Fungal root endophytes influence plants in a species-specific manner that depends on plant's growth stage

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Abstract
1. The mycobiome (fungal microbiome) influences plants—from seed germination to full maturation. While many studies on fungal-plant interaction studies have focused on known mutualistic and pathogenic fungi, the functional role of ubiquitous endophytic fungi remains little explored.

2. We examined how root-inhabiting fungi (endophytes) influence range-expanding plant species. We isolated endophytes from three European intra-continental range-expanders and three congenerics that are native both in the range expander’s original (southern Europe) and new (northern Europe) range. To standardize our collection, endophytes were obtained from all six plant species growing under controlled conditions in northern (new range of the range expander) and southern (native range of the range expander) soils. We cultivated, molecularly identified and tested the effects of all isolates on seed germination, and growth of seedlings and older plants.

3. Most of the 34 isolates could not be functionally characterized based on their taxonomic identity and literature information on functions. Endophytes affected plant growth in a plant species–endophyte-specific manner, but overall differed between range-expanders and natives. While endophytes reduced germination and growth of range-expanders compared to natives, they reduced seedling growth of natives more than of range-expanders.

4. Synthesis. We conclude that endophytic fungi have a direct effect on plant growth in a plant growth stage-dependent manner. While these effects differed between range expanders and natives, the effect strength and significance varied among the plant genera included in the present study. Nevertheless, endophytes likely influence the establishment of newly arriving plants and influence vegetation dynamics.

KEYWORDS
climate change, enemy release hypothesis, fungi, pathogens, plant-soil interactions, range-expanding plant species, root microbiome, soil biodiversity
INTRODUCTION

Many studies have examined the ecology of alien plant species that have been introduced from other continents, as a small proportion of them is highly invasive, changing local biodiversity and ecosystem functioning, and causing significant economic costs (Pimentel et al., 2001; Vilà et al., 2015; Vitousek et al., 1996). However, ecological consequences of climate warming-induced range-expanding plant species have received relatively little attention (De Frenne et al., 2014; van der Putten, 2012), despite the increasing incidents of intra-continental migration of native plant species to higher altitude and latitude due to climate change (Alexander et al., 2015; van der Putten, 2012).

As the growth of almost plants is influenced by the below-ground microbiome (Edwards et al., 2018; Mendes et al., 2013; Shi et al., 2016), micro-organisms may influence the success of plant range expansion as well. Especially those micro-organisms that directly penetrate plant roots have well-acknowledged positive (e.g. mycorrhizal fungi, rhizobial bacteria) and negative (e.g. bacterial, fungal and fungal-like oomycete pathogens) effects on plant growth (Raaljmakers et al., 2009). Among those players, fungi and oomycetes hold key functional roles and include major mutualists (Richardson et al., 2000; van der Heijden et al., 2015) and pathogens (Coats & Rumpho, 2014; Gilbert, 2002; Klironomos, 2002; Mendes et al., 2013; Mills & Bever, 1998).

Indeed, mutualistic micro-organisms are able to promote the success of invasive alien plants (Bever, 2002; Pringle et al., 2008) by enhancing plant abundance. Invasive alien plants even may change community composition of fungal mutualists to their own favour (Mummary & Rillig, 2006; Stinson et al., 2006; Zhang et al., 2010). In contrast to mutualists, many pathogens are limited in their host range (Gilbert & Webb, 2007) and often have a limited biogeographical distribution (Rout & Callaway, 2012). This has likely consequences for plant range shifts, as native plants can become released from their specialized pathogens, a hypothesis predicted by the enemy release hypothesis for introduced alien plants (Keane & Crawley, 2002; Mitchell & Power, 2003; Reinhart et al., 2003; Wolfe, 2002). However, pathogen effects may depend on plant growth stage, with mature plants generally suffering less from pathogens than seedlings or seedlings, with profound importance especially on short-lived herbaceous plant species (Bagchi et al., 2014; Blaney & Kotanen, 2001; Gilbert, 2002; Jarosz & Davelos, 1995; Mordecai, 2015; Packer & Clay, 2000).

Similar to invaders from inter-continental origin, plants that expand their range within a continent may be less exposed to negative feedback from their soil microbiome, and host lower numbers of enemies in the new range (Dostálek et al., 2016; Morriën et al., 2010; van Grunsven et al., 2007, 2010). While experiments to decipher interactions between range-shifting plant species and soil microbiomes have focused on overall plant–soil feedback patterns (Alexander et al., 2015; De Frenne et al., 2014; Dostálek et al., 2016; Engelkes et al., 2008; van Grunsven et al., 2007), the roles of specific pathogens and mutualists have received less attention.

In general, the majority of the plant-associated microbiome components, such as diverse root-inhabiting fungi and oomycetes—in the following termed endophytes—remains functionally unknown (Arnold et al., 2000; Bamisile et al., 2018; Busby et al., 2016; Gange et al., 2019; Jumpson & Trappe, 1998; Porras-Alfaro & Bayman, 2011; Rodriguez et al., 2009; Vandenkoornhuyse et al., 2002). Like pathogens, many endophytes might have a restricted host range especially at the plant species level (Hawksworth & Rossman, 1997; U’Ren et al., 2012, 2019). However, many endophytes might also be generalists in their host preference (Knapp et al., 2012). Overall, endophytes are suggested to be positive for plant growth, especially under stress from insect attack (Gange et al., 2019), salt stress (Gonzalez Mateu et al., 2020) or other challenging factors.

