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Dark septate endophytes and arbuscular mycorrhizal fungi (*Paris*-morphotype) affect the stable isotope composition of 'classically' non-mycorrhizal plants

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Abstract

1. The vast majority of terrestrial plants exchange nutrients with fungal partners forming different mycorrhizal types. The minority of plants considered as non-mycorrhizal, however, are not necessarily free of any fungi, but are frequently colonized by elusive fungal endophytes, such as *dark septate endophytes* (*DSE*) or *fine root endophytes* (*FRE*). While a functional role of *FRE* in the improvement of nutrient gain was recently elucidated, the function of *DSE* is still in discussion and was here addressed for 36 plant species belonging to the families Equisetaceae, Cypereaceae and Caryophyllaceae.
2. Molecular and microscopic staining approaches were conducted to verify the presence of *DSE* in the investigated species. Stable isotope natural abundances of the elements carbon, nitrogen, hydrogen and oxygen and total nitrogen concentrations were analysed for the respective species of the target plant families and accompanying mycorrhizal and non-mycorrhizal (Brassicaceae) plant species.
3. Staining approaches confirmed the presence of *DSE* in all investigated species within the families Equisetaceae, Cyperaceae and Caryophyllaceae. A co-colonization with *Paris*-type arbuscular mycorrhiza (AM) was occasionally found by staining and molecular approaches in species of the Equisetaceae. Species of the Equisetaceae, Cyperaceae and Caryophyllaceae were significantly ^{15}N-enriched in comparison to accompanying plants. In addition, a significant ^{13}C and ^{2}H enrichment and increased total nitrogen concentrations were found for representatives of the Equisetaceae.
4. The ^{15}N enrichment found here for representatives of Equisetaceae, Cyperaceae and Caryophyllaceae provides evidence for a functional role of the ubiquitous *DSE* fungi. *DSE* fungi obviously provide access to ^{15}N-enriched soil organic compounds probably in exchange for organic carbon compounds from plant photosynthesis. As indicated by additional ^{13}C and ^{2}H enrichments, representatives of the Equisetaceae apparently gain simultaneously organic carbon compounds from

their AM fungi of the *Paris*-morphotype. Thus, species of the Equisetaceae have to be considered as partially, or in case of the achlorophyllous fertile *Equisetum arvense*, as fully mycoheterotrophic at least in some stages of their life cycle.
5. So far, mostly underappreciated fungi classified as *DSE* are suggested to occupy an ecologically relevant role similar to mycorrhizae and the occurrence of simultaneous functions of *DSE* and AM fungi in Equisetaceae is proposed.

KEYWORDS
Caryophyllaceae, Cyperaceae, dark septate endophytes, Equisetaceae, mycoheterotrophy, mycorrhiza, stable isotope natural abundance

1 | INTRODUCTION

The vast majority of terrestrial plants live in a mostly mutualistic symbiosis with fungi forming different types of mycorrhizae (Smith & Read, 2008; Tedersoo, Bahram, & Zobel, 2020). Only a small minority of terrestrial plants is considered as non-mycorrhizal. Species belonging to the families Equisetaceae (horsetails), Cyperaceae (sedges) and Caryophyllaceae (carnation family) are classical examples of plants considered as non-mycorrhizal (Brundrett & Tedersoo, 2019; Pressel, Bidartondo, Field, Rimington, & Duckett, 2016; Smith & Read, 2008). The absence of commonly known mycorrhizal partners in plants belonging to these three families, however, does not imply a general lack of fungal root endophytes. In contrary, Equisetaceae, Cyperaceae and Caryophyllaceae are all known to be colonized by *dark septate endophytes* (*DSE*, Ascomycota; Jumpponen & Trappe, 1998) and *fine root endophytes* (*FRE*, Mucoromycotina; Orchard et al., 2017; Walker, Gollotte, & Redecker, 2018). *DSE* form hyaline or melanized inter- and intracellular septate hyphae and microsclerotia (Jumpponen & Trappe, 1998; Mandyam & Jumpponen, 2005). *FRE* form arbuscule-like structures and non-septate fine branching hyphae with small vesicle-like swellings (Hoysted et al., 2019; Orchard et al., 2017). Occasionally, arbuscular mycorrhizae (AM, Glomeromycotina) were also documented in Equisetaceae interestingly forming the *Paris*-morphotype characterized by dense aseptate intracellular hyphal coils (Dhillion, 1993; Dickson, Smith, & Smith, 2007; Fernández, Messuti, & Fontenla, 2008; Hodson, Shahid, Basinger, & Kaminskyj, 2009; Koske, Friese, Olexia, & Hauke, 1985), whereas, if present, in Cyperaceae (Dickson et al., 2007; Druvca-Lusite & Ievinsh, 2010; Velázquez, Biganzoli, & Cabello, 2010) and Caryophyllaceae (Dickson et al., 2007; Druvca-Lusite & Ievinsh, 2010; Shah, Reshi, & Khasa, 2009) mainly the *Arum*-morphotype AM forming aseptate intercellular hyphae along the cortical root cell layers was recorded.

