as an outcome. For example, a study on fungal endophyte communities in six different lines of maize showed that host genetic variation, as determined by maize line, had significant effects on endophytic species richness (Pan *et al.* 2008). Hence, it appears that the host phylogeny may play a role in shaping the fungal endophyte communities and their interactions (Redman *et al.* 2001; Vincent *et al.* 2016). On the other hand, the phylogeny of fungi is another important indicator of the interaction with the host (e.g., determining if they will be pathogens or mutualists). The interactions of fungal endophytes with plants are primarily isolate-dependent (Klironomos 2003; Tellenbach *et al.* 2011; Ranelli *et al.* 2015). For example, *Fusarium circinatum* and *Fusarium graminearum* adopted different symbiotic lifestyles on genetically distant host species and variation can also be found among fungal isolates infecting the same host species (Schulz & Boyle 2006; Berendsen *et al.* 2012). Similarly, root dominant endophytes like DSE and even AM fungi can show isolate-dependent association with plants (Hoeksema *et al.* 2010; Tellenbach *et al.* 2011).

Therefore, it seems that benefits or pathogenicity of root endophytes are revealed only in particular host-endophyte combinations (Mayerhofer *et al.* 2012).

Environmental factors extant in the location of origin of the endophytes, such as precipitation, light intensity and temperature (Hawkes *et al.* 2011; Álvarez-Loayza *et al.* 2011; Mandyam & Jumpponen 2015) may affect the interaction between the endophyte and plants. For instance, it has been shown that light levels can affect the lifestyles of *Diplodia mutila* (Álvarez-Loayza *et al.* 2011) and influence the production of inoculum by *Fusarium* spp. (Tschanz *et al.* 1976). High temperature affects the severity of disease caused by pathogenic *Alternaria* species (Timmer *et al.* 1998, 2000). Presumably, the availability of nutrients in the soil and pH play a role in fungal development stages within the plant (Doohan *et al.* 2003). For example, the pathogenicity of *Rhizoctonia solani*, a worldwide distributed plant pathogenic fungus, is host-specific and dependent on environmental conditions such as soil temperature, moisture, pH and potassium and inorganic nitrogen availability (Zachow *et al.* 2011). In summary, the effects on host plants attributed to endophytes depend on the environment in which the interactions take place (Malinowski & Belesky 2006).

1.6. Approaches and methods to elucidate the ecological role of root endophytes

Considering the enormous phylogenetic diversity of fungal endophytes and their incidence in all environments, the apparently unspecific nature of their interaction with hosts, the variability in their lifestyles and the context dependency of their host interactions, the empirical investigation of the symbiotic functions of endophytes is difficult.

Researchers usually categorize and study fungal endophytes based on their taxonomy, nutritional preferences, and specific morphological characteristics, like the presence of melanized hyphae and microsclerotia (Sieber 2002; Stone *et al.* 2004; Wang *et al.* 2009). Even if this general classification of endophytes is important, the assignment of

endophytes to a fixed group is problematic and insufficient to appreciate their ecological functions. As mentioned before, endophytes can act as latent pathogens or as saprotrophs, and by customizing different physiological features may interact differently with the host in different stages of life. Therefore, in order to classify endophytes into functional categories, we need a broad understanding of their function and strategies of association with plants. For this, a comprehensive phylogenetic, metabolic, molecular and physiological profile of fungi and their life history trade-offs in respect to environmental abiotic conditions should be integrated in trait-based multidimensional studies (Aguilar-Trigueros *et al.* 2014, 2015). The trait-based functional grouping will improve our understanding of associations of fungal endophytes with plants, their population dynamics and the biodiversity patterns in their natural communities. However, to follow this approach, a standard trait dataset with standardized protocols and experimental designs need to be developed to avoid trait variation which may bias the results. In addition, we need to improve our understanding of fungal basic biology, the soil and plant microbiome and effect of abiotic environment and host identity on the function of endophytic fungi (Chagnon *et al.* 2013). For studying endophytes, first we need to detect and isolate fungi from the interior of plant tissues. Second, fungal isolates need to be identified and characterized by different culturing methods and molecular methods. Last, the interactions between endophytic isolates and plants are reproduced under controlled conditions to track features of their association and extrapolate to their function in nature.

1.6.1. Detection and isolation of endophytes

The detection and isolation of fungal endophytes from plant tissues are usually influenced by the sampling procedure, the methods, and culture media used for isolation. Therefore, the results of colonization studies based on isolation data must be carefully interpreted and often need to be complemented with microscopic examinations of the plant tissues to confirm

colonization patterns detected by surface sterilization and selective culturing (Cabral *et al.* 1993). Microdissection (cutting tissue into many small pieces or milling the plant material) and culturing, or maceration of host tissue (Bissegger & Sieber 1994) and serial dilution plating (Bills & Polishook 1994) are used as isolation/detection methods. These well-practiced isolation methods usually start with the utilization of wetting agents, followed by a strong oxidant or disinfectants and accomplished by several sterile rinses. Endophytes are generally isolated after cutting individual plant organs into small segments right after sterilization. These pieces are then transferred onto an appropriate growth medium. Enrichment of media with different carbon or nitrogen substrata and the use of selective and general growth inhibitors and antibiotics may be of value for the isolation of certain groups of endophytic fungi. However, culture media with low levels of nutrients are often used for isolation to prevent overgrowth of rapidly growing fungi (Glynou *et al.* 2016). For isolation of fungal endophytes, commonly malt extract agar co-supplemented with antibiotics like ampicillin, streptomycin and/or kanamycin is used (Stone *et al.* 2004). However, in order to get an optimal isolation result, several media with combination of different antibiotics should be examined (McKinnon *et al.* 2017). To assure that the isolated fungi are indeed endophytic strains, every procedure used for surface-sterilization and culturing has to be optimized for the host, including the plants' organ, age and tissue sensitivity (Arnold & Herre 2003). It is also important to ascertain that the tissue has not been damaged by the strength of the sterilization agents. To check for the effectiveness of sterilization, the imprinting method for treated tissue can be applied (Hallmann *et al.* 2006). After culturing, the incubation temperature and light cycles also may affect the emergence of endophytes. Culture plates are normally incubated in dark rooms and at temperature ranges of 18° to 25°C (Stone *et al.* 2004). However, isolation and identification of fungal endophytes by culturing methods are limited, because there are some non-sporulating and non-culturable endophytic fungi. Therefore, molecular techniques, such as DNA fingerprinting and high-throughput sequencing methods, are lately employed for the detection and identification of endophytic fungi (Sun & Guo 2012).

1.6.2. Identification and characterization of endophytes

The identification of endophytes is often based on examinations of their morphological characteristics, which are usually medium-dependent (Boyle *et al.* 2001; Stone *et al.* 2004; Hallmann *et al.* 2006; McKinnon *et al.* 2017). For direct observation of endophytes, as with other fungal isolates, light microscopy coupled with staining or phase contrast is commonly used (Stone *et al.* 2004; Bloemberg & Carvajal 2006). For this, usually a simple procedure starts with clearing the plant tissues in a solution and followed by staining, then stained tissues are dehydrated and fixed in a permanent mounting medium for microscopy (Brundrett *et al.* 1984; Stone 1987). Additionally, high resolution scanning electron microscopy and epifluorescence microscopy with auto-fluorescent proteins (e.g. inserting green fluorescent protein gene into the fungal genome) are recent methods that can be useful. However, material fixation, sample size, shape and high cost of maintaining and performance are limiting the feasibility of these high resolution methods (Schulz & Boyle 2005; Bloemberg & Carvajal 2006; Zhang *et al.* 2006).

Another method for fungal identification is molecular taxonomy (Schoch *et al.* 2012; Raja *et al.* 2017). Nucleic acid sequencing makes it possible to determine the approximate phylogenetic position of any sterile isolate. Through alignment with homologous nucleotide sequences of known fungi, phylogenetic relationships can be inferred and the unknown sterile strain can be assigned to a taxonomic category (order, family and sometimes genus), without assignment of names. In this way, an approximation of the identity of the endophytes can be obtained. For this DNA barcoding, the ITS (internal transcribed spacer) region is a promising, widely used barcoding region (Stone *et al.* 2004; Schoch *et al*. 2012; Glynou *et al.* 2017). Although DNA barcoding is a valuable element in fungal endophyte identification, the value of existing sequence repositories is often limited due to insufficient annotations, taxonomic naming is not totally reliable and we are in need of accurate public databases.

1.6.3. In vitro *plant colonization bioassays*

In order to understand the ecological function and effects of each endophytic individual on the host plant we need to reproduce the interaction between endophytes and plants, with axenic assays under controlled, simplified artificial conditions. The interactions can be performed with any plant that can be easily grown in artificial conditions. Commonly, model plants like *Arabidopsis thaliana* (Schedel *et al.* 2012; Mandyam *et al.* 2013) or agronomically important plants like barley, corn, rice, or tomato are used (Deshmukh *et al.* 2006; Maciá-Vicente *et al.* 2009; Fakhro *et al.* 2010; Chen *et al.* 2015b; Gond *et al.* 2015; Vergara *et al.* 2017). The plants are grown either on artificial growth media or in artificial substrata like clay or vermiculite, which can be easily sterilized and amended with nutritional solutions suitable for the study goals (Maciá-Vicente *et al.* 2009; Tefera & Vidal 2009; Schedel *et al.* 2012; Banhara *et al.* 2015; Pan *et al.* 2017). For longer-term experiments, inoculated plants can be grown in greenhouse experiments where soil is commonly used as substratum. However, in these cases the soil´s natural microbiota, chemistry, and structure may interfere in the outcome of the plant-endophyte interactions (Stone *et al.* 2004; Schedel *et al.* 2012).

1.6.4. Quantification of root colonization by fungal endophytes

There are several ways of quantifying the degree of colonization of endophytes within plant tissues, but none of these methods is optimal. These methods include direct observation of root colonization density, or indirect quantification by correlating the number of isolates to their colonization density using culture techniques, or relative amounts of fungal DNA as an estimator of the degree of root colonization, using polymerase chain reaction (PCR) based methods like real-time quantitative PCR (qPCR) with fungal-specific primers (Stone *et al.* 2004; Hallmann *et al.* 2006; Tellenbach *et al.* 2010). Indirect quantification using culture techniques can be misleading due to the bias in isolation caused by fast-growing fungi, whereas qPCR—which presumably is the most accurate method for quantification—depends

on highly specific primer design for detecting the fungal species. Therefore, in order to detect and quantify the fungi associated with a plant, plant tissue surface-sterilization and culture-based isolation must be optimized and used in parallel to molecular techniques (Schulz & Boyle 2005; Tellenbach *et al.* 2010; Ko *et al.* 2011; Porras-Alfaro & Bayman 2011).

1.6.5. Summary of the methodologies used in this thesis

In this doctoral thesis, I used a combination of the above mentioned methods to study and characterize the functional diversity of fungal root endophytes. First, I collaborated in isolating and culturing of root fungal endophytes from non-mycorrhizal *Microthlaspi* spp. for generating strain collections. Later, a selection of endophytes were identified and characterized by application of phylogenetic analysis and trait based approaches. Additionally, to reproduce the interaction between endophytes with different plants, inoculation bioassays under several environmental contexts were employed and followed by the detection and quantification of root colonization of endophytes via microscopy, culturing and real-time PCR.

1.7. *Thesis aims and approaches*

The core objective of this thesis is to infer the ecological function of fungal root endophytes based on their taxonomy, their sets of traits, and their interactions with plants. In order to accomplish this objective, I aimed to address the following questions:

1. How do phylogeny and traits influence the interaction of fungal endophytes with host plants?
2. How do abiotic factors of the environment where the interactions between fungal root endophytes and plants take place affect the outcome of their associations?

3. How does co-occurrence of different fungi with different degrees of phylogeny and trait similarity affect the outcome of plant-endophyte interactions?

This thesis comprises six chapters describing my research activities to address the above questions. These activities include a minor contribution in the samplings described in Chapter 3, and a major role in the experiments described in Chapters 4, 5 and 6.

In Chapter 2 I discuss the main highlights of my thesis and provide outlook on how the knowledge generated in my work can help shed light on the yet cryptic ecological functions of non-mycorrhizal fungal root endophytes.

In Chapter 3, I collaborated in the isolation of an extensive collection of isolates of endophytic fungi from roots of several plant species growing in different natural contexts. This activity enabled us to collect numerous fungal strains necessary for subsequent investigations described in this thesis. The results described in this chapter are published in Glynou *et al.* (2016) *Environmental Microbiology* 18, 2418–2434.

In Chapter 4, I studied a selection of strains of endophytic fungi belonging to ten fungal orders, which were isolated from different plants across Europe. To test their effects on the growth of different hosts, the fungal strains were individually inoculated into roots of three plant species and several traits of the endophytes as potential influential factors on the outcome of their interaction with the host were measured. The results described in this chapter address my research question 1 and are published in Kia *et al*. (2017) ISME Journal 11, 777–790.

In Chapter 5, based on observations on the ecology of fungi from Chapter 3 and screening results of Chapter 4, a subset of strains was selected to test the impact of several abiotic factors on the endophytes' interaction with different plants. The results from this chapter address my research question 2 and are published in Kia *et al*. (2018) *FEMS Microbiology Ecology* 94, fix162.

In Chapter 6, a group of endophyte strains, representing the dominant taxonomic groups showing different degrees of phylogenetic and trait similarity, were selected to study their interspecies interactions within plant roots and the impact of their co-occurrence on growth of the host plant. Results described in this chapter address my research question 3 and have been submitted for publication.

2. Discussion

To assess the ecological function of fungal root endophytes based on their interactions with plants and their effect on plant fitness, I tested the influence of fungal strains' phylogeny and morphological and physiological traits on their interaction with different plants. I also evaluated the effect of selected abiotic factors and fungal co-occurrence on the association between endophytes and plants. In general, the simple experimental systems I used for this thesis, consisting of *in vitro* bioassays to co-cultivate hosts and root endophytes, enabled me to address my objectives and to test interactions between plants and a large number of endophytic fungal species. Under these conditions, most endophytes behaved as weak parasites, but their interaction with host plants were host-isolate dependent and variable. These variations in endophytes performance could be partially explained by their morphological traits like growth rates and melanization. This indicates functional complementary of root endophytes and their different niche preferences in natural conditions, which may explain their coexistence in complex communities. In agreement with this hypothesis, the assessment of plant interactions with co-inoculated endophytes with different degrees of phylogenetic and trait similarity showed that there is no extensive competition between strains, even if the abundance of each fungus in roots responds differently to co-inoculation. Interestingly, the effects of plant-endophyte interactions on plant growth were insensitive to the abiotic context, including changes in substrates' nutrient availability, pH and light intensity. In this chapter, my main results concerning the possible role of fungal endophytes in natural ecosystems and additional approaches to further study the ecological functions of fungal endophytes, will be discussed.

2.1. Friend or foe? Multifunctionality and different niches of root fungal endophytes

The results from Chapter 3 show that roots of *Microthlaspi* spp. are dominated by widespread cultivable fungi belonging to the orders Helotiales, Hypocreales and Pleosporales, whose occurrence is associated with latitudinal gradients of precipitation and temperature. However, due to the general pattern of distribution of cultivable endophytes we can assume that these endophytes with saprotrophic potential may have more source options to explore than only plant roots, and this may suggest several ecological niches used by these groups of fungi (Glynou *et al.* 2016). Although dealing with a different group of root symbionts, Martino et al. (2018) also revealed that ericoid mycorrhizal fungi adjust to two different ecological niches as saprotrophs and plant mutualists (Martino *et al.* 2018). This, together with our findings, suggests that in contrast to common beliefs, symbiotic fungi have several recognized niches, and due to genetic or environmental causes may use one niche or even both recognized niches (Selosse *et al.* 2018).

The results from direct examination of individual endophytic strains on plants' fitness in Chapter 4 indicate that the effects of fungal endophytes are variable, and that this variation is partially attributed to fungal taxa. In some cases, variations within strains of the same operational taxonomic unit (OTU) were greater than between OTUs. For example, *Fusarium* sp. strains grouped in the same OTU had different effects, ranging from neutral to detrimental, on plant growth (Kia *et al.* 2017). This may suggest the functional redundancy in endophytes community like substitution of species as an assurance against changing environmental conditions (Maherali & Klironomos 2007). In general, variations in effect of endophytes on plant fitness are highly correlated with fungal traits like mycelial growth rate and melanization and less so with phylogeny, suggesting convergent evolution of traits among fungal lineages and involvement of these traits in functional complementary of endophytes (Steudel *et al.* 2012). However, the corporation of intraspecific trait variation into endophytes functional diversity remains a cryptic issue, which demands further genomic and transcriptomic studies.

The “coexistence stabilizing mechanism theory” argues that different symbionts will coexist when their intraspecific competition is greater than interspecific competition (Chesson 2000; May & Nelson 2014). Yet, it is unlikely that closely related genotypes will be transmitted at the same time in the same tissue due to effective selection (Alizon S. *et al.* 2009). Accordingly, to understand the coexistence strategies of different fungal endophytes with different degrees of trait and phylogenetic similarity, in Chapter 6 the impact of fungus-fungus interactions on plant fitness and root colonization were examined. Surprisingly, the results from these experiments show that, although the abundance of each fungus in roots is affected by co-inoculation, competition between strains was not associated with their trait similarity and phylogenetic lineages. It is likely that dominant fungal root endophytes avoid competition exclusion by occupying different niches within roots (resource partitioning), which could explain the high diversity of root fungal endophytes in healthy, natural plants (Ernst *et al.* 2011).

The observed intraspecific trait variations in Chapter 4 and coexistence ability of different endophytic fungal lineages with plant in Chapter 6 may help to elucidate the adaptive evolutionary processes that generate genetic variation within species for traits that affect interactions among species (Fargione *et al.* 2007; Piculell *et al.* 2008). In this regard, the interactions among endophytic fungi may generate a selection on traits of symbionts such as defensive traits. These traits may have evolved to protect the co-occurring symbionts and the protection of the host could be a by-product of selection (May & Nelson 2014). Therefore, defensive traits may have evolved more to protect the microbe than to protect the host. For instance, the cooperative interaction between pathogenic *Ustilago maydis* and endophytic *Fusarium verticillioides* in resistant maize plants shows that the pathogen does not easily access the host resources due to the presence of secondary compounds, e.g. fusaric acid produced by *F. verticillioides* (Glenn *et al.* 2002, 2007; Rodriguez Estrada *et al.* 2012). On the other hand, growth of *U. maydis* is facilitated by *F. verticilloides*, which enzymatically degrades plant defense compounds, e.g., BOA/MBOA

(Doehlemann *et al.* 2008; Rodriguez Estrada *et al.* 2012). Hence, the cooperative interaction between these species moderates the virulence of either fungus towards their host and assures their colonization. This may suggest that defense traits such as enzymatic activities and secondary metabolites produced by endophytes are affected by fungus–fungus interactions, which finally serve to modify host fitness (May & Nelson 2014). Moreover, below-ground plant endophytes may transmit to aerial parts via vascular system and modulate the phyllosphere microbial community and interactions by changing apoplastic pH in leaves, Ca^{2+} signaling, and resistance induction (Whipps *et al.* 2008; Yang *et al.* 2013). These changes in plant chemistry due to biotic interactions in one compartment may affect other compartments, thereby affecting community assembly and biotic interactions and consequently the changes in community composition may lead to changes in functions and service of terrestrial ecosystem (Wurst & Ohgushi 2015).

Plant-endophyte interactions are increasingly recognized by ecologists, and till now reductionistic approaches and classification schemes made big steps towards understanding fungal-plant interactions. However, to date, little is known about fungal endophytes' lifestyle changes in response to environment and their real niche recognition. By customizing genomics, transcriptomics, proteomics, advanced microscopy tools and trait based approaches, we may shed light on endophytic fungal diversity and their functional role in interactions with host and plant microbiome complex in response to changing environment. This will help us to understand the potential of endophytes in plant community dynamics.

2.2. Conditional outcome and context dependency of endophyte-plant interactions

Any interaction within and among microbial species is variable, depending on the environment where the interaction occurs (Hoeksema *et al.* 2010; Davitt *et al.* 2011; Chamberlain *et al.* 2014). The variation in interactions may result in different degrees of fitness of individual plants and their dynamics in the community (Pringle 2016). The major

question is what the reasons are for these conditional outcomes (Koricheva, Gange & Jones 2009; Partida-Martinez & Heil 2011), and which biotic (e.g. plant and microbe genotype) and abiotic factors modulate the interactions between microbes and plants (Pineda et al. 2010). In this work, I aimed at testing the role of environmental factors in such settings.

Results from Chapter 3 show that distribution patterns of fungal endophytes are associated with bioclimatic factors and soil characteristics (Glynou *et al.* 2016). For instance, strains of Helotiales (e.g. *Cadophora* spp.) depend on soil pH and the occurrence of species of *Alternaria* spp. is affected by climatic and spatial factors. Consequently, considering these ecological data and the observed variable effects of endophytic individuals on different host plants in Chapter 4, three important abiotic factors were selected to evaluate their effects on the interactions between endophytes and plants. The findings described in Chapter 5 suggest that the effects of root endophytes on plant growth are robust to changes along the tolerable gradients of nutrient availability, light intensity and substrate pH, although some strain-specific responses to nutrient availably were observed, mainly leading to pathogenic outcomes (Kia *et al.* 2018). However, it is possible that abiotic factors alone do not determine the outcome of interactions between endophyte and plants and the presence or absence of biotic factors may alter the outcome of current abiotic statues like light and nutrients on their interactions (Lehtonen *et al.* 2004; Hoeksema *et al.* 2010; Álvarez-Loayza *et al.* 2011).

Accordingly, in Chapter 6, I focused on fungus-fungus interactions inside plants as biotic context and tried to assess the importance of biotic interactions on plant fitness. The results show that trait differences observed in Chapter 4 likely favor coexistence of certain endophyte species, thus preventing antagonistic interactions among fungal endophytes and competition for niches within plant tissues. This is in agreement with the “ecological assembly rules”, which proposes that coexistence of species is not a random phenomenon and depends on features of species in the community which survive in the specific environment by traits relevant for their persistence (Weiher & Keddy 1995).

In Chapters 4 and 6, plants inoculated with individual fungal strains showed clear differences concerning their growth across the treatments, while the growth of plants inoculated with pairs of strains was less variable across treatments. More positive response of plants to concurrence of multiple fungal species may indicate the complementarity of functions of microbial species within root tissues and highlight the potential of these communities in the establishment of stable ecosystems (Hart & Reader 2002; Halpern *et al.* 2007; Maherali & Klironomos 2007; Harris 2009; Hoeksema *et al.* 2010). The co-inoculation experiment in Chapter 6 shows little interaction between endophytes within roots, with highest susceptibility of *Alternaria* sp. strain abundance within plant tissue to presence of other species, which were reflected in a moderation of the parasitic effect of the strain onto plant. Rather neutral effects of pairwise inoculations of parasitic strains on plant fitness can be explained by the hypothesis of "conditionally beneficial pathogens", which states that most biotrophic pathogens induce a systemic resistance in the host against other pathogenic infections and triggers production of plants' growth hormones (Conrath *et al.* 2002; Vos *et al.* 2015).

One of the important ecological forces in shaping biotic communities are priority effects or colonization history, which may affect ecosystem processes (Cline & Zak 2015b). Establishment of early fungal colonies alters the colonization of later-arriving fungi via exclusive competition and can affect the historical contingency of a community (Kardol *et al.* 2013). Colonization history of species in communities, including temporal or spatial priority, can affect the interaction of symbionts with plants, because it can alter the community assembly structure (Mack & Rudgers 2008). The strength of priority effects may depend on traits of the first colonizer, e.g., the fast growing organism may gain larger competitive position when they first colonize the habitat (Cline & Zak 2015a). According to trait studies in Chapter 4, the morphological traits like growth rate and sporulation are playing important roles in endophytes' interactions with plant, which are possibly functional traits for better competing and persisting inside of plant tissues. Accordingly, in Chapter 6, I tested the effect

of inter- and intraspecific competition between pairs of fungi with different degrees of trait similarity. However, their arrival order or time interval was not considered and it is a factor that should be investigated further.

Plants have to respond to multiple environmental challenges, so they need to integrate both signals associated with biotic and abiotic stresses in the most appropriate response to survive. That may allow the plant to prioritize the different responses when the plant is facing multiple simultaneous stresses. By understanding how biotic and abiotic factors affect the plant signal-transduction pathways and regulate their responses, we may be able to predict how plant–endophyte interactions will respond to environmental factors. For instance, it is known that plant responses to biotic and abiotic stresses are mainly regulated by phytohormones such as jasmonic acid, salicylic acid and abscisic acid (De Vos *et al.* 2005; Christmann *et al.* 2006; Berendsen *et al.* 2012; Pieterse *et al.* 2012; Vos *et al.* 2015). Through synergistic and antagonistic effects, the so-called "phytohormone crosstalk" allows the plants to prioritize the responses in the case of simultaneous stresses (Spoel *et al.* 2007; Koornneef & Pieterse 2008). Thus, crosstalk at the plant-signaling level may have ecological consequences for plants and may establish a driving force for the dynamics of microbial populations, which merits further considerations in ecological studies of fungal endophytes.

Integrating all this information will be needed to predict the impact of environmental changes on the interaction of plants with microbes. In fact, the induction of tolerance/resistance to stresses may explain the perpetuation of plant–endophyte symbioses in conditions where there are no nutritional benefits for the plant (Smith et al. 2009). It can be argued that symbiotic microbes are beneficial for plants mainly when plants need help and this need will be determined by the occurrence of biotic and/or abiotic stresses.

2.3. Community dynamics of fungal endophytes and stability of their interactions

In every ecosystem, diverse species coexist and interact with each other as a community (Rozdilsky & Stone 2001; Turnbull *et al.* 2013; Vályi *et al.* 2016). Theoretically, the reciprocal interactions like parasitism and mutualism are the main directors of these community dynamics (May 1973). Theoretical studies predict that the communities with reciprocal interactions with asymmetrical signs (the interaction strength of one symbiont is stronger than the other one in a pairwise interaction) like parasitism are more stable than those with symmetrical signs like mutualism and these non-random asymmetric interactions are essential for the stability of ecological communities (Rozdilsky & Stone 2001). Also, a recent theoretical approach shows that unilateral interactions (i.e., ammensalism and commensalism) are more stabilizing for the communities than symmetrical interactions like competition and mutualism (Mougi 2016), suggesting that natural ecosystems are probably stabilized by a balance between asymmetric reciprocal interactions like parasitism and/or by unilateral interactions like commensalism.

The findings from Chapter 3 indicate a significant diversity of root dominating fungi, which according to our studies in Chapter 4 have mostly a parasitic behavior or neutral interactions with the host (possibly commensal). Taking the results from Chapter 3 and 6 together, it can be concluded that a range of fungi co-occur asymptomatically inside of plant tissues without competitive exclusion. One can think that their selection was not random and their concurrence has an effect on community stability. Therefore, the phylogenetic niche conservatism probably promotes coexistence between different lineages to enhance ecosystem function (Maherali & Klironomos 2007, 2012).

The co-inoculation experiment in Chapter 6 shows slight but consistent plant root colonization by the *Cadophora* sp. strain and its neutral interactions with different plants in all experiments. This kind of neutral species co-occurrences can be attributed to cooperative interactions, like facilitation. Facilitation in the community usually occurs to promote the

coexistence and community diversity, for example by enzymatic degradation of plant secondary metabolites (Rodriguez Estrada *et al.* 2012; Bulleri *et al.* 2016). Thereby, the species' niche and their geographic range can expand and the fungi can play important roles in community species richness and stability (Tiunov & Scheu 2005; Bulleri *et al.* 2016). However, in order to reveal the function of fungal strains as facilitators in a given community, the effect of temporal or spatial priority of strains should be studied (Kardol *et al.* 2013). The experiments presented in Chapter 6 were restricted to pair-wise interactions of fungal strains with *A. thaliana*. Due to the diversity of the strains studied, generalizing these results will require caution and further studies such as colonization history of strains and the concurrent interactions between multiple species combinations are needed.

The beneficial interactions between multiple fungal species with plants and diverse soil microbial community may point to the complementarity functions of microbial species and highlight the potential of the plant microbiome for the establishment of a stable ecosystem (Hart & Reader 2002; Halpern *et al.* 2007; Maherali & Klironomos 2007; Harris 2009; Hoeksema *et al.* 2010). Life in a large group can have benefits like cooperative defense and induced tolerance against environmental stresses (Stachowicz 2001). However, the stability of the community depends on the functional species as keystone species (Stachowicz 2001) and characterization of these species awaits further investigation.

2.4. Present situation and future applications of endophytes as biologic agents

The *in vitro* enzymatic activities of the fungi screened in Chapter 4 indicate that a large majority of the endophytes have the potential to solubilize phosphate and, hypothetically, due to a high proteolytic activity will be able to mobilize organic nitrogen for plants. Therefore, these fungi might be potential candidates for plant growth promoter agents via solubilizing/mobilizing and scavenging nutrients for plants, where P and N are not accessible for plants. However, we have only recorded the *in vitro* activities of these hydrolytic

enzymes. Therefore, the *in planta* production of these enzymes should be further studied for future agronomical practice purposes.

Among the studied endophytes, some strains showed persistent positive effects on plant fitness and growth. For instance, the *Alternaria tellustris* strain P1191 had a consistent positive effect on plant growth across repetitions of experiments in Chapter 6. This strain also had the ability to moderate the negative effect of pathogenic *Fusarium tricinctum* strain P2190 in co-inoculation experiments. Therefore, this strain could have potential as a prospective plant growth promoter and plant-protecting fungus. Other laboratory and field studies also mentioned *Alternaria* spp. as biocontrol agent against diseases and pests (Lahlali & Hijri 2010; Kaur *et al.* 2013). However, in order to gain insight into mechanisms of symbiont persistence and mode of action of this strain, additional field assessments and profiling such as molecular and metabolic profiling is needed.

Several studies have shown that many DSE enhance plant performance (Mandyam & Jumpponen 2005; Newsham 2011; Knapp *et al.* 2012) and can be considered as potential biostimulants for plant growth promotion. Among the DSE studied in this work, the strain aff. *Cadophora* sp. P1331 has a consistent abundance in plant tissue and slightly positive effect on plants (although not significant under the experimental conditions tested). This strain apparently is a stable root colonizer and good competitor against *A. tellustris, F. oxysporum*, and *F. tricinctum* strains, suggesting a potential as a biostimulant. To understand the mechanisms behind plant growth promotion by this fungus, additional metabolic profiling and the investigation of hormonal activities of strains are necessary.

Endophytes are considered as promising tools for agriculture as plant growth promoters, and plant protecting agents against pests and diseases (Kiewnick & Sikora 2006; Backman & Sikora 2008; Berg *et al.* 2014b; Banhara *et al.* 2015; Card *et al.* 2015). The lack of knowledge concerning their mode of action as plant-associated protective agents or plant growth promoters, on field shelf-life (maximum time that a microbe as a product can actively

perform in the field) and ecological interactions in nature hampers the development of robust biocontrol and biostimulant agents (Lahlali & Hijri 2010; Saunders *et al.* 2010; Ravensberg 2015; McKinnon *et al.* 2017). However, if the defensive mutualisms stand true for fungal endophytes, it is probable that natural parasites adapt to endophytes used for biological control, decrease the control effectiveness and possibly drive increased virulence towards the host (Duffy *et al.* 2003). Given the potential impacts of endophytes on their host fitness and on the plant microbiome, the population dynamics of endophytes and their symbiotic persistence in agronomic practices should be further investigated (Meyling & Eilenberg 2007).

2.5. Concluding remarks

Despite recognized potential of endophytes to assist plants in development and in tolerance against environmental stresses, the ecological functions played by endophytes in natural communities and ecosystem processes are not well-known. To the best of my knowledge, this doctoral thesis is the first study that comprises diverse groups of fungal endophytes and evaluates their interactions with several plants, not only based on phylogeny but also based on their functional traits under several environmental conditions. The combination of data on the phylogeny of dominant endophytes, their distribution patterns and their life history and functional traits, hint to potential functional interactions of endophytes in nature. Results from this work show that root endophytic fungi are phylogenetically diverse and functionally heterogeneous. They are established in root microbiomes occupying different niches to avoid competition with other members of plant microbiota. These microorganisms show several lifestyles (e.g. saprotroph or weak parasites) and are likely able to use plant tissues as a “waiting room” and under certain circumstances change their interaction strategy and niche to maximize resource access. The influence of root endophytes on plant fitness may be a by-product of microbe-microbe interactions or cross-talk between below and above-

ground communities and their response to the abiotic stressors. Although this work attempted at finding the possible environmental forces influencing the position of endophytes in the mutualism–parasitism continuum, drawing general conclusions about the fitness outcome of their interactions with plants and functional roles in ecosystem await further quantitative *in situ* studies with simultaneous assessment of several environmental factors to define the key species and their functions in endophytic community dynamics.

In general, the findings of this thesis are helpful to group dominant root fungal endophytes based on their traits and develop hypotheses about their ecological role in nature, by pointing to their functional complementary and niche differentiation as coexistence mechanism in a hyperdiverse endophytic community.

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3. Research article 1

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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Co-Autor KG: 20%
Co-Autor MT: 30%
Co-Autor JGMV: 50%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende SHK: 5% fungal isolation and preservation
Co-Autor KG: 70% cultivation of roots, fungal isolation and preservation, DNA extraction, amplification (PCR) and sequencing
Co-Author JGMV: 25% cultivation of roots, fungal isolation and preservation, DNA extraction, amplification (PCR) and sequencing

(3) zur Erstellung der Datensammlung und Abbildungen

Co-Autor KG: 50% collection of root and soil material, curation of fungal collection, collection of sequencing data, collection of bioclimatic data, design of figures
Co-Autoren TA, AKB, SP, XX, AÇ, MT: 20% collection of root material
Co-Autor JGMV: 30% collection of root and soil material, design of figures, submission of sequences to GenBank

(4) zur Analyse und Interpretation der Daten

Promovierende SHK mit Co-Autoren TA, MT: 5%
Co-Autor KG: 50% diversity analysis, statistical analysis
Co-Autor JGMV: 45% diversity analysis, statistical analysis

(5) zum Verfassen des Manuskripts

Promovierende SHK mit Co-Autoren TA, AKB, SP, XX, AÇ, MT: 10%
Co-Autor KG: 50%
Co-Autor JGMV: 40%

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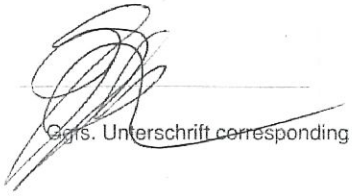

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The local environment determines the assembly of root endophytic fungi at a continental scale

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Summary

Root endophytic fungi are found in a great variety of plants and ecosystems, but the ecological drivers of their biogeographic distribution are poorly understood. Here, we investigate the occurrence of root endophytes in the non-mycorrhizal plant genus *Microthlaspi*, and the effect of environmental factors and geographic distance in structuring their communities at a continental scale. We sampled 52 plant populations across the northern Mediterranean and central Europe and used a cultivation approach to study their endophytic communities. Cultivation of roots yielded 2601 isolates, which were grouped into 296 operational taxonomic units (OTUs) by internal transcribed spacer sequencing of 1998 representative colonies. Climatic and spatial factors were the best descriptors of the structure of endophytic communities, outweighing soil characteristics, host genotype and geographical distance. OTU richness was negatively affected by precipitation, and the composition of communities followed latitudinal gradients of precipitation and temperature. Only six widespread OTUs belonging to the orders Pleosporales, Hypocreales and Helotiales represented about 50% of all isolates. Assessments of their individual distribution revealed particular ecological preferences or a cosmopolitan occurrence. Our findings support a strong influence of the local environment in determining root endophytic communities, and show a different niche occupancy by individual endophytes.

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Introduction

Plant roots establish symbioses with a large diversity of microorganisms, some of which are able to penetrate the outer root boundaries and constitute endophytic assemblages different from those in the surrounding rhizosphere and rhizoplane (Lundberg *et al.*, 2012). Although some are transient colonizers that enter the roots due to stochastic events, others present adaptations that allow them to persist for long periods confined in particular compartments, or to more effectively invade the tissues and establish an active metabolic interaction with the host (Hardoim *et al.*, 2008). A single plant might contain a complex assembly of root endophytic fungi (Vandenkoornhuyse *et al.*, 2002), and plants in all terrestrial ecosystems have these associations. They can reach considerable microbial loads (Maciá-Vicente *et al.*, 2012), thereby constituting an important cost to the host as photosynthetic carbon is diverted to the symbionts. In exchange, some endophytic mycorrhizae provide their host plants with benefits, most prominently assisting in the uptake of nutrients and water, or protecting against stress (Kiers and van der Heijden, 2006; Van Der Heijden *et al.*, 2008; Kiers *et al.*, 2011). Other endophytes constitute a unidirectional sink for plant resources and develop parasitic or pathogenic relationships of varying magnitudes (Tellenbach *et al.*, 2011; Keim *et al.*, 2014; Mandyam and Jumpponen, 2014). Through these processes, endophytic fungi contribute to the functioning of land ecosystems by modulating plant productivity and diversity, alongside their implication in the cycling of soil carbon.

The largest fraction of the endophytic mycobiome remains poorly characterized. Although endophytes are hypothesized to impact plant fitness, experimental work has been unable to assign decisive functions to most of them (Mandyam and Jumpponen, 2005; 2014; Newsham, 2011). Because the function of organisms is necessarily

linked to their habitat, their potential ecological roles can be inferred from their natural occurrence, from the identification of the ecological factors affecting their communities, and from understanding how they affect them. For instance, dominant plant species characteristic of major biomes associate with different types of mycorrhizae, which develop distinct symbiotic functions in relation to the specific soil properties (Read, 1991; Read *et al*., 2004). There is substantial evidence that non-mycorrhizal root endophytes also have preferences towards ranges of hosts and environments (for an extensive review see Sieber and Grünig, 2013). Their local or regional occurrence can be linked to environmental variables like soil type and biotic factors like host phylogeny (Maciá-Vicente *et al*., 2008a; 2012). However, knowledge of the large-scale biogeographic patterns of non-mycorrhizal root endophytes is very limited, at best.

The biogeography of organisms is driven by environmental, geographic and historical factors, together with features intrinsic to them such as their lifestyle, their dispersal capabilities, or their biotic interactions (Prosser *et al*., 2007). As with other microorganisms, fungi were assumed to occur ubiquitously owing to large population sizes and a nearly unlimited ability to disperse (Fitter, 2005). This implies that their diversity is high locally, but comparably low at larger scales because the same species occur across landscapes, as summarized by the tenet 'everything is everywhere, but, the environment selects' (Baas-Becking, 1934). Evidence challenging this view has accumulated and depicts a more complex scenario for the distribution of different fungal guilds (Taylor *et al*., 2006; Amend *et al*., 2010; Tedersoo *et al*., 2014; Van der Gast, 2015). Non-mycorrhizal root endophytes have been suggested not to follow a biogeographic pattern (Queloz *et al*., 2011), as opposed to other aboveground and root-plant symbionts (Arnold and Lutzoni, 2007; Kivlin *et al*., 2011; Tedersoo *et al*., 2012; U'Ren *et al*., 2012). This could indicate that different processes govern the diversity of different fungal functional groups (Tedersoo *et al*., 2012). Alternatively, this could be a consequence of the lesser efforts devoted to study the broad-scale patterns of root endophytes.

Here, we investigate the biogeographic distribution of non-mycorrhizal root fungal endophytes at a continental scale, and evaluate the effects of geographic distance, local environment and the biogeography of their hosts in their community composition, and in the occurrence of dominant phylotypes. As for the host plant, we focus on closely related members of the annual genus *Microthlaspi* F.K. Meyer (Brassicaceae), which were until recently included in the species *Microthlaspi perfoliatum* (L.) F.K. Meyer (Ali *et al*., 2015). These comprise both diploid and polyploid cytotypes that are morphologically similar, but phylogenetically dissimilar. Current data have shown that they represent two distinct species that form predominantly selfing populations (Ali *et al*., 2015). Assessments of the distribution of endophytes have often focused on several unrelated host plants that were not represented in all sampling sites (Arnold and Lutzoni, 2007; Hoffman and Arnold, 2008; Maciá-Vicente *et al*., 2008a; Herrera *et al*., 2010; 2013; U'Ren *et al*., 2012). Because host phylogeny is one of the main factors determining the composition of plant-associated communities (U'Ren *et al*., 2012; Wehner *et al*., 2014), focusing on one host with a widespread occurrence may allow for more accurate biogeographical inferences. *Microthlaspi* has a broad distribution over nearly all of Europe (Meyer, 2003), allowing for samplings across a wide range of environmental gradients. As most Brassicaceae, *Microthlaspi* also lacks classical mycorrhizal associations and alternative adaptations for the efficient capture of soil nutrients because it dwells in habitats where these are not limiting (Fitter, 2005). This could leave additional niches open to other root colonizers with different effects on the host.

The aim of this study is to unravel the broad-scale biodiversity patterns of root endophytes and identify their key ecological drivers. We use *Microthlaspi* as a model host system and rely on a cultivation approach to characterize its endophytic mycobiome. The collection of an extensive inventory of fungal cultures will warrant further phylogenetic and ecological studies on these endophytes and on their interaction with plants.

Results

Diversity of root endophytes

A total of 424 plants were processed for isolation of root endophytic fungi, originating from 52 populations distributed along an area spanning four parallels and five meridians (Table 1). Out of the total plants sampled, 414 (97.4%) yielded endophytic fungal growth in at least 1 of the 10 root pieces plated. We recorded 2601 fungal colonies developing from 2359 out of the total 4240 root pieces, accounting for an overall colonization percentage of 55.6% (i.e., the proportion of root pieces yielding at least one isolate), and an averaged colonization per population of 56.7 ± 18% (mean ± standard deviation).