Yet, under some conditions endophytes can shift, from plant-neutral to mutualistic (Arnold et al., 2003; Clay & Holah, 1999; Clay & Schardl, 2002) or pathogenic (Busby et al., 2016; Hyde & Soytong, 2008; Kia et al., 2017; Rodriguez et al., 2009). Translating this ability to the symbiosis with invasive plants, endophytes may promote the spread of invasive plant species (Molina-Montenegro et al., 2015; Shearin et al., 2018). While endophytes have been studied in the context of inter-continental plant invasions (Klironomos, 2002; Knapp et al., 2012), only one study considered endophytes in the context of climate warming-induced range-expanding plant species. This study showed that range-expanders host different endophyte communities in the new than in the native range, while endophyte communities in congenic natives did not differ between both ranges (Geisen et al., 2017). Yet, the functional role of endophytes in plant growth during this type of range shift remains unknown (Busby et al., 2016).

In order to study how endophytes may influence range-shifting plant species during various stages of their life history, we cultivated fungal and oomycete endophytes from roots of three range-expanding plant species and three congenic natives. All plants were grown in northern soils from their new range (the Netherlands) and southern soil from their native range (Slovenia). We taxonomically identified all endophytes by sequencing the ITS region. Finally, we tested the effects of all cultivated root endophytes on the germination rate, seedling and plant growth of all six plant species. We investigated the following general hypotheses: (1) Range-expanders will have higher germination rates and produce more biomass when inoculated with endophytes isolated from the expanded northern range soil than from the native southern range soil; we did not expect differences for native plant species, as endophytes with negative effects might not have expanded together with the range-expanders but have developed with natives. (2) The effects of endophytes are irrespective of plant growth stages, because we expected a functional conservatism of endophytes.

2 | MATERIALS AND METHODS

2.1 | Plant species

Two plant species in each of the three genera Centaurea (C. stoebe and C. jacea; family Asteraceae), Geranium (G. pyrenaicum and G. molle, family Geraniaceae) and Tragopogon (T. dubius and T. pratensis, family
Asteraceae) were selected for this study. The first of each plant species (C. stoebe, G. pyrenaicum and T. dubius) in the genera originated from south-eastern Europe and expanded its range northwards in the 20th century (named: range-expanders, tRE, Sparrius, 2014). The second of each plant species in the genera is native in Europe in both the original and the expanded new range (named: natives, tNA).

More details are found in Table 1. All six herbaceous plant species co-occur in riverine habitats along the river Waal in the Netherlands, which is the southernmost branch of the river Rhine. Seeds of most plant species were obtained from a seed supplier (Cruydt-Hoeck) that collects seeds from wild plant populations, with the exception of G. molle that we collected ourselves (Table 1).

### 2.2 | Endophyte culturing experiment

To culture root-inhabiting fungi and fungal-like oomycetes (in the present study collectively named ‘endophytes’), we performed a greenhouse experiment using soil collected from three independent sites in Slovenia (southern soil) and three independent sites in the Netherlands (northern soil), where all six plant species commonly occur. We decided to isolate endophytes from roots of greenhouse-grown plants rather than from the field to ensure that root endophytes were collected from all soils under the same environmental circumstances. Soil was collected from the top 3–15 cm of two sublocations in each site in the Netherlands and Slovenia. Soils from the two sublocations from each site were homogenized and sieved using a 4-mm mesh size to create three independent soil samples from two sublocations from each site were homogenized and sieved using a 4-mm mesh size to create three independent soil samples from two sublocations from each site were homogenized and sieved using a 4-mm mesh size to create three independent soil samples from both Slovenia and the Netherlands. Ten per cent of the resulting six independent soil mixes was stored in the dark at 4°C until further use. The remaining soil from the Dutch sites (NL1-3) was combined in equal parts (1:1:1), mixed with sand (2:1) and was gamma-sterilized (20 kGy; Syngenta bv) to be used as sterile background soil. Further details on soil sampling and soil properties are described in the study by Koorem et al. (2018). The procedure of inoculating 10% of alive to 90% background soils was done to ensure that abiotic differences among soil samples were reduced to a minimum (Wilschut et al., 2019; Zhang et al., 2016).

Prior to germination, all plant seeds were surface-sterilized by washing seeds in 0.4% sodium hypochlorite solution for 3 min followed by rinsing with sterile distilled water (H_{2}O_{dest}). Seeds of all plant species were germinated on sterile glass beads in a growth cabinet at 20/10°C (day/night temperature); 16 hr light/8 hr dark conditions. Seedlings were planted individually in 0.8 L pots containing 675 g of the sterilized sandy loam soil supplemented with 75 g of one of the live soil samples. This resulted in a total of 36 pots (2 geographic ranges x 3 sites as true soil replicates from each range x 6 plant species). Individual pots were weighed two times per week and watered with sterile H_{2}O_{dest} to a weight of 750 g, which corresponded to a dry weight-based moisture content of ~60%. The pots were placed on a cart in a greenhouse at 16 hr light, 8 hr dark; 20°C, 15°C and 60% relative humidity. The position of the pots on the carts was randomized weekly. To increase the diversity of potentially plant life stage-dependent endophytes, another seedling of the same species and the same age was added to the same pots 2 and 4 weeks after initiating the experiment. This resulted in three plants of different age per pot.

Six weeks after initiating the experiment, shoots of all three plants per pot were cut, combined and dried at 60°C for 3 days before determining the dry weight. Roots were carefully isolated from the soil and thoroughly washed under running water before cutting into pieces of ~0.5-cm lengths. Fifty randomly selected root pieces per plant individual were placed in 2-ml centrifuge tubes filled with sterile water and stored at 4°C for 1–2 days before endophyte isolation (see below), while the remaining roots were combined and dried at 60°C for 3 days before determining the dry weight.