Recent investigations provide first evidence for potential functional roles of the various fungal root endophytes reported for species of the Equisetaceae, Cyperaceae and Caryophyllaceae. *FRE* were suggested to provide a mycorrhiza-like advantage mainly in grasses (Orchard et al., 2017) as well as in some phylogenetically basal plant groups, like liverworts (Field et al., 2019) and lycopods (Hoysted et al., 2019). Interestingly, Field et al. (2019) described a selective nutritional benefit for a liverwort partner simultaneously colonized by Mucoromycotina (effective nitrogen transfer) and Glomeromycotina (effective phosphorous transfer). *DSE* are ubiquitous root fungi occupying various ecosystems, however, a potential mycorrhizal, that is, beneficial, function for *DSE* is still in discussion and needs to be evaluated (Jumpponen, 2001; Jumpponen & Trappe, 1998; Mandyam & Jumpponen, 2005; Newsham, 2011). Although the first strong indication for an organic N acquisition by *DSE* was recently provided (Hill et al., 2019), in most cases they are treated as saprotrophs on dead roots. Furthermore, the *Paris*-morphotype among AM plants, as occasionally found in representatives of the Equisetaceae, was recently identified as bearing the potential for mycoheterotrophy which is in contrast to plant species forming the *Arum*-morphotype (Giesemann, Rasmussen, Liebel, & Gebauer, 2020).

Analysis of stable isotope natural abundance of the elements carbon (C), nitrogen (N) and hydrogen (H) has proven to be a valuable tool to elucidate organic C and mineral nutrient fluxes between plants and fungi in different types of mycorrhizal associations (Gebauer & Meyer, 2003; Gebauer, Preiss, & Gebauer, 2016; Giesemann, Rasmussen, et al., 2020; Merckx, Stöckel, Fleischmann, Bruns, & Gebauer, 2010; Ogura-Tsujita, Gebauer, Hashimoto, Umata, & Yukawa, 2009; Zimmer et al., 2007). Hundreds of mycoheterotrophic plant species were identified to subvert the usually mutualistic mycorrhizal symbiosis and to utilize their fungal partners as nutrient source, which allows them to produce endospermless dust seeds (initial mycoheterotrophs) or to reduce (partial mycoheterotrophs) or even cease their photosynthetic activity (full mycoheterotrophs; Hynson et al., 2013; Leake, 1994; Merckx, 2013). The draining of fungal nutrients changes the mycoheterotrophs' stable isotope natural abundance pattern towards enrichment in heavy C, N and H isotopes (Gebauer et al., 2016; Hynson et al., 2013; Hynson, Schiebold, & Gebauer, 2016) as also found for fruit bodies of many fungi (Gebauer & Dietrich, 1993; Gleixner, Danier, Werner, & Schmidt, 1993; Mayor, Schuur, & Henkel, 2009; Ziegler, 1995). This 'interlinking' in mutualistic networks has been identified as widely distributed among achlorophyllous as well as photosynthetic plant species forming orchid (Gebauer & Meyer, 2003; Hynson et al., 2013, 2016) and ericoid mycorrhizae

(Hynson et al., 2013, 2016; Lallemand et al., 2017; Tedersoo, Pellet, Kõljalg, & Selosse, 2007; Zimmer et al., 2007) and more recently in AM (Courty et al., 2011; Giesemann, Rasmussen, et al., 2020; Gomes, Merckx, Kehl, & Gebauer, 2020; Hynson et al., 2013; Merckx et al., 2010).