The fungal isolates were grouped into 296 OTUs by sequencing of the internal transcribed spacer (ITS) rDNA region of a subset of representative pure cultures (Fig. 1A). On average we obtained 16.5 ± 6.3 OTUs per population of *Microthlaspi* (Fig. 1B). The overall number of operational taxonomic units (OTUs) obtained was below the maximum expected richness of 344.3 OTUs as assessed by Bootstrap analysis, and the 564.3 OTUs as assessed by the Chao estimator. This translates into an average value of 4 ± 1.8 or 11.7 ± 13.7 OTUs that went undetected in each plant population respectively

Table 1. Description of *Microthlaspi* populations studied in this work, and results of fungal colonization and diversity.

Country	Site	Coordinates	Elevation (m.a.s.l.)	Host's ploidy	n^a	Isolates	Colonization (%)[b]	Observed richness		Estimated richness		Diversity indices	
								S^c	Av. S^d	Boot[e]	Chao[f]	H'^g	J^h
Bulgaria	BG-007	42.50 N / 22.82 E	614	Polyploid	6	60	86.7 ± 13.7	17	5.2 ± 1.9	20.6 (2.3)	62 (30.1)	2.3	0.8
	BG-010	42.70 N / 22.83 E	770	Diploid	9	78	81.1 ± 19	18	4.6 ± 1.3	21.8 (1.7)	27.3 (8.8)	2.4	0.8
	BG-011	42.67 N / 22.84 E	740	Mixed	5	43	76 ± 8.9	14	4.2 ± 1.8	17.2 (2.3)	15.7 (2.2)	2.3	0.9
	BG-012	42.66 N / 22.81 E	773	Polyploid	9	66	67.8 ± 13	25	3.8 ± 1.5	31.8 (3)	38.2 (10.2)	2.9	0.9
	BG-013	42.63 N / 22.73 E	837	Polyploid	9	65	64.4 ± 19.4	23	4.6 ± 2.2	28.5 (3)	62 (30.3)	2.7	0.8
	BG-014	42.59 N / 22.72 E	711	Diploid	4	37	85 ± 10	14	4.8 ± 1	17.6 (2.1)	29 (12.8)	2.0	0.8
	BG-015	42.57 N / 22.69 E	685	Diploid	9	84	77.8 ± 15.6	28	5.6 ± 1.5	34.5 (3)	35.3 (5.7)	2.9	0.9
	BG-023	42.91 N / 22.83 E	621	Polyploid	6	52	80 ± 22.8	14	4.3 ± 1.9	16.3 (2)	14.5 (1)	2.3	0.9
Germany	D-100	49.54 N / 09.34 E	415	Polyploid	10	82	69 ± 12.9	31	5.4 ± 2.2	38.8 (3.6)	48 (10.7)	2.8	0.8
	D-101	49.68 N / 10.00 E	278	Diploid	10	48	44 ± 29.5	20	3.4 ± 2.1	24.8 (2.8)	47.5 (22.7)	2.7	0.9
	D-102	49.45 N / 09.82 E	281	Diploid	10	32	29 ± 24.2	22	3.1 ± 3.2	28.3 (3.8)	46 (16.4)	2.9	1.0
	D-103	49.27 N / 09.84 E	299	Diploid	10	40	39 ± 26	21	3 ± 1.5	27.4 (2.7)	89 (48.6)	2.6	0.8
	D-104	48.61 N / 09.53 E	515	Diploid	10	51	41 ± 23.3	18	3.4 ± 1.6	22.5 (2.4)	40 (17.4)	2.3	0.8
	D-105	48.55 N / 10.12 E	481	Diploid	10	42	40 ± 13.3	23	3.6 ± 0.8	28.5 (2.3)	32.4 (7.2)	3.0	1.0
	D-11a	50.37 N / 07.22 E	504	Diploid	10	73	61 ± 24.7	20	4.1 ± 1.9	24.6 (2.8)	24 (3.9)	2.5	0.8
	D-11b	50.37 N / 07.22 E	504	Diploid	10	61	55 ± 21.2	17	3.7 ± 1.6	20.1 (1.7)	20.8 (4.2)	2.5	0.9
Spain	ES-001	38.04 N / 02.48 W	1630	Polyploid	10	54	50 ± 20	20	3.7 ± 1.2	25.4 (2.2)	46 (20)	2.5	0.8
	ES-002	38.05 N / 02.54 W	1612	Polyploid	10	58	50 ± 20.5	15	3.7 ± 1.6	17.7 (1.4)	25.5 (10.5)	2.3	0.8
	ES-003	38.09 N / 02.56 W	1253	n.d.	10	65	60 ± 18.3	21	3.5 ± 1.6	27.1 (2.6)	34.2 (10.2)	2.3	0.8
	ES-004	37.97 N / 02.45 W	1204	Polyploid	10	85	80 ± 15.6	17	3.6 ± 1.2	20.9 (2)	21.7 (4.5)	1.9	0.7
	ES-005	37.14 N / 03.48 W	1351	Polyploid	10	47	46 ± 23.7	11	2.2 ± 0.9	13.3 (1.6)	11.3 (0.7)	2.1	0.9
	ES-006	37.13 N / 03.43 W	1669	Polyploid	10	59	57 ± 14.9	11	2.9 ± 0.7	13 (1.3)	14.3 (4.1)	1.9	0.8
	ES-010	42.81 N / 04.25 W	1055	Polyploid	10	64	59 ± 18.5	24	3.3 ± 0.8	31.1 (2.5)	69.3 (31.8)	2.5	0.8
	ES-012	42.87 N / 04.15 W	1305	Polyploid	10	62	59 ± 27.7	17	3.2 ± 1.6	20.9 (2.3)	22.3 (5.4)	2.4	0.8
France	F-001	47.41 N / 06.56 E	285	Diploid	10	36	33 ± 25	13	2.2 ± 1.1	16.1 (1.8)	18 (5.5)	2.2	0.9
	F-002	47.14 N / 06.20 E	553	Diploid	10	45	43 ± 15.7	17	3 ± 0.7	20.8 (1.8)	18.3 (1.7)	2.6	0.9
	F-004	47.03 N / 06.33 E	699	Polyploid	10	27	27 ± 13.4	12	2.2 ± 1.2	15 (1.7)	26 (13.1)	2.0	0.8
	F-007	47.11 N / 06.07 E	543	Diploid	9	64	67.8 ± 18.6	16	3.8 ± 1.3	19.1 (1.6)	16.8 (1.3)	2.2	0.8
	F-008	47.08 N / 06.07 E	533	Diploid	9	32	34.4 ± 25.1	11	1.9 ± 1.1	14.2 (1.6)	12.5 (2.2)	2.0	0.8
	F-009	47.18 N / 05.46 E	216	Polyploid	9	77	75.6 ± 14.2	18	4.6 ± 1.6	22.3 (2.1)	23.6 (5.3)	2.3	0.8
	F-010	47.20 N / 05.43 E	198	Diploid	9	56	60 ± 17.3	18	3.8 ± 1.1	22.3 (2.1)	30 (10.7)	2.4	0.8
	F-011	47.32 N / 04.60 E	446	Polyploid	6	29	48.3 ± 23.2	8	2.2 ± 1	10 (1.5)	18 (10.1)	1.3	0.6
	F-013	47.30 N / 03.59 E	215	n.d.	6	35	55 ± 18.7	16	4 ± 1.5	19.7 (2.1)	38.5 (19.2)	2.5	0.9
	F-014	47.19 N / 01.20 E	121	Polyploid	7	62	75.7 ± 5.3	16	4.1 ± 1.1	19.6 (1.8)	18 (2.6)	2.3	0.8
	F-015	46.41 N / 00.22 E	112	Polyploid	9	42	43.3 ± 24.5	26	3.7 ± 2.2	33.5 (4.1)	48.7 (14.9)	3.1	0.9
	F-021	44.58 N / 05.38 E	1260	Polyploid	7	65	74.3 ± 21.5	17	5 ± 2.3	20.1 (2.1)	20.8 (4.2)	2.3	0.8
	F-023	44.49 N / 05.44 E	1095	Polyploid	9	59	57.8 ± 12	30	5 ± 1.7	37.5 (3.8)	47.1 (11.5)	3.2	0.9
	F-024	44.50 N / 05.42 E	734	Diploid	7	42	55.7 ± 23.7	20	4 ± 1.9	25 (2.9)	31.3 (9.5)	2.8	0.9
Greece	GR-001	39.81 N / 20.77 E	1065	Polyploid	10	87	82 ± 13.2	18	4.1 ± 1.9	22.2 (2.6)	33 (12.8)	2.1	0.7
	GR-002	38.94 N / 21.76 E	1410	Polyploid	10	71	61 ± 16	24	4.1 ± 1.4	29.8 (2.6)	37.2 (10.2)	2.7	0.9
	GR-003	38.91 N / 21.74 E	1283	Polyploid	10	57	55 ± 25.5	15	2.8 ± 1.5	18.3 (1.7)	17 (2.6)	2.1	0.8
	GR-004	38.91 N / 21.83 E	905	Polyploid	8	54	62.5 ± 17.5	20	3.9 ± 1.7	24.9 (2.7)	27.5 (6.3)	2.6	0.9
Croatia	HR-021	44.16 N / 15.58 E	795	Diploid	7	12	15.7 ± 17.2	8	1.4 ± 1.4	10.2 (1.4)	11.3 (4.1)	2.0	1.0
	HR-022	44.19 N / 15.52 E	574	Diploid	8	57	61.3 ± 24.2	17	4.3 ± 2	20.5 (2.1)	17.4 (0.9)	2.6	0.9
	HR-023	44.24 N / 15.54 E	760	Polyploid	4	12	27.5 ± 9.6	7	2.3 ± 1	8.9 (1.1)	12 (5.9)	1.7	0.9
	HR-025	44.46 N / 15.40 E	755	Diploid	10	47	46 ± 32	11	2.1 ± 1.2	14.1 (2.1)	18.5 (8.1)	1.6	0.6
	HR-028	44.59 N / 15.44 E	525	Diploid	8	14	17.5 ± 28.7	5	0.9 ± 1.1	6.4 (1.1)	8 (4.4)	1.3	0.8
Turkey	T-024	38.33 N / 30.64 E	1101	Polyploid	3	25	66.7 ± 15.3	9	5.3 ± 2.3	10.3 (1.6)	9.3 (0.9)	2.0	0.9
	T-025	38.39 N / 30.67 E	1180	Polyploid	3	17	53.3 ± 30.6	4	2 ± 1	4.9 (0.9)	7 (4.3)	0.7	0.5
	T-026	38.57 N / 30.58 E	1166	Polyploid	3	34	93.3 ± 11.5	5	3.7 ± 1.5	5.4 (0.7)	5 (0.4)	1.3	0.8
	T-027	38.79 N / 30.28 E	1105	Polyploid	3	20	50 ± 26.5	9	4.3 ± 2.3	10.6 (1.6)	11 (2.9)	2.0	0.9
	T-028	38.86 N / 30.00 E	1210	Polyploid	3	15	46.7 ± 41.6	8	3 ± 3	10.1 (2.3)	11.3 (4.1)	1.8	0.9

a. Number of plants sampled.
b. Percentage of root pieces yielding at least one fungal colony (±SD).
c. Overall observed OTU richness.
d. Mean observed OTU richness (±SD) across plant individuals.
e. Bootstrap incidence-based richness estimator (SE).
f. Unbiased Chao abundance-based richness estimator (SE).
g. Shannon's diversity index.
h. Pielou's evenness index.
n.d., not determined. SD, standard deviation; SE, standard error.

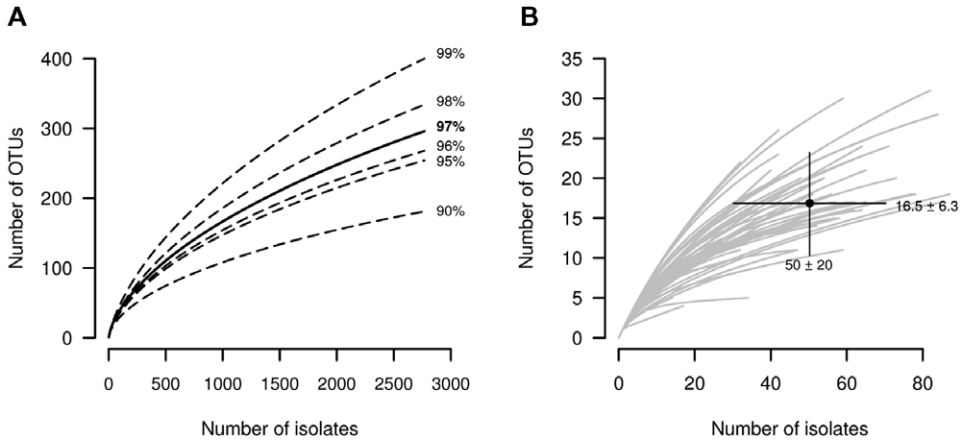

Fig. 1. Rarefaction curves of OTU accumulation with sampling effort, consisting of the total number of isolates developing from root pieces plated in a cultivation medium.
A. Accumulation curves for the entire study showing the effect of different sequence similarity thresholds for OTU definition.
B. Accumulation curves for individual *Microthlaspi* populations. The point with error bars indicate average and standard deviation for the number of isolates obtained per population (*x* axis), and number of OTUs per population (*y* axis), based on individual plant values.

(Table 1). The lack of saturation of the fungal diversity was confirmed by rarefaction curves calculated for the overall survey and for every individual plant population, which in most cases failed to reach an asymptote even when using similarity thresholds to define OTUs as low as 90% (Fig. 1).

Taxonomic classification of isolates

OTUs were classified at varying taxonomic precision by comparing ITS sequences with reference databases. They were assigned to 16 fungal orders, most of them within the Ascomycota (95%; Table S1). The Pleosporales and Hypocreales were the most represented orders, both in terms of the number of OTUs (43.2% and 19.6% respectively) and of the frequency of counts (Fig. 2A). The order Helotiales followed with 14.9% of the OTUs and a frequency of 11.6% of the colony counts, whereas the remaining orders were marginally represented (Fig. 2A; Table S1). Only six OTUs accounted for 50% of the isolates recorded (Fig. 2B). Three of these could be assigned to the order Hypocreales, two within the genus *Fusarium* – with affinities to the species *Fusarium tricintum* and *Fusarium avenaceum* (OTU001) and *Fusarium oxysporum* (OTU003) – and one within the genus *Ilyonectria* (OTU005). Another two of these OTUs belonged to the Pleosporales, the most abundant (OTU002) within the genus *Alternaria* – with close affinity to *Alternaria tellustris* – and another (OTU004) within *Pyrenochaeta* – with closest BLAST hits on *Pyrenochaeta lycopersici*. The sixth OTU in abundance (OTU006) was classified as *Cadophora* sp. Apart of their overall frequency, these OTUs had a widespread distribution and occurred in most plant populations, often representing an important proportion of communities (Fig. 2C). They were followed in abundance mostly by members of the Hypocreales and Pleosporales (Table S1). The remaining OTUs were in general infrequent, with 161 of the total 296 (54.4%) represented by a single isolate, and 47 (15.9%) and 21 (7.1%) by 2 and 3 isolates respectively.

Effect of environmental factors on endophytic diversity

Fungal assemblages differed significantly across populations in OTU richness ($H_{51} = 135.2$, $P < 0.001$), Shannon's diversity ($H_{51} = 121.2$, $P < 0.001$) and Pielou's evenness ($H_{51} = 93.1$, $P < 0.001$; Table 1). We compared endophytes' richness and diversity across environmental factors by using plant averages to correct for the different sampling sizes at each site (Table 1). None of these variables was significantly correlated with latitude (Fig. 3A), but linear regression showed a strong negative relationship of richness and diversity ($P < 0.002$) with various factors related to precipitation (Fig. 3B). These included annual precipitation at each site (Fig. 3B), precipitation of the wettest month, and precipitation of the wettest and coldest quarters of the year. Soil physico-chemical variables had no significant relationships with either richness or diversity of endophytic communities.

Effect of environmental factors on community structure

The unconstrained non-metric multidimensional scaling (NMDS) ordination of Horn–Morisita distances (stress: 20%) revealed a clear structure of endophytic communities along a latitudinal gradient (Fig. 4A). Differences among sites were significant when country of origin or climatic region were used as grouping variables in permu-

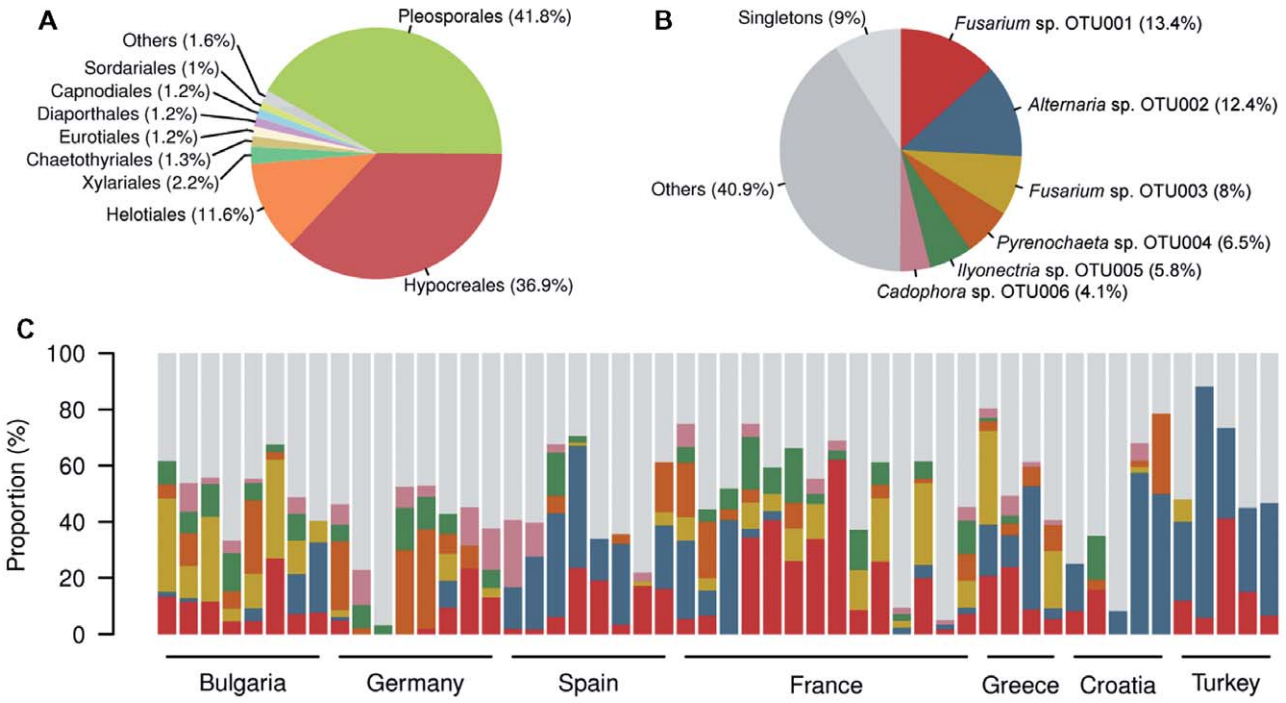

Fig. 2. Relative proportion of fungal taxa among the total number of endophytic isolates.
A. Proportion of isolates belonging to the most frequent fungal orders.
B. Proportion of isolates belonging to the most frequent OTUs.
C. Relative proportion of dominant OTUs across plant populations. Colours as in (B).

tation analysis of variance (PERMANOVA) ($P < 0.001$). All environmental variables retained in the forward selection as potential descriptors of communities had significant correlations with the ordination of sites and were strongly collinear with the latitudinal axis (Fig. 4A). Among these, the only soil variable with a certain degree of correlation with communities was Mg content (pseudo-$F_{40} = 1.8$, $P = 0.046$). We explored other variables not included in the forward selection in an attempt to explain variation in the axis perpendicular to latitude and found that the overall degree of endophytic colonization was the best fitting (pseudo-$F_{51} = 5.7$, $P = 0.001$; Fig. 4A).

An assessment of the distribution of the most common orders showed distinctive patterns of occurrence of Hypocreales in contrast to both Pleosporales and Helotiales (pseudo-$F_{57} = 1.8$, $P = 0.002$). Hypocreales tended to accumulate in communities leftwards in the ordination plot, perpendicular to the main axis of influence of environmental factors and positively correlated with overall colonization (Fig. 4B). Pleosporales and Helotiales on the other hand did not show a clear preference towards any factor.

Variation partitioning was used to assess the individual effect of climatic, spatial and host-related variables on the

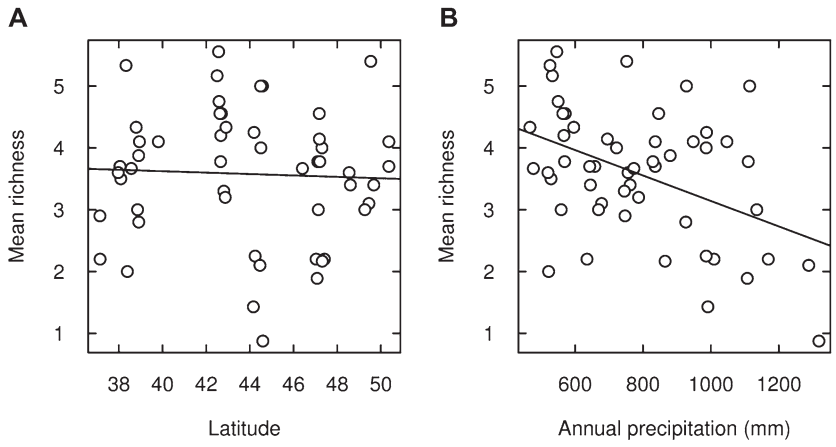

Fig. 3. Relationship between OTU richness in each *Microthlaspi* population, calculated as the average richness observed in each plant of the populations, and the respective latitude (A) and mean annual precipitation (B). Lines denote the linear regression model of interaction between both variables.

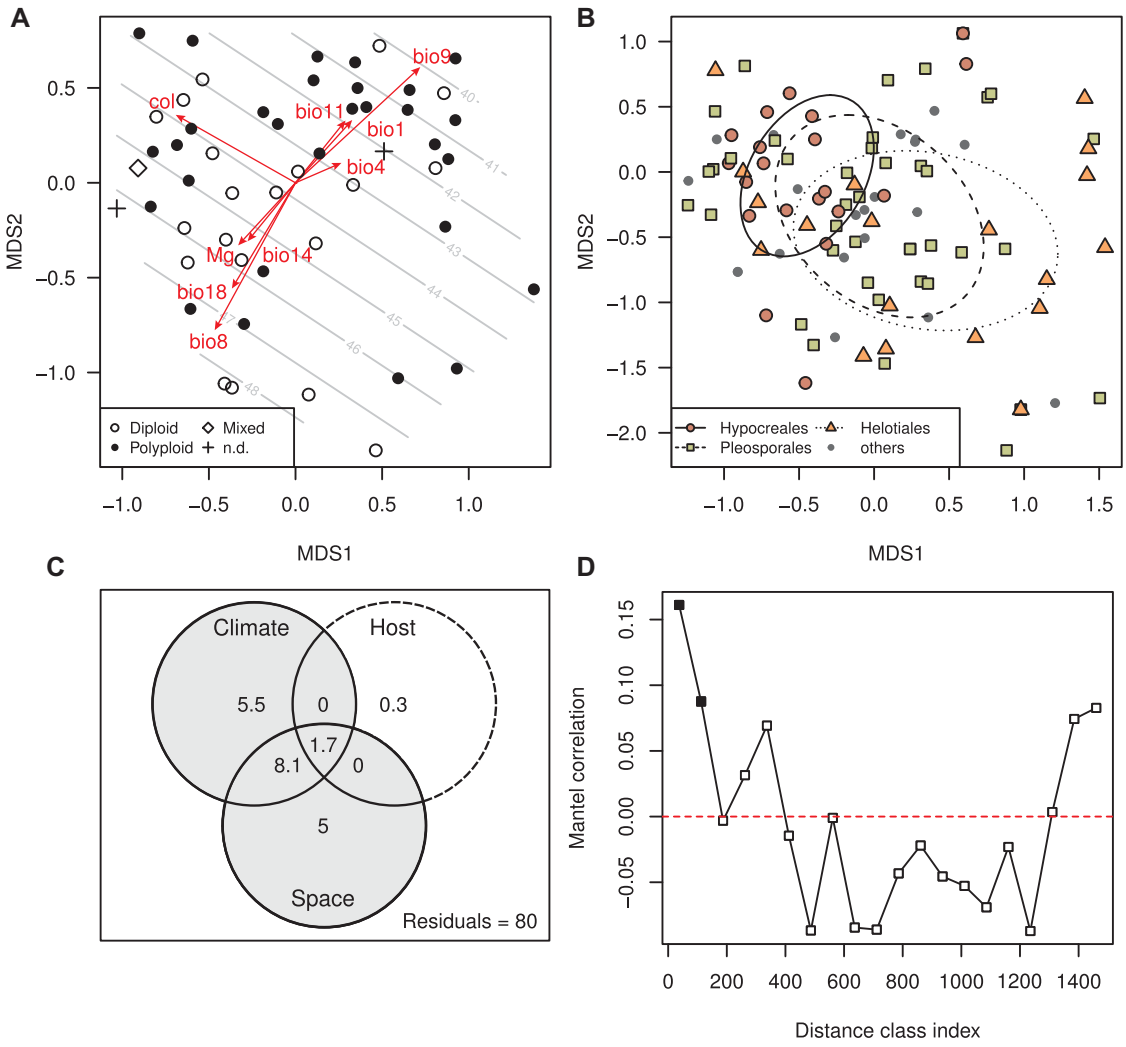

Fig. 4. Effect of ecological factors in whole-community structure of root endophytes.
A. Unconstrained non-metric multidimensional scaling (NMDS) analysis of communities displaying distances among populations, and depicting the relative influence of selected variables (arrows). The latitudinal gradient is represented as surface lines.
B. Species scores of the NMDS ordination in (A), highlighting the three dominant fungal orders. Ellipses delimit 95% confidence intervals around the mean values for each order.
C. Partition of the community variance into a climatic, a spatial and a host component. The numbers inside the sections indicate the percentage of the variation explained. Grey sectors with solid line indicate that the values comprised are significant ($P < 0.05$), whereas the value in an empty sector with dashed lines is not significant.
D. Mantel correlogram showing Mantel correlations among communities across distance classes. Solid symbols denote significant ($P < 0.05$) correlations for each class. Comparisons beyond 1500 km were not calculated due to the low number of samples included beyond this distance.
Abbreviations: bio1, annual mean temperature; bio4, temperature seasonality (standard deviation); bio8, mean temperature of wettest quarter; bio9, mean temperature of driest quarter; bio11, mean temperature of coldest quarter; bio14, precipitation of driest month; bio18, precipitation of warmest quarter; col, mean colonization percentage per population; Mg, magnesium soil content.

structure of endophytic communities (Fig. 4C). These three components explained 20% of the variance, and each accounted for a significant proportion individually according to pseudo-*F* tests ($P < 0.05$). However, the host predicted only a 1.7% of the overall variation ($P = 0.02$), indistinguishable from the contribution by climate or space. Each of the climatic and the spatial components explained individually around 15% of the total community variance, of which about 10% was jointly attributed to both categories (Fig. 4C). Sampling size accounted for a 3.3% of the overall variation, of which 2.4%, 1.8% and 1.1% were undistinguishable from the effect of climatic, spatial and host variables respectively. Sampling size alone explained a 0.7% ($P = 0.15$) of community variance.

Geographic distance had no overall effect on the similarities among communities ($R = 0.02$, $P = 0.32$). To investigate a potential effect across distance classes, we built a multivariate Mantel correlogram (Fig. 4D), which showed

a patchy distribution of communities separated by up to 115 km ($P < 0.002$).

Effect of ecological factors on individual endophyte populations

Maps of OTU occurrence and variance partition of individual fungal populations showed distinctive patterns in their distribution (Fig. 5). Occurrences of *Fusarium* sp. OTU001 (Fig. 5A) and *Pyrenochaeta* sp. OTU004 (Fig. 5D) were unaffected by the ecological components considered, and the models for their distribution were not significant. *Alternaria* sp. OTU002 (Fig. 5B) and *Ilyonectria* sp. OTU005 (Fig. 5E) showed clear but opposed latitudinal gradients of occurrence, mostly driven by confounding climatic and spatial factors. *Cadophora* sp. OTU006 was the only dominant endophyte the occurrence of which was consistent with a local distribution determined by soil factors, especially pH and Mg content (Fig. 5F). In this case, a fraction (5%) of the soil component overlapped with a significant effect ($P = 0.002$) of the sampling size. The contribution of the individual ecological variables to the occurrence of each OTU is shown in Table 2.

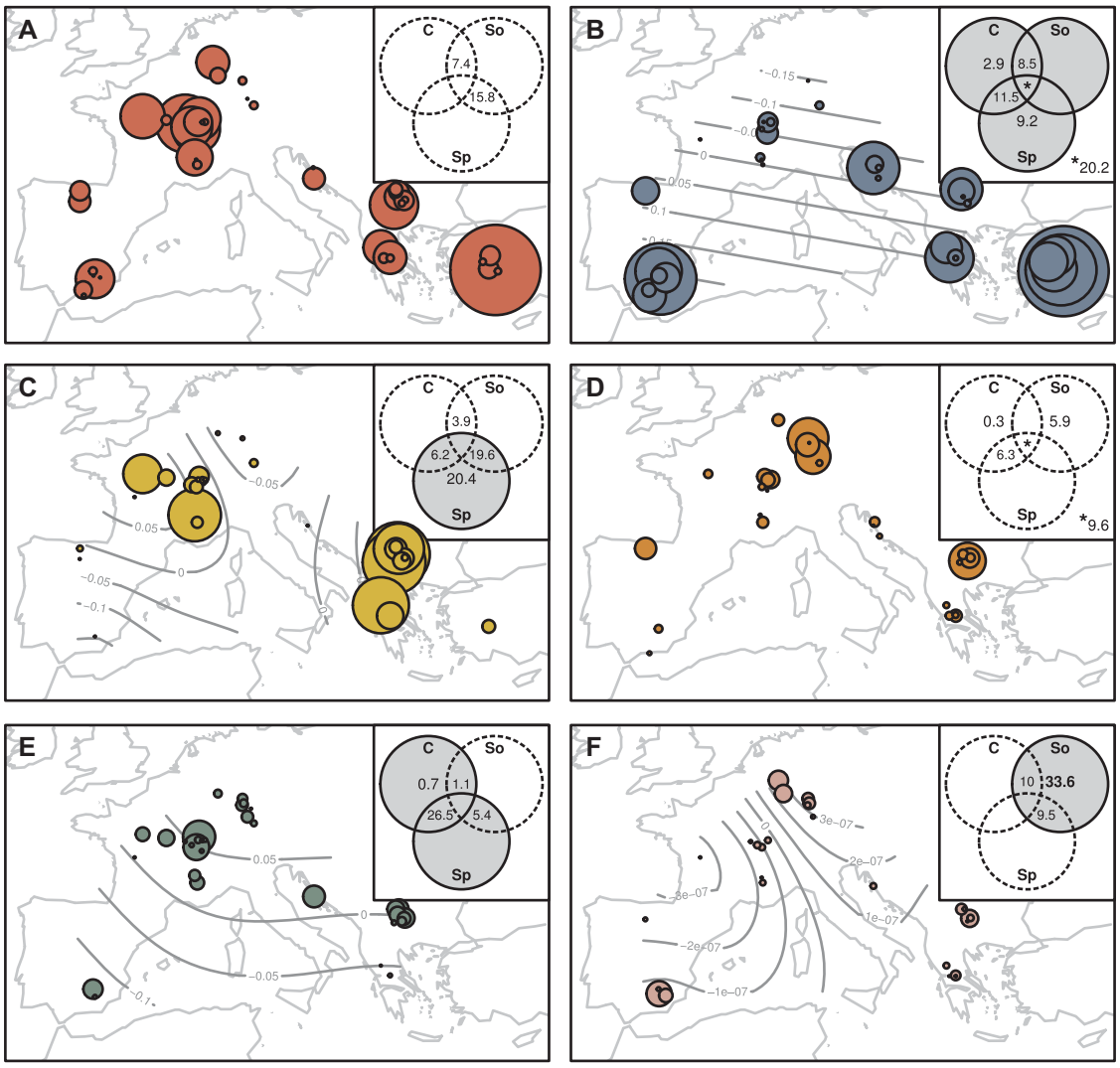

Fig. 5. Distribution and frequency of the six dominant OTUs across the sampling area: *Fusarium* sp. OTU001 (A), *Alternaria* sp. OTU002 (B), *Fusarium* sp. OTU003 (C), *Pyrenochaeta* sp. OTU004 (D), *Ilyonectria* sp. OTU005 (E) and *Cadophora* sp. OTU006 (F). Bubble sizes indicate relative frequency for each OTU at every location, and surface lines represent the fitted scores of redundancy analysis, depicting the variation explained by significant ecological components (samples from Croatia and Turkey not included in models, due to missing data on soil properties). Insets represent the variation partitioning results in a climatic (C), a spatial (Sp) and a soil (So) component. Grey sectors with solid line indicate significant values ($P < 0.05$), and empty sectors with dashed lines are not significant. Sectors without numbers indicate no variance explained at all.

Table 2. Variance partitioning for the occurrence of individual dominant endophytes into ecological categories of factors, including climatic, soil and spatial components.

		OTU001		OTU002		OTU003		OTU004		OTU005		OTU006	
Component	Factor[a]	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*	Adj. R^2	*P*
Climate	Annual temperature range (Bio 7)	–	–	0.2	0.002	–	–	–	–	–	–	–	–
	Mean temperature of wettest quarter (Bio 8)	–	–	0.19	0.004	–	–	–	–	–	–	–	–
	Mean temperature of driest quarter (Bio 9)	–	–	0.25	0.002	–	–	–	–	0.26	0.002	–	–
	Precipitation seasonality (Bio 15)	–	–	0.24	0.001	–	–	–	–	0.22	0.002	–	–
	Precipitation of warmest quarter (Bio 18)	–	–	0.13	0.015	–	–	–	–	0.23	0.002	–	–
Soil	Magnesium soil content (Mg)	–	–	0.14	0.01	–	–	–	–	–	–	0.24	0.003
	pH	–	–	–	–	–	–	–	–	–	–	0.26	0.001
	Latitude	–	–	0.36	0.001	–	–	–	–	0.22	0.003	–	–
Spatial[b]	MEM1	0.11	0.027	–	–	–	–	–	–	0.11	0.024	–	–
	MEM2	–	–	0.21	0.003	0.1	0.03	0.08	0.04	0.21	0.003	–	–
	MEM3	–	–	–	–	0.21	0.004	–	–	–	–	–	–
	MEM5	–	–	–	–	0.08	0.038	–	–	–	–	–	–

a. Ecological factors explaining a significant fraction of the occurrence of at least one fungal OTU, selected via a forward selection of all factors in this study.
b. MEMs (Moran's Eigenvector Maps) represent spatial structures in the data not explained by measured variables.

Co-occurrence and in-plate interactions of dominant endophytes

We found positive co-occurring patterns involving OTU001, OTU003 and OTU005, among each other and between OTU004 and OTU005 (Fig. 6). Relations among OTU001, OTU002 and OTU003 were strongly affected by a positive spatial autocorrelation, but the significance and magnitude of the relations persisted after correcting the spatial effect in linear regressions (slope = 0.30, $P = 0.014$ for the OTU001-OTU003 interaction). There was a strong negative correlation between OTU002 and OTU005 ($P < 0.001$), but this could not be linked with antagonistic interactions in culture (Fig. 6). Only *Pyrenochaeta* sp. OTU004 presented a consistent presence of inhibitory halos with other colonies. Both *Fusarium* strains tended to overgrow other fungi via direct contact between colonies because of their fast radial growth.

Discussion

The diversity and structure of fungal assemblages within roots of *Microthlaspi* were largely determined by the local environment to which plants were subjected. Of the ecological variables directly measured, climatic rather than soil conditions were the best descriptors of the broad-scale structure of endophytic communities. They were also strongly influenced by other factors that were lumped in the so-called spatial effect. This explains non-random spatial structures of the data not accounted for by the variables measured, and might include processes of environmental, historical or biological nature (Peres-Neto and Legendre, 2010; Dray *et al*., 2012). In contrast to the climatic and spatial effect, geographic distance among locations had a negligible influence in defining the composition of communities. The pattern arising when examining the turnover of OTUs across distance classes supported such view because communities that were close or very far away had greater commonalities than communities within intermediate distances. An effect purely due to distance and consequently to a limited dispersal, on the contrary, would imply a steady decay in community similarity with distance (the distance–decay relationship; Green *et al*., 2004; Peay *et al*., 2007).

In line with previous studies (Green *et al*., 2004; Amend *et al*., 2010; Queloz *et al*., 2011; U'Ren *et al*., 2012), our results do not reject the Baas–Becking hypothesis of a ubiquitous dispersal for fungi. Our findings are somewhat surprising because many root endophytes commonly lack specialized structures for dispersal in culture or field conditions (Jumpponen and Trappe, 1998; Sieber, 2002; Addy *et al*., 2005; Maciá-Vicente *et al*., 2008a), and even those able to produce spores have important constraints hindering their long-range dissemination (e.g., due to

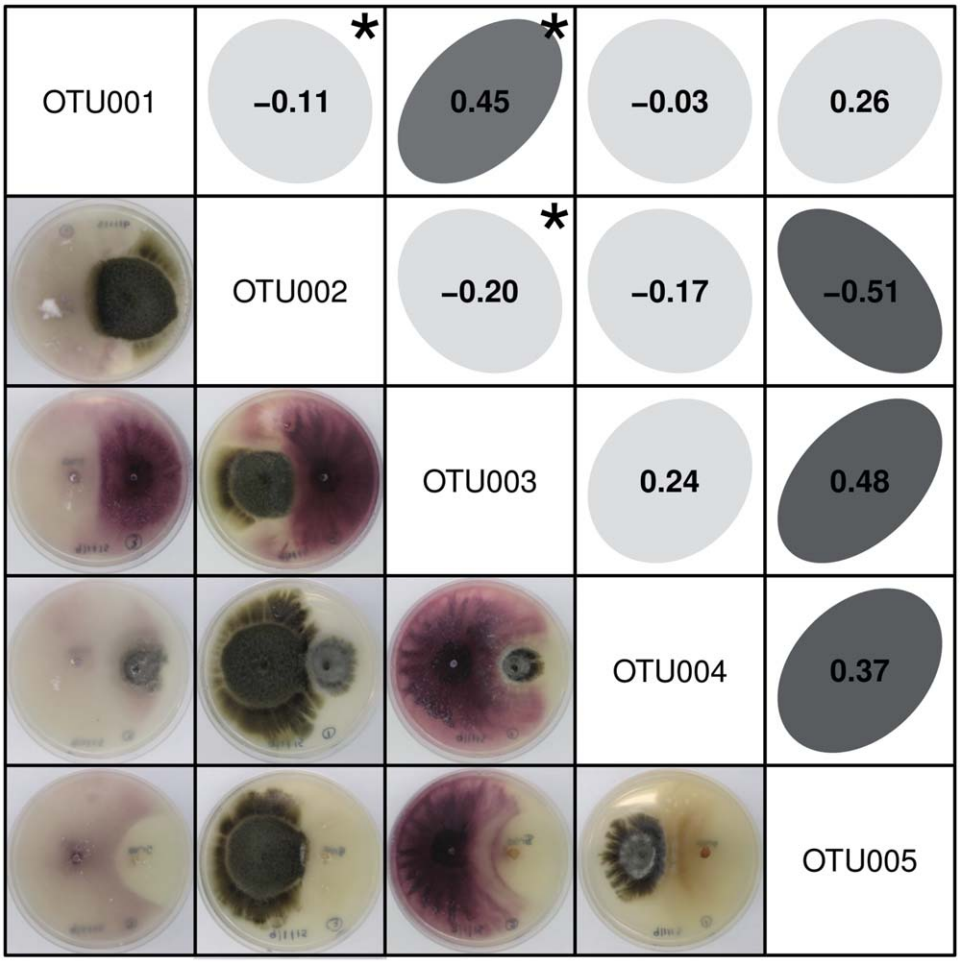

Fig. 6. Co-occurrence patterns and *in vitro* interactions among the five most frequent endophyte OTUs. Boxes with ellipses show the magnitude of the correlation between the co-occurrence of OTU pairs in roots of *Microthlaspi* populations. Values and ellipticity represent Spearman's ρ, and darker ellipses denote significance at $P < 0.05$. Asterisks indicate relationships with a significant autocorrelation, which were further assessed by spatial autoregressive models. Images in the lower diagonal represent interaction between colony pairs in dual culture assays. OTUs in each row are shown to the right in each interaction.

structural characteristics of the spores or their release points; Peay *et al*., 2010). Alternative mechanisms for their efficient dispersal must therefore exist. The hitchhiking with host dispersal could be relevant for endophytes that colonize the plant systemically and reach the seeds or fruits, but this mechanism cannot account for the majority of root endophytes that are restricted to below-ground plant organs (Rodriguez *et al*., 2009; Herrera *et al*., 2010; Maciá-Vicente *et al*., 2012). Other mechanisms of dissemination could imply animal transportation of plant material via herbivory and deposition, especially for some fungal groups that develop resistance structures within the plant tissues like microsclerotia (Currah *et al*., 1993; Porras-Alfaro *et al*., 2008), or processes in common with soil-borne fungi, like wind transportation by carrier soil particles, adhesion to invertebrates or spore washings (Dix and Webster, 1995).