### 2.3 | Isolation and molecular characterization of endophytes

The root pieces stored in the centrifuge tubes were thoroughly washed by transferring three times to new sterile demineralized H_{2}O (H_{2}O_{dest}) in order to minimize the number of root-attached spores. Subsequently, roots were transferred to centrifuge tubes filled with 70% ethanol and incubated for 7 min under occasional mixing before final transfer and washing in a centrifuge tube containing sterile H_{2}O_{dest}. Root pieces were placed on sterile tissue paper to dry the surface under sterile conditions in a flow cabinet. Three individual root pieces per plant and pot were placed apart from each other in ten 10-cm Petri dish filled water agar (WA; 1.6% agar, pH 6.7, ampicillin 50 mg/L), resulting in a total of 30 root pieces per pot and a total of 360 Petri dishes. Plates were stored at 20°C in the dark. Remaining roots per pot were combined and divided into three parts.

### TABLE 1 | Seed origin and status of the experimental plants. All seeds were obtained from plants growing in the Netherlands

| Plant species               | Abbreviation | Status | Seed origin          |
|----------------------------|--------------|--------|----------------------|
| Centaurea stoebe L.        | CS           | RE     | Cruydt-Hoeck         |
| Centaurea jacea L.         | CJ           | NA     | Cruydt-Hoeck         |
| Geranium pyrenaicum Burm. f.| GM           | RE     | Cruydt-Hoeck         |
| Geranium molle L.          | GP           | NA     | Millingerwaard       |
|                           |              |        | natural protected area |
| Tragopogon dubius Scop     | TD           | RE     | Cruydt-Hoeck         |
| Tragopogon pratensis L.    | TP           | NA     | Cruydt-Hoeck         |

Note: Cruydt-Hoeck Wildebloemenzaden (Nijieberkoop, The Netherlands) is a commercial seed supplier that collects seeds from natural populations and grows them in their own fields for seed multiplication. Abbreviations: NA, native congeneric plant species; RE, range-expanding plant species.
Each part was placed on a 6-cm diameter Petri dish filled with a 1:1 mix of sterile pond water and sterile $H_2O_{dest}$ containing dry and sterile grass leaves (Agrostis capillaris, 2–3 cm) for baiting zoospore forming oomycetes (Pettitt et al., 2002). The resulting 108 Petri dishes were placed at room temperature under light/dark conditions. After 4 days, the grass leaves were washed in sterile $H_2O_{dest}$, dried on a tissue, transferred onto WA and grown at room temperature under light/dark conditions.

All WA-containing Petri dishes were checked for growth of fungi and oomycetes 4, 6, 8 and 14 days after placing the roots on WA, whereas plates with grass leaves were checked after 1, 2, 7 and 14 days. Newly formed colonies were transferred to 6-cm Petri dishes containing WA.

The resulting cultures of endophytes were transferred to three additional media, that is, 2% malt extract agar (MEA; Difco) 0.4 × oatmeal agar (OA; Difco), 0.5 × potato carrot agar (PCA; according to Crous et al. (2009)) and 0.5 × potato dextrose agar (PDA; Oxoid). For all media, the pH was adjusted to 6.7. To reduce the number of potentially duplicated isolates, only one endophyte culture isolated from the same plant individual was kept if morphologically indistinguishable from the other isolates on all tested media. This highly conservative filtering resulted in a total of 34 distinct cultures, which were taxonomically identified by BLASTn searches of their ITS region followed by maximum likelihood analyses as detailed in Supporting Information and Section 2. Sequences are accessible at NCBI GenBank under the accession numbers MT242270–MT242299. Four cultures could not be amplified with the ITS primers used leading us to identify partial 18S rRNA gene reads to obtain information on their taxonomic identity (see Supporting Information).

Taxonomic identification revealed that 32 of the 34 cultures (94%) were fungi and two cultures were oomycetes (Table 2). Based on BLASTn searches, most of the cultures (91%) had a ≥99% sequence similarity with fungi or oomycetes existing in GenBank. However, 13% of those cultures with high similarity to sequences present in GenBank only matched with sequences not affiliated to described fungal or oomycete species but with unknown environmental sequences. For those isolates, described taxa showed a sequence similarity ≥96%. This suggests that more than 20% of all of our cultures represent species or even genera that are currently unknown or represent species with so far missing sequences in databases.

2.4 | Inoculation experiment

2.4.1 | Seed germination

Spores and hyphae of endophytes were extracted from well-grown cultures on malt extract agar by adding sterile $H_2O_{dest}$ and carefully suspending endophyte material using a cell scraper. Spore suspensions were equilibrated to $1 \times 10^6$ spores or hyphal pieces per ml as counted under an inverted microscope at 400x magnification. Seeds of all targeted plants were surface-sterilized immediately before use by washing seeds in 10% sodium hypochlorite solution for 3 min (Centaurea and Geranium spp.). We applied this much harsher procedure than the one described above to ensure a complete elimination of all non-endophytic micro-organisms. Yet, tests revealed that this sterilization procedure was not eliminating all seed-attached fungi from Tragopogon species, so we used a harsher sterilization by pre-treating those seeds in 3% HCl for 1 min before washing seeds in 10% sodium hypochlorite solution for 5 min. Surface-sterilized seeds were rinsed with sterile $H_2O_{dest}$.