Due to strong global and local variations in climatic growing conditions, soil conditions and precipitation, isotope abundances of plant tissues vary depending on their growing location, that is, stable isotope abundances are site-dependent and thus, cannot directly be compared across different locations. In order to circumvent this limitation Preiss and Gebauer (2008) suggested a normalization approach referring the isotope abundances of target plants (TPs; e.g. mycoheterotrophs) to a diverse set of putatively autotrophic reference plants growing under identical micro-site conditions and thus, converting traditional isotope abundances (δ values) into enrichment factors ε. Based on this approach, a steadily increasing site-independent enrichment factor database, not only of mycoheterotrophic plants, but also of putatively autotrophic plant species has been established. Interestingly, within the group of putatively autotrophic reference plants (all following the C_3 pathway of photosynthesis), 12 species belonging to the classically as non-mycorrhizal classified families Equisetaceae, Cyperaceae and Caryophyllaceae emerged as conspicuously enriched in the heavy isotope ^{15}N (and in case of the Equisetaceae simultaneously in the heavy isotope ^{13}C).

This finding raised two hypotheses: (1) A ^{15}N enrichment is a general phenomenon among plant species belonging to the families of Equisetaceae, Cyperaceae and Caryophyllaceae. (2) This unique pattern in isotope composition is functionally related to their respective fungal endophytes. In order to test these hypotheses, we generated C and N and in some cases H and oxygen (O) stable isotope abundance data suited for enrichment factor calculations for 24 plant species belonging to the families Equisetaceae, Cyperaceae and Caryophyllaceae. We then combined the data from these 24 species with the 12 species contained in our database in order to test whether unique stable isotope abundance patterns are a general phenomenon among plant species of these three families. Furthermore, we used staining techniques and light microscopy to identify endophytic fungi in the roots of selected species from the three families. Additionally, molecular approaches for the identification of AM fungi were applied for three selected *Equisetum* species. The functional drivers leading to unique stable isotope abundance patterns among the investigated plant species are discussed.

2 | MATERIALS AND METHODS

2.1 | Plant sampling

Target plant material was collected for representatives of the Equisetaceae (six species, $n = 103$), Cyperaceae (12 species, $n = 78$) and Caryophyllaceae (six species, $n = 35$) at six different locations per family distributed in NE-Bavaria (Germany; ~49.50 to 50.20 N, ~11.20 to 11.90 E decimal WGS84). The sampling scheme followed the methodology outlined in Gebauer and Meyer (2003), which includes at least one TP species accompanied by three to six neighbouring plants as references in a 1-m^2 plot ($n = 335$, 126 and 92 reference plants associated with Equisetaceae, Cyperaceae and Caryophyllaceae TPs respectively). The reference plant material represented a variety of C_3 plants belonging to 25 plant families colonized by different mycorrhizal fungal partners (ectomycorrhiza: ECM, arbuscular mycorrhiza: AM, ericoid endomycorrhiza: ErM) or being non-mycorrhizal: NM (Brundrett & Tedersoo, 2019; Wang & Qiu, 2006).

- Plant material of Equisetaceae was sampled from May to August 2005 distributed in agricultural land (*Equisetum arvense* L.), mixed forests (*E. sylvaticum* L.) and within patchy located swamp lands (*E. fluviatile* L., *E. hyemale* L., *E. palustre* L., *E. sylvaticum*, *E. telmateia* Ehrh.) including lateral shoots and thereon scale leaves, stem and root material of the target as well as leaf, stem and root material of respective reference plant species. For *E. arvense* achlorophyllous fertile and chlorophyllous sterile plant individuals were sampled. In 2018, one site was subsequently added, aiming to compare lateral shoots and thereon scale leaf material of *Equisetum* with leaf material of a moss species. Additionally, corresponding root samples for the estimation of colonization by fungal endophytes and temporal development of colonization during the vegetation period were collected at the same sites in 2016 and 2017.
- Leaves of Cyperaceae were sampled during the vegetation periods of 2008 and 2009 in *Fagus sylvatica* dominated forests (*Carex flacca* Schreb., *C. digitate* L.), wet meadows (*C. disticha* Huds., *C. flacca*, *C. hirta* L., *C. nigra* (L.) Reichard, *C. panicea* L., *C. pallescens* L., *C. vulpina* L., *Scirpus sylvaticus* L.), chalk heath (*C. caryophyllea* Latourr) and within a swamp land surrounded by a coniferous forest (*C. nigra*, *C. vesicaria* L., *Eriophorum vaginatum* L.). In addition, corresponding root samples for staining approaches were collected at the same time.
- Leaves of Caryophyllaceae were sampled from spring to early summer 2019 in a public lawn area (*Stellaria media* (L.) Vill.), wet forest riversides (*Cerastium fontanum* Baumg., *Stellaria holostea* L., *Silene dioica* (L.) Clairv.) and vegetation-poor and sunny slopes (*Lychnis viscaria* (L.) Jess., *Saponaria officinalis* L.). Root sampling for the approximation of fungal colonization was conducted at the same time.