Two considerations have to be taken into account with respect to the ubiquitous occurrence of root endophytes found in this study. First, our sampling could not achieve a complete description of the fungal richness constituting communities, and therefore the main results are driven by dominant endophytes. Thus it cannot be ascertained whether rare OTUs detected locally have a truly restricted distribution, or if such a finding would be due to under-sampling. Such under-representation is common in assessments of microbial diversity and has only been tackled by reaching a considerable sampling depth in species-poor habitats (Taylor *et al*., 2014). Second, the definition of fungal OTUs based on ITS similarity might not be sufficient to resolve closely related species (even when using stringent clustering parameters), and thus might mask hidden biogeographic patterns. Queloz and colleagues (2011) could not detect a biogeographic

pattern in an assembly of root endophytes distributed globally, after applying several molecular markers. However, based on observations in other eukaryotic microorganisms (including fungi; Taylor *et al*., 2006; Gazis *et al*., 2011; Ryšánek *et al*., 2015), and given the broad taxonomic diversity uncovered in our study, we do not discard the possibility of cryptic patterns in the distribution of some fungal groups that showed a cosmopolitan occurrence.

Environmental descriptors of community structure

Endophytic communities were clearly structured along a latitudinal gradient. Latitude gathers a set of co-varying historical, abiotic and biotic gradients that have a strong influence on the distribution of all sorts of organisms (Hillebrand, 2004; Mittelbach *et al*., 2007), including soil and plant-associated fungi (Hoffman and Arnold, 2008; Herrera *et al*., 2010; Tedersoo *et al*., 2012; 2014; U'Ren *et al*., 2012). In our study, latitude determined community composition but not richness and diversity, possibly because the latitudinal range covered was shorter than in other works (Amend *et al*., 2010; Tedersoo *et al*., 2012; 2014). Instead, OTU richness and diversity were negatively correlated with various variables reflecting local precipitation. This situation is similar to what has been found in mycorrhizal fungi (Tchabi *et al*., 2008; Tedersoo *et al*., 2012), but not for above-ground endophytes (U'Ren *et al*., 2012), including those in plants within the Brassicaceae, like *Microthlaspi* (García *et al*., 2013). This pattern contradicts well-known positive effects of rainfall on soil fungal richness (Tedersoo *et al*., 2014, but see Hawkes *et al*., 2011), and hence suggests processes of environmental filtering specific for root symbionts. Water deficiency could increase fungal richness within roots by favouring an active hyphal growth towards roots with a higher water content than the surrounding soil, by compromising host defences against fungal colonization through water stress or by a direct functional modulation of rhizosphere microbial consortia (Van Der Heijden *et al*., 2008; Hawkes *et al*., 2011).

Climatic variables collinear with the latitudinal gradient were the strongest determinants of community composition. These factors were related to annual temperature and precipitation ranges that clearly differentiated endophytic communities from southern areas, with hot and dry summers and wet winters, from those in northern temperate regions, characterized by wetter and colder seasons. Both temperature and rainfall are well-known broad-scale descriptors of fungal occurrence (Arnold and Lutzoni, 2007; Amend *et al*., 2010; Herrera *et al*., 2010; Hawkes *et al*., 2011; Tedersoo *et al*., 2012; 2014; Timling *et al*., 2014). They impose physiological constraints to fungal growth with a differential effect across taxa, affecting growth, spore formation and germination (Torres *et al*., 2003). In addition, the effect of bioclimatic variables on fungal communities might be indirect because they are likely to modulate the structure and productivity of plant communities, and this in turn could affect the microbial diversity associated with particular plants (e.g., Mohamed and Martiny, 2011; Blaalid *et al*., 2012).

Soil characteristics had a negligible influence on endophytic communities at the scale of this study. Soil physicochemistry is a well-known determinant of belowground fungal assemblages, with pH being the factor best explaining large-scale differences (Taylor *et al*., 2014; Tedersoo *et al*., 2014; Timling *et al*., 2014). However, soil features are most likely decisive at local and regional scales, where closely adjacent soil patches can have heterogeneous edaphic conditions. This was shown by Maciá-Vicente and colleagues (2012), who described a profound shift in the structure of root endophytic communities of a single plant species along a gradient of soil salinity of only a few meters. Besides, *Microthlaspi* has specific edaphic preferences that determine only a slight variation in soil characteristics across samples (Koch and Bernhardt, 2004), which are unlikely to exceed the ranges of tolerance for the majority of fungi and thus to represent an important selective factor.

Effect of host phylogeography on endophyte assemblages

Host phylogeny is one of the best descriptors of the plant-associated fungal communities, when widely divergent plant species are considered (Wehner *et al*., 2014). However, the biogeographic structure identified in our study appears to be host independent, which may reflect the relatedness of the plants sampled. While the host genotype had a weak effect in determining whole community structure, it was collinear with other latitude-associated climatic factors that better explained fungal occurrence. Diploid and polyploid *Microthlaspi* species have divergent biogeographic distributions owing to different climate preferences (Ali *et al*., 2015). These explain a somewhat latitudinal distribution of cytotypes (Koch and Bernhardt, 2004), with polyploid *M. perfoliatum* having a wider distribution but preferentially occurring in southern regions, and diploid *M. erraticum* occurring in cooler regions.

Niche occupancy by dominant root endophytes

Our data reveal a clear pattern of distinctive preferences for specific niches or ecological conditions by individual endophytes. Niche occupancy is not only delimited by the distribution of relevant environmental properties, but is also driven by the interaction with competitor species (Silvertown, 2004). In the current study, however, positive

or negative co-occurrences among dominant endophytes seemed to reflect their shared or opposing ecological needs, rather than direct interactions. For example, *Alternaria* sp. OTU002 and *Ilyonectria* sp. OTU005 had latitudinally opposed distributions, which were largely determined by the climate, while their colonies showed a neutral interaction *in vitro*. An alternative explanation for their exclusive presence could be the competition of both groups for the same resources. *Fusarium* sp. OTU001 and *Pyrenochatea* sp. OTU004 had a cosmopolitan occurrence, which was unaffected by environmental or spatial variables. The distribution of *Fusarium* sp. OTU003 was likewise independent from the environment and purely driven by spatial autocorrelation, which defined two apparent foci of occurrence. Interestingly, *Cadophora* sp. OTU006 was the only endophyte the distribution of which was largely determined by soil conditions, showing a negative interaction with pH. Dark septate endophytes within the Helotiales – to which *Cadophora* belongs – are often associated with acidic soils (Sieber and Grünig, 2013), and a recent work found several accessions phylogenetically similar to OTU006 to be strongly correlated with soil properties, including pH (Taylor *et al*., 2014).

Taxonomic identity of Microthlaspi *endophytes*

The largest proportion of root endophytes in *Microthlaspi* belonged to the phylum Ascomycota, consistent with findings for most plants (except for ecto-mycorrhizal trees) based on both cultivation-based and molecular approaches (Sieber, 2002; Porras-Alfaro *et al*., 2008; Herrera *et al*., 2010; Maciá-Vicente *et al*., 2012; Pecoraro *et al*., 2012; Obase and Matsuda, 2014; Wehner *et al*., 2014). The dominance in diversity and frequency of Pleosporales and Hypocreales, the latter with a high proportion of Fusaria, also reflects common patterns of fungal occurrence in roots (Maciá-Vicente *et al*., 2008a; 2012; Márquez *et al*., 2010). Both constitute species-rich orders containing functionally versatile species adapted to a variety of habitats, and their relative presence appears to be modulated by the environment (Maciá-Vicente *et al*., 2008a; Porras-Alfaro *et al*., 2008). Because the occurrence of hypocrealean endophytes was correlated with overall root colonization, it is possible that cultivation methods positively bias towards them, since they often have fast growth rates and easily overgrow other fungi in the isolation plates. Alternatively, this could indicate a systemic colonization of roots by these fungi, which would possibly explain their growth from most of the root pieces plated. The order Helotiales, being the third-most frequent order, contains instances of dark septate root endophytes that predominate in woody hosts in temperate and boreal regions (Sieber, 2002; Sieber and Grünig, 2013).

Several of the frequent OTUs found here overlap with those in a previous description of the cultivable root mycobiota of *Microthlaspi*, from specimens collected in Germany 1 year prior to our sampling (Keim *et al*., 2014). This included species of *Fusarium*, *Ilyonectria*, *Alternaria*, *Pyrenochaeta* and multiple others related to strains identified in our sampling, suggesting a temporal stability of the fungal communities associated with this plant. Remarkably, a large proportion of the endophytic diversity that can be found in healthy wild plants is from genera containing known plant pathogens, many of which are of economic importance in crops. Our work complements previous studies that disclose a cryptic biology of fungi traditionally considered as *bona fide* pathogens because they were first described from diseased plants or are prevalent in agricultural systems (Malcolm *et al*., 2013). This could hint at a switch to pathogenicity because of the highly artificial environment created by intensive forms of agriculture. Besides, it also highlights the problem of oversimplifying the functional roles of root-associated fungi, which is frequent in ecological studies (Aguilar-Trigueros *et al*., 2014) and might lead to the erroneous interpretations of the participation of the fungal biodiversity in the functioning of ecosystems.

Conclusions

Understanding the distribution patterns of fungal root endophytes will help infer the potential functions they play in natural ecosystems, which are as yet largely cryptic. This information will be essential for the long-term monitoring of the global fungal biodiversity, especially in the context of current environmental threats. Here, we show that the distribution of fungal endophytes in roots of an annual plant is determined by the local environment at a continental scale. Geographic distance was a poor descriptor of community structure, suggesting efficient mechanisms for dispersion in this group of fungi. The large-scale changes are principally driven by climatic factors that define a latitudinal gradient of community structure, while soil conditions and host factors appear to have little or a locally restricted effect. Our results also demonstrate particular ecological preferences by individual groups of endophytes, suggesting that they play different functional roles in the ecosystems. To date, there is a limited number of studies on the biogeography of non-mycorrhizal root endophytes. Additional studies based on cultivation-free molecular approaches are ongoing and will provide a more comprehensive view of the spatial scaling of the endophytic fungal diversity. Lastly, the availability of an extensive collection of endophyte strains will warrant the performance of laboratory ecological studies that will help draw a link between their distribution and their potential functional roles.

Experimental procedures

Sample collection

Microthlaspi plants were collected from 52 sites distributed across six European countries (Spain, France, Germany, Croatia, Greece and Bulgaria) and Turkey (Table 1). The samplings were performed in 2013, from mid-April up to early June, roughly corresponding to the flowering period of the plant, and consisted of several field campaigns. Sites were selected according to the presence of an individual *Microthlaspi* population, defined as a cluster of several plant individuals. Populations were separated from one another by a minimum of 2 km. The only exception were populations D-11a and D-11b (Table 1), which grew adjacently but formed clearly different clusters, each with a particular accompanying vegetation. We collected 3–10 healthy-looking and medium-sized plants per population (Table 1), which we carefully uprooted to minimize disruption of roots and stored in cool conditions in food-grade plastic bags until their processing in the laboratory.

Acquisition of environmental and host data

In 42 out of the 52 sites we collected soil samples to characterize the chemical properties of the substrate in which the plants grew. For each site we took multiple soil subsamples from points covering the area of distribution of the plant population, and then pooled them in a single sample. Soils were analysed for pH, conductivity, organic/inorganic carbon and content of macronutrients (N, P, K, S, Na, Mg and Ca; Table S2) by the Soil Science Laboratory Unit of the Goethe University (Frankfurt am Main, Germany). Besides, for all sites we gathered data on elevation and geographic coordinates, which were used to retrieve several bioclimatic variables from the WorldClim (http://www.worldclim.org/; Hijmans *et al*., 2005) and the Consortium for Spatial Information (CGIAR-CSI; Trabucco and Zomer, 2009) data sets. The data set includes 19 variables derived from temperature and precipitation measurements (O'Donnell and Ignizio, 2012), and the degree of aridity (Table S2).

The genotype of the host plants was considered as an additional factor likely to influence the distribution of endophytes. The ITS regions of the ribosomal DNA were sequenced for up to three representative plants of most populations. DNA ploidy levels of these representative plants were determined by flow cytometry calibrated by chromosome counts for reference *Microthlaspi* specimens and ITS sequence comparisons (Ali *et al*., 2015). The estimated ploidy levels were used as a categorical variable in later analyses. Additionally, one representative ITS sequence per population was used to generate a matrix of pairwise genetic distances among populations to include host phylogeny as a numeric variable in statistical analyses. Selection of only one sequence per population was done after assessing a high sequence similarity within populations. One mixed population containing both cytotypes of *Microthlaspi* was excluded from analyses aimed at testing the effect of host on endophytic communities.

Isolation of endophytic fungi from roots

The processing of samples in the laboratory took place in most cases within 72 h after their collection. Roots from every plant were detached and treated individually in every step of the process to isolate endophytic fungi. We chose a mild surface-sterilization protocol for the elimination of microbial epiphytes to avoid over-disinfecting the roots, given their reduced thickness. The protocol consisted of a first wash under running tap water to remove adhered soil particles, and then a surface-sterilization with a 0.5% (v/v) sodium hypochlorite solution for 1 min, followed by three rinses with sterilized deionized water. Roots were then dry-blotted onto sterilized filter paper and cut into *c*. 3-mm long pieces. Ten randomly picked root pieces per plant were plated on a Petri plate containing 0.5% (w/v) Malt Extract Agar (AppliChem, Darmstadt, Germany) supplemented with 0.5 g l^{-1} chloramphenicol to minimize development of bacteria, and with 0.1% (v/v) Triton X-100 (Amresco, Solon, OH, USA) to restrict the spread of fast-growing fungi. We tested the effectiveness of the surface-sterilization protocol by imprinting one third of all root pieces (representing all samples) in the same medium before plating them into the final cultivation plates (Hallmann *et al*., 2006). This yielded fungal growth in 1.7% of the imprints, which we considered acceptable given the large number of root pieces handled and the overall variability in root morphology among individual plants and populations.

The plates with root pieces were incubated at room temperature for a period of 2 months. During this time we recorded the occurrence of fungal colonies as they emerged, and we classified them into morphotypes. We isolated in pure culture one representative colony from each morphotype per plate, yielding a total of 2006 cultures representing 2601 colony counts. All isolates have been deposited in the living fungal cultures collection of IPF hosted at Goethe University, and are available upon request from the authors.

Molecular characterization of strains

We processed 1998 isolates representing all morphotypes for sequencing of their ITS rDNA region. Genomic DNA was extracted from all cultures using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) on a KingFisher Flex 96 robotic workstation (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The ITS region was amplified with the fungal-specific primer pair ITS1F and ITS4 (White *et al*., 1990; Gardes and Bruns, 1993) in 20 μl of polymerase chain reactions containing 1 μl of DNA template, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of each primer, and 0.5 U Taq polymerase (VWR International, Darmstadt, Germany). Temperature cycles were carried out in a Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany) and consisted of an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension step of 72°C for 5 min. The size of amplicons ranged between 500 and 600 bp in most cases, although some reactions yielded products of up to 1000 bp. The amplified products were sequenced using the same primers by the sequencing laboratory of the Biodiversity and Climate Research Centre (Frankfurt am Main, Germany).

Isolates were assigned to OTUs according to pairwise similarities of ITS sequences, as calculated with the BLASTCLUST tool (ftp://ftp.ncbi.nih.gov/blast/documents/blastclust.html) from the NCBI-BLAST package (Altschul *et al*., 1990), using cut-off values that ranged from 90% to 99%. For subsequent analyses, we selected the data set based on the clustering at 97% similarity because it has been shown to provide a good approximation to biological species in studies spanning wide fungal diversities (Taylor *et al*., 2014). Besides, in our case this clustering matched well individual identification of strains, and downstream analyses with data sets based on 98% and 99% cut-offs yielded similar results. The classification of strains was based on comparisons of all sequences with NCBI GenBank entries using BLAST, and with the curated UNITE database (Kõljalg *et al*., 2013) using the Naïve Bayesian classifier running under MOTHUR v1.34.4 (Wang *et al*., 2007; Schloss *et al*., 2009), with a bootstrap support of 80%. Additionally, we repeated the latter approach with an in-house database with sequence and taxonomic data for all fungal ITS sequences in GenBank identified to species level, formatted for its use in MOTHUR. A consensus taxonomy was built for every OTU by either method considering a within-group sharing of at least 51% of the hits at each taxonomic level. Finally, we combined all taxonomic data for a definitive assignment of OTUs up to genus level. Conflicting assignments between the UNITE and the in-house databases were checked manually against GenBank using BLAST. When this was not conclusive, the lowest taxonomic level at which both databases agreed was selected. The taxonomic assignment for each OTU and the GenBank accessions for all sequences are shown in Table S1.

Dual plate assays

To understand potential in-culture interactions among different fungal groups that could have biased the root isolation results, we performed a dual-plate assay as described in Maciá-Vicente and colleagues (2008b). Briefly, representative cultures of the five most frequent fungal OTUs found were confronted in all pairwise combinations in the same medium used for their isolation. Assays were performed in triplicate, and plates were incubated for 1 month after which we recorded presence/absence of inhibitory interactions between colonies (e.g., formation of inhibition halos).

Data analysis

Fungal diversity. All analyses were carried out in R v3.0.2 (R Core Team, 2013) using relevant packages. To analyse fungal diversity and community data, we mostly relied on the package VEGAN v2.2-1 (Oksanen *et al*., 2015). OTU count records for individual root pieces were first assembled into a data matrix containing group-wise colonization percentages per plant, calculated as in Fröhlich and colleagues (2000). This was used to compute overall and averaged values of OTU frequency, richness and diversity indices and richness estimators (Magurran and McGill, 2011) for each plant population. Statistical support for these comparisons was determined with the Kruskal–Wallis Rank sum test (Hollander *et al*., 2013) at a significance level of 0.05. Potential links among richness and diversity data, frequencies of individual OTUs and ecological factors were explored by the calculation of pairwise correlations with the Spearman's rank statistic and linear regression. Because the sampling design included clusters of sites closely spaced and unevenly separated, our data were sensitive to spatial autocorrelation that could inflate type I error in significance tests and invalidate them (Peres-Neto *et al*., 2006). Therefore, we estimated autocorrelation in all bivariate tests using Moran's *I* (Li *et al*., 2007) and corrected it when present using spatial autoregressive models (Dormann *et al*., 2007).

Community analyses. To compare overall fungal communities across plant populations, we calculated dissimilarities in OTU composition among assemblages using the Horn–Morisita index (Horn, 1966). Prior to this we removed singletons (defined as OTUs occurring in only one plant specimen over the survey), and then square-root-transformed the data to reduce the weight of dominant OTUs in the dissimilarities. The utilization of other distance indices or transformation methods yielded similar downstream results, and hence we considered these parameters appropriate. All environmental variables recorded were fitted to the dissimilarities among samples to investigate potential relationships, and significance of these correlations was tested with PERMANOVA (Anderson, 2001). Distances among samples and their correlation with significant factors were visualized by means of an NMDS. The effect of geographic distance on communities was investigated with a Mantel test and a Mantel correlogram by comparing ecological and geographical distances among sites at different ranges.

Variation partitioning. To determine the contribution of ecological factors as predictors of the endophytic community, we used the variance partitioning method following procedures described in Borcard and colleagues (2011) and Legendre and Legendre (2012). This was used to decompose the variation of OTU assemblages into four independent components gathering climatic, soil, spatial and host factors. The spatial component – accounting for unmeasured processes, either intrinsic to the organisms (e.g. dispersal) or environmental (Peres-Neto and Legendre, 2010) – was obtained by the calculation of Moran's eigenvector maps (MEMs; Dray *et al*., 2006), which represent the multivariate structure of the data at all scales covered by the sampling. MEMs were the resulting ordination axes of a principal coordinate analysis (PCoA) of geographic distances among sites, following a weighted Delaunay triangulation connectivity matrix. The geographic coordinates of sites were also included in the spatial component, because MEMs do not cover linear trends associated with latitude and longitude. On the other hand, we excluded elevation from this component because of its strong collinearity with latitude (Spearman's $\rho = -0.86$, $P < 0.001$). The host component included the categorical variable ploidy, and vectors representing the phylogenetic relationship among populations, obtained as the resulting axes of a PCoA ordination of genetic distances in a manner similar to MEMs (Desdevises *et al*., 2003).

Variation partitioning of the community data relied on constrained redundancy analyses (RDAs). We first transformed the singleton-free community matrix using a Hellinger conversion (Legendre and Gallagher, 2001), and then included it

as response variable. The explanatory matrices included factors that individually explained a significant proportion of the variation of the community data, as determined by a forward selection using the R package PACKFOR v0.0-8. We decided to exclude the soil component from these analyses because it only predicted a marginal proportion of the variation, and it reduced considerably the number of observations due to missing data. To assess potential effects of the number of plants collected at each site on the observed structure of communities, we included it as an additional explanatory variable and reported its contribution to the variance explained by ecological components. After variance partitioning, the significance of the variance fractions explained by each component was assessed using constrained RDA with pseudo-*F* tests.

Distribution of dominant OTUs. A modification of the above procedure was used to determine the contribution of the ecological components to the individual variation of the six most frequent OTUs (Peres-Neto and Legendre, 2010). In this case, RDA is equivalent to multiple linear regression because only one response variable is included. We forward-selected factors individually for each OTU and retained all those selected at least once, which were then used as explanatory variables. We excluded the host component from these analyses because of its poor contribution to the variation, and instead we included soil factors at the expense of reducing the number of observations in the models because we deemed them important in explaining the occurrence of particular fungi. Repetition of these analyses with climate and spatial effects alone yielded similar results for these components (data not shown). Fitted scores for linear models representing significant fractions of the variance were represented as surfaces in distribution maps for each OTU.

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Author contributions

MT and JGMV conceived the study. KG, TA, AKB, SP, XX, MT and JGMV collected plant and soil samples. AÇ helped to coordinate samplings in Turkey. KG, SHK and JGMV isolated and processed fungal endophytes. KG and TA characterized plant genotypes. MT and JGMV contributed material/reagents. KG and JGMV analysed the data. KG and JGMV wrote the manuscript with contributions from the other authors.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sequence accessions, taxonomic information, and isolation results for the OTUs described in this study.
Table S2. Ecological factors used in this study.

4. Research article 2

Kia, S.H., Glynou, K., Nau, T., Thines, M., Piepenbring, M. & Maciá-Vicente, J.G. (2017). Influence of phylogenetic conservatism and trait convergence on the interactions between fungal root endophytes and plants. *ISME J.*, 11, 777–790.

Anlage 2

Erklärung zu den Autorenanteilen an der Publikation / an dem Manuskript (Titel):

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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Promovierende SHK: 25%
Co-Autoren MT, MP: 15%
Co-Autor JGMV: 60%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende SHK: 70% plant-fungus co-inoculation assays, measuring of fungal traits, microscopical analyses
Co-Autoren KG, TN: 15% isolation and molecular characterization of strains
Co-Autor JGMV: 15% plant-fungus co-inoculation assays, measuring of fungal traits, microscopical analyses

(3) zur Erstellung der Datensammlung und Abbildungen

Promovierende SHK: 80% collection of bioassays and fungal trait data
Co-Autoren KG, TN: 10% collection of sequencing data
Co-Autor JGMV: 10% collection of bioassays and fungal trait data

(4) zur Analyse und Interpretation der Daten

Promovierende SHK: 50%
Co-Autoren KG, MP, MT: 5%
Co-Autor JGMV: 45%

(5) zum Verfassen des Manuskripts

Promovierende SHK: 50%
Co-Autoren KG, MP, MT, TN: 10%
Co-Autor JGMV: 40%

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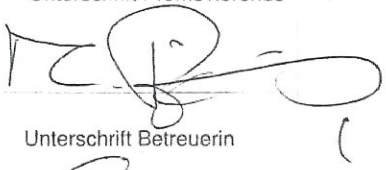

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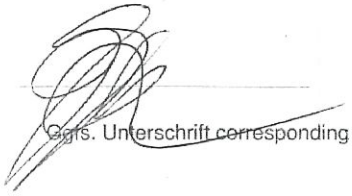

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ORIGINAL ARTICLE

Influence of phylogenetic conservatism and trait convergence on the interactions between fungal root endophytes and plants

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Plants associate through their roots with fungal assemblages that impact their abundance and productivity. Non-mycorrhizal endophytes constitute an important component of such fungal diversity, but their implication in ecosystem processes is little known. Using a selection of 128 root-endophytic strains, we defined functional groups based on their traits and plant interactions with potential to predict community assembly and symbiotic association processes. *In vitro* tests of the strains' interactions with *Arabidopsis thaliana, Microthlaspi erraticum* and *Hordeum vulgare* showed a net negative effect of fungal colonization on plant growth. The effects partly depended on the phylogenetic affiliation of strains, but also varied considerably depending on the plant-strain combination. The variation was partly explained by fungal traits shared by different lineages, like growth rates or melanization. The origin of strains also affected their symbioses, with endophytes isolated from *Microthlaspi* spp. populations being more detrimental to *M. erraticum* than strains from other sources. Our findings suggest that plant–endophyte associations are subject to local processes of selection, in which particular combinations of symbionts are favored across landscapes. We also show that different common endophytic taxa have differential sets of traits found to affect interactions, hinting to a functional complementarity that can explain their frequent co-existence in natural communities.

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Introduction

Root endosymbiotic fungi have impacts on ecosystem functioning through their effects on plant productivity and community assembly (Bever *et al.*, 2010, 2012). Some, such as mycorrhizal fungi, develop mutualistic interactions that allow plants to exploit habitats that would otherwise be inaccessible to them, and to boost their competitiveness over plants lacking these associations (Bever *et al.*, 2010). Others have evolved pathogenic lifestyles and can reduce considerably their hosts' fitness, thus contributing to the diversity of plant communities (Van der Putten *et al.*, 1993; Wardle *et al.*, 2004; Mangan *et al.*, 2010). In addition to these relatively well-defined symbionts, healthy plant roots harbor a broad diversity of other fungi, referred to as root endophytes (Rodriguez *et al.*, 2009; Sieber and Grünig, 2013). The effects of root endophytes on their hosts' development are poorly known, and hence their function in natural ecosystems remains cryptic (Mandyam and Jumpponen, 2005).

Non-mycorrhizal endophytes represent the largest fraction of the fungal diversity within roots, and they are found in all plants and land ecosystems (Vandenkoornhuyse *et al.*, 2002; Sieber and Grünig, 2013). They form polyphyletic ensembles seemingly adapted to the root environment, as their structure and composition differ from those in the neighboring soil and plant organs (Maciá-Vicente *et al.*, 2012; Coleman-Derr *et al.*, 2016). Because the endophyte concept constitutes a catchall classification encompassing all symbionts in the interior of healthy plant tissues (Rodriguez *et al.*, 2009), it is likely to lump together fungal lineages with heterogeneous ecological roles. For example, it is argued that endophytes develop symbioses ranging from parasitic to mutualistic (Mandyam and Jumpponen, 2015) or that depend on the trade-off of particular resources (Newsham, 2011). Moreover, the occurrence of particular endophytes depends on host identity and environmental conditions (for example, Maciá-

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Vicente *et al.*, 2008a, 2012; Wehner *et al.*, 2014; Glynou *et al.*, 2016), they can occupy different root compartments, or follow distinctive patterns of colonization (Maciá-Vicente *et al.*, 2008b, 2009a, 2012; Peterson *et al.*, 2008; Atsatt and Whiteside, 2014). A comprehensive characterization of the symbiotic roles played by endophytes is necessary to understand the evolutionary processes determining the plant-associated fungal diversity and its contribution to the feedbacks that sustain natural communities (Bever *et al.*, 2012).

Many studies have aimed to assess the natural function of endophytes by reproducing their interaction with plants under controlled conditions (for example, Usuki and Narisawa, 2007; Maciá-Vicente *et al.*, 2008b, 2009a,b; Tellenbach *et al.*, 2011; Keim *et al.*, 2014; Mandyam and Jumpponen, 2014, 2015). They provide examples of specific associations between particular fungal and plant genotypes, but they are difficult to extrapolate to general scenarios owing to a high intra-specific variability of the interactions (Tellenbach *et al.*, 2011; Mayerhofer *et al.*, 2012) and to the difficulty in detecting responses in either symbiont (Mandyam and Jumpponen, 2005). Alternative approaches based on the measurement of fungal traits have been proposed to unravel the implication of fungi in ecosystem dynamics (Aguilar-Trigueros *et al.*, 2014, 2015). Classifications of species based on their sets of traits have been used to define major life history strategies, which, in turn, can predict patterns of biodiversity, community assembly and natural associations (Chagnon *et al.*, 2013).

Trait-based approaches have proven valuable to identify relationships between life history and functional traits of arbuscular mycorrhizal fungi and their plant interactions (for exampl, Powell *et al.*, 2009; Maherali and Klironomos, 2012; Chagnon *et al.*, 2013). For example, differences across arbuscular mycorrhizal fungal lineages in rates of nutrient exchange with hosts, sporulation and biomass allocation to mycelial compartments, are linked with their association and interaction with particular plants, their biogeographic and successional patterns, and their community structure (Chagnon *et al.*, 2013). Distinctive traits have also been used to define groups of non-mycorrhizal endophytes, like in the so-called dark-septate endophytes (DSE; Jumpponen and Trappe, 1998). But how these traits are relevant for the symbiosis is seldom known, and systematic studies on the patterns of distribution and evolution of characters across endophytic lineages are lacking (Aguilar-Trigueros *et al.*, 2014).

In this study, we examine the influence of phylogeny and traits of root-endophytic fungi on their interaction with plants. We employ a collection of strains isolated from different plant species, geographical locations and habitats. Most of them originate from a screening of the non-mycorrhizal plant *Microthlaspi* spp. (Brassicaceae) across Europe (Glynou *et al.*, 2016), which harbored a broad diversity of endophytes. In it, a few endophytes with disparate phylogenetic affiliations, like *Fusarium* spp., *Alternaria* spp. and *Cadophora* spp., were ubiquitous and co-existed frequently in the same root communities, but displayed distinctive distribution patterns and ecological preferences. Therefore, our collection provides a basis to assess patterns of trait variation across fungal lineages, geography and ecological conditions. Here, we measure life history traits of endophytes, such as growth rates and sporulation capacity, as well as traits proposed to be potentially functional for the symbiosis, like hyphal melanization and production of intraradical microsclerotia—defining characters of DSE—and enzymatic activities that can facilitate host nutrient uptake or assist fungal penetration of plant tissues (Mandyam *et al.*, 2010). In addition, we assess the effect of strains on the growth of *Microthlaspi erraticum*, its confamilial *Arabidopsis thaliana* and the gramineous *Hordeum vulgare* (Poaceae). Our aim is to test how the interactions between root-endophytic fungi and plants are influenced by phylogenetic conservatism, as well as by convergent traits and ecological origins of fungi that are unrelated to phylogeny.

Materials and methods

Fungal strains and plant material

One hundred and twenty-eight fungal strains isolated from roots of different plant species and geographical locations were used in this study. The majority originate from *Microthlaspi* spp. (Glynou *et al.*, 2016), whereas others were isolated from *Salicornia* spp. (Amaranthaceae). Endophytes were isolated in culture after the surface-sterilization of roots as described by Maciá-Vicente *et al.* (2012), and selected prior to their identification by choosing morphologically divergent strains from different plants/locations. In addition, we obtained *Serendipita indica* (syn: *Piriformospora indica*) CBS 125645 from the KNAW-CBS Fungal Biodiversity Centre. *S. indica* has been thoroughly studied as a model endophyte with a mutualistic interaction with multiple plants (Banhara *et al.*, 2015). A description of all strains is provided in Supplementary Table S1.

The plants *A. thaliana* ecotype Col-0, *M. erraticum* and *H. vulgare* cv. Barke (barley) were used as hosts in plant–endophyte interaction assays. Seeds of *A. thaliana* were provided by the Laboratory of Plant Physiology of Wageningen University. Seeds of *M. erraticum* were collected from a field population in Germany (Mp_K11; Ali *et al.*, 2016). Barley seeds were provided by the company Saatzucht Josef Breun GmbH & Co. KG (Herzogenaurach, Germany).

Molecular characterization of strains

We obtained the sequences of the ribosomal DNA internal transcribed spacer regions (ITS) of all

strains. ITS sequences from most strains were already available from Glynou *et al.* (2016), and the rest were obtained as described therein. We also followed the procedures in Glynou *et al.* (2016) to assign the strains to taxa and to group them into operational taxonomic units (OTUs). In brief, genomic DNA was extracted from fungal mycelia using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) on a KingFisher Flex 96 robotic workstation (Thermo Fisher Scientific, Waltham, MA, USA). ITS sequences were amplified and sequenced using the primer pair ITS1F/ITS4 (White *et al.*, 1990; Gardes and Bruns, 1993), and they were then classified at different taxonomic precisions with the Naive Bayesian classifier tool of Mothur v1.34.4 (Wang *et al.* 2007; Schloss *et al.* 2009), based on comparisons with the UNITE database of curated fungal ITS sequences (Kõljalg *et al.*, 2013). Strains were grouped into OTUs according to ITS pairwise similarity of at least 97%, using the BLASTClust program (Altschul *et al.*, 1997). The taxonomic classification of strains and the GenBank accession numbers of all sequences are provided in Supplementary Table S1.

We built a molecular phylogeny with the ITS sequences using Bayesian inference. The ITS1, 5.8S and ITS2 regions were independently aligned using MAFFT v7.123b (Katoh and Standley, 2013), and ambiguously aligned regions were removed using Gblocks v0.91b (Castresana, 2000). Two parallel MCMC analyses, using the GTRGAMMA model with independent parameter estimates for each partition, were run in MrBayes v3.2.2 (Huelsenbeck and Ronquist, 2001) for 10 M generations with sampling every 100th generation and 30% burn-in. An ultrametric majority-rule consensus tree was used in subsequent analyses. Whereas the ITS regions are not suitable for phylogenies involving distantly related taxa owing to their variability, our trimmed alignment consisted mostly of the conserved 5.8S gene. The latter has been used in phylogenies of highly divergent taxa (Redecker *et al.*, 1999), and our resulting tree reflected the OTU relationships among strains (Supplementary Figure S2).

Morphological and physiological characterization of strains

The strains were maintained in triplicate cultures on corn meal agar (CMA, Sigma-Aldrich, St. Louis, MO, USA) and malt extract agar (MEA, Applichem, Darmstadt, Germany). We recorded the presence/absence of conidia and darkly pigmented (dematiaceous) mycelia in cultures for up to 3 months. Radial growth rates were measured three days after plating on each medium and reported as millimeters of colony expansion per day. We measured the production of extracellular enzymes using custom plate assays. Cellulase activity was assessed by the clearing halo produced by 7-day-old colonies on Czapek-Dox agar with 0.5% (w/v) carboxymethylcellulose sodium salt as sole carbon source (Johnsen and Krause, 2014). Peptidase, pectinase, laccase and peroxidase activities were measured following the methods described by Basiewicz *et al.* (2012). In addition, the ability of strains to solubilize mineral phosphate was measured as in Zavala-Gonzalez *et al.* (2015). Cellulase, pectinase and phosphate solubilization activities were measured as the proportional width of the clearing halo respect to colony diameter. Peptidase, laccase and peroxidase activities were rated according to a 1–4 semi-quantitative scale.

Arabidopsis and microthlaspi inoculation assays

We tested the effect of colonization by individual strains on the development of *A. thaliana* and *M. erraticum* using an *in vitro* assay (Supplementary Figure S3). Surface-sterilized seeds were plated on half-strength Murashige-Skoog basal salt solid medium (MS, Sigma-Aldrich; Murashige and Skoog, 1962), stratified for 2 days at 4 °C in the dark, and then incubated for 7 days at 23 °C under continuous illumination (80 $\mu mol\, m^{-1}\, s^{-1}$). Upon emergence of the first true leaves, seedlings were transferred to 24-well plates containing MS medium and maintained in the same incubation conditions. After 10 days, plants were inoculated with individual strains or left uninoculated in controls. Because many strains did not sporulate in culture, inoculation was performed by puncturing the margin of actively growing colonies on CMA with a sterilized toothpick to collect a small amount of mycelium, and then transferring it a few millimeters from the crown of plants by inserting it in the agar. Ten days after inoculation, the development of mycelium in roots was confirmed under a stereomicroscope. Symptoms of chlorosis and/or necrosis in leaves were rated on a semi-quantitative scale (0 = none, 1 = up to 30% chlorotic/necrotic leaves, 2 = 30–60%, 3 = >60%, 4 = dead plants), and the fresh weight of the aerial tissues was measured. Every treatment consisted of five replicates, performed simultaneously in a separate 24-well plate each. The layout of treatments was randomized within wells to minimize potential effects on the data owing to position. Experiments were performed in batches including 23 strains and a control treatment each, and measurements for each fungal treatment were compared only to its respective control. To assess the reproducibility of assays, we repeated them for 34 strains in *A. thaliana* (Supplementary Figure S4).

Barley inoculation assays

The effects of root colonization on barley were assessed using a standard *in vitro* assay (Dufresne and Osbourn, 2001; Maciá-Vicente *et al.*, 2008b). In brief, 2-day-old seedlings obtained from

surface-sterilized seeds were planted in glass tubes with 30 ml sterilized, hydrated vermiculite. Four 5-mm-diameter plugs taken from the margin of actively growing colonies on CMA were used as inoculum, by placing them 2–3 cm deep in the vermiculite. Control tubes were mock-inoculated with sterile CMA plugs. Plants were grown under long day conditions (16 h:8 h, light:dark, 80 μmol m^{-1} s^{-1}) at 23 °C. The fresh weight of roots and shoots was measured after 10 days. In this case, we did not score symptoms because they were seldom evident in leaves, and detrimental effects were manifested by reductions on biomass (Supplementary Figure S3). Treatments consisted of 10 replicates, which were performed in batches of three to nine strains and one un-inoculated control treatment each. We repeated these assays for five strains to test their reproducibility (Supplementary Figure S4).

We assessed the endophytic colonization of roots by cultivation and microscopy methods. In the first case, one to two roots per plant were surface-sterilized for 1 min with 0.5% sodium hypochlorite, rinsed with sterilized deionized water, and cut into 0.5 cm pieces. Ten root pieces per plant were randomly selected, dry-blotted onto sterilized paper and plated on CMA. The efficacy of the sterilization procedure was assessed in a subset of 30 root pieces per treatment with the imprint method (Hallmann *et al.*, 2006). The percentage of root pieces colonized was recorded 5–7 days later. For the microscopical observation of root colonization, we randomly selected three barley plants per treatment. One entire seminal root per plant was cleared overnight in a 1 M KOH solution, stained with acidified lactophenol blue and kept in acidified glycerol until observation. Samples were observed in squash preparations, in which epiphytic and/or endophytic root colonization, and the presence of microsclerotia were recorded (Supplementary Figure S5). The latter was considered as a fungal trait in subsequent analyses.

Statistical analyses

Data organization. Statistical analyses were performed using R v3.0.2 (R Core Team, 2013). Data from the inoculation assays were first assessed for normality and homoscedasticity, and then treatments were compared using analysis of variance or the Kruskal–Wallis test. Subsequent pairwise comparisons of each fungal treatment against its respective control were done by either *t*-tests or Wilcoxon tests with a Holm–Bonferroni correction. In order to incorporate these data into further analyses, we calculated the effect size of biomass variables from each treatment respect to its un-inoculated control. Effect sizes with 95% confidence intervals were calculated according to the Cohend's *d* statistic (Cohen, 1988) using function *cohen.d* in package *effsize* v0.5.4 (Torchiano, 2015), which measures the difference in means and standardizes it by their pooled s.d.

Fungal identifications resulted in several strains isolated from the same plant population being assigned to the same OTU. We considered them likely to belong to the same genets. In order to avoid repeated observations that could inflate the significance of tests, we thinned our datasets to 115 strains representing unique potential genets (Supplementary Figure S2). Pairs of strains within each potential genet often showed similar effects on plant growth (Supplementary Figure S6), and hence alternative selections of strains hardly changed results in downstream analyses.

Analysis of fungal traits. We used principal component analysis with standardized morphological and physiological trait data to summarize the differences among the 115 selected strains. We assessed the goodness of fit of the principal component analysis using the broken-stick criterion, which tests the cumulative percentage of variance explained respect to a random breakdown of variance. Individual variables significantly contributing to axes were identified using the equilibrium circle method (Legendre and Legendre, 2012).

Measurement of phylogenetic signal. We calculated the phylogenetic signal in the response of plant growth to fungal inoculation with the K statistic (Blomberg *et al.*, 2003), using function *phylosig* in package *phytools* v0.5 (Revell, 2011). The method is used to assess conservation of traits among species, with $K=0$ indicating absence of a phylogenetic signal, and $K<1$ or $K>1$ resemblance lower or higher than expected under Brownian motion evolution. Because simultaneous inferences of phylogenetic signal between species and within species are difficult to interpret (Blomberg *et al.*, 2003), we only included in these analyses individual values for each OTU as the mean of the effects by its strains. Sampling errors of within-OTU variability were incorporated following Ives *et al.* (2007), assuming variances for OTUs with only one strain equal to the mean of the overall within-OTU variance. The significance of K was assessed by comparing with a random shuffle of values at the tips of the phylogenetic tree. We also tested for phylogenetic signal in the interactions of strains within the orders Pleosporales, Hypocreales or Helotiales, separately.