The germination experiment was conducted in 10-cm diameter Petri dishes filled with 0.5% $H_2O$ agar in November 2015. Pre-tests revealed that T. pratensis did not germinate on this medium. Therefore, T. pratensis seeds were placed in Petri dishes (9 cm diameter) with filter paper above and below the seeds and 2 ml sterile $H_2O_{dest}$ was added. Surface-sterilized seeds of each plant species were dipped into a spore solution of individual endophyte cultures isolated from the same plant species or individual endophyte cultures isolated from the congener species. For example, the seeds of C. jacea were inoculated with each endophyte culture isolated from C. jacea and C. stoebe plants, the seeds of C. stoebe were inoculated with the endophytes isolated from C. jacea and C. stoebe plants, and so on (Figure 1). Controls were initiated by dipping surface-sterilized seeds into sterile $H_2O_{dest}$. Fifteen inoculated seeds were placed in each Petri dish. Each treatment was replicated four times, resulting in a total of 296 Petri dishes ([34 cultures × 2 plant groups (RE and NA) + 6 controls] × 4 replicates). After sealing Petri dishes with Parafilm, they were incubated in a fully randomized order for 11 days at 16 hr 20°C (day) and 8 hr 15°C (night) in a controlled plant growth chamber. Seeds from all plant species started to germinate at latest 2 days after placing on agar. At the 11th day the number of germinated seeds was counted to determine the total % of germinated seeds. Roots and shoots of all germinated seeds in each of the Petri dishes were harvested. Lengths of all fresh roots was measured to determine the average root length. Then, the roots and shoots were separately dried at 60°C for 36 hr and weighed.

2.4.2 | Plant growth

We germinated surface-sterilized seedlings of all six plant species (described above) on sterile glass beads in a growth cabinet at 20/10°C; 16 hr light/8 hr dark conditions. Roots of 10-day-old seedlings of each plant species were dipped in the same spore suspensions of the respective endophytes and sterile $H_2O_{dest}$.
**TABLE 2**  Fungal endophytes recovered from roots the different plant species *Tragopogon dubius* (Td), *T. pratensis* ssp *pratensis* (TP), *Centaurea jacea* (Cj), *C. stoebe* (Cs), *Geranium mole* (Gm) and *G. pyrenaicum* (Gp) and from soils from the Netherlands (Nl) and Slovenia (S); ID: identity. Note that more detailed descriptions of the culture including the potential function are likely biased by previous studies that often focused on agricultural settings.

| Endophyte | Phylogroup | GenBank accession # of highest match | % similarity with best BLASTn hit | Origin of the best BLASTn hit and those with same % similarity | Details on other BLASTn hits with similar or lower % similarity | Genus (phylogenetic placement) | Comments on genus |
|-----------|------------|--------------------------------------|----------------------------------|--------------------------------------------------------------|---------------------------------------------------------------|-------------------|------------------|
| TdS1      | Fungus     | JQ964802                             | 100                              | Biocollection agent                                          | Others with same % ID include plant-associated ones, for example, KP174676 as an endophyte in tree root | Chaetomium | Common endophytes with known roles for plant disease control through the production of various secondary metabolites |
| TdS2      | Fungus     | KM030576                             | 100                              | Isolated from bark                                           | Other *C. globosum* with same % ID; several potential human or mammal pathogen! | Chaetomium | Common endophytes with known roles for plant disease control |
| TpS3      | Fungus     | KP641158                             | 100                              | Endophyte in *Pistacia vera*                                 | Other *Acremonium* spp., for example, *A. sclerotigenum* (endophyte in *Pistacia vera*) and *A. alternate* | Acremonium | Mostly saprophytic but some with reported role as human pathogens |
| TpS4      | Fungus     | GQ131885                             | 100                              | Soils                                                        | Other *Humicola* spp as well, mostly from soils, but can be pathogenic or endophytic | Humicola | Humicola spp. also endophytic, mycoparasitic and with suggested plant disease control |
| TpS5      | Oomycete   | AY455696                             | 100                              | Unknown                                                      | Also *P. pleroticum* and also *P. minus*, for example, HQ643695; some isolated from soybean (AY590279), others from soil | Pythium | Mostly plant pathogens |
| TpS6      | Fungus     | AB625588                             | 100                              | Soils/Endophytes                                            | Other *Humicola* spp same ID; often from soils, but can be pathogenic or endophytic | Humicola | Common endophytes with known roles for plant disease control |
| TpN7      | Fungus     | KF494159                             | 100                              | Tomato roots and rhizosphere                                 | Some *P. cucumerina* Leaf Spot-causing agent | Plectosphaerella | Most are plant pathogens |
| TpN8      | Fungus     | KF494159                             | 100                              | Tomato roots and rhizosphere                                 | Some *P. cucumerina* Leaf Spot-causing agent | Plectosphaerella | Most are plant pathogens |
| CjS9      | Fungus     | EU035984                             | 100                              | Dog parasite                                                | *Phialemoniospis curvata* (human skin pathogen) same % ID | *Phialemonium* | Mammal pathogen |
| CjS10     | Fungus     | KF494089                             | 100                              | Endophytes in roots of *tomato* and *Sesamum indicum*       | Also other *F. oxysporum* spp. same % ID; for example, KC304803 causing wilt in clementine | Fusarium | Most of this strain plant pathogens or endophytes |
| CjS11     | Fungus     | JF755896                             | 100                              | Endophyte in *Holcus lanatus*                                | Similar %ID with Lasiosphaeriaceae HM007093, a root endophyte of *Stipa grandis*; next known *Cercophora coprophila* with 92% ID | Unknown genus, related to *Cercophora* | Unknown |
| CjS12     | Fungus     | KJ528986                             | 99                               | Soils/Endophytes                                            | Other *Humicola* spp. with 99% ID; some endophytic and pathogenic | *Humicola* | Common endophytes with known roles for plant disease control |
| CjS13     | Fungus     | JF508361                             | 100                              | Root endophyte of *Astragalus adsurgens* Pall.              | Other strains (also *Slopeolomyces = Gaeumannomyces*) which are root endophytes in different plants | *Gaeumannomyces* | Likely non-pathogenic with potential biocontrol, some of the genus are pathogens |