All root samples for the identification of fungal partners were treated equally by washing procedures, followed by fixation in 70% ethanol and storage at 4°C until further analysis.

2.2 | Stable isotope natural abundance and nitrogen concentration analysis

Plant material for isotope abundance analysis was washed with deionized water, oven-dried overnight (105°C) and ground to a homogenous powder in either a ball mill (Retsch Schwingmühle MM2) or a micro-dismembrator (B. Braun Biotech International). The samples were stored in desiccators filled with silica gel until further

processing. Isotope ratio mass spectrometry (IRMS) was applied to analyse natural relative abundances of stable C ($^{13}C/^{12}C$), N ($^{15}N/^{14}N$), H ($^{2}H/^{1}H$) and O ($^{18}O/^{16}O$) isotopes. An elemental analyser (NA 1108, Carlo Erba Instruments) coupled to an IRMS (delta S, Finnigan MAT) via a Conflo III interface (Thermo Fisher Scientific) was applied for C and N isotopes, while a thermal conversion device (HTO, HEKAtech) coupled to an IRMS (delta V advantage, Thermo Fisher Scientific) via a Conflo IV interface (Thermo Fisher Scientific) were used for H and O isotopes. Acetanilide (Merck KGaA) was used to calibrate the obtained C/N concentrations. Standard gases (Riessner) were calibrated according to international standards (CO_2 vs. V-PDB, N_2 vs. N_2 in air, H_2 and CO vs. V-SMOW), applying reference substances provided by the International Atomic Energy Agency, Vienna, Austria (ANU sucrose, CH6, CO8, NBS18 and NBS19 for the C isotopes, N1 and N2 for the N isotopes, CH7, V-SMOW and SLAP for H isotopes and IAEA601 and IAEA602 for the O isotopes). A memory bias (see Gebauer et al., 2016) was avoided by analysing H isotope samples four times. All samples were analysed plot-wise in identical batches to minimize an atmospheric bias through a potential H atom exchange within the samples with ambient air. Reproducibility of isotope measurements was always within ±4‰ for δ^2H, ±0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, and ±0.6‰ for $\delta^{18}O$. The resulting relative isotope abundances follow the rules of the δ-notation: $\delta^{13}C$, $\delta^{15}N$, δ^2H and $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1{,}000$ (‰), whereby R is the ratio of the heavy to the respective light isotope.

In order to complement the obtained field survey in NE-Bavaria, our database survey added stable isotope enrichment factors for eleven species belonging to the Cyperaceae (n = 131, *C. conica* Boott, *C. flava* L., *C. distachya Desf.*, *C halleriana* Asso, *C. remota*, *C. siderosticta* Hance, *Machaerina* sp., *Rhynchospora alba*, *Rhynchospora* sp., *Trichophorum cespitosum* (L.) Hartm. and an unidentified *Carex* species) from 10 published datasets and three unpublished field works and references ($n_{Cyperaceae}$ = 372). Additionally, the data base added one species of the Caryophyllaceae (n = 13, *Dianthus arenarius* L.) from two published datasets and one unpublished field work and references ($n_{Caryophyllaceae}$ = 73). The database survey added 28 plant families to our field survey (in total 53). Only data following the sampling design described here were included. The detailed coordinates of the sampling areas and the studies complementing our analysis can be obtained from the supplement (Tables S2, S4, S6). A complete list of plant individuals can be obtained from the data repository (Giesemann, Eichenberg, et al., 2020).