Contribution of strain features to plant interactions. To assess the influence of phylogeny, traits and origin of strains on plant growth, we applied the variation partitioning method described by Desdevises *et al.* (2003) with function *varpart* in package *vegan* v2.2–1 (Oksanen *et al.*, 2015). We performed the tests independently for each plant species, including as a response variable the effect size of their biomass as affected by every fungus. The

variation was decomposed into three independent explanatory matrices gathering variables related to the strains' traits, origin (geographical coordinates, and natural host as *Microthlaspi* spp. or others) and phylogeny (principal coordinates (PCs) obtained from the phylogenetic tree). We retained only 19 PCs that were significantly correlated in linear regressions ($P<0.05$) with the effects on at least one plant, representing a combination of early (PCs 1 and 2) and late phylogenetic divergences. The significance of the variance fractions explained by each component was tested using permutation tests with pseudo *F*-ratios.

To estimate the contribution of each fungal lineage to tree-wide variation in traits, we calculated their contribution indices (Moles *et al*., 2005) using the *aotf* function in the program *phylocom* v4.2 (Webb *et al*., 2008). The index measures the proportional contribution of individual nodal divergences along the phylogeny to extant trait variation. Statistical support is assessed by comparing the values with those obtained by a random shuffle of traits at the tree tips. A trait can be considered conserved if more variation is explained by ancient than by recent divergences (Maherali and Klironomos, 2012).

We tested for potential relationships between individual trait/origin variables and the effects on plant biomass, using phylogenetic generalized least squares to account for phylogenetic signal in the data. Phylogenetic generalized least squares estimates regression parameters weighted by phylogenetic signal measured as Pagel's λ (with 0 and 1 indicating no or strong signal, respectively; Pagel, 1999), and it is equivalent to an ordinary least squares model when the signal is absent in the residuals (Symonds and Blomberg, 2014). These analyses were carried out using function *pgls* in package *caper* v0.5.2 (Orme *et al*., 2011).

Results

Taxonomic classification of strains

The strains were classified in 54 OTUs and ascribed to 17 families in 11 orders. Among the 115 strains representing likely independent genets, 111 (96.5%) were species of *Ascomycota* and four (3.5%) of *Basidiomycota*. The most frequently encountered orders were Pleosporales and Hypocreales, with 56 (48.7%) and 39 (34%) strains belonging to 26 (48.1%) and 11 (20.4%) OTUs, respectively (Supplementary Figure S1). They were followed by Helotiales, with 10 strains (8.7%) in seven OTUs (13%), whereas other orders were represented by two or less strains each (Supplementary Figure S1). Within Pleosporales, 29 strains (25.2%) in seven OTUs (13%) belonged to the family Pleosporaceae and were mainly represented by OTUs related to *Alternaria* spp. Other OTUs within Pleosporales were designated as family *incertae sedis* or remain unclassified. Most members of Hypocreales belonged to Nectriaceae, with 31 strains (27%) out of which 29 belonged to six OTUs classified as *Fusarium* spp. The most frequent of these were OTU001 with affinities to *Fusarium tricintum* and *Fusarium avenaceum*, and OTU003 related to *Fusarium oxysporum*. Other species of Hypocreales were assigned to *Emericellopsis* and *Ilyonectria*, with families *incertae sedis*. Six Helotiales strains (5.2%) had ITS affinities with the genus *Cadophora* with family *incertae sedis*, and are referred to as *Cadophora*-like onwards (Supplementary Figure S1).

Characterization of strain traits

We measured variables of morphology, growth rates and enzymatic and phosphate solubilization activities in all fungal strains (Supplementary Table S1; Supplementary Figure S5). A principal component analysis ordination of the trait data explained an overall 48.4% of the variance (Figure 1), larger than that explained by a random breakdown of variance (36%). The first component collected most information related to the strains' growth rates and clearing halos (Figure 1a). The second component mainly represented variation in peroxidase activity, pigmentation and production of intraradical microsclerotia (Figure 1a). The ordination of strains reflected their phylogenetic affinities (Figure 1b), with a clear separation of Hypocreales, Pleosporales and *Cadophora*-like strains along the first axis. Members of Nectriaceae formed a compact cluster clearly separated from other Hypocreales and most other fungi (Figure 1b). Strains within Pleosporaceae showed a tendency toward a high peroxidase activity and the formation of dematiaceous mycelia and microsclerotia, although they also showed a wide variability in these characters (Figure 1b).

Effect of fungal strains on plant growth

Inoculation assays of individual strains in *A. thaliana*, *M. erraticum* and *H. vulgare* yielded a wide range of growth responses, ranging from a strong inhibition to a moderate stimulation of plant biomass production in comparison to un-inoculated controls (Figure 2; Supplementary Figure S3). The overall effect of fungal inoculation was negative for all host species ($W=395$–1685, $P<0.001$), but it was less marked in barley. Similar results were obtained when considering variables of plant development other than total biomass, because they were strongly collinear with it (Pearson's $r<-0.74$, $P<0.001$ for symptoms data in both Brassicaceae; $r>0.97$, $P<0.001$ for the effects on shoot and root biomass in *H. vulgare*). Moreover, similar effects were observed in repetitions of these experiments with subsets of strains (Supplementary Figure S4).

We only found conservatism in the response of *M. erraticum* to fungal inoculation (Table 1). OTUs within the Hypocreales had a conserved effect on

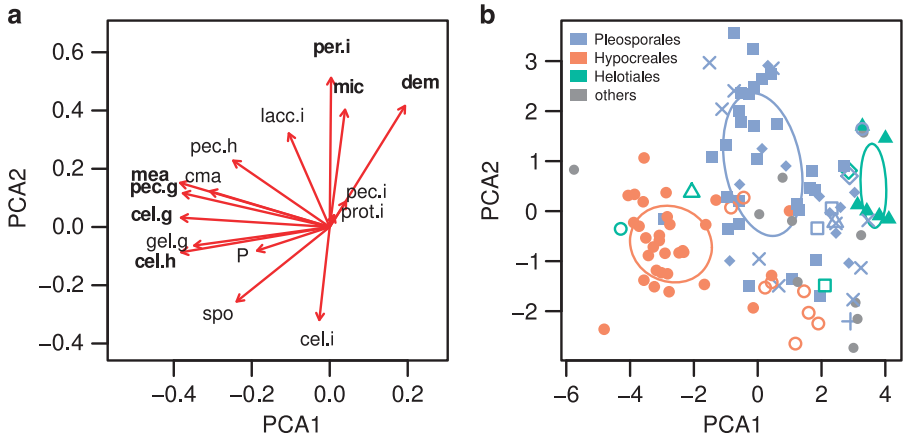

Figure 1 Principal component analysis (PCA) ordination of fungal endophytic strains according to their physiological and morphological traits. The two axes represent 36.2% and 12.2% of the data variance, respectively. (**a**) PCA scores showing the contribution of each trait to the separation of the strains, as indicated by the direction and magnitude of the respective arrows. Variables in bold contributed significantly to the variance, according to the equilibrium circle method. (**b**) Ordination of strains according to their traits. Strains belonging to the three most represented fungal orders are shown in different colors (see color key). Different symbols within each of these orders indicate strains belonging to different families, or to paraphyletic groups at that taxonomic level. Ellipses delimit 95% confidence intervals around the strains of Nectriaceae (solid circles), Pleosporaceae (solid squares) and Helotiales *incertae sedis* (=*Cadophora*-like, solid triangles). Abbreviations: *cel.g*, growth rate on cellulose; *cel.h*, degradation halo on cellulose; *cel.i*, cellulase activity; *cma*, growth rate on CMA; *dem*, pigmentation; *gel.g*, growth rate on gelatin; *lacc.i*, laccase activity; *mea*, growth rate on MEA; *mic*, production of microsclerotia; *P*, phosphorus solubilization; *pec.g*, growth rate on pectin; *pec.h*, degradation halo on pectin; *pec.i*, pectinase activity; *per.i*, peroxidase activity; *prot.i*, protease activity; *spo*, production of conidia.

A. thaliana but non-significant signals in their interactions with *M. erraticum* and *H. vulgare* (Table 1). The responses to fungal inoculation varied considerably across plants and fungal lineages. *M. erraticum* and *H. vulgare* were most negatively affected by *Fusarium* spp. strains, whereas the strongest negative effects on growth of *A. thaliana* were caused by members of the Pleosporaceae (Figure 2, Supplementary Figure S7). Fungal OTUs with the strongest overall virulence towards either plant species, such as *Fusarium* spp. OTU001 and OTU003, and *Alternaria* sp. OTU008, also showed a broad within-group variability that spanned the entire range of interactions (Figure 2, Supplementary Figure S7). The effects of *Cadophora*-like strains were always close to neutrality (Figure 2, Supplementary Figure S7).

Fungal colonization of roots

Fungal root colonization was detected in most plants at the moment of sampling. In *H. vulgare*, we quantified the degree of colonization in culture, and we often observed it directly by light microscopy (Supplementary Figure S5). We did not find a significant phylogenetic conservatism in the fungal colonization of barley roots ($K=0.7$, $P=0.9$). Root colonization was negatively correlated, after controlling for phylogenetic signal, with the effect on total plant biomass of each strain (slope $=-0.023\pm0.003$, adj. $R^2=0.27$, $F_{124}=32.82$, $P<0.001$, $\lambda=0$). A similar result was obtained for the effect on shoot and root biomass (Supplementary Table S2). Moreover, root colonization was positively correlated with all variables of fungal growth ($P<0.01$).

Contribution of strain features to plant interactions

We evaluated the contribution of strain variables related to phylogeny, traits and origins, to the effect of fungal inoculation on plant growth (Figure 3). Models for each plant explained a significant proportion of the variation in their growth response to fungal inoculation (48–59%, $P<0.001$). The phylogeny of strains predicted the largest fraction of the variance in *A. thaliana* and *M. erraticum* (45.1 and 35.7%), but it was less informative than the strains' traits in *H. vulgare* (25.1% respect to 33%). Some fungal clades contributed greatly to the overall phylogenetic signal, mainly representing late divergences in the phylogenetic tree at the OTU level (Figure 4). *Fusarium* sp. OTU001 had the largest contribution to overall variance in the responses of *M. erraticum* and *H. vulgare*, whereas divergences in growth of *A. thaliana* were most affected by several pleosporaceous OTUs (Figure 4). These late divergences contrast with those obtained for mycelial traits, which tended to be greater earlier in the phylogeny (Figure 4, Supplementary Figure S8). The response of both Brassicaceae had little dependence on the strains' traits alone (4–8%), which had an effect partly indistinguishable from that of the strains' phylogeny in *M. erraticum* (Figure 3). Fungal traits explained a significant amount of the interactions with *H. vulgare* (33.1%). Only the response of *M. erraticum* to colonization was significantly correlated with the strains' origin (Figure 3).

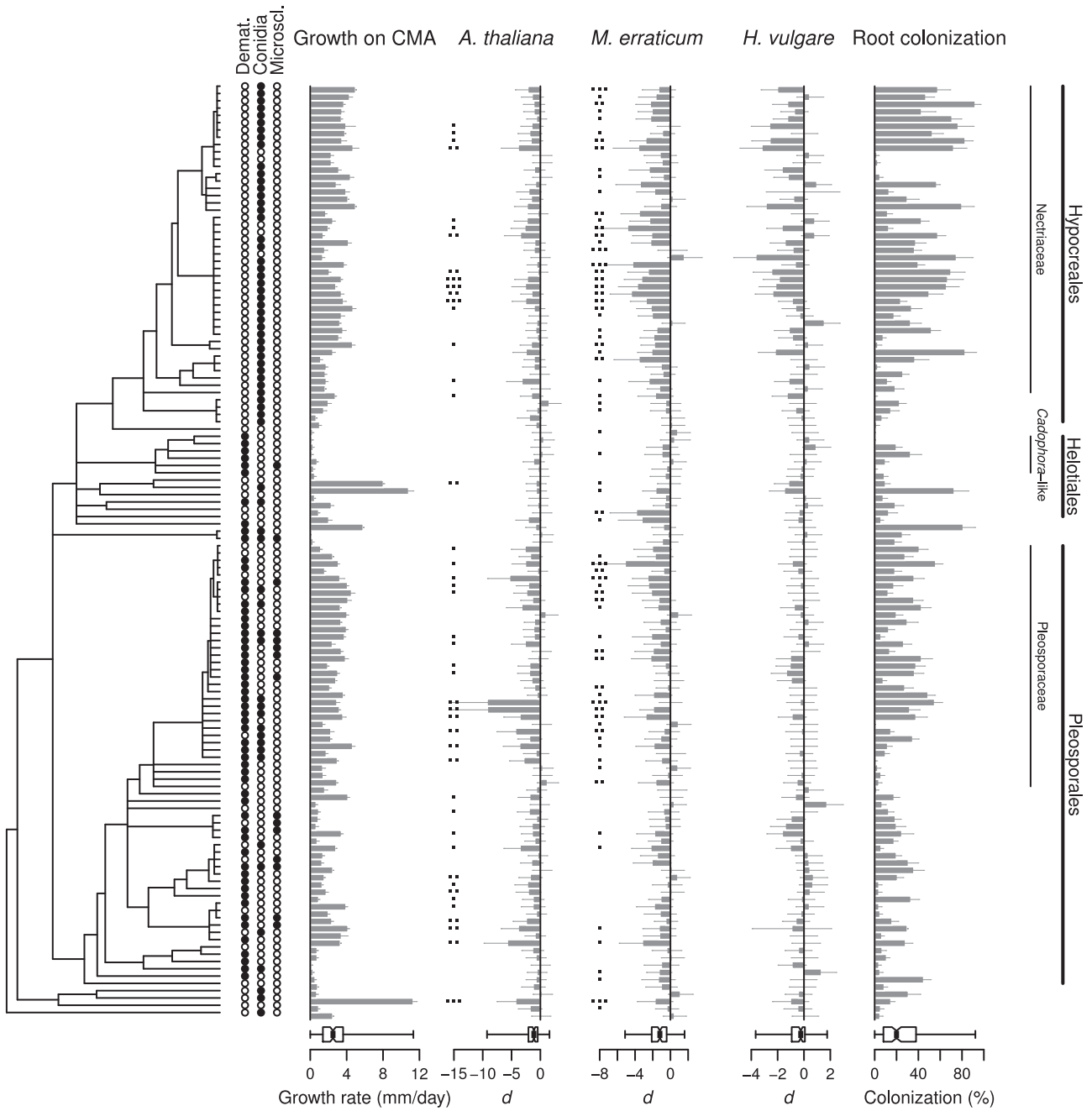

Figure 2 Interactions between 128 fungal endophytic strains and the plants *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare*. Selected fungal traits are also shown. Bars represent effect sizes (Cohend's *d*) ± 95% confidence intervals for the interactions with plants, and mean values ± s.e. for the growth rates of strains on corn meal agar (CMA) and barley root colonization. Boxplots at the bottom of graphs represent the overall data distribution for each variable. Points next to bars for *A. thaliana* and *M. erraticum* indicate average scores >1 in a semi-quantitative scale of symptoms. Solid and empty bullets in the qualitative traits of dematiaceous mycelium (Demat.), production of conidia, and production of microsclerotia (Microscl.) indicate presence or absence of the character, respectively.

Phylogeny-independent determinants of plant–endophyte interactions

We assessed the correlation between individual strain variables included in the variation partition and the response of plants to fungal colonization using phylogenetic generalized least squares to subtract phylogenetic signal. All descriptors of hyphal growth were strongly associated with negative effects on the development of the three plants (Table 2). Of the physiological characteristics of strains, only laccase and pectinase activities showed a significant association with biomass of *H. vulgare* (Table 2). Production of dematiaceous mycelium and conidia had contrasting positive and negative relationships with the development of individual plant species, respectively (Table 2).

Table 1 Phylogenetic signal in the growth responses to fungal inoculation of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare*, according to Blomberg's *K*.

Fungal group[a]	A. thaliana		M. erraticum		H. vulgare	
	K[b]	P	K	P	K	P
All fungi	0.8	0.8	**0.9**	**0.029**	0.7	0.5
Pleosporales	0.6	0.4	0.9	0.9	0.9	0.8
Hypocreales	**0.9**	**0.048**	0.2	0.7	0.2	0.7
Helotiales	0.9	0.5	1	0.8	0.6	0.7

[a]Phylogenetic signal was tested in the plant interactions with all strains, and with strains within Pleosporales, Hypocreales, or Helotiales alone.
[b]$K=0$ indicates random evolution of traits, and $K=1$ indicates trait evolution under Brownian motion.
Significant values of K ($P<0.05$) are shown in bold face.

Interestingly, strains originally isolated from *Microthlaspi* spp. showed a stronger virulence than strains from other sources toward the congeneric *M. erraticum* (Table 2).

Discussion

We provide evidence that the effects of non-mycorrhizal fungal root endophytes on plant growth are strongly influenced by the phylogeny of fungi. However, the phylogenetic signal is mostly explained by recent divergences that indicate little conservatism in the evolution of interactions. Moreover, particular fungal traits shared by phylogenetically dispersed taxa affected to a different extent the plant responses to fungal inoculation. These effects always followed a similar trend in different plant species, suggesting a direct relation of the traits with specific types of associations, or their linkage with other characters relevant to the symbiosis (Treseder and Lennon, 2015). The collation of these traits across strains allows a rough functional classification of the fungal diversity included in our study, and to hypothesize about their influence in the assembly of natural root-endophytic communities.

Effect of fungal colonization on plant development

The net effect of fungal colonization on plant biomass was negative, consistent with previous results based on the controlled inoculation of plants with root endophytes (Tellenbach *et al*., 2011; Mayerhofer *et al*., 2012; Keim *et al*., 2014; Mandyam and Jumpponen, 2015). Our experimental system included the plant as the sole carbon source to sustain fungal growth, which conditioned a strong negative correlation between fungal development and plant biomass, similar to that reported in other endophytic interactions (Tellenbach *et al*., 2011). However, the negative responses were often small, and strong compromises of plant growth and development of symptoms were scarce. Most root endophytes do not seem an important burden to their hosts, suggesting that their parasitism may be easily compensated by slight enhancements of plant fitness in their natural habitat. Mutualistic interactions depend on a balance between net costs and benefits provided by symbionts, whereby they can become parasitic in the absence of the ecological factors that drive the relationship. There are multiple instances of non-mycorrhizal endophytes providing their hosts with benefits when exposed to external factors, such as pathogens (Maciá-Vicente *et al*., 2008b), environmental stress (Rodriguez *et al*., 2008) or nutrient shortages (Usuki and Narisawa, 2007; Behie *et al*., 2012; Hiruma *et al*., 2016). Endophytes are likely implicated in different yet unknown context-dependent trade-offs associated with conditions not reproduced in our system. A blind testing of multiple environmental factors was out of the scope of our study, but further work in this direction may help to unravel context-dependent symbioses.

Phylogenetic conservatism of plant–endophyte interactions

Plant responses to endophytic colonization partly depended on the phylogenetic relations of strains, suggesting the evolution of distinctive strategies for the interaction with hosts. These effects varied markedly across plant species, in line with studies evidencing a large dependency of plant–fungus symbioses on the specific combination of partners (Klironomos, 2003; Mandyam and Jumpponen, 2015). Much of the variation was associated with negative feedbacks with fungi related to well-known pathogens, particularly species within *Fusarium*, and Pleosporaceae like *Alternaria* spp. These lineages often dominate roots of healthy wild plants (Maciá-Vicente *et al*., 2008a, 2012; Knapp *et al*., 2012; Sánchez Márquez *et al*., 2012; Glynou *et al*., 2016), where their detrimental effects appear to be mitigated by environmental conditions and/or interactions with extant microorganisms. But the linkage between particular responses and well-defined fungal clades was not clear-cut, because several strains in the most virulent groups had little impact on plant growth. It would be reasonable to expect a clear differentiation in the associations involving particular fungal lineages, given the diverse life histories that determine distinctive physiological and morphological adaptations. Such trends have been identified in endophytes (Mayerhofer *et al*., 2012), but they are generally diffuse owing to a high intraspecific variability that often exceeds the variation between species (Tellenbach, *et al.* 2011; Mandyam and Jumpponen, 2014). Fungal determinants of the symbiosis might be subject to rapid and recent change over evolutionary time, perhaps separating different genetic populations within species. For example, *Fusarium* species show a wide intraspecific variability in their virulence because they have pathogenicity genes subject to strong diversifying pressure or that can be horizontally transferred

with mobile chromosomes (Ma *et al*., 2010; Sperschneider *et al*., 2015). Further, Cheikh-Ali *et al*. (2015)) found that root endophytes of a same phylotype isolated from distant localities expressed divergent morphological and physiological characters. The high intra-specific variation of plant–

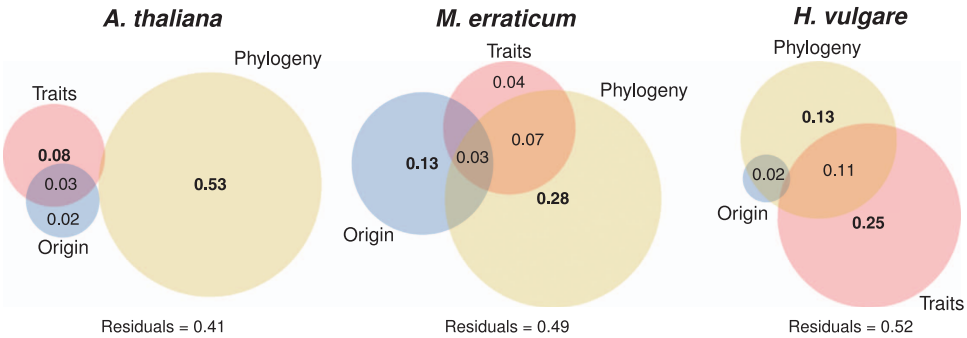

Figure 3 Euler diagrams of variation partitioning analysis, showing the effects of traits, origin and phylogeny of the endophytic strains on the growth of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare*. Values indicate the proportion of the variation explained (adjusted R^2) by each fraction, corresponding to the pure effects of explanatory variables, or their shared effects (overlapping fractions). Values in bold are significant ($P<0.05$). Zero and negative values are not shown.

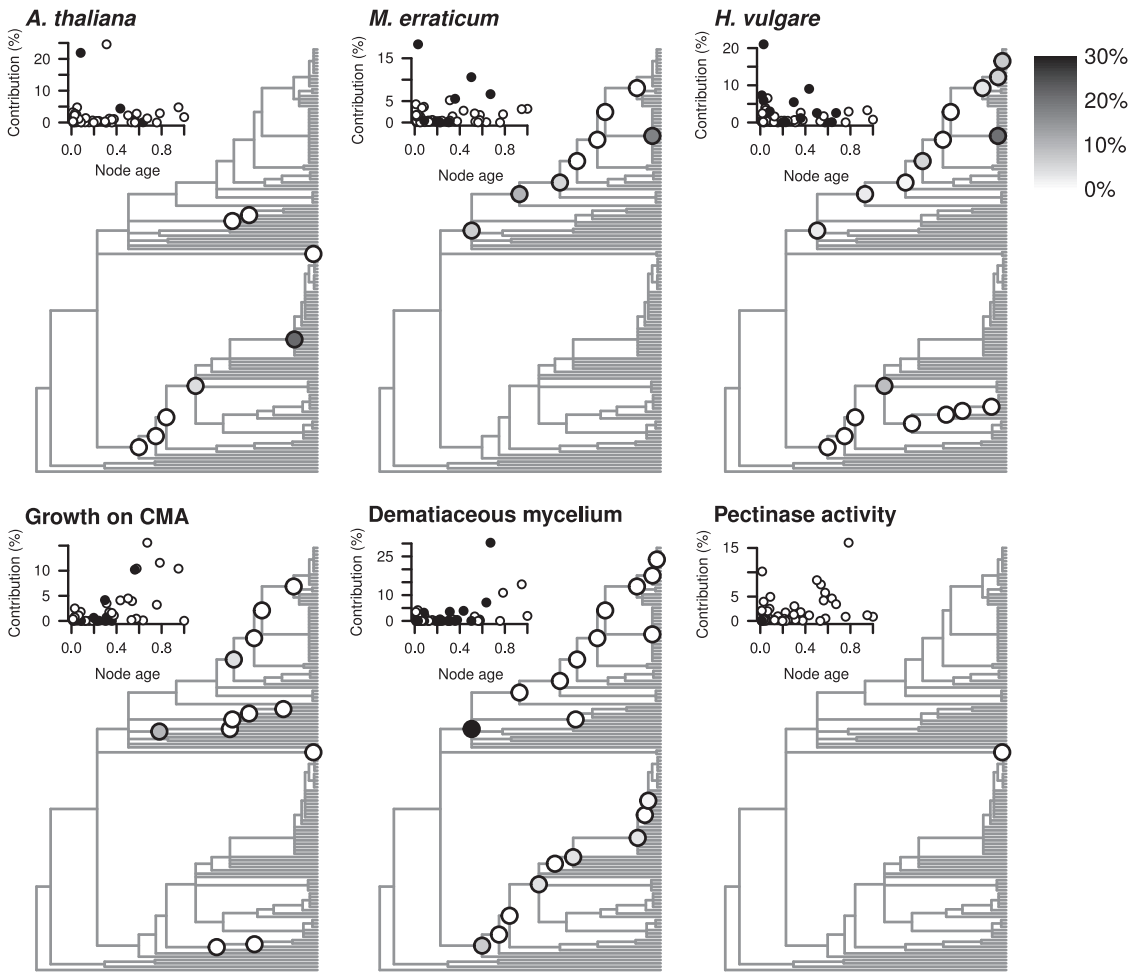

Figure 4 Nodal contributions to tree-wide variation in the response of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare* to fungal inoculation, and to selected fungal traits relative to their morphology and physiology. Circles in the tree nodes indicate contribution indices that are significant ($P<0.05$), as compared with a distribution of 999 values calculated by a random shuffle of trait values across the tips of the phylogeny. Shading of circles represents the relative contribution of individual nodes to extant trait variation (see key). Scatterplots in insets show the relationship between the contribution indices and the respective age of nodes, with black and white circles indicating contribution indices that are significant or not, respectively.

Table 2 Phylogenetic generalized least squares regression models of the relations between plant growth responses to fungal inoculation, and variables of the strains' traits, geographical origin and natural host

Variable	A. thaliana[a]				M. erraticum				H. vulgare			
	Slope (± s.e.)	*Adj.* R^2	P-*value*	λ	*Slope (± s.e.)*	*Adj.* R^2	P-*value*	λ	*Slope (± s.e.)*	*Adj.* R^2	P-*value*	λ
Growth rate on corn meal agar	−0.24 ± 0.07	0.08	0.002	0	−0.18 ± 0.07	0.05	0.008	0.1	−0.16 ± 0.04	0.09	0.001	0.1
Growth rate on malt extract agar	−0.29 ± 0.08	0.09	<0.001	0.1	−0.29 ± 0.07	0.14	<0.001	0	−0.25 ± 0.04	0.21	<0.001	0
Growth rate on cellulose	−0.03 ± 0.01	0.15	<0.001	0.1	−0.02 ± 0	0.2	<0.001	0	−0.02 ± 0	0.21	<0.001	0
Growth rate on gelatin	−0.4 ± 0.13	0.07	0.003	0.1	−0.38 ± 0.11	0.09	0.001	0	−0.4 ± 0.07	0.22	<0.001	0
Growth rate on pectin	−0.37 ± 0.09	0.12	<0.001	0.1	−0.29 ± 0.08	0.11	<0.001	0	−0.27 ± 0.05	0.18	<0.001	0
Cellulase activity	—	—	—	—	—	—	—	—	—	—	—	—
Protease activity	—	—	—	—	—	—	—	—	—	—	—	—
Laccase activity	—	—	—	—	—	—	—	—	−0.17 ± 0.07	0.04	0.022	0.2
Pectinase activity	—	—	—	—	—	—	—	—	0.36 ± 0.16	0.04	0.022	0.2
Peroxidase activity	—	—	—	—	—	—	—	—	—	—	—	—
Phosphorus solubilization	—	—	—	—	—	—	—	—	—	—	—	—
Production of conidia	−0.64 ± 0.31	0.03	0.042	0.1	−0.53 ± 0.24	0.03	0.03	0	—	—	—	—
Production of microsclerotia	—	—	—	—	—	—	—	—	—	—	—	—
Pigmentation	—	—	—	—	0.62 ± 0.24	0.05	0.011	0	0.59 ± 0.17	0.09	0.001	0
Geographic latitude	—	—	—	—	—	—	—	—	—	—	—	—
Geographic longitude	—	—	—	—	—	—	—	—	—	—	—	—
Natural host (*Microthlaspi* vs others)	—	—	—	—	−1.11 ± 0.3	0.1	<0.001	0.1	—	—	—	—

Only model data of variables with a significant effect ($P<0.05$) are shown.
[a]Results of PGLS models show the slope (± s.e.) of the fitted line representing the correlation between variables, the coefficient of determination, the *P*-value of the model, and the estimate of the phylogenetic signal associated with the regression as Pagel's λ.

endophyte interactions has likely ecological implications for the local assembly of natural communities, because it might be a consequence of adaptations to local conditions. This would promote selection mosaics across landscapes in which particular combinations of symbionts are favored by their joint response to extant conditions (Thompson, 2005; Piculell *et al*., 2008).

The broad variability in plant–endophyte interactions contrasts with patterns of evolution in functional traits of arbuscular mycorrhizal fungi, which appear to be phylogenetically conserved at the family level (Powell *et al*., 2009; Chagnon *et al*., 2013). The opposing patterns between mycorrhizal and non-mycorrhizal fungal endosymbionts probably reflect large differences in their specialization for the symbiosis. Unlike mycorrhizas, most endophytes are not bound to their plant hosts and can be found as saprotrophs in other substrata. Therefore, their evolution might be less subjected to constraints imposed by the symbiotic lifestyle.

Phylogeny-independent determinants of plant–endophyte interactions

The growth responses to fungal colonization varied among plant species. Interactions involving either brassicaceous host had a great dependency on the phylogenetic relations of strains, whereas in *H. vulgare* convergent fungal traits were more important in explaining the growth responses to inoculation. The distinctive results across plants could have been determined by methodological differences in the bioassays. Nevertheless, *A. thaliana* and *M. erraticum* were tested using a similar setup yet they had divergent responses to particular fungal groups like fusaria or Pleosporaceae strains, whereas growth patterns of *M. erraticum* had commonalities with those of barley. Besides, only *M. erraticum* showed a significant degree of conservatism in its response to close fungal relatives, which can be indicative of mutual adaptations between partners, given the affiliation of the plant with the natural host of most strains. Therefore, it seems likely that the variation in individual plant–endophyte combinations largely reflects actual specificities across partners.

In all cases, a significant amount of the variation was explained by traits shared by dispersed fungal clades, which were correlated with the outcome of interactions in a similar manner. Among these, the growth rates of strains were strongly associated with reductions of plant biomass, probably owing to the tendency of these strains to colonize host tissues systemically and to their larger demands on plant carbon. Likewise, strains capable to sporulate in culture tended to be more detrimental to plants, perhaps owing to the linkage of this trait with fast rates of mycelial growth.

Dematiaceous fungi were less prone to develop detrimental symbioses. Melanized hyphae, in combination with lack of spores in culture and the production of intraradical microsclerotia are defining attributes of DSE. These form a polyphyletic

group of fungi frequently regarded as potential mutualists based on their high prevalence and ubiquity in roots (Mandyam and Jumpponen, 2005), although their symbiotic function is still elusive. Newsham (2011) detected a net positive effect of DSE on plant performance, associated with increments in nutrients uptake in the presence of soil organic matter. The hydrolytic capabilities of several DSE have been previously described and suggest that they are able to access detrital nutrient pools as saprotrophs (Caldwell *et al*., 2000; Mandyam and Jumpponen, 2005; Mandyam *et al*., 2010). Our inoculation assays did not include organic sources of nutrients available for the fungi other than the plant, but the saprotrophic capabilities of DSE might entail in nature a fitness benefit to hosts that could easily overcome their weak parasitism.

We were unable to detect direct substantial effects of the strains' physiological activities on the outcome of interactions. Laccase and pectinase activities were the only traits somewhat associated with plant performance in barley. The expression of hydrolytic activities was highly variable in our assays, perhaps reflecting different substrate specificities and inducing conditions (Basiewicz *et al*., 2012), or unspecificities in the detection of particular activities (Johnsen and Krause, 2014). But this variation also highlights large differences among and within fungal taxa that suggest a broad diversity of potential interactions in response to the availability of substrates. This could be ultimately confirmed by comparing genomic traits relative to these activities and the assessment of their expression *in planta* (Lahrmann *et al*., 2015).

The original host of strains had a strong impact on their interactions with *M. erraticum*, in which strains isolated from congeneric plants were more virulent. This could indicate a certain host specificity of these strains that is backed by their phylogenetically conserved effect on this plant. Similar effects have been described for other root-endophytic (Tellenbach *et al*., 2011), pathogenic (Sacristán and García-Arenal, 2008) and mycorrhizal symbioses (Klironomos, 2003; Hoeksema and Thompson, 2007), what supports the hypothesis of symbiotic partners co-evolving in response to each other. Interestingly, stronger adaptations of root endophytes to their hosts often lead to an increased virulence (Tellenbach *et al*., 2011), as opposed to those involving mutualistic mycorrhizas (Hoeksema and Thompson, 2007). This hints to parasitism as the main lifestyle adopted by many root endophytes in nature.

Trait-based classification of strains

The grouping of strains based on the similarity of their traits clearly separated endophytic lineages that frequently dominate and co-exist in roots, particularly those related to *Fusarium*, Pleosporaceae and *Cadophora*-like (Glynou *et al*., 2016). The clustering was influenced by life history traits associated with the plant's response to infection, suggesting a differential niche occupancy by groups of endophytes likely to condition their spatial distribution (Violle *et al*., 2007; Violle and Jiang, 2009). *Fusarium* spp. clearly differed from other taxa by fast growth and production of conidia. These characters are associated with an efficient ability of dispersal and resource colonization, which is consistent with the broad distribution across Europe observed for OTUs in this group (Glynou *et al*., 2016). Pleosporaceous and *Cadophora*-like strains, on the other hand, had slower growth rates and exhibited traits typical of DSE, and their geographical distribution apparently is constrained by environmental factors such as climatic and soil variables (Glynou *et al*., 2016). Spatial distribution is often used as a proxy of niche breadth, but this principle has been shown to be less applicable to microbes than to macroorganisms (Carbonero *et al*., 2014). Microorganisms highly specialized for a particular factor can have broad distributions if the latter is widespread, because they are less affected by other conditions than other generalist species. Consequently, the wide spatial occurrence of *Fusarium* spp. independently of other environmental factors could be a result of their efficient adaptations to colonize roots.

Fungal traits as determinants of community assembly

Our selection of strains represents well the composition and structure of endophytic assemblages associated with *Microthlaspi* spp. and other plants (Maciá-Vicente *et al*., 2008a, 2012; Sieber and Grünig, 2013; Keim *et al*., 2014), which are often co-dominated by species related to *Fusarium*, Pleosporaceae and *Cadophora*. The distinctive traits of these lineages are suggestive of different niche occupancies, therefore it is possible to associate the phylogenetic diversity of endophytic communities with processes of competition or complementarity among species. The co-occurrence of endophytes not sharing functional characteristics is indicative that competition is a main driver of community assembly, because functional complementarity reduces competition and promotes co-existence (Maherali and Klironomos, 2007, 2012). Conversely, communities shaped by environmental filtering usually show phylogenetic clustering of species with similar traits selected by the limiting factors. This could explain the low diversity in root-endophytic communities subject to salt stress, where pleosporaceous endophytes become enriched while otherwise dominant fusaria are absent (Maciá-Vicente *et al*., 2008a, 2012). Although functional complementarity among species enhances ecosystem function, the trait similarity of phylogenetically related endophytes (for example, different *Fusarium* spp. co-occurring in the same root) can lead to functional redundancy. This has shown to provide stability to plant–endophyte symbioses, because it prevents the loss of symbiotic functions with the replacement of

fungal species across environmental gradients (Maherali and Klironomos, 2007).

Conclusions

Non-mycorrhizal fungal endophytes are pervasive in roots, hence they are likely to affect plant abundance and productivity in natural communities. Although our experimental system was artificial, it was adequate to address our objective to test interactions between plants and a large number of endophytic species. Under these conditions most endophytes behaved as weak parasites, but their performance varied across plant species and fungal taxa. Diverging endophyte lineages have evolved distinct strategies of plant symbiosis, but their associations were often variable, suggesting that they are subject to local processes of selection. Part of the variation in the interactions was explained by convergent fungal traits that differentiate categories of endophytes with potentially distinct niches. The functional complementarity of strains belonging to different groups is predicted by the structure of natural root-endophytic communities. The characterization of the endophytic diversity into potential functional groups will aid in the testing of further questions about their role in ecosystems. In particular, the assessment of the responses of plant–endophyte interactions to (a)biotic factors, including combinations of endophytes with different degrees of trait similarity and shared evolutionary history, will help unravel context-dependent symbioses adaptive under natural conditions.

Conflict of Interest

The authors declare no conflict of interest.

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Influence of phylogenetic conservatism and trait convergence on the interactions between fungal root endophytes and plants

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Supplementary Figures

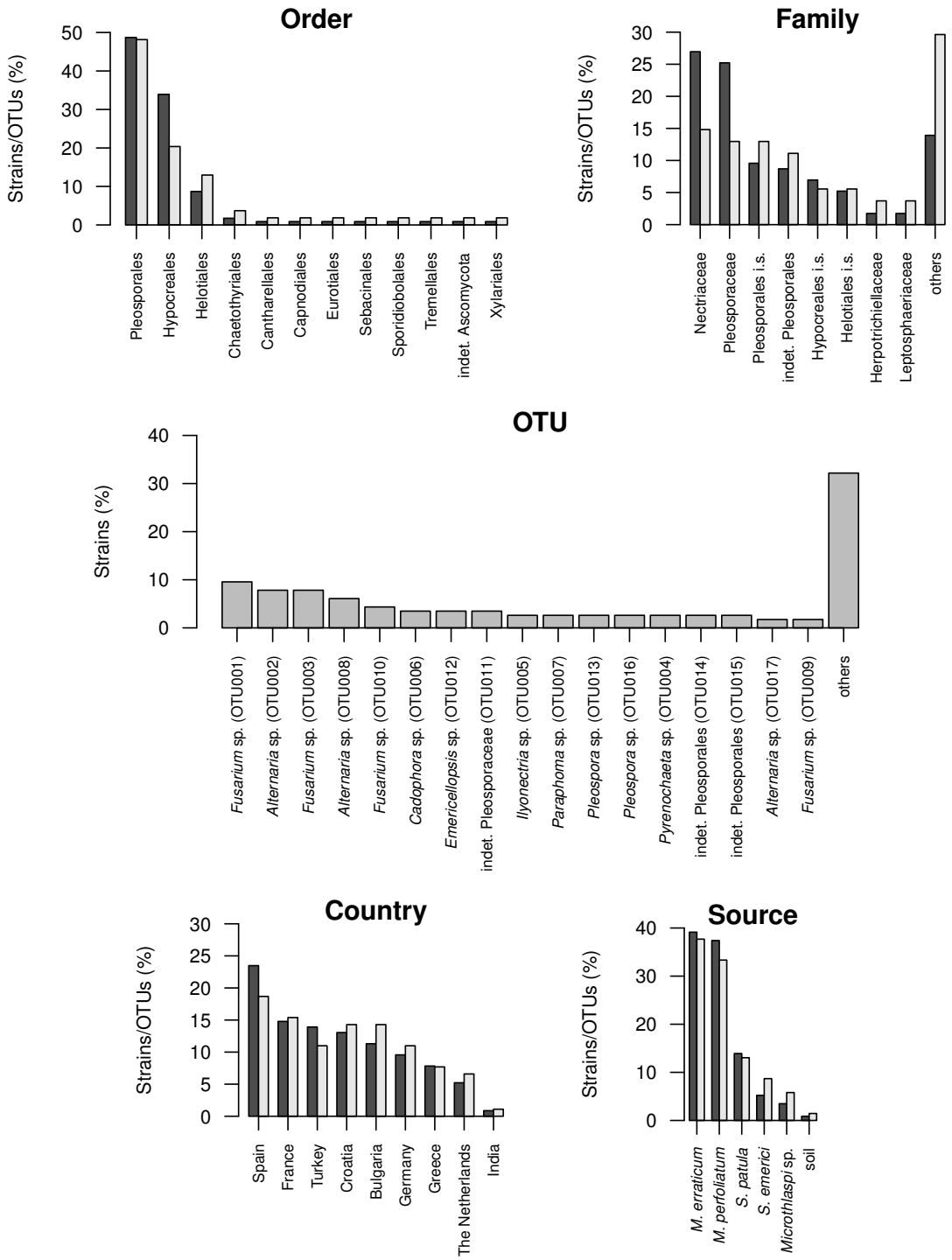

Figure S1: Summary of systematic positions, geographic, and host origins of fungal endophytic strains. Dark or light bars represent the percentage of operational taxonomic units (OTUs) or strains in each category, respectively. i.s. denotes *incertae sedis* categories.