(Continues)
| Endophyte | Phylogroup | GenBank accession # of highest match | % similarity with best BLASTn hit | Origin of the best BLASTn hit and those with same % similarity | Details on other BLASTn hits with similar or lower % similarity | Genus (phylogenetic placement) | Comments on genus |
|-----------|------------|-------------------------------------|---------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|-----------------------------|------------------|
| CsS14     | Fungus     | JQ435795                            | 97                              | Human pathogen                                               | below 90% ID; other Clohesomyces also pathogens of plants and humans | Clohesomyces               | Pathogens of animals and plants; also free-living aquatic taxa |
| CsN15     | Fungus     | KP641158                            | 100                             | Endophyte of *Pistacia vera*                                 | Similar % ID with *Trichothecium roseum* EU622273            | Acremonium                 | Mostly saprophytic but some with reported role as human pathogens |
| GpN16     | Fungus     | JX139038                            | 85                              | Too divergent to make reliable suggestions                  | 18S rRNA gene sequence - low phylogenetic resolution          | Unknown genus; sister to family Sordariaceae | Unknown |
| GpS17     | Fungus     | KM268718                            | 100                             | Endophyte of tobacco                                         | Other *Chaetomium* spp. also similar % ID                    | Chaetomium                 | Common endophytes with known roles for plant disease control |
| GpN18     | Fungus     | FN397268                            | 99                              | Soil                                                        | Other uncultured similar % ID that are endophytic; *Podospora* spp. ≥96% ID-sometimes fungal parasites | Podospora                  | Saprophytes |
| GpS19     | Fungus     | GQ922547                            | 99                              | Unknown                                                     | Other uncultured same % ID that are endophytic or from soils or fungal parasites | Podospora                  | Saprophytes |
| GpS20     | Fungus     | EU035984                            | 100                             | Dog parasite                                                | *Phialemoniopsis curvata* (human skin pathogen) same % ID     | *Phialemonium*              | Mammal pathogen |
| GpS21     | Fungus     | KJ528986                            | 99                              | Soils/Endophytes                                            | Other *Humicola* spp. with 99% ID; some endophytic and pathogenic | *Humicola*                 | Common endophytes with known roles for plant disease control |
| GpS22     | Fungus     | KF313106                            | 99                              | Soils/Endophytes                                            | Same match with other strains, root endophytes and undescribed *Verticillum* spp | *Simplicillium*             | Common endophytes with known roles for plant disease control |
| GpS23     | Fungus     | FJ004599                            | 100                             | Soils                                                       | 18S rRNA gene sequence - low phylogenetic resolution         | *Penicillium*               | Unknown |
| GmS24     | Fungus     | AF108480                            | 99                              | Soils/Endophytes                                            | Similar to other root endophytes                             | *Simplicillium*             | Common endophytes with known roles for plant disease control |
| GmN25     | Fungus     | FJ536208                            | 100                             | Root endophyte                                              | And other *P. macrosponosa* same % ID; most endophytes in different roots | *Periconia*                 | Plant pathogens and saprophytes |
| GmN26     | Fungus     | KP230824                            | 99                              | Rhizosphere soil of *Panax notoginseng*; potential endophytes | Similar % ID with *Alternaria* spp. (isolated from orchid roots) | *Pyrenochaeta*              | Human and plant pathogens, endophytes |
| GmS27     | Fungus     | KJ767117                            | 99                              | Soil                                                        | Several *Humicola* spp same ID; often from soils, but can be pathogenic or endophytic | *Humicola*                 | *Humicola* spp. also endophytic, mycoparasitic and with suggested plant disease control |
| GmN28     | Fungus     | KM247477                            | 100                             | Endophyte in orchids                                        | Next matches much lower % ID; next known *Sebacina* sp. 84% ID | Unknown genus; sister to *Lophioistoma* | Unknown |

(Continues)
that were used in the germination experiment. Seedlings were randomly placed in wells of a 140 multi-well plate pre-filled with 50 g of the same sterile soil that was used in the endophyte culturing experiment. Due to logistics reasons, for each treatment we used eight replicates for *Centaurea* and *Tragopogon* spp., and seven replicates for *Geranium* spp., resulting in a total of 552 wells.

The multi-well plates were incubated in a plant growth chamber under the same conditions as described above. Plants were watered two times per week with 5 ml of sterile H$_2$O$_{dest}$ in the first 2 weeks after planting, and 7.5 ml in the last 2 weeks of the experiment. We conducted this experiment starting from the seedlings stage without reaching maturation to capture a phase during plant growth where plants might still acquire endophytes from their surrounding soils and where effects might affect plant growth initially. Therefore, we harvested after 25 days by clipping shoots, and carefully washing roots under running H$_2$O$_{dest}$. Shoot and root biomass was determined after drying at 60°C for 36 hr.

### 2.5 | Data analysis

All statistical analyses were carried out in R statistical language, version 3.6.1 (R Core Team, 2019).

#### 2.5.1 | Endophyte culturing experiment

The effects of soil origin (south or north) and plant status (range explorer: tRE or native: tNA), on plant biomass were analysed using ANOVA with plant genus (*Centaurea*, *Geranium*, and *Tragopogon*), soil origin (north or south), status of the tested plant (tRE or tNA) and all their interactions as fixed factors.