2.3 | Root staining

Roots of Equisetaceae, Cyperaceae and Caryophyllaceae were stained according to Phillips and Hayman (1970) and following recommendations from Vierheilig, Schweiger, and Brundrett (2005). In principle, the ethanol fixed roots were washed in deionized water at least three times. Afterwards, roots were cleared applying a 10% KOH (w/v) solution for 30 min at 70°C in a water bath with continuously slight panning (Köttermann 3043, Köttermann GmbH & Co.) or under carefully inverting the staining tube several times manually. If necessary, pigmented roots were bleached applying 5% (v/v) H_2O_2 and 0.5% (v/v) NH_3 solution following the protocol from Fernández et al. (2008) for 10–30 min under room temperature depending on the pigmentation of the root sample. The durations of the clearing and bleaching treatments were adjusted for several individuals if necessary, e.g. for very dark pigmented roots of Equisetaceae or very fine roots of some Cyperaceae and Caryophyllaceae plant species. Root samples were washed with deionized water after bleaching and clearing several times. Before staining, Equisetaceae and Caryophyllaceae roots were acidified with 1% (v/v) HCl and Cyperaceae were acidified with 2% (v/v) lactic acid for 5–10 min respectively. A 0.05%-1% (v/v) trypan blue staining solution in 33% (v/v) acidic glycerol and 33% (v/v) lactic acid and 33% deionized water was applied for staining over-night at room temperature. Stained roots were stored in a refrigerator in a solution of glycine/lactic acid/distilled water (3:1:3) until further analysis. Fungal colonization was determined via light microscopy and documented with either an Olympus BH (Olympus Deutschland GmbH) equipped with an Olympus C-330 camera (Olympus Deutschland GmbH) or applying BA210LED trino (Motic) equipped with the 3MP Moticam 3+. Images were observed with Cell^A 2.6 (Olympus Soft Imaging Solutions GmbH) or Fiji ImageJ 1.51n (Schindelin et al., 2012).

In principle, the quantification of fungal root colonization followed Brundrett, Bougher, Dell, Grove, and Malajczuk (1996) by the evaluation of the presence or absence of fungal structures intersecting the hair-cross of the object lens. For Equisetaceae, ten 1-cm long root fragments were observed in three replicates per species. The presence/absence of fungal structures intersecting with the hair-cross were noted. This procedure was repeated for three (*E. sylvaticum*, *E. palustre*, *E. arvense*, *E. telmateia*) to five (*E. hyemale*) observations per species in one vegetation period. The number of colonized fields of views relative to the total number of fields of views (100 per individual) represented the colonization rate. Caryophyllaceae were treated equally, applying five root fragments (50 field of views per individual). The total number of fungal structures intersecting the hair-cross were counted. For Cyperaceae, four root fragments were observed and the presence/absence of fungal structures were evaluated for the whole root fragment (50 field of views per individual). Fungal structures were blue-stained aseptate hyphae, brownish and blue-stained septate hyphae, brownish microsclerotia, blue-stained vesicles, spores and arbuscules (Figure 1).

2.4 | Molecular approaches

In order to test roots of Equisetaceae for the occurrence of AM fungi in addition to *DSE* molecular approaches were applied for three *Equisetum* species. Root tips were cleaned with water and stored on 2% CTAB buffer at −20°C until further processing. Fungal DNA