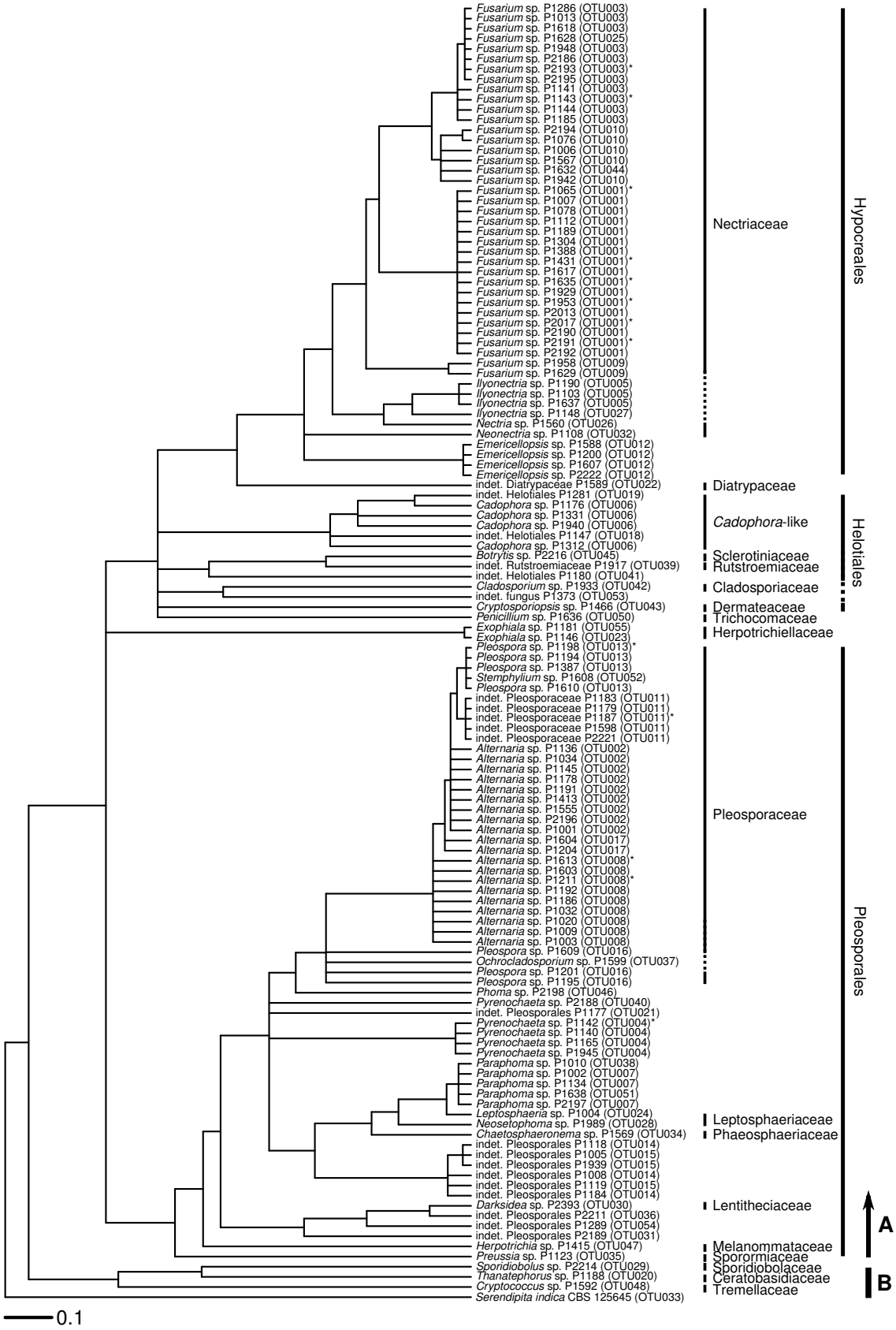

Figure S2: Phylogenetic relations of fungal endophytic strains in this study, based on a Bayesian inference of the ITS1 and 2, and 5.8S rDNA regions. Brackets delimit strains at different taxonomic levels. All families to which strains were assigned are shown, plus the monophyletic incertae sedis clade termed *Cadophora*-like; other *incertae sedis* or undetermined groups are not labelled. Only the three most represented orders are shown. A and B denote Ascomycota and Basidiomycota, respectively. Strains marked with an asterisk were excluded from analyses because they represent likely duplicates of genetic populations.

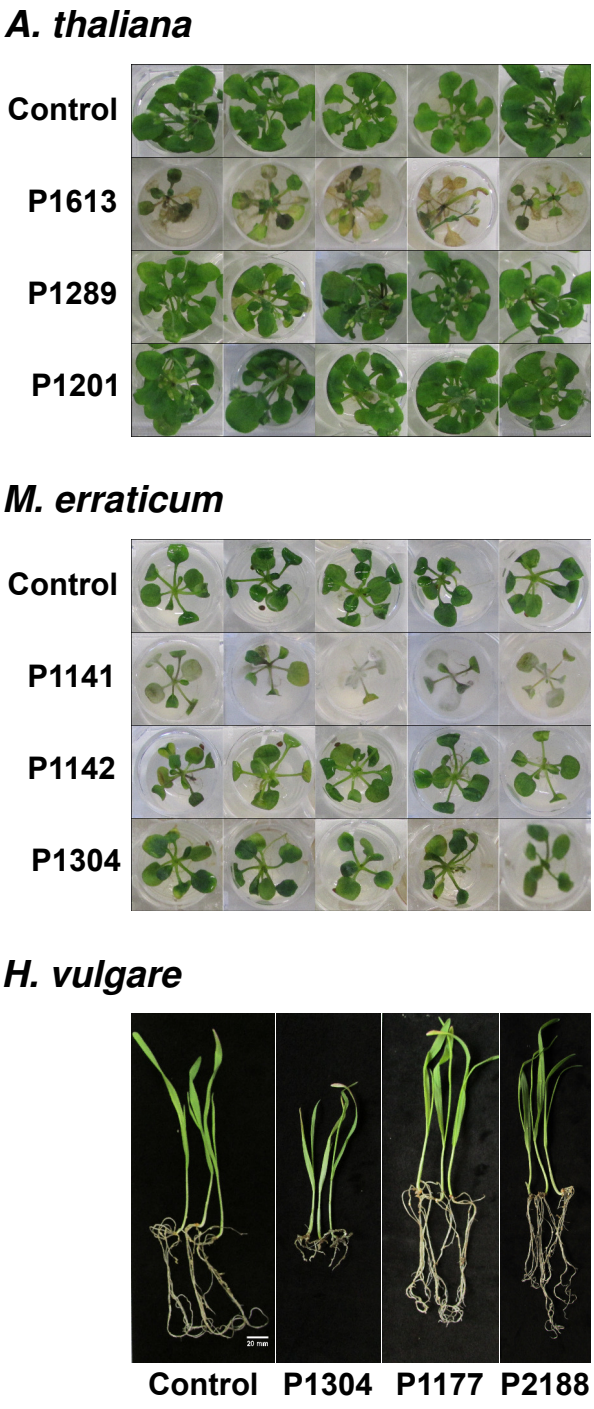

Figure S3: Examples of interactions between strains of fungal endophytes and *Arabidopsis thaliana*, *Microthlaspi erraticum*, and *Hordeum vulgare*. For each plant, uninoculated controls, neutral, and negative interactions with strains are shown. Each replicate in assays with *A. thaliana* consisted of two plants growing per well, while only one plant was used for *M. erraticum* due to a lesser availability of seeds. For *H. vulgare*, only three representative plants are shown, out of ten used in each treatment.

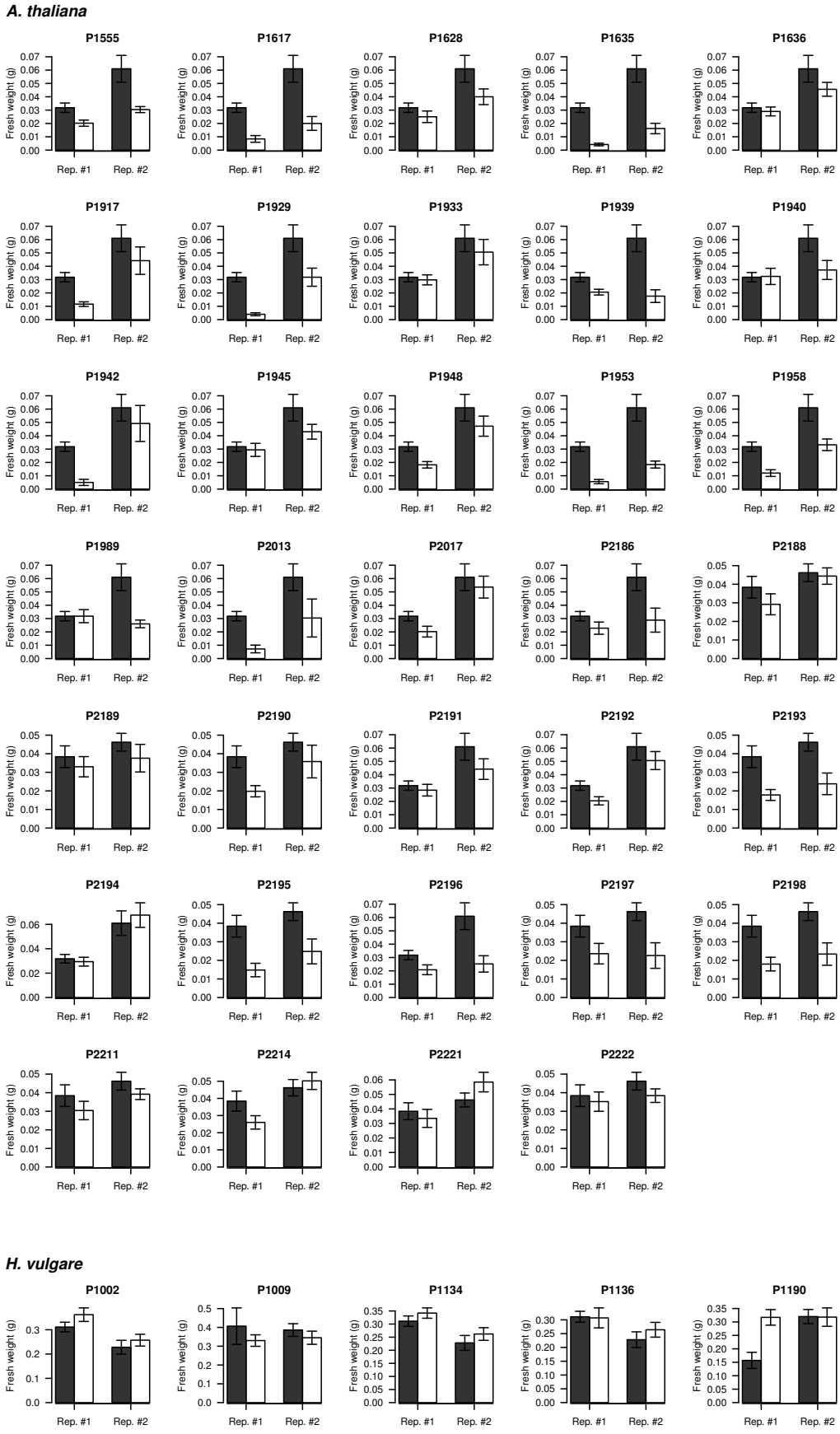

Figure S4: Repetition of inoculation bioassays in *Arabidopsis thaliana* for 34 fungal strains, and in *Hordeum vulgare* for five strains. Only measurements of total fresh weight are shown. Solid bars represent uninoculated control treatments, and empty bars represent fungal treatments. Error bars represent standard error of the mean.

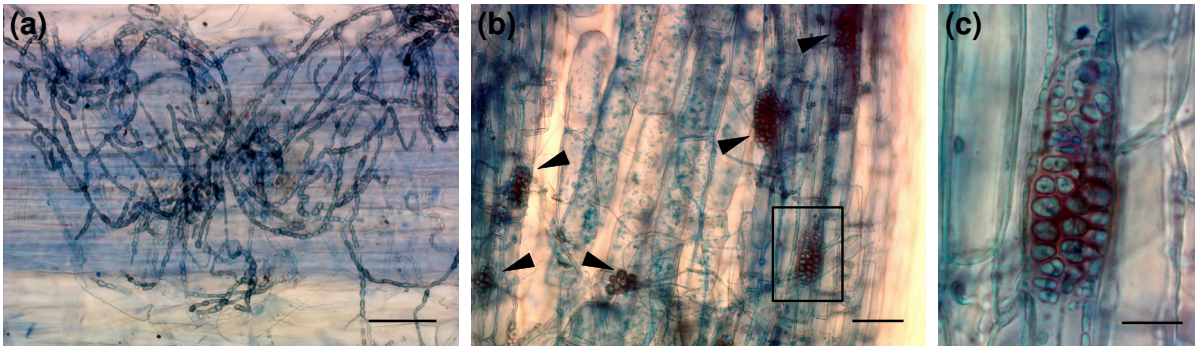

Figure S5: Light microscopy images of *Hordeum vulgare* root colonization by endophytic strains. **a**, Abundant development of hyphae of *Fusarium* sp. P1141 (OTU003) on the surface of a root. Scale = 50 µm. **b**, Microsclerotia (arrowheads and inset) formed by the unidentified Pleosporales strain P1008 (OTU014) within root tissues. Scale = 30 µm. **c**, Detail of a microsclerotium in **b** (inset). Scale = 10 µm.

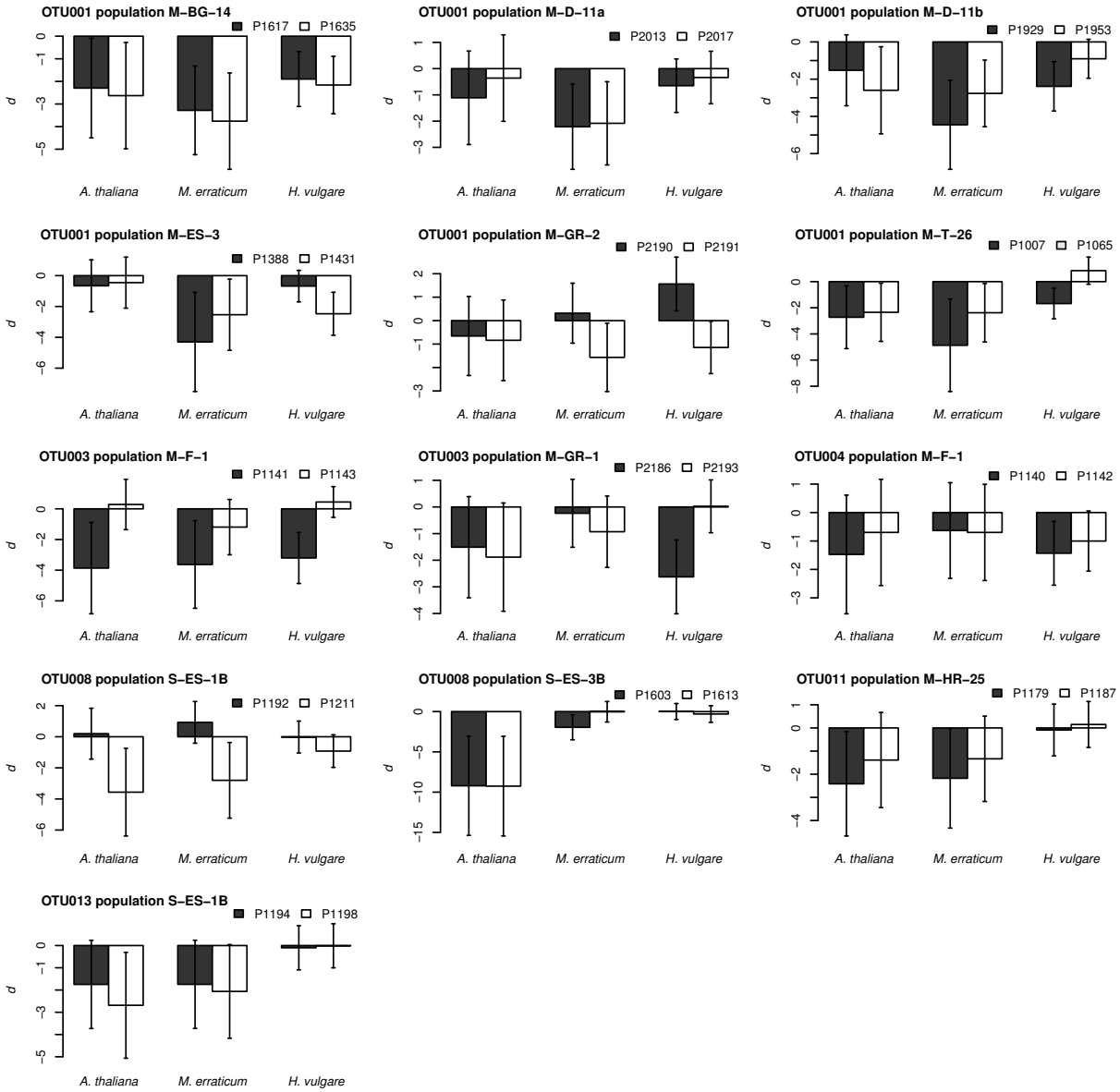

Figure S6: Results of inoculation bioassays in *Arabidopsis thaliana*, *Microthlaspi erraticum*, and *Hordeum vulgare* of fungal strains considered to be potential clonal individuals. Each plot corresponds to two strains assigned to the same OTU that were isolated from the same plant population. Bars represent effect sizes (Cohend's *d*) $\pm$ 95% confidence intervals for the interaction with each plant. Strains represented by white bars were excluded from analyses.

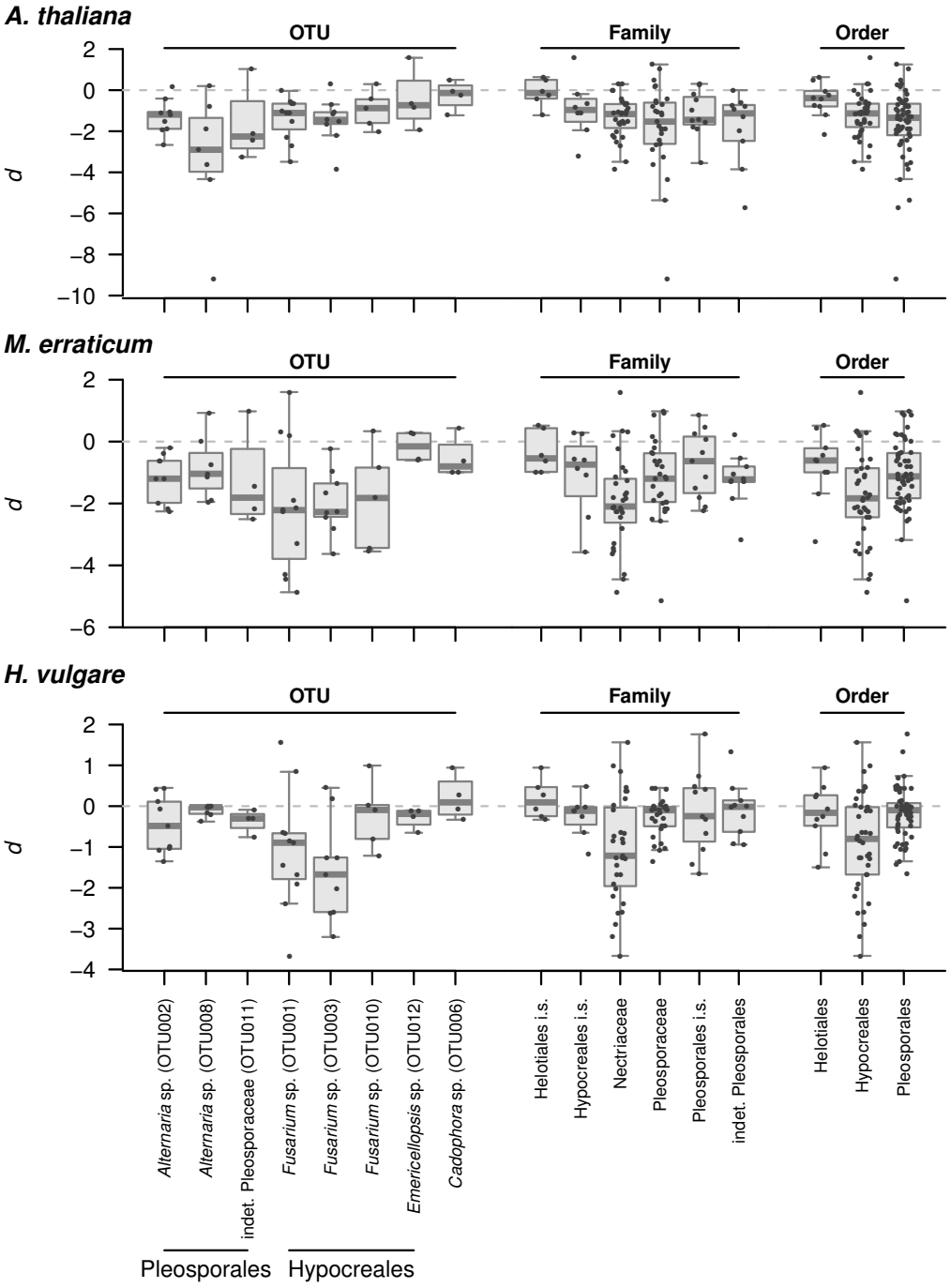

Figure S7: Effects on growth of *Arabidopsis thaliana*, *Microthlaspi erraticum*, and *Hordeum vulgare* of individual endophytic strains from the most represented taxonomic categories in this study. Categories at the OTU, family, and order level, represented by at least three strains are shown. Boxplots represent the effect size values (Cohend's *d*) for each category and plant, while points indicate the values of individual strains within each group. i.s. denotes *incertae sedis* categories.

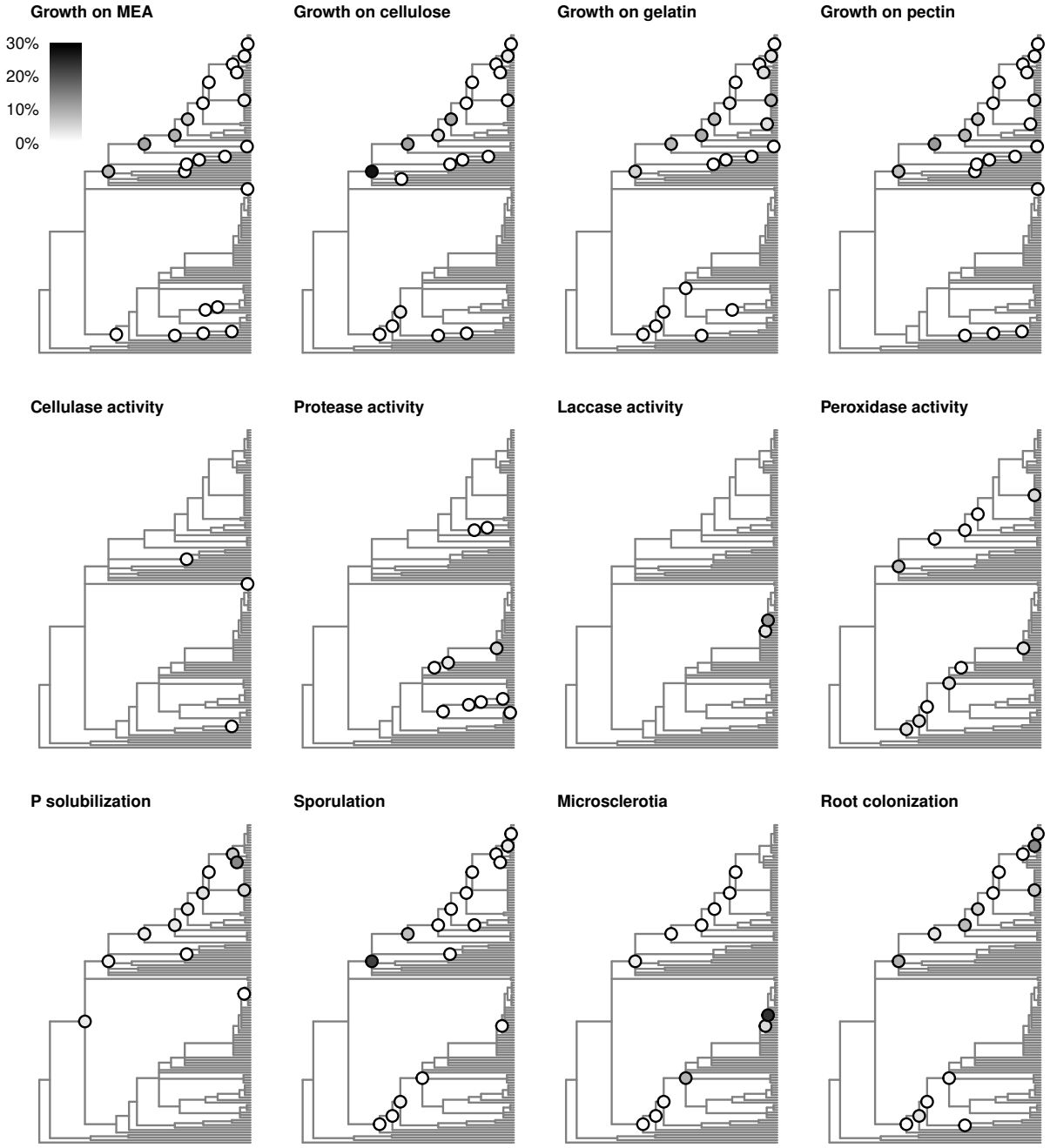

Figure S8: Nodal contributions to tree-wide variation in traits of fungal endophytic strains not shown in Fig. 3. Circles in the tree nodes indicate contribution indices that are significant ($P < 0.05$), as compared to a distribution of 999 values calculated by a random shuffle of trait values across the tips of the phylogeny. Shading of circles represents the relative contribution of individual nodes to extant trait variation (see key).

5. Research article 3

Kia, S.H., Jurkechova, M., Glynou, K., Piepenbring, M. & Maciá-Vicente, J.G. (2018). The effects of fungal root endophytes on plant growth are stable along gradients of abiotic habitat conditions. *FEMS Microbiol. Ecol.*, 94: fix162.

Anlage 3

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(1) zu Entwicklung und Planung

Promovierende SHK: 45%
Co-Autor MP: 5%
Co-Autor JGMV: 50%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende SHK: 70% plant-fungus co-inoculation assays and root colonization quantification
Co-Autor MJ: 15% plant-fungus co-inoculation assays and root colonization quantification
Co-Autoren KG, JGMV: 15% plant-fungus co-inoculation assays and root colonization quantification

(3) zur Erstellung der Datensammlung und Abbildungen

Promovierende SHK: 70% collection of bioassays and colonization data
Co-Autor MJ: 15% collection of bioassays and colonization
Co-Autoren KG, JGMV: 15% collection of bioassays and colonization

(4) zur Analyse und Interpretation der Daten

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(5) zum Verfassen des Manuskripts

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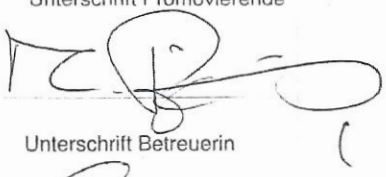
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Research Article

RESEARCH ARTICLE

The effects of fungal root endophytes on plant growth are stable along gradients of abiotic habitat conditions

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One sentence summary: Fungi living within plant roots mainly have weak detrimental effects on their hosts' growth, and these effects barely change along habitat changes in nutrient availability, light intensity or substrate pH.

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ABSTRACT

Plant symbioses with fungal root endophytes span a continuum from mutualistic to parasitic outcomes, and are highly variable depending on the genotype of each symbiont. The abiotic context in which interactions occur also seems to influence the outcome of plant–endophyte symbioses, but we lack understanding of its relative importance. We aimed to assess if changes in abiotic variables determine the effects of fungal root endophytes on plant growth. We used *in vitro* co-cultivation assays to test the impact of a selection of endophytic strains from diverse lineages on the growth of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare* along gradients of nutrient availability, light intensity or substrate pH. Most fungi showed a negative but weak effect on plant growth, whereas only a few had persistent detrimental effects across plants and conditions. Changes in abiotic factors affected plant growth but had little influence on their response to fungal inoculation. Of the factors tested, variation in nutrient availability resulted in the most variable plant–endophyte interactions, although changes were feeble and strain-specific. Our findings suggest that the effects of root endophytes on plant growth are robust to changes in the abiotic environment when these encompass the tolerance range of either symbiont.

Keywords: context dependency; environmental gradients; plant–fungus interactions; root; endophytes; symbiosis

INTRODUCTION

The interactions between species result in diverse effects on the fitness of each organism. Depending on whether the net effects are negative or positive, the interactions commonly are positioned along a continuum between parasitism and mutualism (Ewald 1987). However, the outcomes of interspecies interactions are not static over space and time, as they are affected by the ecological context in which they occur, a process that is frequently termed context dependency of the interactions (Bronstein 1994; Chamberlain, Bronstein and Rudgers 2014). The variation in the outcome of interactions is largely affected by abiotic factors such as temperature or illumination (Davitt, Stansberry and Rudgers 2010; Daskin and Alford 2012), or by biotic factors such as the presence of other species in the community (Agler *et al.* 2016; Laitinen, Hellström and Wäli 2016), to the extent that the net result can change in direction—from mutualism to parasitism, or vice versa—for at least one of the interacting partners. For example, the associations between plants

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and mycorrhizal fungi are often beneficial for both symbionts, in that the fungus assists the host in the uptake of nutrients and receives organic carbon in return. But these associations have been shown to shift from mutualism to parasitism when soil nutrients are not limiting and the trade-offs between the costs and benefits are reversed (Smith and Read 2010; Andreo-Jimenez *et al.* 2015). Likewise, legume–rhizobacteria symbioses that have been historically considered as mutualistic display differential outcomes depending on the availability of soil nitrogen, as well as on the genotypes of the interacting partners (West *et al.* 2002; Heath and Tiffin 2007). Another widespread and diverse interaction in nature is that occurring between non-mycorrhizal fungal endophytes and plants. Endophytes have been frequently deemed to benefit their hosts through enhancing their resistance and tolerance toward environmental stresses (Clay 1991; Kannadan and Rudgers 2008; Maciá-Vicente *et al.* 2008; Rodriguez and Redman 2008). Most experimental evidence suggests that the outcomes of these associations are very variable across the symbiotic continuum depending on the biotic/abiotic context (Saikkonen *et al.* 1998; Mandyam and Jumpponen 2015; Hiruma *et al.* 2016). However, only a few comprehensive studies have described the range of outcomes and context dependency of endophytic symbioses, and these have largely focused on interactions above-ground (Davitt, Stansberry and Rudgers 2010; Davitt, Chen and Rudgers 2011; Laitinen, Hellström and Wäli 2016). In plant roots, studies on context dependency have mostly dealt with mycorrhizal symbioses (Hoeksema *et al.* 2010), but comparably little is known on the variability of plant associations with non-mycorrhizal root endophytes across environmental gradients.

In a recent study, we assessed how the interactions between a diverse array of fungal root endophytes and the three plant species *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare* depend on the traits and the phylogenetic affiliations of the fungal partners (Kia *et al.* 2017). Under the assayed conditions, most fungal strains behaved as weak parasites, but their effects on plant growth were strain-dependent and could be partly explained by their morphological, physiological and ecological traits. The study, however, did not afford clues as to how the particular interactions respond to changes in the environmental conditions to which they are subjected. Understanding not only how groups of endophytes differently impact plant fitness, but also how these effects vary across environmental conditions is a necessity to unravel the as yet cryptic role of this fungal guild in ecosystems. For example, there are multiple instances of endophytes phylogenetically related to plant pathogens that provide benefits to their hosts only under particular environmental circumstances (Redman *et al.* 2002; Rodriguez *et al.* 2008; Hiruma *et al.* 2016). Whether these conditional interactions suppose general phenomena with an essential role in the functioning of certain natural systems is not yet clear.

Here, we use a selection of the fungal endophytic strains tested in Kia *et al.* (2017) to assess how their interactions with plants are affected by the abiotic environment. The selection of strains is based on their phylogenetic affiliations, their ecological origins and their observed differential traits and effects on plant growth. We reproduce the interactions of the strains with *A. thaliana*, *M. erraticum* and *H. vulgare* as in Kia *et al.* (2017), but this time we subjected the symbiotic system to gradients of nutrient availability, light intensity and substrate pH. The selected factors are easy to modify under laboratory conditions, and they are also likely to impact plant–microbe interactions. It is well known that in mycorrhizal associations, fitness of both symbionts depends on abiotic soil conditions (e.g. Piculell, Hoeksema and Thompson 2008), in which levels of macronutrients are important predictors of the plant response to fungal colonization. Nutrient availability may also alter the plant associations with non-mycorrhizal endophytes, with a suspected tendency toward mutualistic interactions in nutritionally limited environments, and to parasitic associations in those that are nutritionally rich (Thrall *et al.* 2007; Newsham 2011; Hiruma *et al.* 2016). Another important factor affecting endophyte symbioses is light availability, which determines plant productivity and hence the availability of photosynthates for plant-associated microbes. There are previous studies showing an interaction of available light and the outcome of plant–endophyte symbioses that show a trend toward parasitism under high light intensities (Bereau *et al.* 2000; Davitt, Stansberry and Rudgers 2010; Álvarez-Loayza *et al.* 2011). Finally, soil pH has been also found to be an important determinant of soil microbial communities and of the performance of plant-associated microbes (Marx and Zak 1965; Wang *et al.* 1993; Belesky and Fedders 1995; Rousk *et al.* 2010).

In this study we aimed to assess the relative importance of the selected environmental variables as predictors of the plant response to endophytic inoculation. As indicative measure of the interaction outcomes, we measured changes in the sign and the strength of the endophyte's effect on plant growth. Specifically, we aimed at answering the following questions: (i) are the outcome of interactions between plants and fungal root endophytes stable across abiotic contexts? (ii) Is the outcome of context-dependent interactions strain-dependent? (iii) Is the outcome of context-dependent interactions stable across host plant species?

MATERIALS AND METHODS

Fungal strains and plant material

A set of 23 strains of endophytic fungi isolated from roots of different plants and geographical locations were selected for this study (Table 1). Most strains originate from a study on the root endophytic diversity associated with *Microthlaspi* spp. (Glynou *et al.* 2016). In addition, one strain was isolated from *Salicornia* sp. roots, and *Serendipita indica* (syn. *Piriformospora indica*) strain CBS 125645 was obtained from the KNAW-CBS Fungal Biodiversity Centre. Most strains belong to orders Pleosporales, Hypocreales and Helotiales (the most frequent orders found by Glynou *et al.* 2016), and their selection for this study was based on their observed differential combination of morphological and physiological traits, their effects on plant growth (Kia *et al.* 2017), as well as on the association of their natural occurrence with particular ecological factors (Glynou *et al.* 2016). The fungal strains were maintained on corn meal agar medium (CMA, Sigma-Aldrich, St Louis, MO, USA) at approximately 25°C.

The Brassicaceae *Arabidopsis thaliana* ecotype Columbia (Col-0) and *Microthlaspi erraticum* (Mp_K11), and the Poaceae *Hordeum vulgare* cv. Barke (barley) were used as host plants in co-cultivation experiments with fungal endophytes. Seeds of *A. thaliana* were provided by the Laboratory of Plant Physiology of Wageningen University. Seeds of *M. erraticum* were collected from a field population in Germany (Ali *et al.* 2016). Barley seeds were provided by the company Saatzucht Josef Breun GmbH & Co. KG (Herzogenaurach, Germany).

Experimental design

Nine independent experiments were set up to test the effects of fungal strains on plant growth under different abiotic conditions. Each experiment was performed using one of the

Table 1. Details of the fungal endophytes included in this study, and of their use in experiments involving different plant hosts and abiotic factors.

	Identification				Origin		Experiment[b]		
Strain	Proposed classification	OTU[a]	Order	ITS accession	Country	Host plant/source	*A. thaliana*	*M. erraticum*	*H. vulgare*
P1188	*Thanatephorus* sp.	OTU020	Cantharellales	KT268504	Croatia	*Microthlaspi erraticum*	n, l, p	n, l, p	n, l, p
P1176	*Cadophora* sp.	OTU006	Helotiales	KT268493	Croatia	*Microthlaspi perfoliatum*	n, l, p	n, l, p	n, l, p
P1312	*Cadophora* sp.	OTU006	Helotiales	KT268607	Spain	*Microthlaspi perfoliatum*	p	—	—
P1331	*Cadophora* sp.	OTU006	Helotiales	KT268626	Spain	*Microthlaspi perfoliatum*	n, l, p	n, l, p	n, l, p
P1686	*Cadophora* sp.	OTU006	Helotiales	KT268959	Bulgaria	*Microthlaspi perfoliatum*	p	—	—
P1866	*Cadophora* sp.	OTU006	Helotiales	KT269135	Bulgaria	*Microthlaspi perfoliatum*	p	—	—
P1940	*Cadophora* sp.	OTU006	Helotiales	KT269207	Germany	*Microthlaspi perfoliatum*	p	—	—
P2800	*Cadophora* sp.	OTU006	Helotiales	KT269998	Germany	*Microthlaspi perfoliatum*	p	—	—
P1190	*Dactylonectria* aff. *macrodidyma*	OTU005	Hypocreales	KT268506	Croatia	*Microthlaspi erraticum*	n, l, p	n, l, p	n, l, p
P1076	*Fusarium incarnatum-equiseti* species complex	OTU010	Hypocreales	KT268395	Turkey	*Microthlaspi perfoliatum*	n, l, p[c]	n, l, p	n, l, p
P1141	*Fusarium oxysporum* species complex	OTU003	Hypocreales	KT268459	France	*Microthlaspi erraticum*	n	n	n
P1185	*Fusarium oxysporum* species complex	OTU003	Hypocreales	KT268501	Croatia	*Microthlaspi erraticum*	n, l, p	n, l, p	n
P1304	*Fusarium tricinctum* species complex	OTU001	Hypocreales	KT268599	Spain	*Microthlaspi perfoliatum*	n, l, p[c]	n, l, p	n, l, p
P1020	*Alternaria* aff. *alternata*	OTU008	Pleosporales	KT268339	Turkey	*Microthlaspi perfoliatum*	n, l, p	n, l, p	n, l, p
P1603	*Alternaria* aff. *alternata*	OTU008	Pleosporales	KU933996	Spain	*Salicornia patula*	n	n	n
P1191	*Alternaria tellustris*	OTU002	Pleosporales	KT268507	Croatia	*Microthlaspi erraticum*	n, l, p[c]	n, l, p	n, l, p
P1008	unidentified Pleosporales	OTU014	Pleosporales	KT268327	Turkey	*Microthlaspi perfoliatum*	n, l, p	n, l, p	—
P1177	unidentified Pleosporales	OTU021	Pleosporales	KT268494	Croatia	*Microthlaspi erraticum*	n, l, p	n, l, p	n
P1004	*Leptosphaeria* sp.	OTU024	Pleosporales	KT268323	Turkey	*Microthlaspi perfoliatum*	n, l, p	n, l, p	n, l, p
P1134	*Paraphoma* sp.	OTU007	Pleosporales	KT268452	France	*Microthlaspi perfoliatum*	n, l, p	n, l, p	n, l, p
P2188	*Pyrenochaeta* sp.	OTU040	Pleosporales	KT269451	Greece	*Microthlaspi perfoliatum*	n, l, p	n, l, p	n
P2093	*Roussoella* sp.	OTU043	Pleosporales	KT269356	France	*Microthlaspi perfoliatum*	p	—	—
CBS 125645	*Serendipita indica*	OTU033	Sebacinales	DQ411527	India	Rhizospheric soil	n, l, p	n, l, p	n

[a]Classification into operational taxonomic units as defined by >97% ITS sequence similarity, as described by Kia *et al.* (2017).
[b]Experiments in which fungal strain were used, involving either inoculations in *A. thaliana*, *M. erraticum*, or *H. vulgare*, under different regimes of nutrient availability (n), light intensity (l), or substrate pH (p).
[c]Treatments removed due to contamination of the batch controls.

three plant species and one of the three abiotic factors: nutrient availability (four levels), light intensity (three levels), and substrate pH (four levels). A core number of fungal strains representing all orders and most genera were used in all cases, but others were only included in particular experiments (Table 1). In particular, several isolates identified as *Cadophora* sp. OTU006 were included in experiments with *A. thaliana* and pH, because their natural occurrence was found to be associated with soil pH (Glynou *et al.* 2016), and this association has also been frequently observed for other phylogenetically close endophytic fungi (Sieber and Grünig 2013; Taylor *et al.* 2014).

Due to the number of combinations of fungal strains, plant hosts and abiotic factor levels, most experiments were divided into different experimental batches tested at different times, as described in Kia *et al.* (2017). In each batch, several fungal treatments were tested simultaneously and compared with a single control treatment consisting of non-inoculated plants. A summary of the experimental designs, including the factor levels and the number of strains tested in each batch, is provided in Supplementary Table S1.

Plant inoculation assays and growth conditions

Plant–endophyte co-cultivation bioassays were performed in a Binder KBW400 growth chamber (Binder Gmbh, Tuttlingen, Germany) as described in Kia *et al.* (2017), with modifications to include gradients in the abiotic variables (details of the experimental set-up for each variable are described in the following subsections). In brief, *A. thaliana* and *M. erraticum* were grown on half-strength (except in nutrient availability assays) Murashige–Skoog basal salt solid medium (MS; Sigma-Aldrich; Murashige and Skoog 1962) at 23°C under continuous illumination (80 μmol m^{-2} s^{-1} [photosynthetic photon flux; PPF], except in light intensity assays), on individual wells of 24-well plates, and they were inoculated with the selected fungal strains from CMA colonies as previously described (Kia *et al.* 2017). Treatments for each fungal strain or uninoculated control plants per abiotic factor level consisted of five replicates, each performed in a separate plate placed in random well positions. Ten days after fungal inoculation, root colonization was assessed via direct observation under a stereomicroscope, and the fresh weight of the plant shoots was measured using a precision scale.