#### 2.5.2 | Inoculation experiments

The percentage of germinated seeds was calculated as $P = \frac{100 \times (\text{number of germinated seeds} + 0.5)}{\text{total number of seeds} + 1}$ to guard against 0 or 100% values. We corrected the obtained values of percentage of germination using the mean values of the corresponding controls. We call this corrected values 'ratio to control'. For example, for the % seed germination, the ratio was calculated as % germination with endophyte/mean % germination in control treatment. If the resulting value is below 1, the endophytes had a negative effect; a value above 1 indicates a positive effect of the endophyte; and a value of 1 indicates that there was no effect of endophytes. As each pair of species was inoculated with a different set of endophytes, the effects of endophytes for each pair of congeners were analysed separately. The ratios were analysed using mixed-effects models. Prior to analysis, the ratios were log-transformed as $\ln(ratio + 0.5)$ to meet the assumptions of homogeneity of variances. The fixed effect structure of the full model for *Geranium* Endophyte Phylogroup is shown in Table 2.

### Table 2 (Continued)

| Endophyte | Phyla | GenBank accession # of highest match | % similarity with best BLASTn hit | Origin of the best BLASTn hit and those with same % similarity | Details on other BLASTn hits with similar or lower % similarity | Genus (phylogenetic placement) | Comments on genus |
|-----------|-------|-------------------------------------|---------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|-------------------------------|------------------|
| GmN29     | Fungus| JF508361                            | 99                              | Root endophyte of Astragalus adscens Pall.                  | Same match with other strains (also *Sloepolomyces = Gaeumannomyces*) which are root endophytes in different plants | Gaeumannomyces               | Likely non-pathogenic with potential biocontrol, some of the genus are pathogens |
| GmN30     | Oomycete| KF861548                            | 100                             | Soil/endophyte                                              | Other *P. pleroticum* and *P. minus* spp., for example, *AY990279* (soybean endophyte) same % ID | Pythium              | Mostly plant pathogens |
| GmN31     | Fungus| GQ924005                            | 98                              | Endophyte in *Bouteloua gracilis*                          | Likely new genus/family/order; next best match (95% ID) with uncultured fungi (GQ929202), rest lower than 90%; best known *Lophiostoma* with 86% ID | Unknown genus; sister to *Lophiostoma* |
| GmN32     | Fungus| KJ689788                            | 99                              | Endophyte in *Quercus suber*                                | Next best matches (98% ID) with endophytic *Xylaria* spp. and other uncultivated taxa | *Xylaria* or *Halorosellinia* | Saprophytes and endophytes |
| GmN33     | Fungus| KT367526.                           | 99                              | Soil/endophyte                                              | Other endophytic/pathogenic *Saccharicola* similar % ID     | *Saccharicola* | Plant pathogen |
| GmN34     | Fungus| KF468126                            | 100                             | Endophyte                                                  | Other uncultured taxa same % ID, most endophytic: *Alternaria* sp. with 99% | *Alternaria* | Plant pathogen or endophytes |
pair was: endophyte soil origin (eSouth or eNorth), endophyte plant origin (eRE or eNA), status of the tested plant (tRE or tNA) and all possible interactions. Because we were not able to initiate cultures from all plant species and soils, it was not possible to include endophyte soil origin by endophyte plant origin and three-way interactions in the full models for Centaurea and Tragopogon pairs. Furthermore, as only one endophyte was cultivated in the Southern soil from both Centaurea species, we excluded the factor endophyte soil origin from their models. Within all mixed models, Petri dish (or Well) or endophyte identity was treated as random effect to account that plants exposed to the same fungal culture in a Petri dish or Well are pseudo-replicates. Model selection was performed using the corresponding full model as a starting point. The best performing models were selected using an information theoretical approach (Akaike information criterion, AIC, Burnham & Anderson, 2004) and likelihood ratio tests. The detailed description of the model selection procedure for each congeneric pair is described in the Supporting Information. The final selected models for each congeneric pair and their numerical outputs are shown in Table S2 (in Supporting Information).

The assumption of the homogeneity of variance and normality were checked graphically by inspecting the residuals plotted against fitted values, and against each explanatory variable in or outside the model. The Cook’s distance values (Cook & Weisberg, 1982) were used to detect any influential observations. Two observations from two different datasets had much smaller residuals compared to the rest of the data points. After inspecting the raw data and confirming that these data points were largely differing from the rest in the same group, the two points were deleted from the analyses.

The mixed models were fitted using lmer function (in lme4 package; (Bates et al., 2015)). To obtain the p-values presented in the results, we used Type III Wald F tests with Kenward-Roger correction for degrees of freedom from the car package (Fox & Weisberg, 2019). The factors were set to sum-to-zero contrasts (contr. sum), which compare each level of the factor to the average of the other levels.

In the Section 3, we only show those figures that were directly linked to our hypotheses (Hypothesis 1: Range-expanders perform best with endophytes from northern soils while natives do not show such a pattern; Hypothesis 2: endophytes affect plants in the same way throughout plant growth).

3 | RESULTS

3.1 | Endophyte culturing experiment

In total, 34 unique fungal and oomycete endophyte cultures were obtained from roots of all six plant species grown in both northern and southern soils (Table 2; Table S1). Plants grown in northern soil produced on average more total biomass than in southern soil ($F_{1,24} = 4.45, p = 0.045$; Figure S1a).