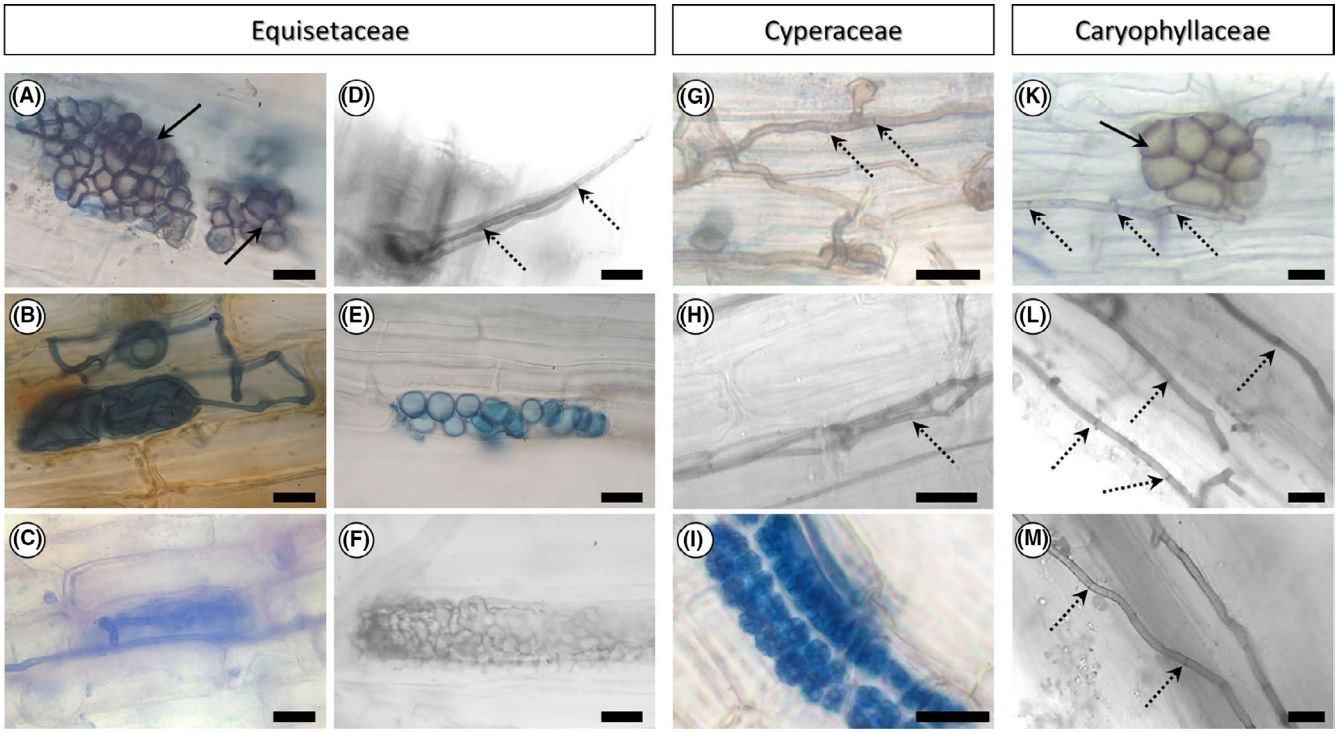

FIGURE 1 Structures of fungal root endophytes investigated in representatives of the plant families Equisetaceae, Cyperaceae and Caryophyllaceae. Dark septate fungal microsclerotia were found in all three plant families (e.g. A, K; bold arrow). Hyphae of dark septate fungal endophytes (dashed arrow) were either blue-stained or brownish and present in Equisetaceae (D), Cyperaceae (G, H) and Caryophyllaceae (L, M). Arbuscular mycorrhizal hyphae were present in Equisetaceae (e.g. B, *Paris*-type coils; C, F, *Paris*-type like structures and E vesicles- or spore-like structures). Occasionally, big colonies of the intracellular structures of Chytridiomycota fungi (I) were found in Cyperaceae and Caryophyllaceae. Scale bar: 20 μm

was extracted from root samples with the KingFisher Flex Magnetic Particle Processors (Thermo Fisher Scientific) using the NucleoMag 96 Plant Kit (Machery-Nagel). Each of the 96 aliquots in the well was assigned a unique Multiplex Identifier (MID) barcode sequence. Subsequently, amplicon libraries were created using barcode-tagged primers for the internal transcribed spacer 2 (ITS2), using the fungal specific primer fITS7 (Ihrmark et al., 2012) and ITS4 (White, Bruns, Lee, & Taylor, 1990). PCR products were purified using 0.9× NucleoMag NGS Clean-Up and Size Select beads (Macherey-Nagel) according to the manufacturer's instructions. The concentration of the individual indexed amplicons was measured with the QIAxcel using the DNA Screening kit (Qiagen), and normalized and pooled equimolar using the QIAgility robot (Qiagen). Sequencing was performed on a MiSeq Illumina platform using the paired-end 300 bp kit at BaseClear. Sequence processing was performed following the UNOISE3 algorithm (Edgar, 2016b) implemented in USearch v.11 (http://www.drive5.com/usearch/). Paired reads were assembled and passed the quality filter allowing for reads with maximum error <1.0. Followed by dereplication and filtering out singletons, the resulting sequences were used to create zero radius operational taxonomic units (zOTU), resulting in 183 zOTUs (93,883 reads) with putative chimeras removed (Blaalid et al., 2013). The taxonomic rank was assigned by BLAST search against the UNITE 8.2 database (Abarenkov et al., 2020) and with the *sintax* algorithm implemented in USearch (Edgar, 2016a) in conjunction with the UNITE database.