Experiments with barley were carried out in glass tubes filled with water-saturated sterilized vermiculite (except in nutrient availability assays), as in Kia *et al.* (2017). Fungal inoculations were performed by adding four 5-mm plugs taken from the margin of growing fungal colonies on CMA to the substrate. Two-day-old barley seedlings were planted on the vermiculite and grown under half-day conditions (12 h:12 h, light–dark, 80 μmol m^{-2} s^{-1}, except in light intensity assays) at 23°C. Treatments consisted of 10 replicates each implemented in several batches, as described above. After 10 days of growth, the fresh weight of the plants' shoots and roots were measured and used to calculate total plant weight. In this case, endophytic root colonization in most treatments was assessed in a subset of five randomly selected plants, except for treatments in which the fungus had a strong detrimental effect on roots that prevented the sampling of enough replicates. For every plant, a 10-cm-long root section was surface-sterilized for 1 min in 0.5% (v/v) sodium hypochlorite and washed thrice with sterilized water. Sterilized roots were then ground in 0.5 ml of 0.1% (w/v) water agar using a Retsch MM200 bead beater (Retsch, Haan, Germany), and 200 μl of the resulting suspension was plated on CMA supplemented with antibiotics (25 mg ml^{-1} chloramphenicol and 50 mg ml^{-1} streptomycin) and 0.1% (v/v) Triton X-100. Three to seven days later, development of colony forming units of the respective fungi was assessed to confirm fungal colonization of roots.

Nutrient availability assays

Experiments of fungal inoculation in *A. thaliana* and *M. erraticum* were subjected to gradients of nutrient availability by modifying the strength of the MS medium, using the following levels: full MS, 1/2 MS, 1/4 MS and 1/10 MS. In barley, a similar procedure was used by saturating the vermiculate with 20 ml of water, or with full, 1/50, or 1/100 dilutions of Hoagland's plant nutrients solution (Sigma-Aldrich).

Light intensity assays

Gradients of light intensity for experiments with all three plant species were applied by modifying the number of active fluorescent daylight tubes in different shelves of the growth chamber. These changes resulted in three levels of light intensity at 80.8, 49.2 and 26.5 μmol m^{-2} s^{-1}, corresponding to five, three or one active tubes out of a maximum of five, respectively. The light tube cassettes were equipped with a reflector material to maximize light diffusion on the shelves.

pH assays

In assays with *A. thaliana* and *M. erraticum*, gradients of pH of 5.7, 6.5, 7 and 7.5 were achieved by modifying the pH of the MS medium before planting the 7-day-old seedlings. These pH levels encompass the natural range of soil pH covered by the samplings described in Glynou *et al.* (2016) from where most strains originate, and correspond to soil categories from moderately acidic to slightly alkaline (Ditzler, Scheffe and Monger 2017). The same range of pH values was used for barley assays, in this case by saturating the vermiculite with different solutions of 0.1 M sodium phosphate buffer obtained by mixing solutions of monosodium phosphate and disodium phosphate at different ratios, so that the net number of phosphorus atoms remained constant.

Statistical analyses

All statistical analyses were performed using R v3.0.2 (R Core Team 2016). The data files and the script with the R command lines for the data analysis have been deposited in Figshare (https://figshare.com/s/6009e8e26a5aff0c55f7, http://dx.doi.org/10.6084/m9.figshare.5240572). We first investigated the changes in plant biomass upon fungal inoculation across abiotic conditions by calculating the effect sizes of each fungal treatment with respect to its respective uninoculated control. Effect size is useful to easily detect changes in the sign and strength of the interactions. Before calculations, measurements from control plants showing fungal contamination were removed from the data. This caused the removal of data from three strains in the experiment of *A. thaliana* and pH (Table 1), which belonged to a batch where all control plants were removed. Effect sizes with 95% confidence intervals were calculated according to the Cohend's *d* statistic (Cohen 1988) using the function cohen.d in the R package effsize v0.5.4 (Torchiano 2014), which measures the difference in means and standardizes it by their pooled standard deviation. In order to investigate general patterns of variation in the fungal impact on plant growth

across abiotic conditions, we compared the effect sizes at different abiotic factor levels using the Kruskal–Wallis rank sum test.

We further investigated the variation of the plant interactions with endophytic strains across conditions using linear fixed-effects models. First, to assess the overall effects of endophytes and abiotic conditions on plant growth for each experiment, we built linear models with plant biomass as a response variable, and the abiotic factor and fungal treatment as explanatory fixed-effect variables including an interaction term. In those experiments performed over the course of different batches, we included the factor experimental batch as an additional explanatory variable. Statistical significance in the effects of explanatory variables and in the interaction term was assessed by means of analysis of variance (ANOVA), after checking that the model's residuals did not strongly deviate from normal distributions. The statistical power of these tests was evaluated using the R package pwr v1.2-1 (Champely 2017), represented as the minimum effect size likely to be detected at $P < 0.05$ with a power of 95%. We carried out a second set of analyses to summarize the individual variation across conditions of individual plant–strain combinations, by repeating the above linear models independently for each experimental batch, so as to represent variation due to each fungus as compared exclusively with its respective control treatment. The model coefficients with confidence intervals for each fungal treatment were extracted from these models using the function sjp.lmer of the package sjPlot v2.3.1 (Lüdecke 2015). *P*-values from fitted model objects were calculated with the same function, based on conditional *F*-tests with Kenward–Roger approximation for the degrees of freedom.

RESULTS

Inoculation of plants with endophytic strains resulted in a consistent fungal colonization across experiments, as assessed by direct observation under a dissecting microscope for experiments with *A. thaliana* and *M. erraticum* (99–100% of plants colonized), and by cultivation of root samples in *H. vulgare* (60–93% of plants colonized). A few uninoculated control plants were colonized by fungal contaminants, in which case they were excluded from further analyses.

The effect sizes of plant growth in response to fungal inoculation were most often negative (Fig. 1), with overall median values ranging between -2 ± 2 (SD) and -0.05 ± 0.5, indicating a consistent reduction of plant biomass in fungus-inoculated *versus* uninoculated plants. In all cases, effect sizes showed little overall variation across the abiotic factors tested, namely, nutrient availability, light intensity and substrate pH (Fig. 1). Of all experiments, only those involving *A. thaliana* and light intensity, and *M. erraticum* and substrate pH showed a significant variation in effect size across levels of each condition ($\chi^2 = 8.9$, df $= 2$, $P = 0.006$ and $\chi^2 = 39.7$, df $= 3$, $P < 0.001$, respectively), although the magnitude of the changes was negligible and neither were clear trends in the direction of the variation. Changes in the magnitude of effect sizes were evident in individual treatments, and mainly ranged between approximately neutral and highly negative values (Fig. 1).

We used linear regression analyses to assess the influence of the abiotic factors on the interactions between plants and endophytes. An additional explanatory variable was included in analyses for experiments performed in different batches, to account for the data variation among independent assays. Power analyses revealed the capability of tests to detect effect sizes of 10.5–17% in assays with *A. thaliana* and *M. erraticum* and of 4.9–9.5% in those with *H. vulgare*, with a power of 95% (Supplementary Table S2). All experiments showed a significant variation across fungal treatments, and the same was true for all abiotic factors in all plant species with the exception of *A. thaliana* and pH (Table 2). Overall, there was a positive effect of nutrient availability on plant growth, with model estimates ranging from 0.02 ± 0.005 g (SE) to 0.3 ± 0.02 g. In comparison, the overall effects of light intensity and pH were close to neutrality. The interaction between the factors abiotic condition and fungal inoculation were only consistently significant across plant species in the nutrition experiment, indicating that nutrient availability can systematically affect plant–endophyte interactions (Table 2). In addition to these, growth of *H. vulgare* in response to endophytes also varied significantly with light availability and substrate pH (Table 1). It is possible, however, that model estimates are somewhat biased in experiments involving *A. thaliana* in pH and *H. vulgare* in nutrient and light availability conditions, where different experimental batches were included that had a significant impact in data variation (Table 2). Results obtained in experiments with *H. vulgare* were similar when using as response variables either total plant weight (Table 2) or shoot and root weights separately (Supplementary Table S3), although in the latter cases light intensity alone had little effect on both variables, and the effect of pH appeared to be strong in roots but not in shoots (Supplementary Table S3).

An assessment of the model coefficients for estimates of each variable supported observations based on effect sizes that most fungal strains negatively impact plant growth (Fig. 2, Supplementary Fig. S1). In this case, the effect of experimental batch was excluded by obtaining coefficients from models independently performed for treatments in each batch, and hence values solely represent the effect of strains as compared with uninoculated plant treatments. These analyses also confirmed the strong interaction between the variables nutrient availability and fungal strain, since they showed a wide variability across strains (Fig. 2, Supplementary Fig. S1). Comparatively, the significant interactions found between light availability or pH and fungal inoculation in *H. vulgare* showed little variation and hence we deemed them to be marginal (Fig. 2). A visual inspection of the interactions between the three plant species and the fungal strains with effects on plant growth that significantly varied with nutrient availability shows that treatments changing positively with this variable had a rather trivial magnitude and/or did not follow a steady pattern (Fig. 3, Supplementary Fig. S1). On the other hand, interactions varying negatively with nutrient availability seemed to be due to the increase in the gap between the fungus-inoculated and the uninoculated treatments due to fungal parasitism or pathogeny (Fig. 3). In these cases, the increase in plant growth with increasing nutrient availability does not occur in plants hosting fungi that severely compromise their development. We could not detect a tendency of strains within particular fungal lineages to trigger the same responses on plant growth across treatments, but there were individual strains that had a similar impact on plant growth irrespective of the host species or abiotic condition (e.g. *Thanatephorus/Rhizoctonia* sp. P1188 or *Fusarium tricinctum* P1304; see Supplementary Fig. S2 for results of all assays). We did not detect a correlation between the overall effect of strains on plant growth and the interaction of these effects with nutrient availability (Spearman's $\rho = -0.14$ to 0.22, $P > 0.4$).

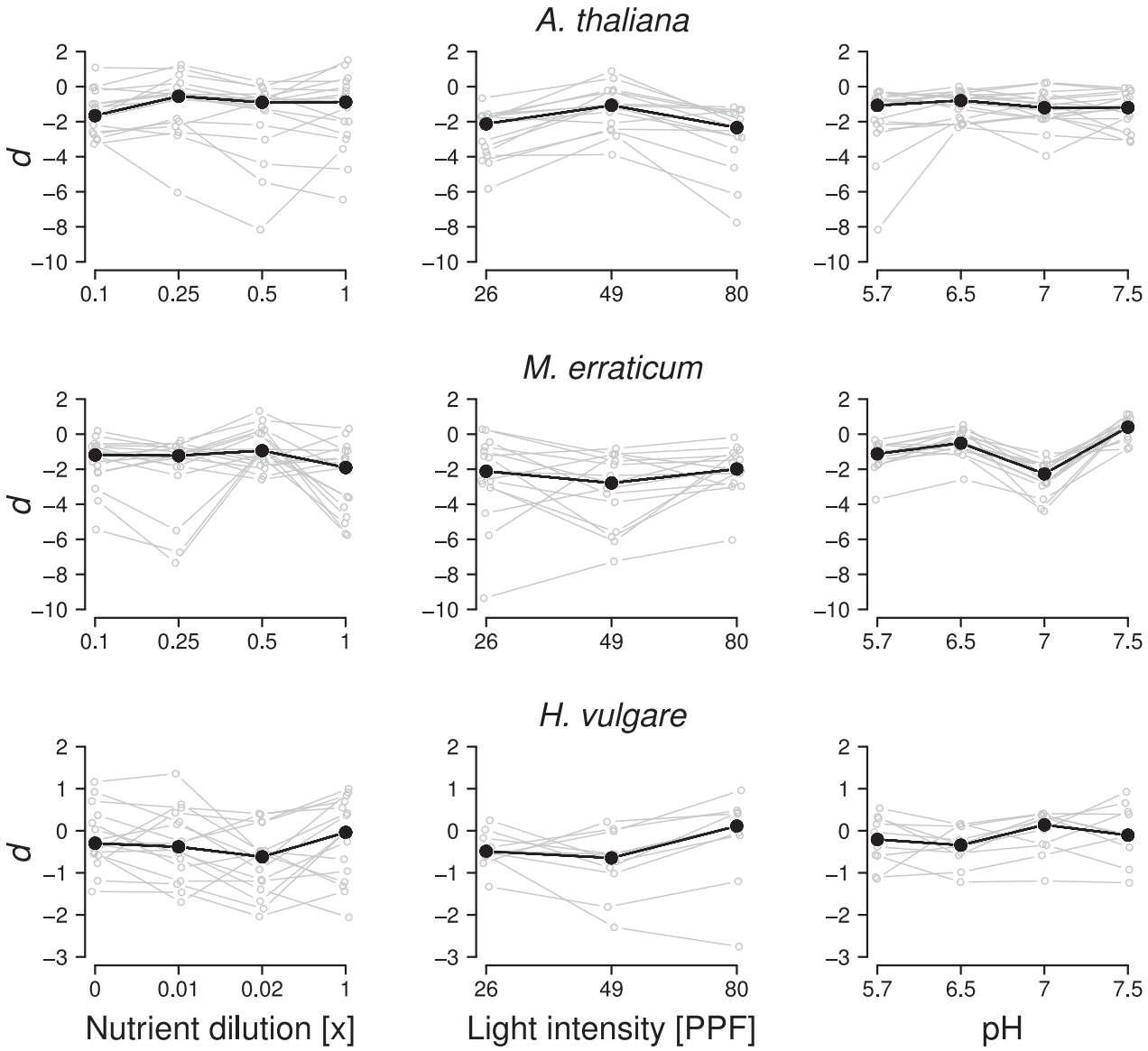

Figure 1. Effect sizes measured as Cohen's *d* showing the influence of nutrient availability, light intensity and substrate pH on the interactions between fungal root endophytes and the plants *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare*. For each plot, gray lines with points represent the variation in effect sizes for individual fungal strains over the abiotic conditions tested, and the black line with points denote the median value for all strains within the experiment. Note that x-axes are represented as factors, and are not proportional to the variable values.

DISCUSSION

We have tested the effect of abiotic conditions—including nutrient availability, light intensity and substrate pH—on the interactions between three different plant species and a diversity of root–endophytic fungi, comprising a variety of taxonomic lineages and geographical origins. Our results show that the effects of fungal root endophytes on the growth of their hosts mainly consist of reductions in plant biomass that are robust to changes in abiotic environmental conditions. No general trends were found in the variation of the magnitude or the direction of the fungal effects across plant species, ruling out a common response to specific abiotic factors by diverse root-colonizing fungi. When changes in the outcome of the symbioses were observed, these occurred in particular combinations of hosts and fungal strains, similarly to what has been found for the interaction between plants and pathogenic fungi (Laine 2007). The differential response of individual plant–fungus combinations is concordant with the frequently reported high variability in the outcome of fungal endophytic symbioses, even when phylogenetically related fungi are compared (Tellenbach, Grünig and Sieber 2011; Mayerhofer, Kernaghan and Harper 2012; Reininger and Sieber 2013; Kia *et al.* 2017). Altogether, our findings suggest that the interplay between the genotypes of the plant host and the root-colonizing fungi is an important determinant of variability in plant growth and likely to affect the host's fitness, which could entail the selection of particular combinations of symbionts in locations under different environmental conditions, as proposed by the geographic mosaic theory of coevolution (Thompson 2005).

As expected, both the different abiotic factors and the inoculation with fungal strains had independent effects on plant growth, as estimated by measurements of above-ground or total fresh biomass. In the case of fungal inoculation, the effects

Table 2. Summary statistics for the ANOVA of the effects of abiotic factors and fungal inoculation on plant fresh weight.

		Nutrients			Light			pH		
Plant species	Effect	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
A. thaliana	Abiotic factor	1,342	**124.1**	**<0.001**	1,203	**5.9**	**0.016**	1,358	0.7	0.35
	Fungus	17,342	**21.1**	**<0.001**	15,203	**10.9**	**<0.001**	18,358	**65.9**	**<0.001**
	Experimental batch	1,342	0.5	0.5	n.d.	n.d.	n.d.	1,358	**1.3**	**<0.001**
	Abiotic factor × fungus	17,342	**5.5**	**<0.001**	15,203	0.7	0.8	18,358	1.3	0.21
M. erraticum	Abiotic factor	1,338	**68.3**	**<0.001**	1,208	**27.5**	**<0.001**	1,234	**48.8**	**<0.001**
	Fungus	17,338	**13.2**	**<0.001**	15,208	**5.6**	**<0.001**	15,234	**3.2**	**<0.001**
	Experimental batch	1,338	3.2	0.07	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Abiotic factor × fungus	17,338	**2.5**	**0.001**	15,208	0.9	0.6	15,234	0.4	0.97
H. vulgare	Abiotic factor	1,777	**89.5**	**<0.001**	1,329	**5.4**	**0.021**	1,488	**12.4**	**<0.001**
	Fungus	16,777	**7.8**	**<0.001**	10,329	**10.8**	**<0.001**	10,488	**6.2**	**<0.001**
	Experimental batch	4,777	**19.1**	**<0.001**	1,329	**22.2**	**<0.001**	2,488	0.6	0.5
	Abiotic factor × fungus	16,777	**3.8**	**<0.001**	10,329	**2.3**	**0.013**	10,488	**8.1**	**<0.001**

Significant values ($P < 0.05$) are shown in bold. n.d., not determined.

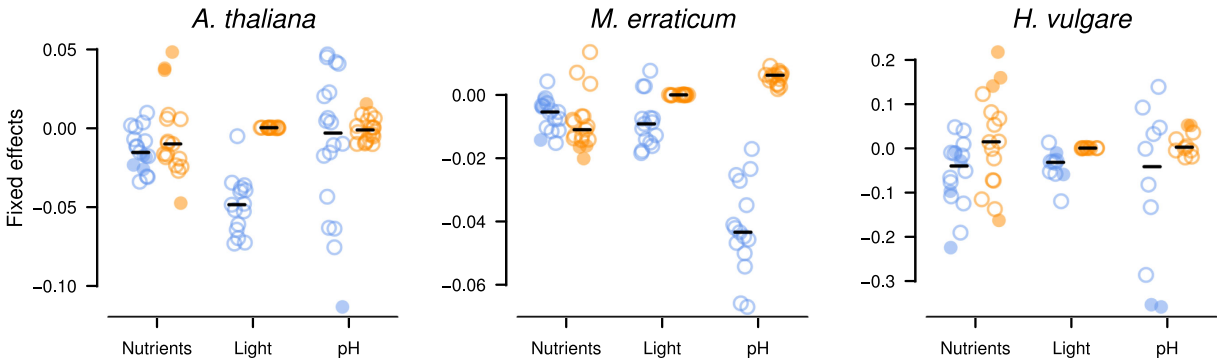

Figure 2. Effects of fungal inoculation on growth of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare* under varying conditions of nutrient availability, light intensity and substrate pH, as extracted from linear fixed effects models. For each plant species and abiotic condition, data points on the left represent the effects of individual fungal strains respect to uninoculated control plants, whereas points on the right represent the interaction effects of the fungal strain and the abiotic variable. Solid points represent values significant at $P < 0.05$, based on conditional *F*-tests with Kenward–Roger approximation for the degrees of freedom. Horizontal lines represent the median values for all data points in each condition. Additional information for this figure, including the correspondence between data points and fungal strains as well as 95% confidence intervals, is provided in Supplementary Fig. S1.

of most strains were negative, indicating a parasitism toward the host that is in agreement with previous studies based on *in vitro* assays of plant–endophyte interactions (Tellenbach, Grünig and Sieber 2011; Keim *et al.* 2014; Mandyam and Jumpponen 2015; Kia *et al.* 2017). The negative impact on plant growth is unsurprising given the trophic dependence of endophytic fungi on the host's resources and the simplicity of the co-cultivation system used, which lacked alternative sources of organic carbon to sustain fungal growth. It is noteworthy, however, that the effects in such conditions mainly consisted of reductions of biomass not accompanied by strong disease symptoms like wilting or chlorosis, and that apparently did not compromise plant survival, with few exceptions in strains related to well-known pathogens such as *Thanatephorus*/*Rhizoctonia* sp. or *Fusarium* sp. The weak plant-parasitic behavior of most root endophytes has already been described for the strains used in this work and others, in bioassays with the same three host plant species also used here (Kia *et al.* 2017). Kia *et al.* (2017) speculated that the relatively small difference in growth between the endophyte-inoculated and the uninoculated plants *in vitro* may be readily overcome in natural conditions by changes in the fungal impact on plant growth in response to the environmental context. This is supported by empirical evidence that mutualistic interactions with endophytic fungi can develop in the presence of abiotic or biotic environmental stress (Faeth 2002; Maciá-Vicente *et al.* 2008; Rodriguez *et al.* 2008; Redman *et al.* 2011; Hiruma *et al.* 2016; Almario *et al.* 2017), or that mycorrhizal symbioses tend to be more positive when the accompanying soil microbial communities are more complex (Hoeksema *et al.* 2010). A similar rationale was adopted by Chamberlain, Bronstein and Rudgers (2014) to hypothesize that interactions with weak effect sizes, such as mutualism, are more prone to be context-dependent than other types of symbioses because their outcomes are likely to swing around a neutral effect. Nevertheless, Chamberlain, Bronstein and Rudgers (2014) did not find a strong difference in the context dependency of mutualistic interactions with respect to other types of interactions like competition. Likewise, the low degree of variability that we found in this study is surprising, and suggests that the physiological changes triggered by the tested factors on both the fungus and the plant did not affect the interaction between the two organisms. It must be noted that the ranges applied for each condition were not extreme and encompass magnitudes within the tolerance limit of either symbiont for the short duration of the experiment. It can be expected that use of more severe conditions, either by limitation or excess, would have resulted in a stronger impact in the symbiosis by surpassing the tolerance growth breadth of at least one symbiont.

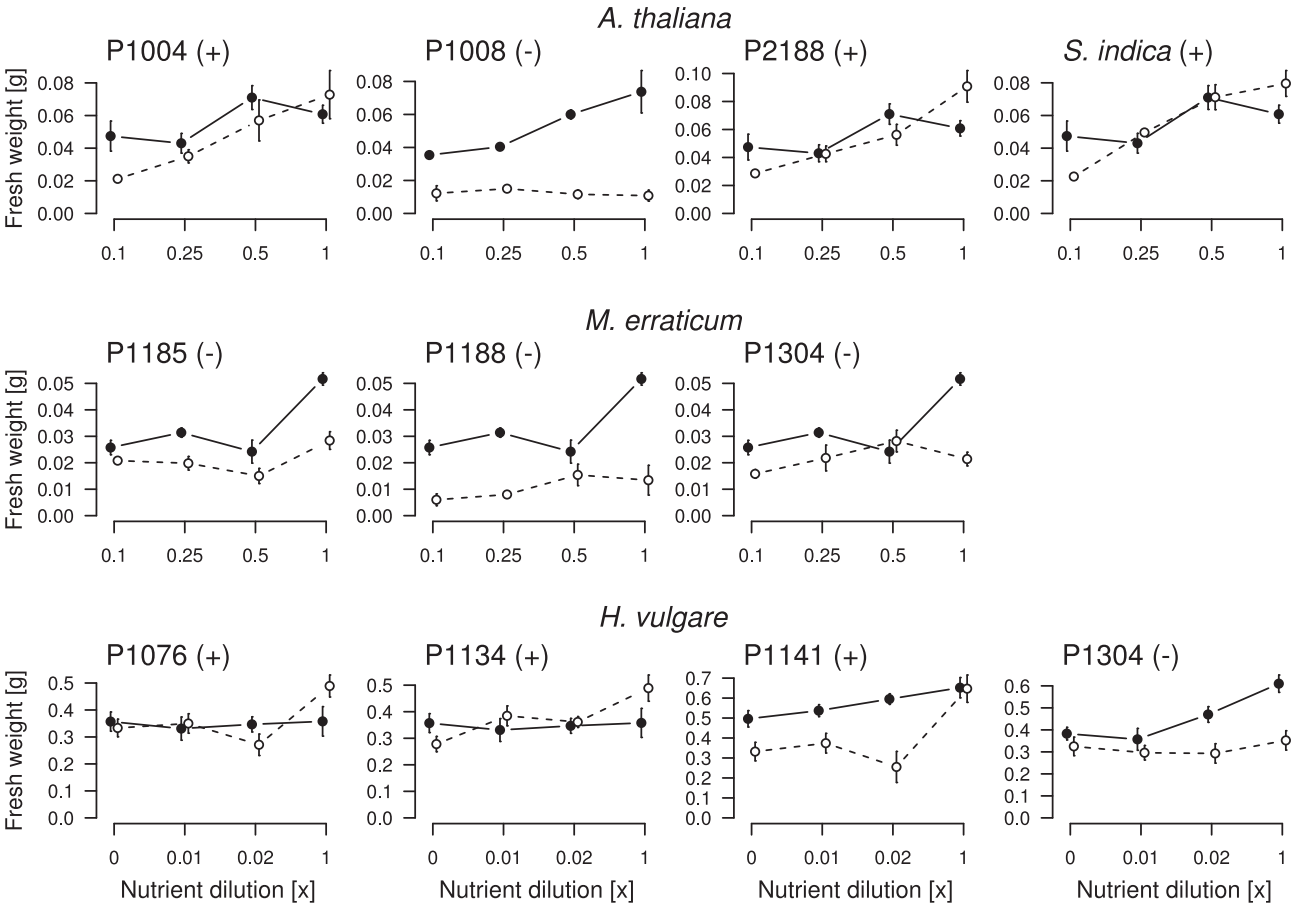

Figure 3. Variation in the effect of fungal inoculation on total plant biomass of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare* under varying conditions of nutrient availability, as compared with uninoculated controls. Interactions shown correspond to fungal strains with an effect on plant growth that was significantly affected by nutrient availability (see Fig. 2). Solid points with continuous lines represent mean weight values for uninoculated control plants, and open points with dashed lines represent values for fungus-inoculated plants. Error bars represent standard errors. Positive or negative symbols next to the strain names indicate the direction of the variation in the effect of fungi on plant growth with increasing nutrient availability, as obtained by linear models regression analysis.

Of the three abiotic gradients tested, the change in nutrient availability had the strongest impact on plant–endophyte interactions. The content of nutrients in the substrate where host plants grow, especially of nitrogen and phosphorus, is well known to impact plant–microbe symbioses. For example, in arbuscular mycorrhizas, mutualistic interactions with a mycorrhizal fungus are preferentially established under phosphorus starvation conditions (Andreo-Jimenez *et al.* 2015), although nitrogen content has also been shown to be relevant for a conducive mutualism (Hoeksema *et al.* 2010). A similar dependency on nutrients has been reported in a few cases for symbioses with non-mycorrhizal fungal endophytes (Behie, Zelisko and Bidochka 2012; Hiruma *et al.* 2016; Almario *et al.* 2017). Newsham (2011) suggested that plant relationships with root endophytic fungi can become beneficial when organic nutrients are present in soil, owing to the ability of fungi to saprotrophically break down complex organic molecules and mobilize sequestered nutrients. In our experiments, no organic nutrients were present, nor did we observe a tendency of endophytes to enhance plant growth under limiting nutrients, which excludes the conditional translocation of particular compounds to the plant under starvation. It seems more likely that the differences in effect sizes triggered by some fungi in response to different nutrient concentrations are related to other kind of physiological changes in either symbiotic partner, such as modifications in the susceptibility to fungal infection in the plant or in the virulence of the fungus, as suggested by Laine (2007).

In comparison with nutrient availability, light intensity and pH had little impact on the variability of the interactions. Several studies have reported light intensity as an important factor determining the sign of the interaction between plants and fungal endophytes, in which low light intensities seem to be conducive for more beneficial associations (Davitt, Stansberry and Rudgers 2010; Álvarez-Loayza *et al.* 2011). However, these interactions have been described in leaves, where a strong exposure to light exists, which could drive physiological changes in the fungus important in determining pathogenicity, such as the build-up of reactive oxygen species (Egan *et al.* 2007; Álvarez-Loayza *et al.* 2011). In the case of pH, the differential effects on growth between shoots and roots of *H. vulgare* indicates that it affects more evidently below-ground tissues, in direct contact with the substratum. In fungi, whereas pH can influence mycelial growth, most fungi can sustain similar growth rates across broad pH ranges (Wheeler, Hurdman and Pitt 1991; Grum-Grzhimaylo *et al.* 2015).

The *in vitro* systems used in this study are artificial and fall short in representing the complex context in which

natural plant–endophyte interactions occur. However, simplified systems such as the ones used here have proven adequate to reproduce both beneficial and detrimental interactions between root-colonizing fungi and host plants (e.g. Sesma and Osbourn 2004; Hiruma *et al.* 2016; Almario *et al.* 2017; Venneman *et al.* 2017). Moreover, such systems are necessary to isolate the effects of the study factors from the many biotic and abiotic variables that may confound results in less managed set-ups (Jessup *et al.* 2004). Surprisingly, in spite of such tight control over the non-target sources of variation—and possibly due to it as well—laboratory studies have been shown to yield the highest variability in the interaction outcomes in context-dependency studies, as compared with greenhouse or field studies (Chamberlain, Bronstein and Rudgers 2014). In natural conditions, potential variations in the effects of particular endophytes on the host due to changes in the abiotic environment may become diluted by the combined effects of co-occurring microorganisms and other abiotic and biotic variables. Given the relatively weak variation in the effects of endophytes observed here, it seems unlikely that context-dependent interactions between plants and root endophytes may result in significant changes in the host's fitness when both symbionts grow under conditions within their tolerance ranges.

In conclusion, our results do not show an overall strong effect of nutrient availability, light intensity or substrate pH on the interactions between fungal root endophytes and plants, indicating that the outcome of these plant–fungus relationships may be robust to changes in the abiotic environment in the field. Variations in plant growth were observed in interactions between particular fungi and plant species, especially in response to nutrient availability, but these did not seem consistent across fungal lineages or plant species. These different outcomes may ultimately depend on the interplay between the genotypic characteristics of each symbiont, adding to the general variability observed in the interactions between different endophytes and plants (Tellenbach, Grünig and Sieber 2011; Kia *et al.* 2017). Whereas the abiotic conditions studied here seem unlikely determinants of the outcome of plant–endophyte interactions in nature, further research is necessary to assess their importance by assaying more extreme condition and longer term interactions, as well as the implication of biotic factors such as microbe–microbe interactions within the root microbiome.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of Interest. None declared.

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The effects of fungal root endophytes on plant growth are stable along gradients of abiotic habitat conditions

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Supplementary Figures:

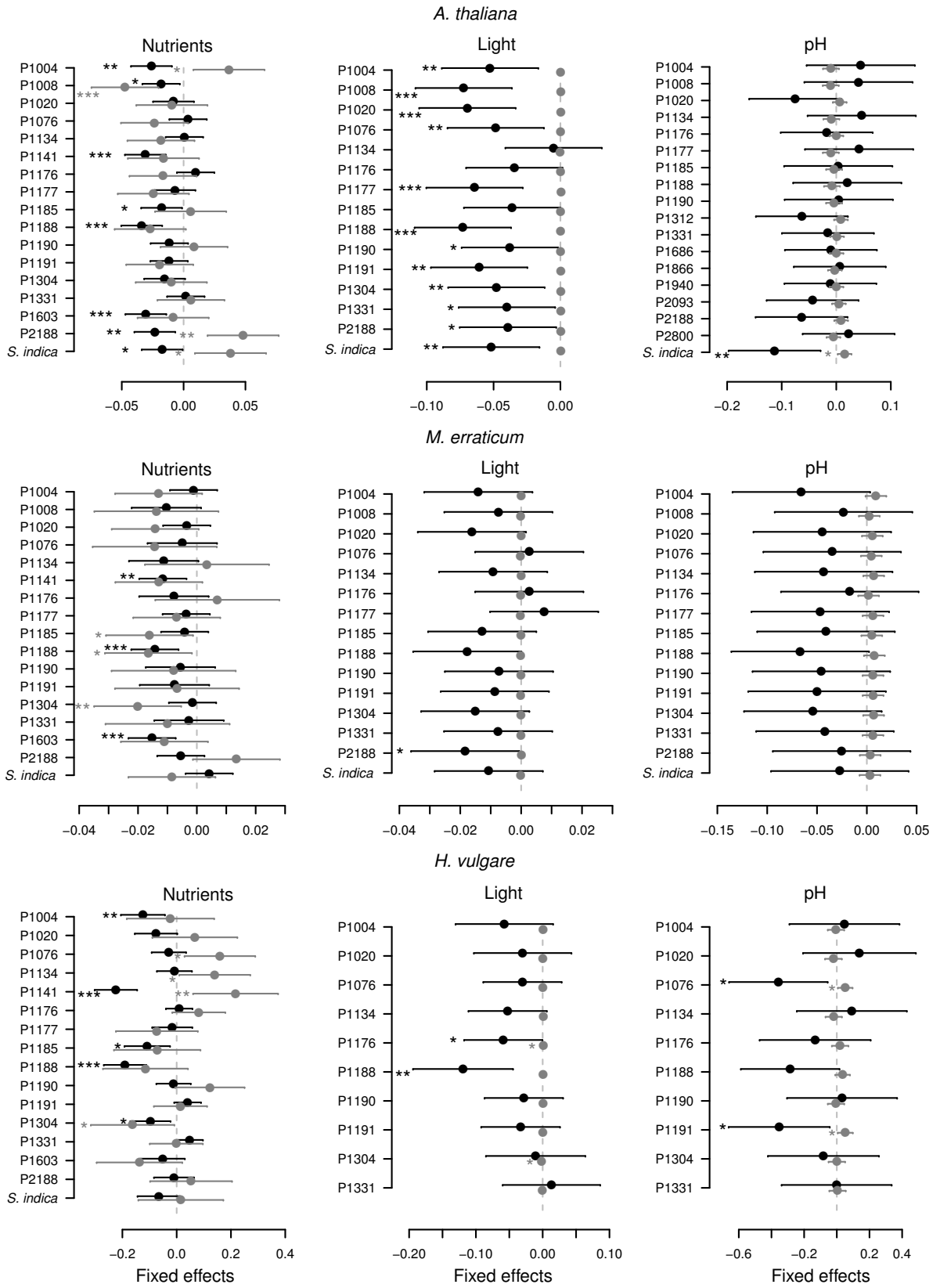


Figure S1: Effects of fungal inoculation on plant growth of *Arabidopsis thaliana*, *Microthlaspi erraticum* and *Hordeum vulgare* under varying conditions of nutrient availability, light intensity and substrate's pH. Points with error bars represent fixed effect values ± 95% confidence intervals as extracted from linear fixed effects models, with values in black showing the overall effects of each strain, and values in gray their interaction term with the respective abiotic variable. One, two or three asterisks next to values indicate significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, based on conditional F-tests with Kenward-Roger approximation for the degrees of freedom.

(a)

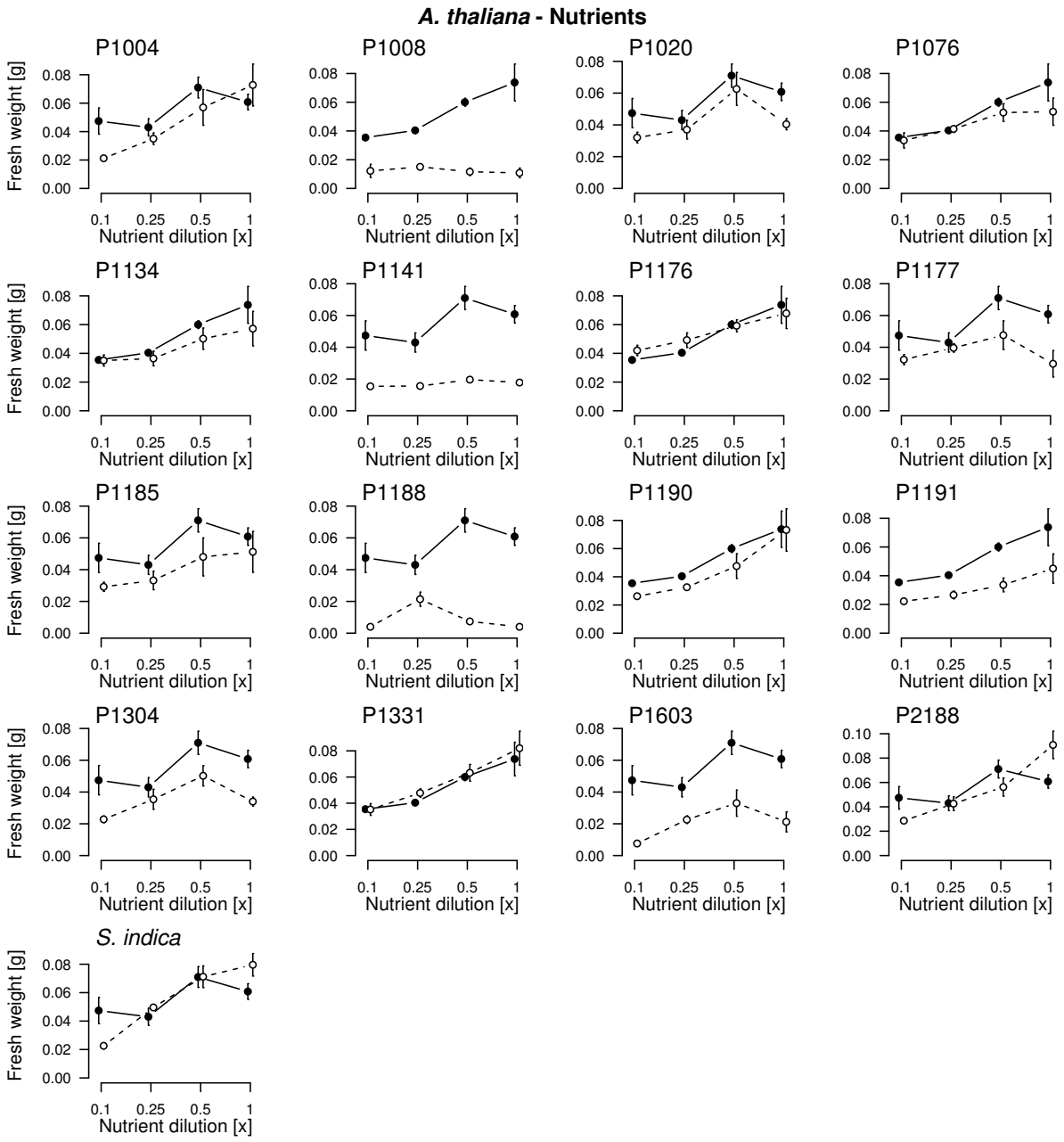
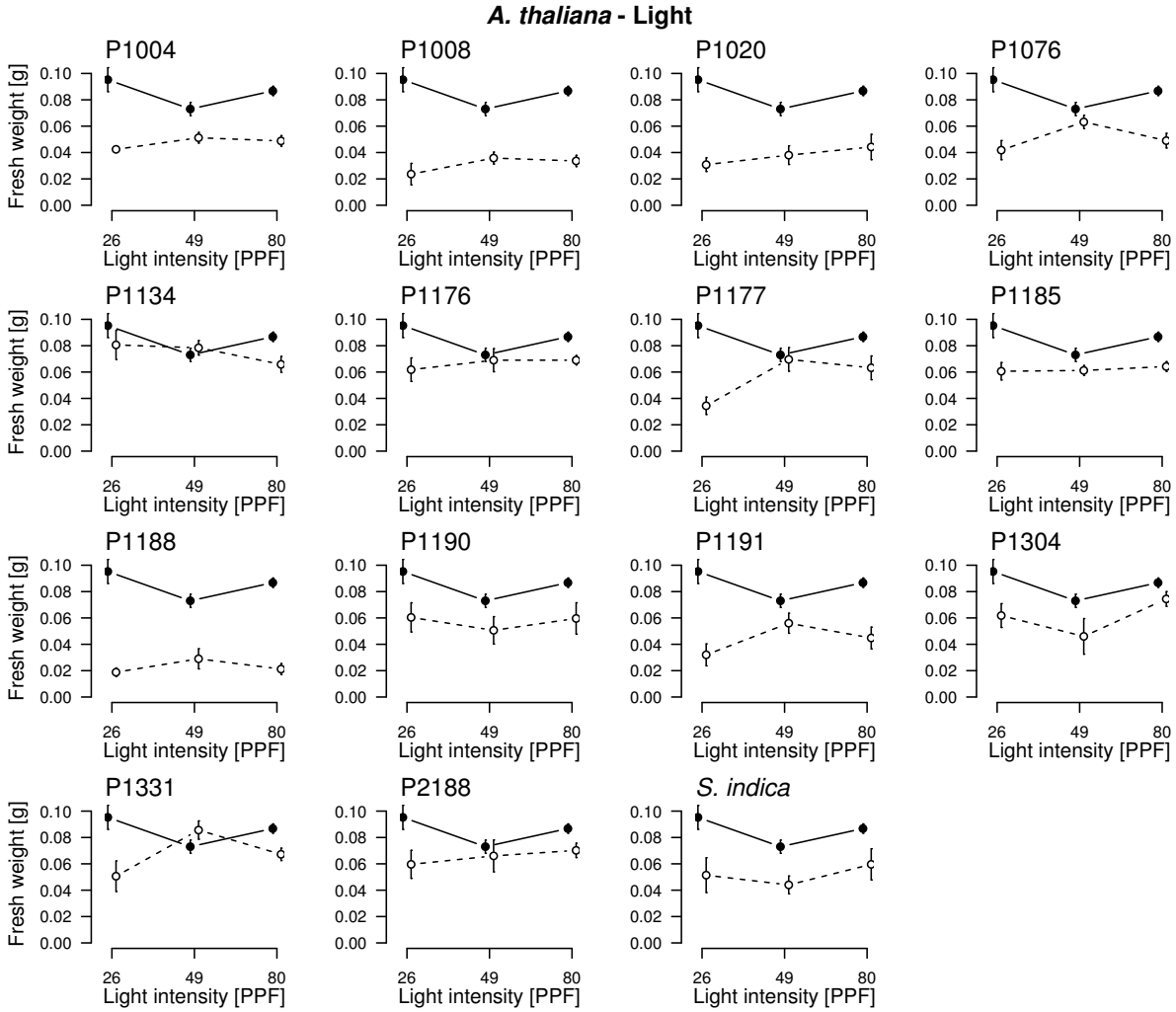
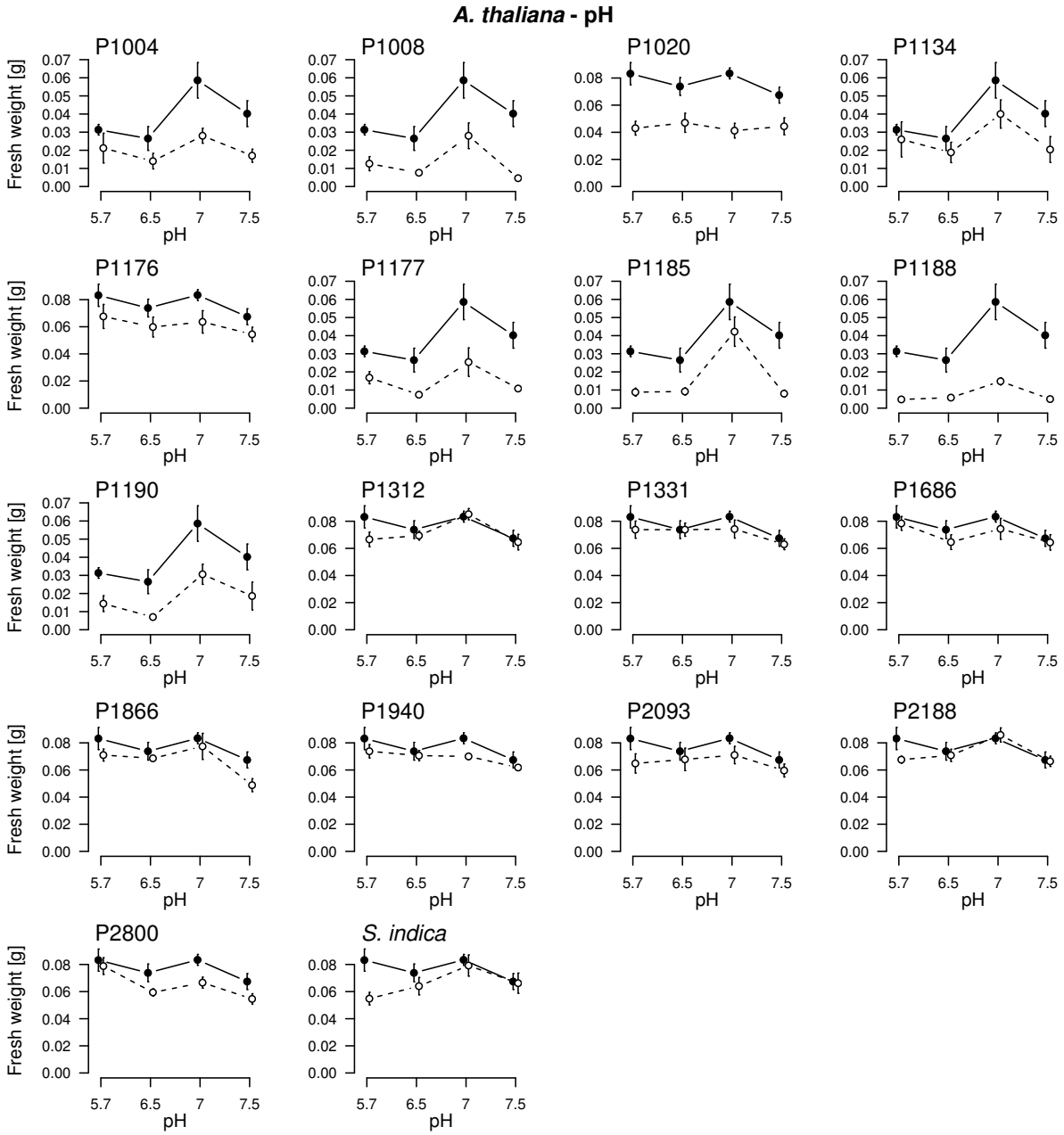