3.2 | Inoculation experiments

3.2.1 | Germination

Seed germination of range-expanding Geranium was on average more negatively affected by the inoculated endophytes than its native congener ($F_{1,36} = 27.94; p < 0.001$; Figure 2A). Seed germination of range-expanding and native Centaurea species was not affected by the endophytes ($p > 0.05$ in all cases, Table S2). Seed germination of range-expanding Tragopogon species was on average more negatively affected by the endophytes than its native congener, but the latter effect depended on whether the endophyte originated from the northern or southern soil (Figure 2B). Specifically, seed germination of the native Tragopogon was positively affected by endophytes
retrieved from the northern soil when compared to the southern soil ($F_{1,54} = 6.53; p = 0.014$; Figure 2B).

### 3.2.2 Seedling biomass

Range-expanding *G. pyrenaicum* seedlings produced more root biomass when exposed to the endophytes than the native *G. molle*, but this effect depended on the endophyte’s soil origin ($F_{1,32} = 4.17; p = 0.049$, Figure 3A). Endophytes originating from southern soil promoted the root biomass of the range-expanding *G. pyrenaicum* seedlings more than the endophytes from the northern soil, whereas native *G. molle* root biomass was not affected by origin of the endophyte (Figure 3A). A similar interactive effect of endophyte soil origin and status of the tested plant was found for the shoot biomass of *Geranium* species, but this effect was marginally significant ($F_{1,32} = 4.04; p = 0.053$,
FIGURE 4 Ratio of root biomass of the plants inoculated with endophytes to the averaged root biomass in control treatment. Means of the ratios ± standard errors are shown. Top (A–C): Difference in plant root biomass of native (tNA) and range-expanding (tRE) Geranium (left A and D), Centaurea (middle B and E) and Tragopogon (right C and F) species exposed to endophytes originating in northern (eNorth) and southern soils (eSouth). Bottom (D–F): Difference in root biomass based on whether endophytes were isolated from native (eNA) or range-expanding (eRE) plant species. Black solid points depict average responses of plants exposed to the same fungal culture. The number of endophyte cultures is given in Table S1. Black-dashed line indicates no effect of endophytes. Different letters indicate significant differences based on linear model analysis.

FIGURE 5 Ratio of shoot biomass of the plants inoculated with endophytes to the averaged shoot biomass in control treatment. Means of the ratios ± standard errors are shown. Top (A–C): Difference in plant shoot biomass of native (tNA) and range-expanding (tRE) Geranium (left A and D), Centaurea (middle B and E) and Tragopogon (right C and F) species exposed to endophytes originating in northern (eNorth) and southern soils (eSouth). Bottom (D–F): Difference in shoot biomass based on whether endophytes were isolated from native (eNA) or range-expanding (eRE) plant species. Black solid points depict average responses of plants exposed to the same fungal culture. The number of endophyte cultures is given in Table S1. Black-dashed line indicates no effect of endophytes. Different letters indicate significant differences based on linear model analysis.
Figure 3B). The root biomass of Centaurea seedlings was not affected by inoculated endophytes \((p > 0.05\) in all cases, Table S2), whereas shoot biomass of native \(C. jacea\) was negatively affected by the endophytes compared to the range-expanding congener \((F_{1,12} = 68.55; p < 0.001, \text{Figure 3C})\). The root biomass of native \(T. pratensis\) was negatively affected by the endophytes compared to the range-expanding congener \((F_{1,12} = 18.05; p = 0.001, \text{Figure 3D})\), whereas the shoot biomass of \(Tragopogon\) species was not affected by the endophytes \((p > 0.05\) in all cases, Table S2). Overall, we conclude that endophytes had variable effects on seedling growth that depended on the source of the endophyte and plan genus studied.

### 3.2.3 | Plant biomass

The root biomass of Geranium species was affected by the endophyte plant origin \((F_{1,35} = 4.72; p = 0.037; \text{Figure 4})\). Here, endophytes isolated from native Geranium molle plants more negatively affected root biomass of Geranium species than those isolated from range-expanding \(G. pyrenaicum\). In contrast, the shoot biomass of range-expanding Geranium pyrenaicum was more negatively affected by the cultivated endophytes than the native congener \((F_{1,246} = 10.22; p = 0.0016; \text{Figure 5})\). In particular, the root biomass of Geranium plants (both Geranium species combined) was more negatively affected by endophytes that were isolated from native plants than from range-expanders. Plant shoot and root biomass of range-expanding and native Centaurea species was not affected by the endophytes \((p > 0.05\) in all cases, Figures 4 and 5; Table S2). The root biomass of the range-expanding \(Tragopogon\) species was positively affected by endophytes retrieved from northern soil compared to endophytes from the southern soil, whereas root biomass of the native Tragopogon was not affected by origin of the endophytes \((F_{1,104} = 5.56; p = 0.02; \text{Figure 4})\). The shoot biomass of range-expanding and native \(Tragopogon\) species was not affected by the endophytes \((p > 0.05\) in all cases, Figure 5; Table S2).

## 4 | DISCUSSION

Here we show that effects on plants of fungal and oomycete root endophytes isolated from range-expanding and congenere native plant species are variable depending on plant life stage.