2.5 | Statistical analysis

Following the recommendations of Preiss and Gebauer (2008), enrichment factors ε were calculated from measured δ values by using the TP subtracted by the mean δ values of the reference plants (RP; for $\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$ and $\varepsilon^{18}O$: $\varepsilon_{TP} = \delta_{TP}$-mean δ_{RP}), or by using already published δ values. The reference plants describe the habitat conditions and ε values reflect the relative differences to our TPs, corrected for any variations in heavy isotope enrichment due to site-specific peculiarities. This ensures that, across all samples, enrichment factors ε are independent of spatial variation; therefore, ε values can be compared across sampled populations.

The relative amount of C received from their fungal source by each single *Equisetum* individual was approximated applying the two-source linear mixing model (Gebauer & Meyer, 2003; Giesemann, Rasmussen, et al., 2020; Hynson et al., 2013). There, the reference plants are assumed to exclusively obtain C through photosynthesis while the achlorophyllous fertile stages of *E. arvense* are considered as covering their entire C demand from a fungal source. The relative amount of C received from their fungal source is shown as mean values and standard deviations for *Equisetum* species and averaged across all *Equisetum* individuals.

Data analyses were performed with RSTUDIO 1.2 (R Core Team, 2019) and SIGMAPLOT 11.0 (Systat Software, 2008). Effect sizes *d* were additionally checked according to Lenhard and Lenhard (2017).

An effect size $d \geq 0.8$ is considered as large (Cohen, 1992). Stable isotope abundances were tested for normality via Shapiro–Wilk test and homogeneity of variances via Levene test. The nonparametric tests, one-tailed Mann–Whitney *U* and one-tailed Kruskal–Wallis *H*, had to be applied for pairwise comparisons or comparisons across multiple groups respectively. In the case of a significant Kruskal–Wallis result, a post-hoc Dunn's test for multiple comparison was applied (*Z*; Dinno, 2017). *P*-values were corrected according to the sequential Holm-Bonferroni method. The critical level of significance was set to $\alpha = 0.05$. Data expression is in mean values with standard deviations ($\bar{x} \pm SD$).

3 | RESULTS

3.1 | Equisetaceae

A variety of fungal endophytes were visually documented for *Equisetum* species with *DSE* approximately more than 80% (Figure 1A–F). *DSE* remained either brown or were blue-stained but septa were very clear. Occasionally, AM forming *Paris*-type coils and vesicles were documented. The sequencing data indicated a diversity of unidentified fungi from the order Helotiales representing 51%, 40% and 29% of the reads in *E. arvense*, *E. palustre* and *E. sylvaticum* (see Figure S1 and Table S8). In addition, zOTUs identified as the *septate endophytes Phialocephala fortinii* (6% of the total reads), *Cladophialophora chaetospira* and *Tetracladium* spp. (1% of the total reads) while also AM Glomeromycotina were documented (<1% of the total reads). The total colonization rate of fungal endophytes across all here investigated *Equisetum* species was 25 ± 14%. While *Equisetum hyemale* had almost constantly low colonization rates during the year, a trend from high colonization towards almost no fungal colonization from May to August was observed for *E. palustre*, *E. sylvaticum*, *E. arvense* and *E. telmateia* (Figure S2).

By definition of the enrichment factor ε, the reference plants clustered around zero with a calculated standard deviation of ±1.1‰ for lateral shoots including scale leaves, ±1.1‰ for stems, ±1.3‰ for roots in ^{13}C and of ±1.5‰ for lateral shoots including scale leaves, ±1.1‰ for stems, ±1.3‰ for roots in ^{15}N (Figure 2). The reference plants forming AM, ECM or ErM clustered together within the range of the standard deviation of all reference plant species. The NM plant species *Brassica napus* and *Capsella bursa-pastoris* (Brassicaceae) were similar to the mycorrhizal reference plant species in ^{13}C and ^{15}N.