Figure S2: Results from the effects of inoculation with all fungal endophytic strains in this study on total plant biomass of *Arabidopsis thaliana* (a–c), *Microthlaspi erraticum* (d–f) and *Hordeum vulgare* (h–j) under varying conditions of nutrient availability (a, d, h), light intensity (b, e, i) and substrate's pH (c, f, j). Solid points with continuous lines represent mean weight values for uninoculated control plants, and empty points with dashed lines represent values for fungus-inoculated plants. Error bars represent standard errors. *(Continued in following pages).*

(b)


A. thaliana - Light
P1004
P1008
P1020
P1076
P1134
P1176
P1177
P1185
P1188
P1190
P1191
P1304
P1331
P2188
S. indica
Fresh weight [g]
0.00
0.02
0.04
0.06
0.08
0.10
26
49
80
Light intensity [PPF]

(c)


A. thaliana - pH
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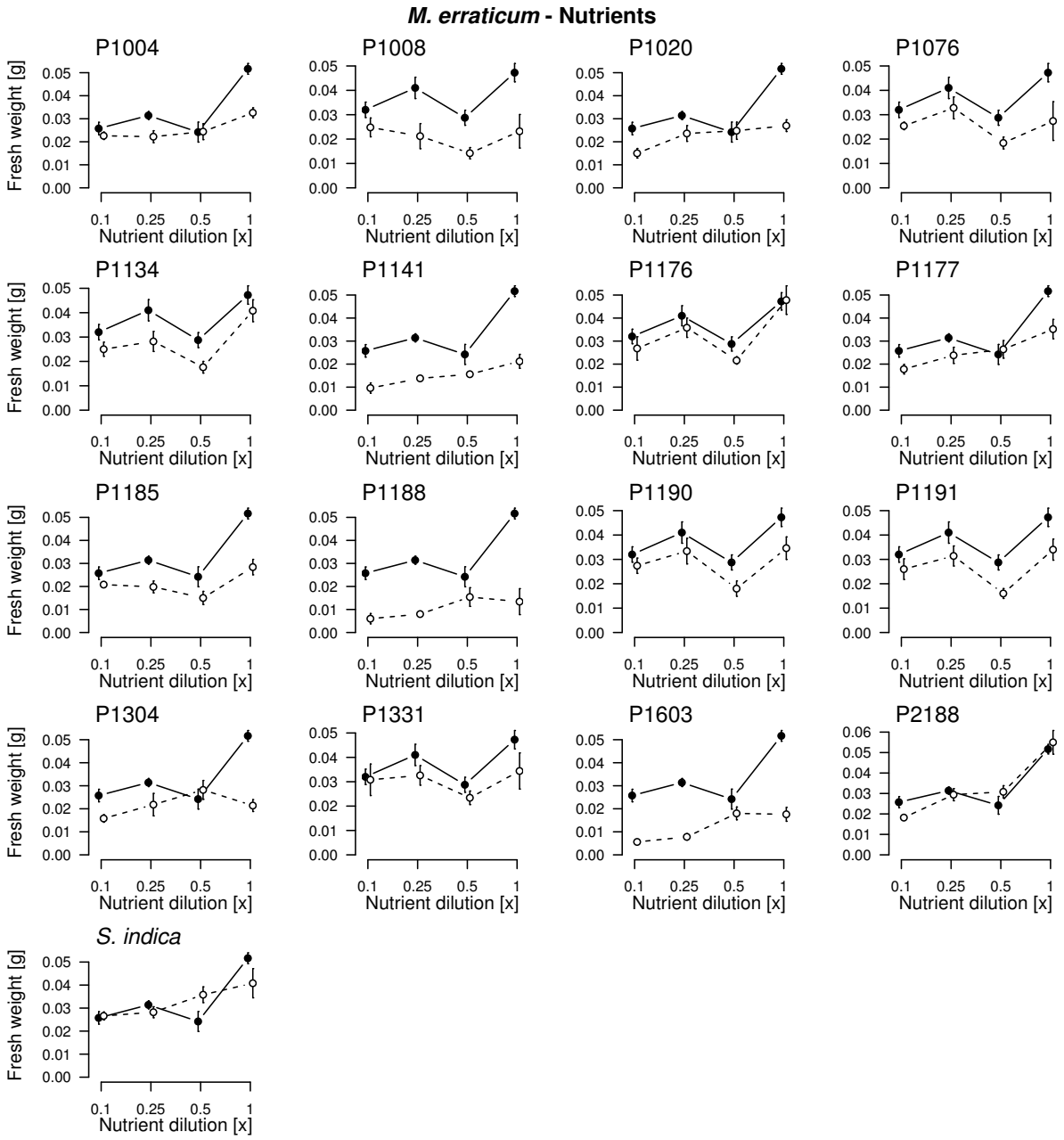
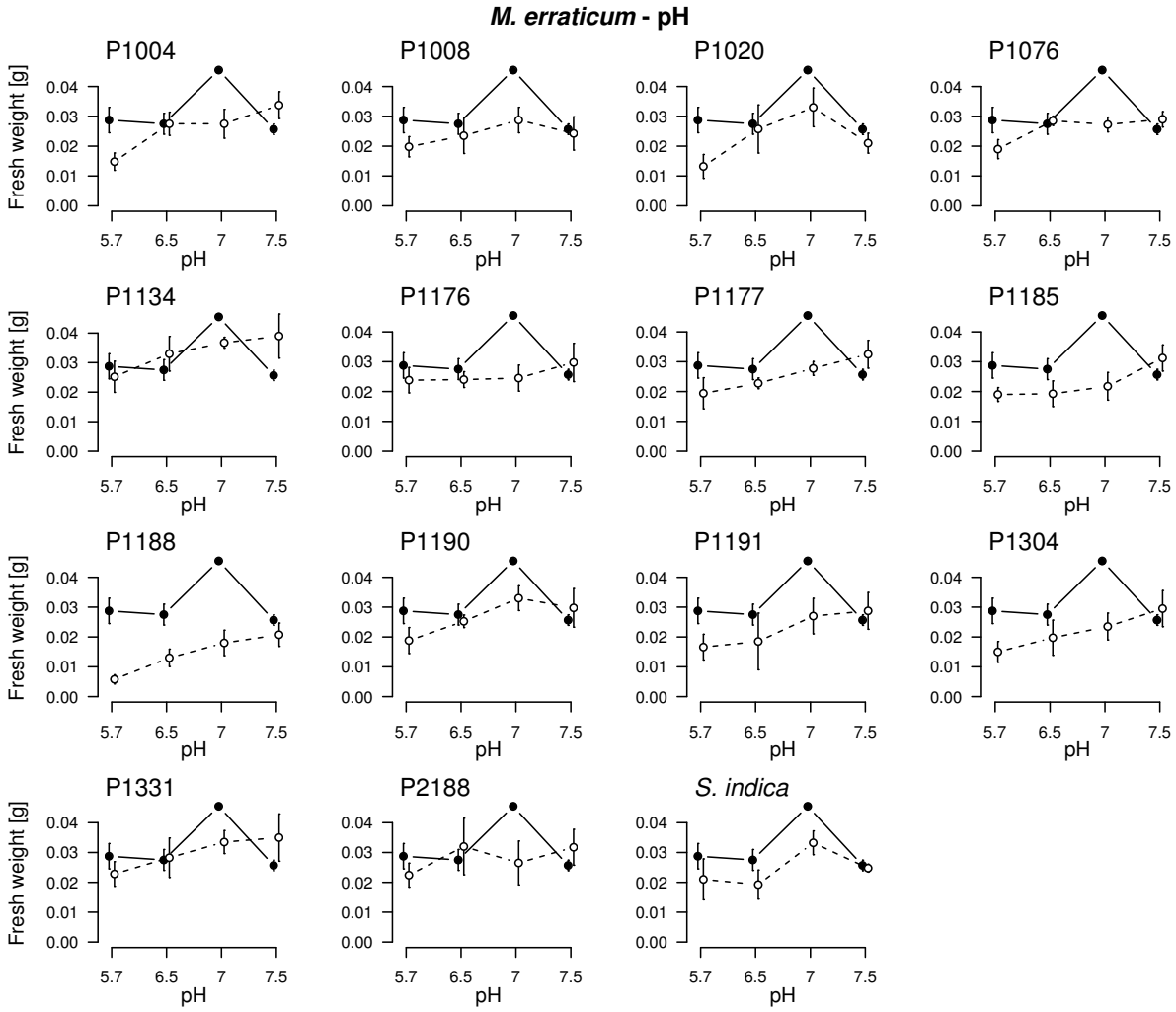
P1008
P1020
P1134
P1176
P1177
P1185
P1188
P1190
P1312
P1331
P1686
P1866
P1940
P2093
P2188
P2800
S. indica
Fresh weight [g]
pH
5.7
6.5
7
7.5

(d)


M. erraticum - Nutrients
P1004
P1008
P1020
P1076
P1134
P1141
P1176
P1177
P1185
P1188
P1190
P1191
P1304
P1331
P1603
P2188
S. indica
Fresh weight [g]
Nutrient dilution [x]
0.1 0.25 0.5 1

(e)


M. erraticum - pH
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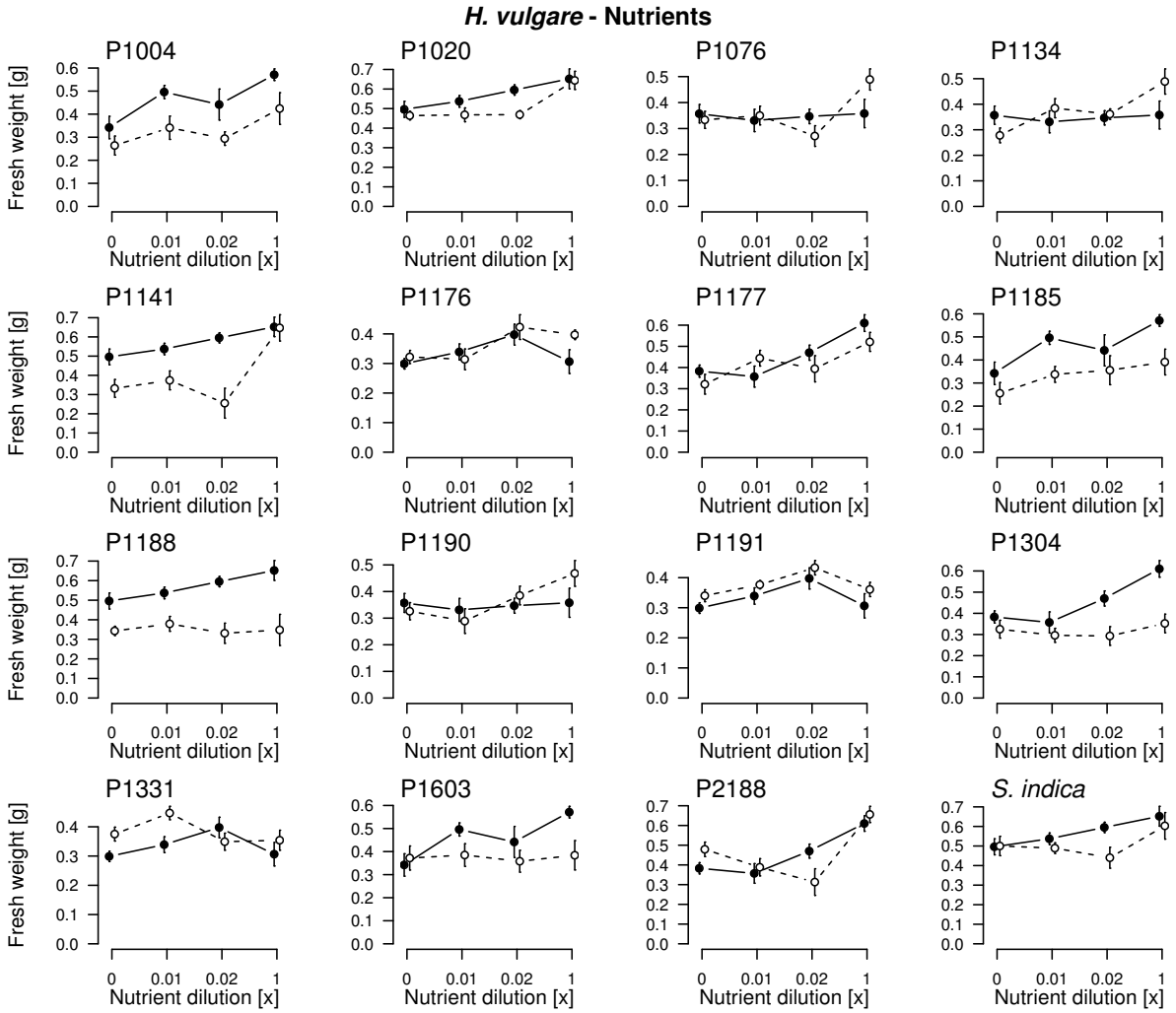
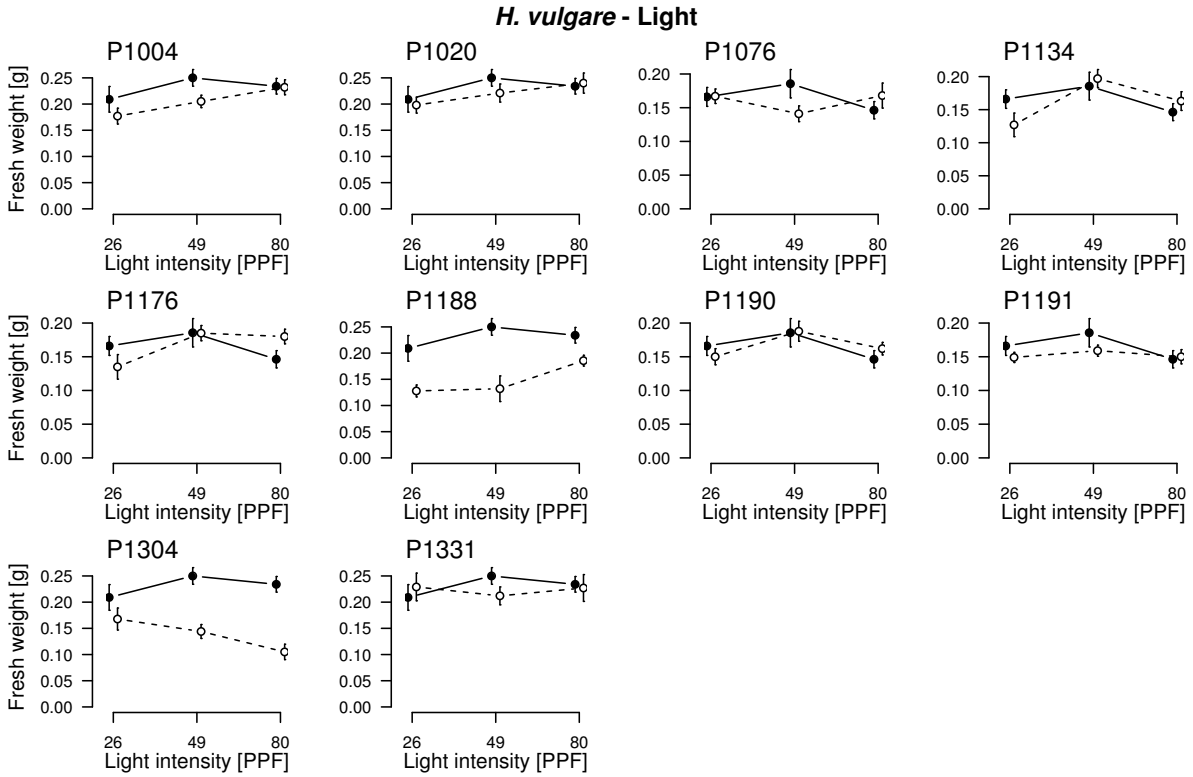
P1008
P1020
P1076
P1134
P1176
P1177
P1185
P1188
P1190
P1191
P1304
P1331
P2188
S. indica
Fresh weight [g]
0.00
0.01
0.02
0.03
0.04
5.7
6.5
7
7.5
pH

(f)


H. vulgare - Nutrients
P1004
P1020
P1076
P1134
P1141
P1176
P1177
P1185
P1188
P1190
P1191
P1304
P1331
P1603
P2188
S. indica
Fresh weight [g]
Nutrient dilution [x]
0
0.01
0.02
1

(g)


H. vulgare - Light
P1004
P1020
P1076
P1134
P1176
P1188
P1190
P1191
P1304
P1331
Fresh weight [g]
Light intensity [PPF]
26
49
80

(h)

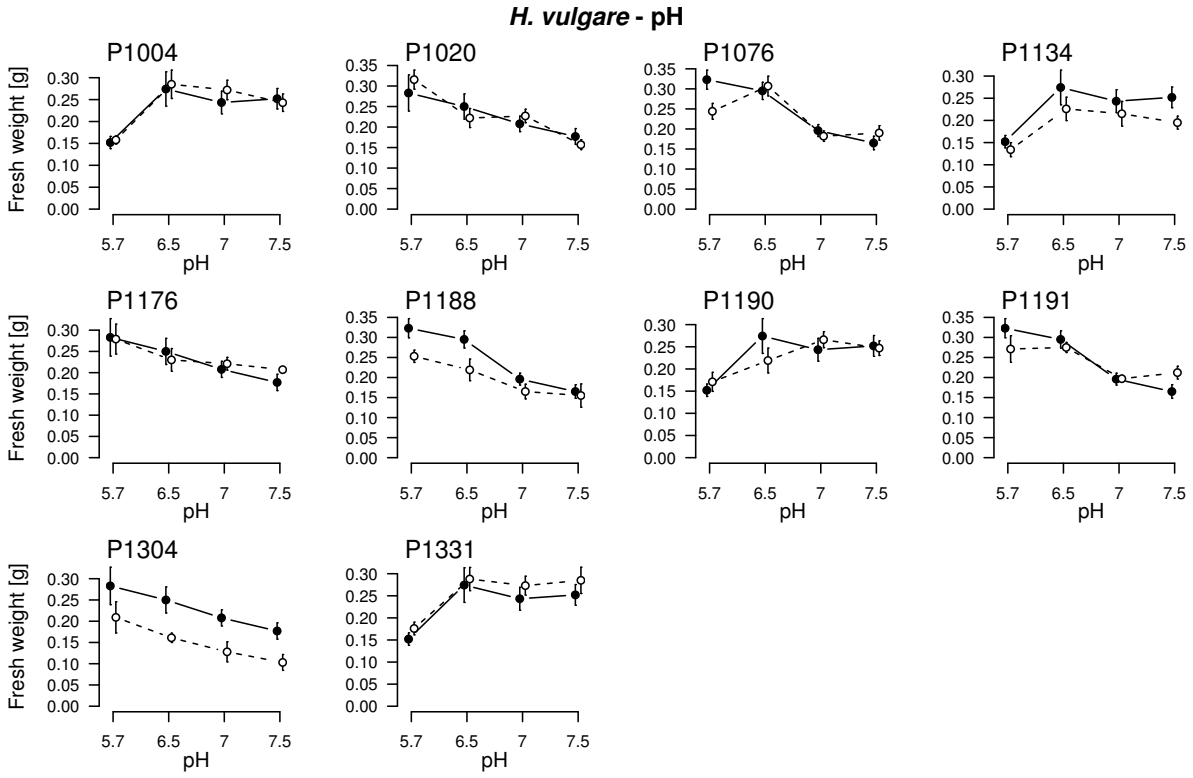

H. vulgare - pH
P1004
P1020
P1076
P1134
P1176
P1188
P1190
P1191
P1304
P1331
Fresh weight [g]
pH
5.7
6.5
7
7.5

Table S1. Description of the experimental designs to test interactions between fungal strains and the three host plants across abiotic conditions, including the number and description of the factor levels and experimental batches used in each experiment.

		Nutrients		Light		pH	
Plant species	Factors	Number	Description	Number	Description	Number	Description
A. thaliana	Levels[1]	4	0.10, 0.25, 0.50, 1.00 (x MS)	3	26, 49, 80 (µmol m^{-2} s^{-1})	4	5.7, 6.5, 7.0, 7.5 (pH)
	Batches[2]	2	11, 8 (n)	1	16 (n)	3	12, 5, 8 (n)
M. erraticum	Levels	4	0.10, 0.25, 0.50, 1.00 (x MS)	3	26, 49, 80 (µmol m^{-2} s^{-1})	4	5.7, 6.5, 7.0, 7.5 (pH)
	Batches	2	11, 8 (n)	1	16 (n)	1	16 (n)
H. vulgare	Levels	4	0.00, 0.01, 0.02, 1.00 (x Hoagland's)	3	26, 49, 80 (µmol m^{-2} s^{-1})	4	5.7, 6.5, 7.0, 7.5 (pH)
	Batches	4	4, 4, 5, 4, 4 (n)	2	6, 6 (n)	3	4, 4, 5 (n)

[1] Number and description of abiotic factor levels, including the units in parentheses.

[2] Number of experimental batches in which each experiment was split. In the description, the number of fungal treatments (including uninoculated controls) tested in each batch is shown.

Table S2. Statistical power of linear fixed-effects models used to analyze the variation of plant interactions with endophytic strains across conditions.

	Abiotic factor		
Plant species	Nutrients	Light	pH
A. thaliana	10.8	17	10.5
M. erraticum	10.9	16.6	14.8
H. vulgare	4.9	9.5	6.5

The values indicate the effect size (%) that each analysis can detect considering a power of 95% and $P < 0.05$.

Table S3. Summary statistics for the ANOVA of the effects of abiotic factors and fungal inoculation on *Hordeum vulgare*'s shoot and root fresh weight.

		Nutrients			Light			pH		
Variable	Effect	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
H. vulgare shoot weight	abiotic factor	1, 777	**429.8**	**< 0.001**	1, 329	0.5	0.5	1, 488	3.2	0.07
	fungus	16, 777	**5.8**	**< 0.001**	10, 329	**11**	**< 0.001**	10, 488	**5.6**	**< 0.001**
	experimental batch	4, 777	**6.8**	**< 0.001**	1, 329	**11.6**	**< 0.001**	2, 488	1.6	0.2
	abiotic factor x fungus	16, 777	**8.9**	**< 0.001**	10, 329	**2.8**	**0.002**	10, 488	**4.6**	**< 0.001**
H. vulgare root weight	abiotic factor	1, 777	**24.5**	**< 0.001**	1, 329	1.3	0.25	1, 488	**23**	**< 0.001**
	fungus	16, 777	**10.4**	**< 0.001**	10, 329	**9.3**	**< 0.001**	10, 488	**5.7**	**< 0.001**
	experimental batch	4, 777	**28.2**	**< 0.001**	1, 329	1.9	0.17	2, 488	1	0.36
	abiotic factor x fungus	16, 777	**2.5**	**0.001**	10, 329	**3.4**	**< 0.001**	10, 488	**11.4**	**< 0.001**

Significant values ($P < 0.05$) are shown in bold face.

6. Research article 4

Kia, S.H., Pallesch, S., Piepenbring, M. & Maciá-Vicente, J.G. (2018). Complementary niche occupancy within plant roots enables the coexistence of dominant fungal endophytes. *Submitted*

Anlage 4

Erklärung zu den Autorenanteilen an der Publikation / an dem Manuskript (Titel):

Complementary niche occupancy within plant roots enables the coexistence of dominant fungal endophytes

Status: *Submitted*

Name der Zeitschrift:

Beteiligte Autoren:
SHK: Sevda Haghi Kia
SP: Sascha Pallesch
MP: Meike Piepenbring
JGMV: Jose G. Maciá-Vicente

Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(1) zu Entwicklung und Planung

Promovierende SHK: 45%
Co-Autor MP: 5%
Co-Autor JGMV: 50%

(2) zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierende SHK: 60% molecular characterization of fungal strains, plant-fungus co-inoculation assay, plant DNA extraction, quantification of root colonization with q-PCR
Co-Autor SP: 5% plant-fungus co-inoculation assay
Co-Autor JGMV: 35% molecular characterization of fungal strains, strain specific primer design, quantification of root colonization with q-PCR

(3) zur Erstellung der Datensammlung und Abbildungen

Promovierende SHK: 60% collection of sequencing, bioassay and q-PCR data
Co-Autor SP: 5% collection of bioassay data
Co-Autor JGMV: 35% collection of sequencing and q-PCR data

(4) zur Analyse und Interpretation der Daten

Promovierende SHK: 45%
Co-Autor JGMV: 55%

(5) zum Verfassen des Manuskripts

Promovierende SHK: 50%
Co-Autoren MP, SP: 10%
Co-Autor JGMV: 40%

Zustimmende Bestätigungen der oben genannten Angaben:

13.6.2018
Wismar

Datum/Ort

Unterschrift Promovierende

12.6.2018
Frankfurt / Main

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Unterschrift Betreuerin

12.6.2018, Frankfurt a.M.

Datum/Ort

Ggfs. Unterschrift corresponding author

Complementary niche occupancy within plant roots enables the coexistence of dominant fungal endophytes

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Abstract

1. Roots are associated with fungal communities that affect plant growth and health. Individual root-associated fungi have different effects on plant performance, from detrimental to beneficial, but it is barely known how their inter-species interactions determine plant fitness.
2. Here, we evaluate *in planta* interactions among dominant root-colonizing fungi with different degrees of phylogenetic and trait similarity, and study the impact of their co-occurrence on their respective ability to colonize roots and their effects on plant growth.
3. An *in vitro* bioassay with *Arabidopsis thaliana* as host plant was used for the co-cultivation with individual or paired combinations of fungal strains. Root colonization by strains was monitored using real-time quantitative PCR, and the effects on host's growth were estimated by measuring plant biomass.
4. Strains had variable effects on plant growth upon root colonization, although these effects were slight and were little affected by the presence of other fungi. Abundance of each fungus in roots responded differently to co-inoculation, but competition between strains was not associated with their similarity in functional traits.

5. Our findings suggest that dominant fungal root endophytes avoid competition by occupying different niches within roots, which could explain the high diversity of fungi internally colonizing healthy hosts in natural conditions.

Keywords: competition, endophytes, fungi, microbiome, roots, symbiosis

Introduction

Root-associated fungi are important determinants of plant diversity and health (Van Der Heijden, Bardgett, & Van Straalen, 2008), and they are being increasingly recognized as promising tools for sustainable agriculture (Bender, Wagg, & van der Heijden, 2016; Berg, 2009). However, these fungi share their habitat and interact with a large diversity of microorganisms that influence their relationships with the host plant (Agler et al., 2016). Plant-fungus interactions can also be greatly affected by the abiotic environment and host plant identity, to the extent that mutualistic associations, like those formed by mycorrhizal fungi, may become parasitic depending on the environmental conditions (Argüello et al., 2016; Chamberlain, Bronstein, & Rudgers, 2014; Hoeksema et al., 2010; Klironomos, 2003). Therefore, making predictions about the outcome of the association between particular fungal symbionts and plants is challenging, as is already acknowledged in the case of microorganisms used for agricultural applications (Berg, 2009). Such predictions are especially difficult in the case of root symbionts with uncertain ecological functions like the endophytic fungi, which colonize the internal tissues of host plants without causing symptoms, but which also lack other evident effects on plant performance (Mandyam & Jumpponen, 2005; Mayerhofer, Kernaghan, & Harper, 2012). In spite of their cryptic symbiotic functions, root endophytes likely are key determinants of the structure and role of root microbiomes, since they are prevalent and often dominate fungal communities inside plant roots (Bonito et al., 2014; Glynou, Nam, Thines, & Maciá-Vicente, 2017; Vandenkoornhuyse, Baldauf, Leyval, Straczek, & Young, 2002). Many root endophytic

communities from healthy wild plants are often dominated by fungi phylogenetically related to well-known crop pathogens (Glynou et al., 2016; Malcolm, Kuldau, Gugino, & Jiménez-Gasco, 2013), suggesting that they may have detrimental effects on hosts under conditions that limit diversity and competition within root microbiomes (Duhamel & Vandenkoornhuyse, 2013). Gaining insight into how the biotic and abiotic contexts influence plant-fungus interactions will further our understanding of the role of root-associated fungi in natural ecosystems and their implementation in sustainable agricultural production.

Non-mycorrhizal Brassicaceae, and in particular *Arabidopsis thaliana*, are suitable models to study plant-microbe interactions because they are easy to grow *in vitro*, numerous methodological resources are available, and they are economically important (Bulgarelli et al., 2012; Lundberg et al., 2012; Wagner et al., 2016). In the case of root-associated fungi, they offer the additional benefit of lacking classical mycorrhizas while being able to establish mutualistic associations with non-mycorrhizal endophytes (Almario et al., 2017; Fesel & Zuccaro, 2016; Hiruma et al., 2016), allowing for focused research on these fungi under field conditions. Recent samplings of fungal root endophytes of non-mycorrhizal Brassicaceae show a consistent and widespread dominance of fungi belonging to Hypocreales, Pleosporales and Helotiales (Glynou, Ali, Kia, Thines, & Maciá-Vicente, 2017; Glynou, Nam, et al., 2017). Species in these orders are usually among the most abundant fungi within roots, and they frequently coexist in endophytic communities regardless of geographical or environmental constrains (Glynou, Ali, et al., 2017). An *in vitro* characterization of isolates representative of these groups showed that they have differential effects on growth of various host species, and described complementary sets of functional traits that suggest different niche preferences (Kia et al., 2017; Fig. 1). Yet, the degree of niche overlap or complementarity among these groups remains unknown. Assessing how these fungi interact in plant roots may help determine their niche breadths and the relative importance of competition vs. facilitation processes in endophytic community assembly.

Kia and colleagues (2017) described different sets of traits for the main groups of fungal endophytes and the link of these traits with the endophytes' effects on plant growth. Among the most frequent fungi within the orders mentioned above, species of *Fusarium* (Hypocreales) showed rapid mycelial growth and frequent conidiation, and mainly had negative but highly variable effects on plant growth. In contrast, helotialean endophytes phylogenetically related to *Cadophora* had typical dark septate endophyte (DSE) traits, including slow-growing sterile mycelium and the production of intra-radical microsclerotia (Jumpponen & Trappe, 1998), and had consistent neutral effects on plant growth. *Alternaria* spp. (Pleosporales) and related species displayed intermediate sets of traits, with dark and relatively fast-growing mycelium, sporadic conidiation, and variable effects on plant growth. We hypothesized that fungal endophytes with distinct traits can colonize and persist in plant roots without affecting one another, while fungi with high similarity of functional traits likely compete for the same resources within roots. According to this, it can be expected that strongest competition occurs between strains within the same order, whereas strains of *Fusarium* spp. and helotialean DSE may not affect each other. *Alternaria* spp., given their intermediate sets of traits, would likely have a certain degree of competition with both *Fusarium* spp. and the Helotiales fungi (Fig. 1d).

Here, we aimed to evaluate *in planta* interactions among fungi dominant in root endophytic communities and how these interactions affect host's health. We considered that neutral interactions between fungi in roots do not affect each other's abundance (i.e., their extent of root colonization). On the other hand, interacting fungi may show changes in their abundance, with an increase in the case of facilitation, and a decrease in case of competition between species. In this study, we used an *in vitro* system based on the model plant *A. thaliana* as host to track the root colonization by endophytic fungi, either alone or in combination with other fungi, and to assess their effects on plant growth.

Materials and Methods

Fungal strains and plant material

Fungal strains used in this study were obtained as root endophytes from individuals of the brassicaceous plant *Microthlaspi* spp. growing in different European locations (Glynou et al., 2016). The fungi were selected from a large collection of strains according to their phylogenetic affiliation, as well as to their differential traits and effects on plant growth (Kia et al., 2017; Tables 1 and S1, Fig. 1). Six of the strains represent pairs of phylogenetically related fungi from the three dominant orders in fungal communities associated with roots of *Microthlaspi* spp.: two strains pertaining to the *Fusarium tricinctum* species complex (*F. tricinctum* onwards), two *Alternaria tellustris* strains, and two unidentified Helotiales strains. In addition, one strain in the *Fusarium oxysporum* species complex (*F. oxysporum* onwards) was selected for assays involving pairwise inoculations of roots with fungal strains (see *Quantification of fungal root colonization*). Strains were maintained as cultures on corn meal agar (CMA, Sigma-Aldrich, St. Louis, MO, USA) and used for plant inoculation when they were 2–3 weeks old. They were grown on malt extract agar (MEA, Applichem, Darmstadt, Germany) and potato dextrose agar (PDA, Applichem) for morphological characterizations.

Arabidopsis thaliana ecotype Col-0 was used as host plant in inoculation assays. For plant propagation, seeds were surface-sterilized by washings with 70% (v/v) ethanol followed by 2% (v/v) sodium hypochlorite, and then plated on half strength Murashige-Skoog medium; (Murashige & Skoog, 1962; Ref. M5519, Sigma-Aldrich). Seeds were stratified for two days at 4°C in the dark and later incubated for seven days at 23°C under a 12 h:12 h (light:dark, 80 µmol m^{-1} s^{-1}) photoperiod, until they developed the first true leaves.

Molecular characterization of strains

Data on morphological and physiological traits of the strains in this study, as well as their effects on the growth of different plant species, have been previously obtained (Kia et al., 2017) and are provided here in Table S1. Sequences of the rDNA internal transcribed spacers (ITS) and the gene for the translation elongation factor 1α (*TEF-1α*) of some of the strains were already available in GenBank (Glynou et al., 2016; Glynou, Ali, et al., 2017; Table 1). Additionally, we obtained sequences from the rDNA large small subunit (LSU) using the primers pair LR0R/LR7 (Hopple & Vilgalys, 1994), and from the *TEF-1α* of *Fusarium* strains with primers ef1/ef2 (Geiser et al., 2004).

Phylogenetic relationships among strains within each fungal order were investigated using maximum likelihood (ML) phylogenetic inference. In each case, a selection of available representative sequences of fungal species closest to the strains in BLAST searches were retrieved from GenBank. Sequences were aligned using the G-INS-i option of MAFFT v7.123b (Katoh & Standley, 2013) and trimmed with Gblocks v0.91b (Castresana, 2000). ML phylogenies were built using RAxML v8.2.10 (Stamatakis, 2014) with 1000 bootstrap replicates and the GTRGAMMA model of nucleotide substitution and rate heterogeneity. In concatenated alignments, independent ML estimates were allowed for each sequence partition.

Root inoculation assay with individual strains

The co-cultivation of plants with individual fungal strains was done in an axenic growth system slightly modified from the method described by (Schedel, Camehl, & Oelmüller, 2012). In brief, Magenta™ vessels (GA-7-3, Sigma-Aldrich) were filled with 150 ml of Seramis® clay granules (Seramis GmbH, Mohendorf, Germany) and autoclaved for 20 min at 121°C. The sterilized clay was then hydrated with 30 ml of half strength Hoagland's nutrient solution (Ref. H2395, Sigma-Aldrich). One *A. thaliana* plantlet was planted per

vessel, and plants were maintained at 23 °C under a 12 h:12 h (light:dark, 80 μmol m^{-1} s^{-1}) photoperiod for 10 days. Experimental treatments consisted of un-inoculated control plants, and plants inoculated with each of strains P1304, P2190, P1191, P1555, P1176, and P1331. Each treatment comprised five replicates, each consisting of an independent vessel with an (un-)inoculated plant. Fungal strains were inoculated in roots by burying a 5-mm-diameter CMA plug taken from the margin of a colony next to the plant's crown. Un-inoculated control treatments received an un-colonized CMA plug. After the inoculation, plants were let grow for an additional month under the previously described conditions, with weekly watering with 20 ml of half strength Hoagland's solution. After that time, the fresh and dry weights of the aerial plant parts were measured. Roots from each plant were collected and independently processed for assessments of fungal root colonization using cultivation and species-specific real-time quantitative PCR (qPCR).

Pairwise inoculation assay

Co-cultivation assays of *A. thaliana* with pairs of different fungal strains were performed as described above, but each plant was inoculated with two CMA plugs, each from a different colony. Un-inoculated control treatments received two un-inoculated CMA plugs. Additionally, treatments with individual inoculations with each strain were also performed, and received one un-colonized and one colonized CMA plug.

In this experiment we included *F. oxysporum* P1141 in order to explore interactions between *Fusarium* strains in the same root system, because this allowed us to track each individual's abundance using species-specific primers in qPCR. For pairwise combination of strains, we selected both strains of *F. tricinctum*, and one strain from each of the other species. In the later cases, we selected the strain with the strongest effect on plant growth as compared to the un-inoculated control plants in the individual co-cultivation assay. We considered that this would allow to better detect potential changes in plants' growth due to

the endophytic interactions between strains. Pairwise inoculations of roots were done so that each representative strain was combined with all representative strains from other species (Fig. 1d).

This experiment was performed in triplicate, with each repetition performed at a different dates. As in the experiment with individual strains, each repetition comprised five replicates per treatment for a final sample size of 15. Biomass measurements and cultivation-based assessments of root colonization were taken independently for all plants, as described above. Non-independence of measurements owing to observations clustered by repetitions was taken into account during the data analysis by using linear mixed-effects models. qPCR quantifications of fungal root colonization were only performed on all plants included in the first repetition of the experiment. For each of the strains tested alone and pairwise, abundance in roots was quantified using species-specific primers in all plants where the fungus had been inoculated, plus in the non-inoculated control plants.