### 4.1 | Endophytes with unknown functions dominate root cultures

We found a wide taxonomic diversity of endophytes that include fungal and oomycete species in roots of the six plant species. Many species of oomycetes are known to be notorious plant pathogens of numerous plant species and are commonly found in soils and the plants rhizosphere (Arcate et al., 2006; Geisen et al., 2015). Despite applying an oomycete-optimized isolation technique in half of the cultivation efforts (De Cock & Lévesque, 2004), only 5.8% of our unique endophyte cultures resembled oomycetes, and only 14.7% of all endophytes most closely resembled potential plant pathogens. Based solely on a priori functional classification, most microbes cultured in both range-expanding and related native plants were non-pathogenic. Further studies are needed to confirm this pattern as this assumption is based on the comparably low number of 34 cultures and assumes an equal cultivability which selects against obligate rather than facultative plant pathogens. Interestingly, those presumable pathogens based on a priori assignments often did not negatively affect plant growth. This is partly due to the fact that there is a lack of functional knowledge of endophytes in non-crop plant species suggesting that a priori functional assignment in the little studied endophytes can be misleading especially in natural plant species (Lofgren et al., 2018; Malcolm et al., 2013). A limited cultivation efficiency of endophytes prevented a thorough investigation of differences in endophyte infection between plant species or locations—a pattern that exists for some plant species (Bickford et al., 2018; Glynou et al., 2016, 2017). Yet, we believe that our rigorous cultivation approach resulting in few cultivated endophyte species is biased in the same way across treatments. Thus, the functional tests, as discussed next, are reliable but might miss some patterns that exist along the ones tested here.

### 4.2 | Endophytes differentially affect range-expanding and native plant species in a life stage-dependent manner

Range-expanding and congenere native plant species differed in their responses to endophytes, but the responses strongly depended on plant growth stages. This result opposes Hypothesis 2 (endophytes affect plants in the same way throughout plant growth). The observed patterns were also more complex than assumed in Hypothesis 1 (Range-expanders perform best with endophytes from northern soils while natives do not show such a pattern), as there were no differences between range-expanders and congenere natives in their responses to cultures obtained from northern than southern soils. All observed differences depended on plant genus, as well as on soil and plant origin of the endophytes.

The effect of endophytes on plant growth was the most pronounced in the earliest life stages: seed germination and seedling growth. These results support the idea that the impact of plant host-associated organisms depends on plant life stages, being most strong in early plant growth stages (Bagchi et al., 2014; Blaney & Kotanen, 2001; Gilbert, 2002; Jarosz & Davelos, 1995; Mordecai, 2015; Mordical, 2015; Packer & Clay, 2000). Nevertheless, in many experimental studies plant performance is measured as an integration of growth from seedlings to mature plants. Indeed, differences in germination success might explain the success of invasive exotic plant species when they germinate faster than related native plant species (Hirsch et al., 2012).

Our experimental test revealed that endophytes may play a key role in affecting plants especially during early growth stages such as...
during germination and seedling establishment and that these effects differ between native and range-expanding plant species. However, the effects of the endophytes on plant growth in this study are not caused by known plant pathogens, but by endophytes with an unknown a priori functioning (Arnold et al., 2000; Rodriguez et al., 2009). Functions of most of these endophytes remain largely unknown and previous studies showed contradictory effects on plant growth ranging from positive (Newsham, 2011) to negative (Kia et al., 2017; Mayerhofer et al., 2012). Our results support those observations using endophyte culture-dependent analyses. The positive effects of endophytes on plant growth might be attributed to stimulated nutrient exchange comparable to that of mycorrhizal fungi (Arnold et al., 2003; Rodriguez et al., 2009). Positive endophyte effects, however, have been suggested to occur predominantly under stress, for example through secondary metabolite production that confers resistance against herbivores (Cosme et al., 2016), while negative effects can be more common under non-stress conditions (Kia et al., 2017). Our results suggest that both positive and negative effects of endophytes on plant growth are common and depend on the partners that were interacting. As endophytes influenced plant growth during germination and seedling establishment, we conclude that endophytes are not functionally neutral, but affect plant species growth at various growth stages. Furthermore, the plant species-specific effects ranging from positive to negative may provide a mechanism of endophyte–plant interactions depending on plant and closely related congeneric natives. Furthermore, we show that resulting effects of endophyte–plant interactions depend on plant growth stage highlighting the importance of the timing of this interaction in determining plant growth (Sikes et al., 2016). Studies that determine plant growth after a single inoculation or single sampling point might therefore uncover only part of the ecologically relevant interactions between plants and (soil) biota. However, it has to be noted that we conducted the seed germination and seedling growth analyses under artificial conditions on agar plates, while plant growth was determined in sterilized soils. Therefore, patterns observed might deviate under more complex conditions in soils. Nevertheless, we aimed at uncovering potential interactions and therefore believe that our approach provides a valid model system for this purpose (Crowther et al., 2018). Yet, the generality of our findings that individual endophytes have differential effects on range-expanders and related natives may require further testing with other plant species and environmental conditions before conclusions may be generalized.

In conclusion, our findings suggest that a wide range of endophytes can directly impact plant growth, in addition to the known, often positive effects of AMF and negative effects of pathogenic fungi. Particularly, the impact on seed germination and seedling establishment was profound and may need to be investigated as well in agricultural settings, both to improve plant growth directly, as well as to enhance plant growth under environmental stress from drought, pathogens and other factors. While the present study shows the potential for an increased availability of biotic resources for bioengineering purposes, further steps are needed in order to explore the full consequences and possible solutions offered by fungal or fungo-like endophytes.

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**Authors’ Contributions**

S.G., F.C.t.H. and W.H.v.d.P. designed the study; S.G. and F.C.t.H. performed the experiment; S.G., O.K. and L.B.S. analysed the data; S.G., O.K. and W.H.v.d.P. drafted the manuscript supplemented with comments from L.B.S. and F.C.t.H.

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DATA AVAILABILITY STATEMENT
Sanger sequences are accessible at NCBI GenBank under the accession numbers MT242270–MT242299. Further data associated directly with this article are available from Dryad Digital Repository https://doi.org/10.5061/dryad.jq2bq87v (Geisen et al., 2020).

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