Quantification of fungal root colonization

We assessed fungal colonization of *A. thaliana* roots both by cultivation and qPCR methods. In the first case, a 10-cm-length root sample from each plant was disrupted in 0.5 ml of 0.1% (w/v) agar solution, using steel beads in a Retsch MM 200 mixer mill (Retsch GmbH, Haan, Germany). 200 µl of the root suspension were plated on CMA supplemented with 25 mg ml^{-1} chloramphenicol, 50 mg ml^{-1} streptomycin and 0.1% (v/v) Triton X-100, and incubated for seven days at 25°C. Colony forming units (CFUs) were then counted and reported as CFUs cm^{-1} of root length. Remaining root material from each plant was stored at -20°C until processing for qPCR analyses.

For qPCR quantification of fungal root colonization, root samples were disrupted in 1.5 ml tubes with conical pestles, aided by sterilized washed quartz sand. Total genomic DNA from roots was extracted using the DNeasy® plant mini kit (QIAGEN, Hilden,

Germany), quantified with a Nanodrop 2000 (NanoDrop, Wilmington, USA) and brought to a concentration of 2 ng μl^{-1}.

Fungal DNA in each extract was quantified with species-specific primers in qPCR reactions (Table 2). The primers were retrieved from literature or designed with the primer designing tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). We attempted to design strain-specific primers in order to differentiate strains from the same species within samples. However, this was not possible and hence we did not combine conspecific strains. Instead, we included strain *F. oxysporum* P1141 in pairwise inoculation experiments with other fusaria, to test effects between phylogenetically related endophytes. In addition to fungal primers, we detected *A. thaliana* DNA with plant-specific primers to normalize the amount of fungal DNA across samples (Table 2). The specificity of primers for each set of strains was tested by conventional PCR using genomic DNA extracted from pure cultures of different fungi (Fig. S1a). In addition, to validate the specificity of the primer sets for the pairwise inoculation assays, we tested them by qPCR using DNA extracts from roots including target and non-target fungi (Fig. S1b,c).

qPCR amplifications were performed in 20 µl of reactions containing 10 µl of 2x SensiFAST SYBR® Hi-ROX master mix (BIOLINE, Luckenwalde, Germany), 1 µl of each primer at 10 µM, and 2 µl of DNA template. Each sample was included in two independent reactions to detect fungal and plant DNA, respectively, and each reaction was performed in duplicate. Thermal cycles and fluorescence detection were performed in a PeqSTAR 96Q real-time cycler (PEQLAB/VWR, Darmstadt, Germany) with the following conditions: 95°C for 3 min, 40 cycles of 95°C for 5 s, 65°C for 10 s and 72°C for 20 s, followed by a melting profile analysis of amplicons. Calibration curves for each combination of primer pairs and strain or host plant were included in every qPCR run. For constructing the curves, genomic DNA from each organism was quantified with a Qubit flourometer (Thermo Fisher Scientific, Waltham, MA, USA) and used in final amounts of 10, 1, 0.1 and 0.01 ng of total DNA. The curves were used to estimate the total amount of DNA from each strain per sample, and to

refer it to the total amount of plant DNA. Moreover, the slope of each calibration curve was used to calculate amplification efficiency.

Statistical analyses

Statistical analyses were performed using R v3.3.3 (R Core Team, 2016). For each independent experiment, plant biomass and root colonization data were first assessed for normality and homoscedasticity using diagnostic plots and with the Shapiro-Wilk and Levene's tests. Differences between treatments were then compared using one-way analysis of variance (ANOVA), the Kruskal-Wallis rank sum test, or the Wilcoxon rank sum test.

Plant biomass data obtained from the three repetitions of the pairwise inoculation assay were jointly analyzed using a linear mixed-effects model, to account for the non-independence of observations across dates. The model was built using function *lmer* of the R package lme4 v1.1–17 (Bates, Mächler, Bolker, & Walker, 2014), by including square-root-transformed (to fulfill model assumptions) plant biomass as independent variable, fungal treatment as the explanatory fixed-effect, and random intercept and slope and intercepts for the effects of experiment repetition on fungal treatment. Significance of the fungal treatments effect was obtained by the likelihood ratio test of the full model against a reduced model without the fixed effect.

Results

Phylogenetic relationships among strains

Sequences from different loci were compared among strains of *Fusarium*, *Alternaria* or the unidentified Helotiales. Strain P1141 belongs to the *Fusarium oxysporum* species complex, while the other two *Fusarium* strains clustered within the *Fusarium tricinctum* species

complex (Fig. 1a). However, the two latter strains clustered with separate species within the complex, and showed clearly dissimilar colony morphologies (Fig. 1a). The *Alternaria* strains P1555 and P1191 are very similar according to all loci sequences and present similar colony morphologies (Fig. 1b). The helotialean strains P1331 and P1176 had similar colony morphologies but belong to different although related clades. These strains could not be assigned to a species due to the lack of related representative sequences in public databases (Fig. 1c).

Interactions of A. thaliana *with individual fungal strains*

Inoculation of fungal strains in *A. thaliana* roots mostly did not have strong effects on plant growth except for *F. tricinctum* P1304, which stunted the host plants (Fig. 2a,c). Overall significant differences were found in above-ground plant biomass across treatment ($F_{6,28}$ = 5.8, $P < 0.001$). The effects on plant fresh biomass varied between strains of *Fusarium* and *Alternaria*, but not between the two Helotiales. Whereas both fusaria reduced plant growth, differences between strains were most clear by their effect sizes respect to un-inoculated controls. In *Alternaria*, strain P1191 slightly increased plant biomass, while P1555 had no evident effect on growth (Fig. 2). The observed effects on plant growth were maintained when plant dry weight was measured (Fig. S2a).

Colonization of all root samples by the respective fungi in all treatments, or lack thereof in the un-inoculated controls, was confirmed by cultivation (Fig. S2b). In this case, strain P1304 showed a particularly high number of CFUs per unit of root length. All fungi were also detected by qPCR with specific primers (Fig. 2b). All calibration curves had R^2 above 0.97, and in most cases the slopes in standard curves indicated a high amplification efficiency (Table 2). Some degree of amplification with fungal primers was detected in several un-inoculated control plants, but this occurred in the last 2–3 qPCR cycles and always accounted for quantifications several orders of magnitude below that of plants

inoculated with fungi, and melting curves indicated that amplicons from control plants corresponded to unspecific PCR products.

Values of fungal colonization as ratios of fungal to plant DNA ranged from $4.3 \cdot 10^{-4}$ to 0.46, with only two inoculated plant replicates in which no fungus was detected (one for each P1304 and P1176). Colonization values assessed by qPCR are only comparable between samples amplified with the same set of primers. No significant differences in root colonization were observed for any of the strain pairs within each genus (W = 9–21, P > 0.05; Fig. 2b).

Interactions between fungal strains in A. thaliana *roots*

Plants inoculated with individual fungal strains and with pairwise combinations of them showed a wide variation in above-ground biomass across treatments (Fig. 3). Whereas results obtained in the three repetitions of the experiment varied notably for some treatments, no overall significant differences were obtained across repetitions when these were considered as a fixed effect alongside fungal treatment in a two-way ANOVA ($F_{2,235}$ = 0.55, P = 0.58). A linear mixed-effects model in which variation among repetitions was accounted for did not show a significant effect of fungal treatments on plant weight in a likelihood ratio test (X^2 = 17.6, df = 16, P = 0.35). Nevertheless, the variation pattern for individually inoculated strains mirrored that obtained in the previous experiment: plants inoculated with P2190, P1191 and P1176 tended to weight more than those inoculated with P1304, P1555 and P1331 for *F. tricinctum*, *A. tellustris* and the helotialean fungi, respectively (Figs. 2a and 3a). *Fusarium oxysporum* P1141, which was not included in the first experiment, had a consistent detrimental effect on the host. Growth of plants inoculated with pairs of strains appeared to be less variable through treatments, although plants co-inoculated with *F. tricinctum* P1304 and either of *F. oxysporum* P1141 or *A. tellustris* P1555 showed relatively consistent detriment in their growth (Fig. 3).

Colonization of *A. thaliana* roots by different strains was confirmed in all cases by cultivation. In this case, CFUs were not quantified because it was difficult to differentiate the strains in mixed inoculation treatments. Using qPCR, we did not observe large differences in abundance of individual strains in roots from the first experiment repetition when they were either alone or in combination with other strains (Fig. 4). The only exception was *A. tellustris* P1555, for which a significant reduction of its abundance was observed when mixed with all other strains ($F_{4,20}$ = 8.8, P < 0.001). In both *F. tricinctum* strains there was an apparent decrease in their abundance when in combination with *F. oxysporum* P1141, although these changes were not significant (P1304: X^2 = 3.7, df = 3, P = 0.3; P2190: X^2 = 7.2, df = 3, P = 0.06). *Fusarium oxysporum* P1141 abundance was rather constant, although it decreased when in combination with *A. tellustris* P1555 (X^2 = 10.5, df = 3, P = 0.03). Abundance of strain P1331 was constant across treatments (X^2 = 2.1, df = 3, P = 0.71).

Discussion

We have used a bioassay with the model plant *A. thaliana* as a host to test the effects of fungal root endophytes on plant growth and to evaluate the root colonization dynamics of different fungi. The assays included the assessment of within-root interactions between fungi with different degrees of phylogenetic relatedness and trait similarity. All tested fungi colonized the roots and had variable effects on plant biomass, which became less variable upon co-inoculation with different strains. Root colonization by each fungus had a differential response to the presence of other strains, although in most cases co-occurrence did not imply marked changes in their abundance. Our findings suggest that interspecific interactions between dominant root-colonizing fungi may be important determinants of their effects on plant fitness whereas having a limited effect on their assembly within endophytic communities.

Contrarily to what we hypothesized, root-colonizing fungi with similar traits—suggestive of a similar niche occupancy—did not interact more strongly with each other than with fungi less alike. Phylogenetically close species tend to share ecological preferences and exploit similar resources, hence they are expected to compete more intensely (Cavender-Bares, Kozak, Fine, & Kembel, 2009; Miller, Farine, & Trisos, 2017). We hypothesized that *Fusarium* strains would affect each other's establishment in roots while having little interaction with the Helotiales fungi, given their respective trait resemblance or dissimilarity (Kia et al., 2017). *Alternaria tellustris* would have an intermediate response to coexistence with these groups due to its partial sharing of traits with either of them (Kia et al., 2017).

In our tests, *A. tellustris* appeared to be the worst competitor against all fungi, since its degree of root colonization decreased when confronted with all other fungi. *Alternaria* spp. species are frequent endophytes in plant roots (Glynou et al., 2016; J. G. Maciá-Vicente et al., 2008), but they are also common as saprotrophs in other substrata (Thomma, 2003) and as airborne fungi (Skjøth et al., 2016). Glynou and colleagues (2017) found that root-endophytic *A. tellustris* have a low genotypic diversity across broad spatial areas, suggesting an efficient ability to disperse and colonize diverse environments. It is possible that in this species there is a trade-off between a pioneer and a competitive life history, so that in roots it becomes easily displaced by more specialized root colonizers (Chagnon, Bradley, Maherali, & Klironomos, 2013). Contrarily to *A. tellustris*, abundance of the Helotiales fungus was indifferent to the presence of other species. *Cadophora* and related taxa appear to be specialized root colonizers showing typical DSE traits (Rodriguez, White Jr, Arnold, & Redman, 2009), as well as features potentially indicative of niche specialization such as slow growth and lack of spores (Chagnon et al., 2013; Mandyam & Jumpponen, 2005). They have specific adaptations for root symbiosis like microsclerotia (Kia et al., 2017), and related fungi have shown to be ericoid mycorrhizas (Leopold, 2016) and to translocate soil nutrients to the host (Almario et al., 2017). The helotialean DSE could likely avoid competition with other endophytes by occupying a unique niche within roots, either by colonizing particular

compartments or by using specific plant resources unavailable for other species. Fusaria were in general little responsive to other genera, and interspecies interactions within the genus were variable. *Fusarium* spp. usually have life history traits that may provide competitive advantages, such as a fast mycelial growth and profuse conidiation (Kia et al., 2017), which could explain their frequent and abundant occurrence in natural vegetation worldwide (Glynou et al., 2016; Knapp, Pintye, & Kovács, 2012; Lofgren et al., 2018; J. G. Maciá-Vicente et al., 2008; J. G. Maciá-Vicente, Ferraro, Burruano, & Lopez-Llorca, 2012).

Interactions between plant-associated microbes can be driven by several processes, including competition for space and/or nutrients (Mitri & Foster, 2013), production of antimicrobials (Kusari, Hertweck, & Spiteller, 2012), ability to invade plant tissues (Compant, Clément, & Sessitsch, 2010; Knapp & Kovács, 2016), direct parasitism/predation (Brenner, You, & Arnold, 2008), or efficiency in obtaining host nutrients (Kiers et al., 2011). Our results do not enable to specify the interaction processes involved in our assays, although we can probably exclude a direct parasitism since none of the species are known mycoparasites, and they have not shown mutual exclusions in *in vitro* tests. The three fungal lineages studied here have differential substrate utilization profiles, with helotialean DSE related to *Cadophora* sp. showing the highest number of active enzymes, which could support their utilization of exclusive nutrients (Kia et al., 2017; Knapp & Kovács, 2016). The assessment of a complementary compartment occupation would require applying microscopy with species-specific labelling markers. Regardless of the outcome of interspecies interactions in roots, all endophyte species always coexisted, indicating that they are not complete competitors (Hardin, 1960). Even if the inferior competitor becomes displaced or deprived from particular root resources, there may be specific microhabitats or niches still available, or that are more efficiently exploited by this fungus (Bennett & Bever, 2009; Wardle, Parkinson, & Waller, 1993).

The fungi tested had variable effects on plant growth, both when considering strains from close or distant lineages, following previously described trends (Kia et al., 2017).

However, we also found a strong variation in the effects across repetitions of the experiments, particularly in the magnitude of detrimental effects by *Fusarium* and *Alternaria* strains. Unexpectedly, simplified laboratory studies such as the one here have shown to deliver more variable results than greenhouse or field studies (Chamberlain et al., 2014). Given the simplicity of the bioassay, factors that are difficult to be controlled—such as the physiological status of the inoculum or plant, or small heterogeneities in the cultivation substrate—may reflect in a great variation in the effects' magnitude. Combination of fungal strains in the same root systems tended to reduce the variation of the effects on plant biomass, likely due to the mitigation of one fungus' effect by the other. This mostly consisted in a reduction of detrimental effects on plant growth, as has been previously reported in dual fungal inoculation tests in plant roots (Aimé, Alabouvette, Steinberg, & Olivain, 2013; Fravel, Olivain, & Alabouvette, 2003; J. g. Maciá-Vicente, Rosso, Ciancio, Jansson, & Lopez-Llorca, 2009; Olivain & Alabouvette, 1997; Reininger & Sieber, 2013). Besides competition for space or resources, mitigation of detrimental effects could result from a priming of the plants' innate immunity towards microbial infections (Hacquard, Spaepen, Garrido-Oter, & Schulze-Lefert, 2017). Such attenuation of pathogenic effects on plant growth could explain the frequent occurrence of well-known fungal pathogens in root microbiomes of healthy wild plants (Glynou et al., 2016; Malcolm et al., 2013).

Conclusions

In conclusion, our results suggest that interspecies interactions between endophytes that frequently dominate and coexist in root-associated fungal communities of natural vegetation may influence microbiome impacts on plant fitness, even though their effects on community assembly remain uncertain. Further assessment of priority effects (Cline & Zak, 2015) and the differential exploitation of root compartments and/or nutrients is necessary to better understand the importance of microbe-microbe interactions in the plants' microbiome structure and function.

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Authors' contributions

SHK and JGMV conceived the ideas and designed methodology; SHK, SP and JGMV collected the data; SHK and JGMV analyzed the data; SHK and JGMV led the writing of the manuscript; all authors contributed critically to the drafts and gave final approval for publication.

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Table 1. Proposed classification, GenBank accessions and source information of strains included in this study.

Strain	Proposed classification	Order	Source		GenBank sequence accessions [a]		
			Country	Host plant	ITS	*LSU*	*TEF-1α*
P1304	*Fusarium tricinctum* species complex	Hypocreales	Spain	*M. perfoliatum*	KT268599	**MG570087**	**MG570084**
P2190	*Fusarium tricinctum* species complex	Hypocreales	Greece	*M. perfoliatum*	KT269453	**MG570088**	**MG570085**
P1141	*Fusarium oxysporum* species complex	Hypocreales	France	*M. erraticum*	KT268459	**MG570089**	**MG570086**
P1555	*Alternaria tellustris*	Pleosporales	Bulgaria	*M. erraticum*	KT268849	**MG570090**	KX361676
P1191	*Alternaria tellustris*	Pleosporales	Croatia	*M. erraticum*	KT268507	**MG570091**	KX361659
P1331	unidentified Helotiales	Helotiales	Spain	*M. perfoliatum*	KT268626	**-**	-
P1176	unidentified Helotiales	Helotiales	Croatia	*M. perfoliatum*	KT268493	**-**	-

[a] GenBank accession numbers in bold face correspond to sequences obtained in this study.

Table 2. Characteristics of quantitative PCR primers used for quantifying targeted strains in plant tissue.

		Primer pairs					
Organism	Strains	Name	Sequence (5'– 3')	Target region	Amplicon length (bp)	Amplification efficiency (%)	Reference
Fusarium tricinctum	P1304, P2190	tri1	CGTGTCCCTCTGTACAGCTTTGA				
		tri2	GTGGTTACCTCCCGATACTCTA	IGS [a]	215	94 ± 12	Kulik (2008)
Fusarium oxysporum	P1141	FOF1	ACATACCACTTGTTGCCTCG				
		FOR1	CGCCAATCAATTTGAGGAACG	ITS [b]	340	87 ± 0	Mishra, Fox, & Culham (2003)
Alternaria tellustris	P1555, P1191	OTU002-1F	AACGCAGCGAAATGCGATAC				
		OTU002-1R	ACCAAGCAAAGCTTGAGGGT	ITS	138	89 ± 10	This study
Helotiales	P1331, P1176	OTU006-3F	AAGCTCGGTCCTGAACTCC				
		OTU006-3R	TTTCGCTGCGTTCTTCATCG	ITS	199	99 ± 10	This study
Arabidopsis thaliana	Ecotype Col-0	AtUBQ5-F	CCAAGCCGAAGAAGATCAAG				
		AtUBQ5-R	ACTCCTTCCTCAAACGCTGA	Ubiquitin	157	105 ± 30	Wang et al. (2014)

[a] rDNA intergenic spacer.
[b] rDNA internal transcribed spacers.

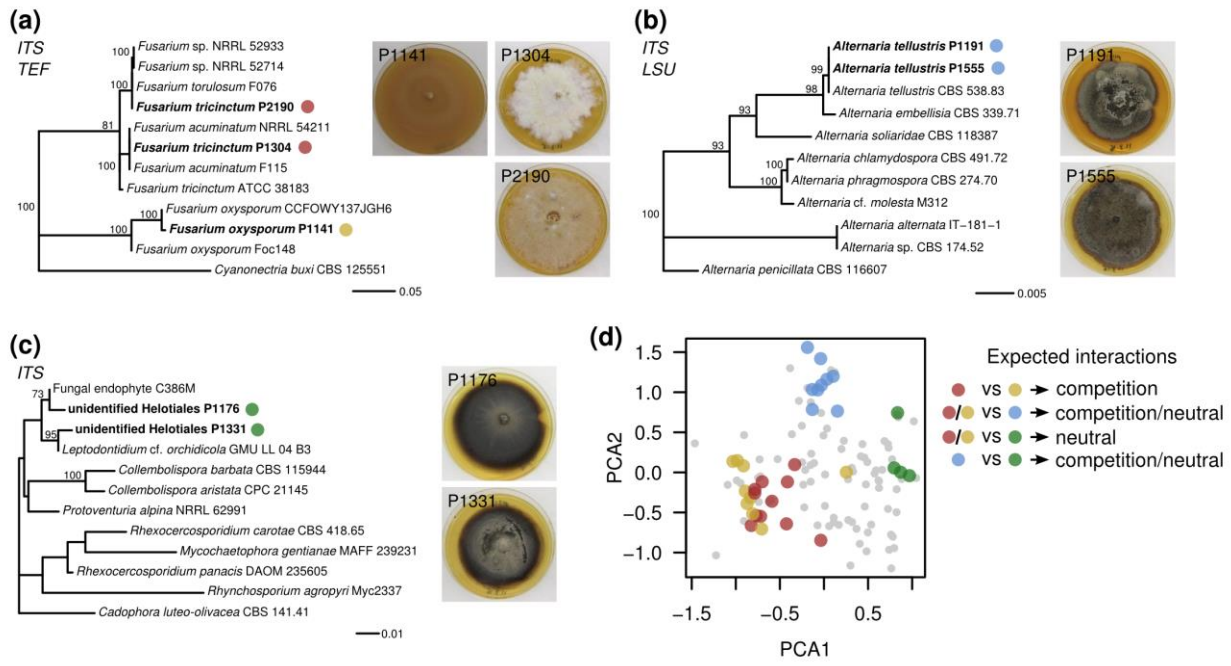

Figure 1. Phylogenetic relations and traits of strains in this study. Trees in **a**–**c** represent maximum likelihood phylogenies for strains within each fungal order, based on different combinations of loci. Trees for *Fusarium* spp. (**a**) and *Alternaria* spp. (**b**) are based on concatenated sequence alignments, while that for helotialean strains (**c**) is based on single ITS alignment. Pictures show colonies of each fungus on malt extract agar after one month of growth. Plot in **d** shows a principal components analysis ordination of several fungal strains including the ones in this study, according to their morphological and physiological traits (see Kia et al., 2017). Colored points correspond to strains belonging to the same phylotypes as the fungi included in this study (see color keys in trees), whereas gray points represent other fungi from different lineages. The diagram next to the plot in **d** summarizes the *in planta* combinations of strains tested in this study and the expected outcomes of their interactions. Plot in **d** modified from Figure 1b in Kia et al. (2017), reproduced with permission from Springer-Nature.

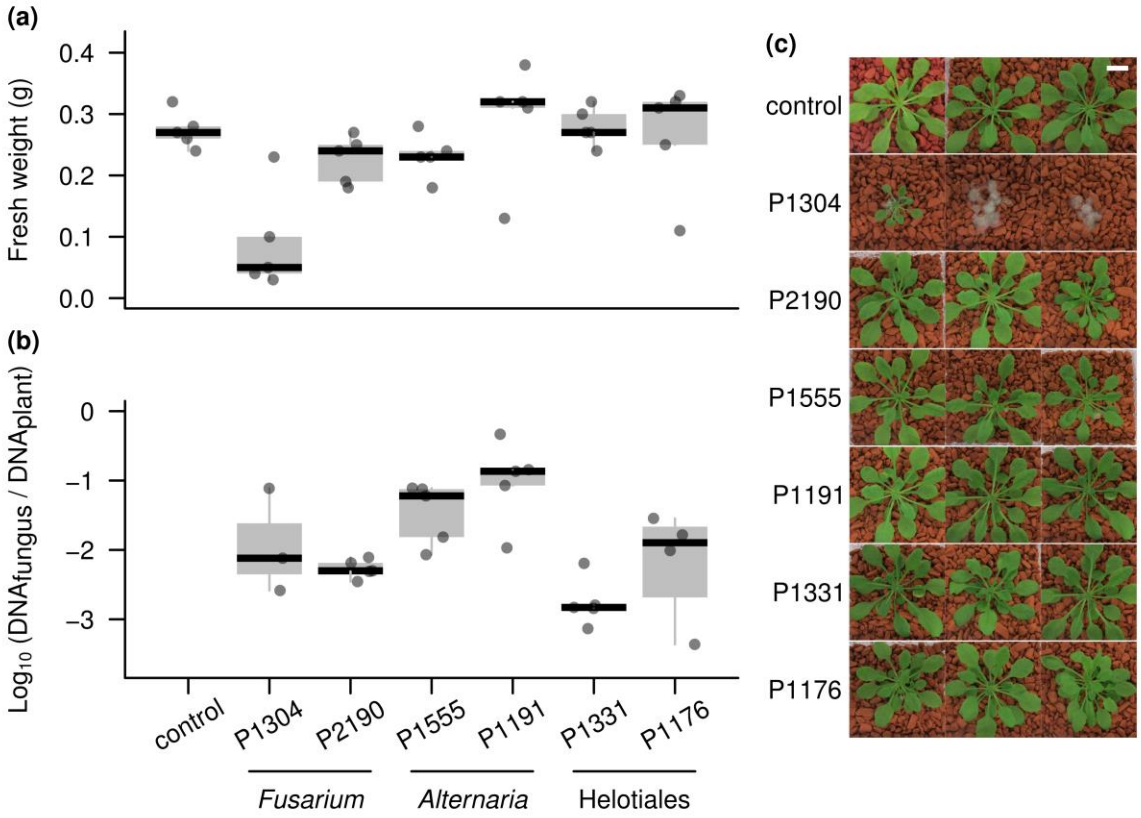

Figure 2. Interactions of fungal strains and *Arabidopsis thaliana*. **a**, Box-and-whisker plot representing the median and interquartile range of the fresh weight of aerial parts of *A. thaliana* from each treatment. Points indicate the individual values (*n* = 5). Values extending further than 1.5 times above or below the boxes are considered outliers. **c**, Box-and-whisker plot showing the root colonization of *A. thaliana* by each strain, as quantified by quantitative PCR. Values represent the logarithm of the ratio between fungal and plant DNA. **c**, Pictures of three representative *A. thaliana* plants from each treatment before sampling. Bar size: 1 cm.

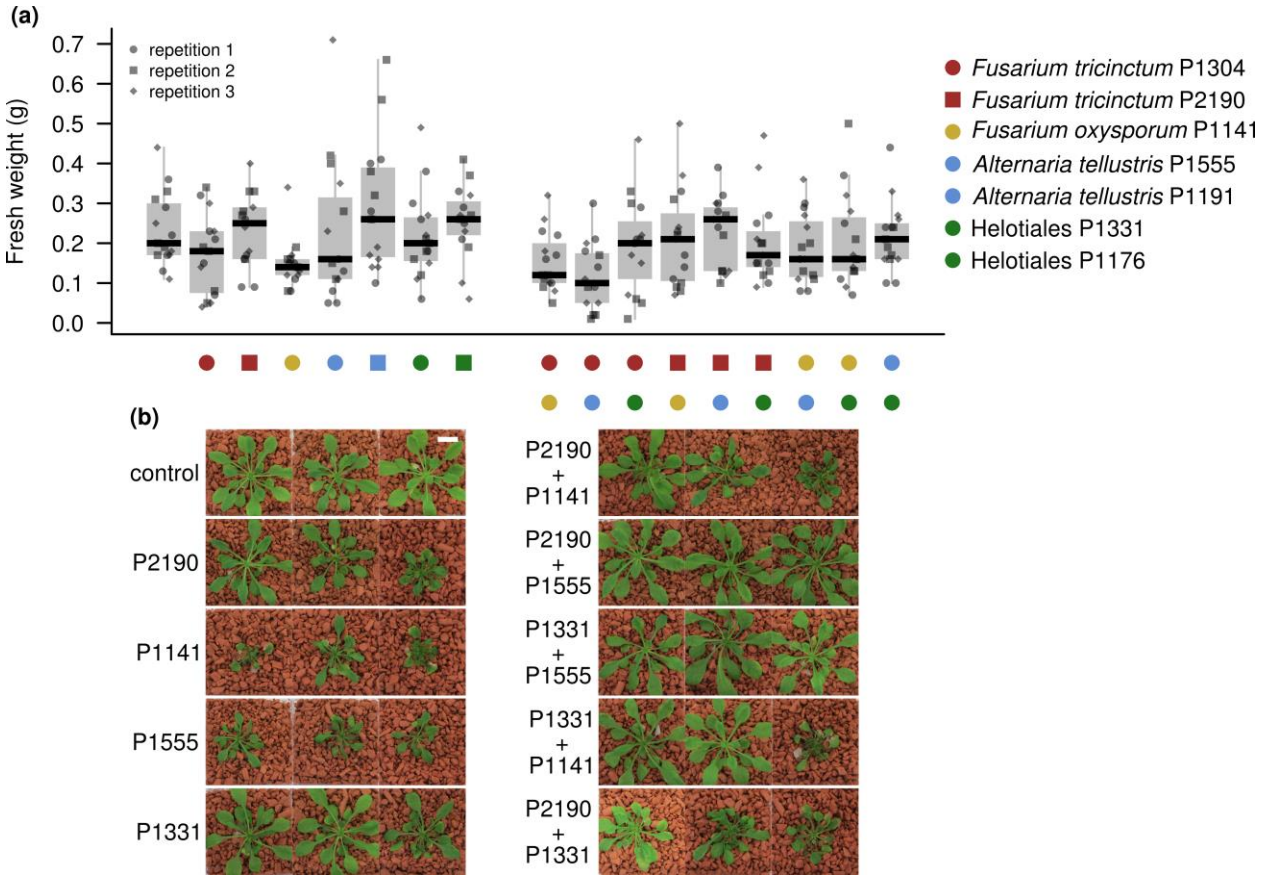

Figure 3. Effect on plant growth of root fungal colonization by individual strains, or different pairwise combinations of them. **a**, Boxbox-and-whisker plot representing the effects of fungal treatments on the fresh biomass of plant aerial parts. Points with different symbols indicate the individual values for each of the three repetitions of the experiment ($n = 3 \times 5$). Values extending further than 1.5 times above or below the boxes are considered outliers. **b** and **c**, Pictures of three representative *Arabidopsis thaliana* plants from a selection of treatments, showing the effect of fungal inoculation with single (**b**) or combinations (**c**) of strains on host's growth. All pictures correspond to the first repetition of the experiment. Bar size: 1 cm.

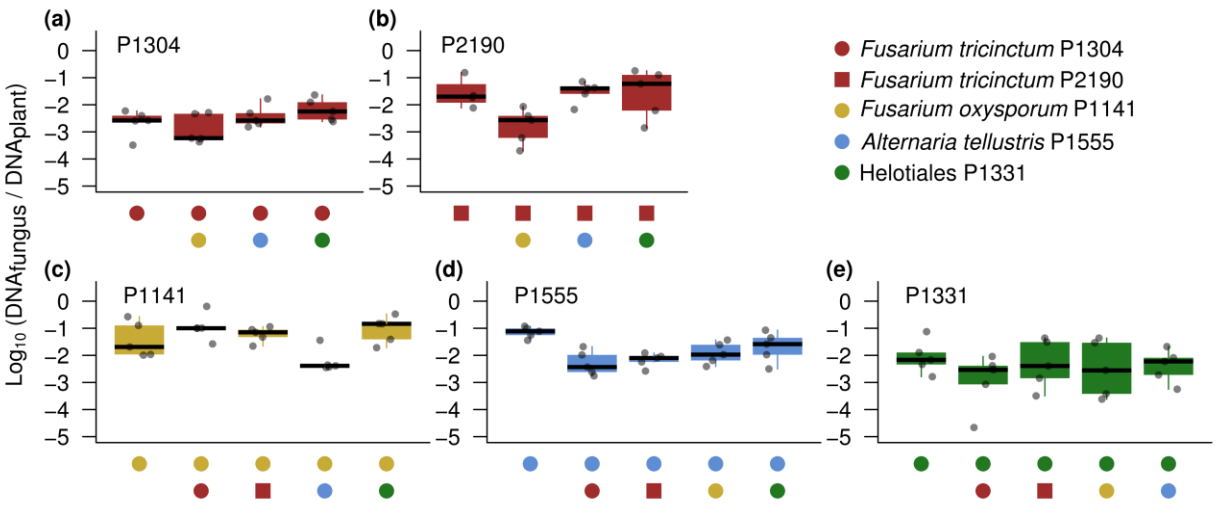

Figure 4. *Arabidopsis thaliana* root colonization by different fungal strains, alone or in combination with other fungi. Each box-and-whisker plot represents the root colonization by a particular strain, either in single (leftmost treatment) or pairwise inoculation with other strains (three/four treatments rightwards). Values represent the logarithm of the ratio between fungal and plant DNA as quantified by quantitative PCR (*n* = 5).

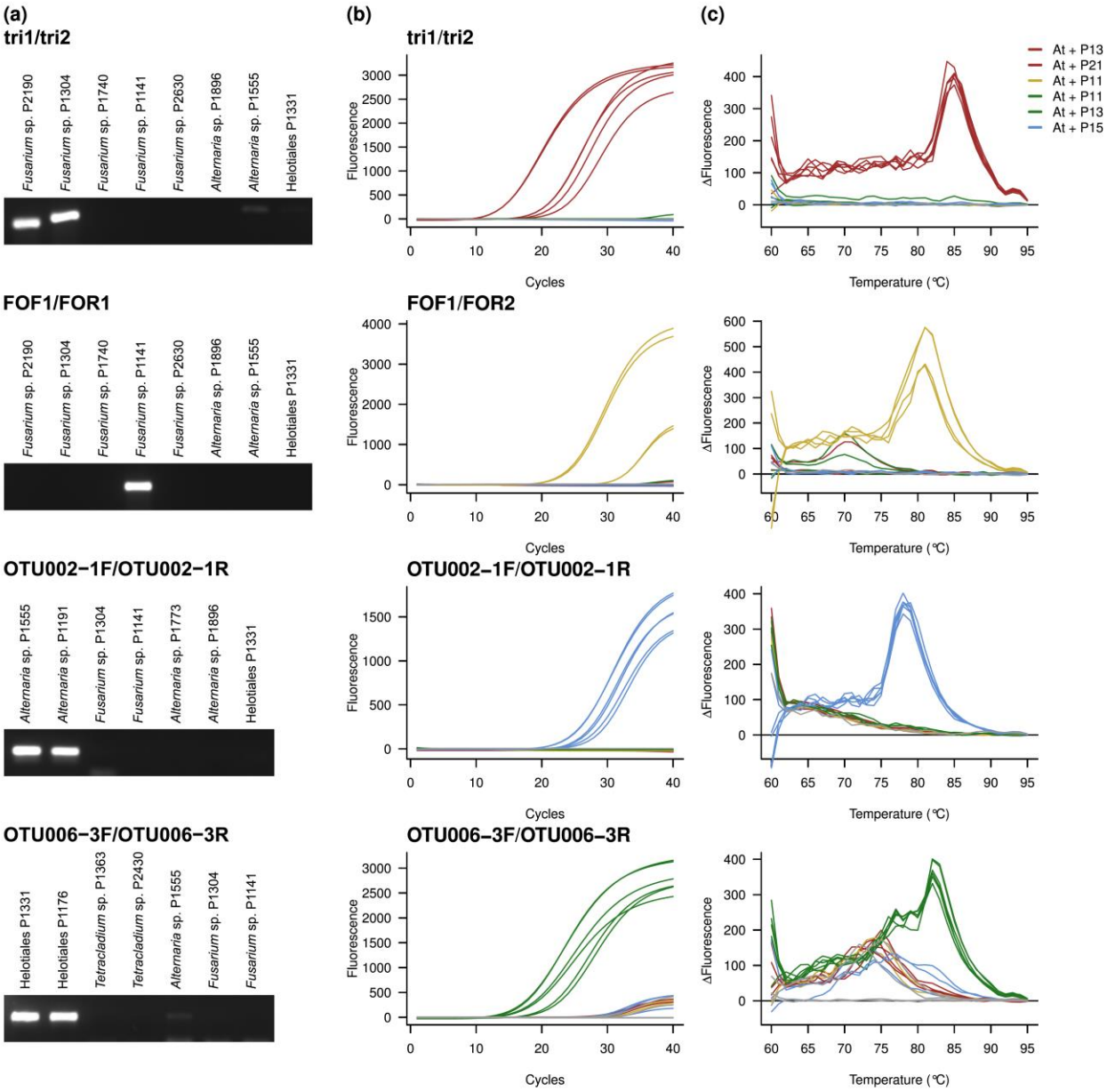

Figure S1. Specificity of primers used for real-time quantitative PCR. **a**, Agarose gel electrophoreses of PCR products for each set of primers using target and non-target fungi. **b**, qPCR amplification plots for each primer set using DNA extracts from *Arabidopsis thaliana* roots inoculated with the fungal strains tested in the study. The same root DNA extracts were included in all qPCR reactions, and a mycelial DNA extract for a target fungus was included for each primer set as a positive control. Negative template controls (NTC) included water instead of DNA. Every reaction was performed in duplicate. **c**, Melting curves corresponding to the qPCR reactions shown in **b**.

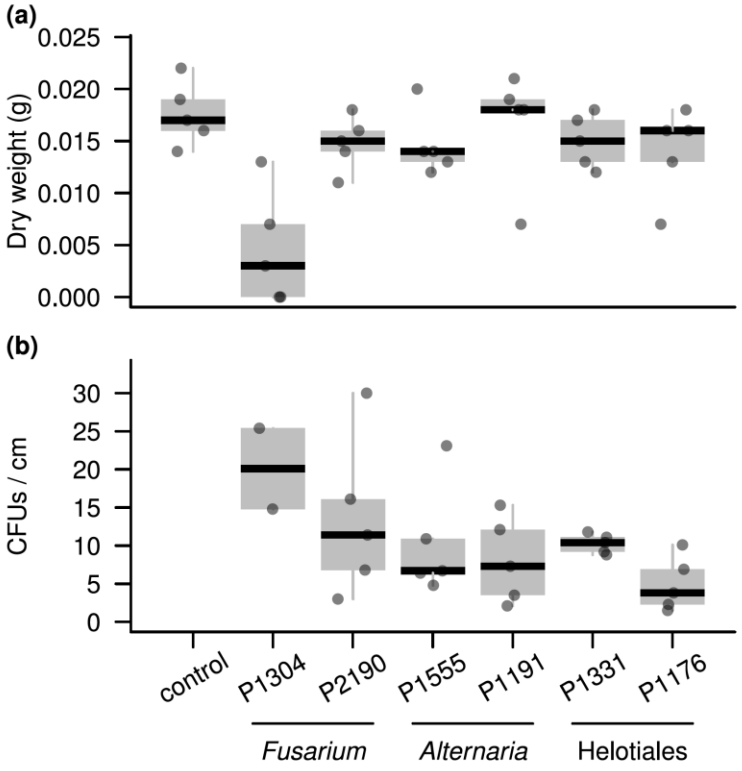

Figure S2. Dry weight of *Arabidopsis thaliana* in inoculations with single fungal strains (**a**), and root colonization as assessed by a cultivation method (**b**). Box-and-whisker plots represent the median and interquartile ranges for each treatment. Points indicate the individual values (n = 5). Values extending further than 1.5 times above or below the boxes are considered outliers. Values of root colonization are shown as colony-forming units (CFUs) per cm of plant root.

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06.2016, (GRADE), Frankfurt, Germany

Project management in Biotech industries
06.2016, (GRADE), Frankfurt, Germany

Negotiation skills for young scientists
05.2016, (GRADE), Frankfurt, Germany

Grant application
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Haghi Kia, S., Jurkechova, M., Glynou, K., Piepenbring, M., Maciá-Vicente, J. G. (2018). The effects of fungal root endophytes on plant growth are stable along gradients of abiotic habitat conditions. FEMS Microbiology Ecology 94, fix 162..

Glynou, K., Ali, T., **Haghi Kia, S.**, Thines, M., Maciá-Vicente, J. G. (2017). Genotypic diversity in root-endophytic fungi reflects efficient dispersal and environmental adaptation. Molecular Ecology 26, 4618–4630.

Haghi Kia, S., Glynou, K., Nau, T., Thines, M., Piepenbring M., Maciá-Vicente, J. G. (2017). Influence of phylogenetic conservatism and trait convergence on the interactions between fungal root endophytes and plants. The ISME Journal 11, 777–790.

Glynou, K., Ali, T., Buch, A., **Haghi Kia, S.**, Ploch, S., Xia, X., Çelik, A., Thines, M., Maciá-Vicente, J. G. (2016). The local environment determines the assembly of root endophytic fungi at a continental scale. Environmental Microbiology 18, 2418–2434.

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Conference Abstracts

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Haghi Kia, S., Macia-Vicente, J.G. (2014). Defining the effect of strain origin and host species in the plant-endophyte interaction. Conference of Deutschen Gesellschaft für Mykologie.

Macia-Vicente, J.G., Glynou, K., **Haghi Kia, S.**, Nau, T. (2014). Genetic and chemical diversity of fungal root endophytes: insights from their ecology and Interaction with plants. The 10th International Mycological Congress.

Macia-Vicente, J.G., Glynou, K., **Haghi Kia, S.** (2014). Finding patterns in the occurrence and plant interactions of fungal root endophytes: towards a better understanding of their ecological significance. Joint meeting of cost FA1206 (WP3) & FA 1103: Exo- & Endogenous signaling.

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Haghi Kia, S., Wewer, V., Hoelzl, G., Schulz, M. (2014). Assessing the effect of Benzoxazolinone on the plant-associated zygomycete *Actinomucor elegans* and its possible role in a weed's tolerance to allelochemicals. The 10th International Mycological Congress.

Schulz, M., **Haghi Kia, S.**, Sicker, D., Paetz, C., Schneider, B., Mondani, L., Ganimede, C., Tabaglio, V., Marocco, A. (2014). *Abutilon theophrasti´*s root associated microorganisms support hydroxylation-dependent benzoxazolinone detoxification and degradation. The 7th World Congress on Allelopathy.