Six out of seven *Equisetum* species were significantly enriched in ^{13}C and ^{15}N (Figure 2) and had higher total N concentrations relative to accompanying reference plants (Table 1). This observation held true for lateral shoots including scale leaves, stems and roots (Table 1); ^{13}C enrichment increased from 2.6 ± 1.2‰, 3.2 ± 1.7‰ to 3.6 ± 1.2‰, while ^{15}N enrichment gradually decreased from 4.6 ± 3.0‰, 3.2 ± 2.8‰ to 2.3 ± 2.5‰ for lateral shoots including the scale leaves, stem and root respectively (Figure 2). The achlorophyllous, fertile stems of *E. arvense* were most enriched in ^{13}C

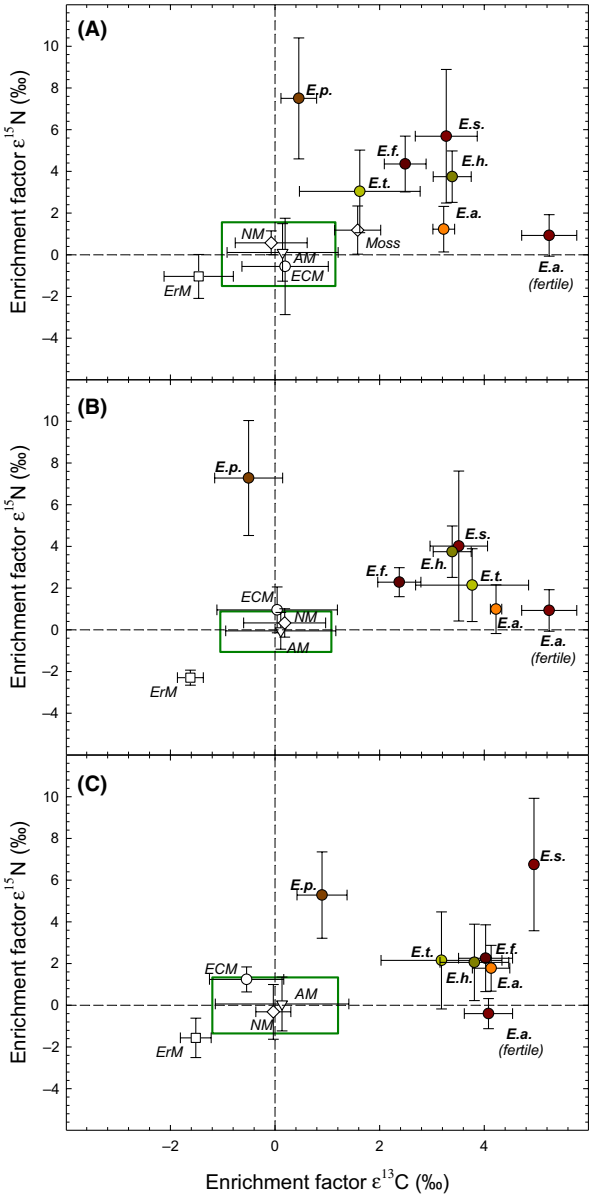

FIGURE 2 Carbon and nitrogen stable isotope enrichment factors ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) in lateral shoots including the scale leaves (A: *Equisetum* spp.: $n = 34$, reference: $n = 125$), stems (B: *Equisetum* spp.: $n = 39$, reference: $n = 125$) and roots (C: *Equisetum* spp.: $n = 30$, reference: $n = 135$) of six chlorophyllous *Equisetum* species and of achlorophyllous fertile *Equisetum arvense* samples and of reference plants comprising arbuscular mycorrhizal (AM, white triangles), ectomycorrhizal (ECM, white circles), ericoid mycorrhizal (ErM white squares) and non-mycorrhizal (NM white diamonds) plant species. The green frames represent standard deviations of all reference plants. Each coloured symbol represents a single species belonging to the Equisetaceae. In the case of *E. arvense*, two different states in the life cycle (chlorophyllous sterile individuals and achlorophyllous fertile individuals) are presented separately. All data are shown with mean values and standard deviations. E.a. *Equisetum arvense* (separated by chlorophyllous sterile and achlorophyllous fertile individuals), E.f. *E. fluviatile*, E.h. *E. hyemale*, E.p. *E. palustre*, E.s. *E. sylvaticum*, E.t. *E. telmateia*. The plant lateral shoots including the scale leaves and the plant stem were pooled for *E. arvense* (fertile) and *E. hyemale*, respectively, thus shown in A and B

TABLE 1 Test for differences in enrichment factors ε of ^{13}C (‰) and ^{15}N (‰) and in total N concentrations (mmol/g_{dw}) of six chlorophyllous *Equisetum* species and their reference plants separately for three different plant organs. Mann–Whitney *U* test. Significances are highlighted in bold

Organ (N_{Equi}, N_{Ref})	$\varepsilon^{13}C$		$\varepsilon^{15}N$		Total N	
	TS	*P*	TS	*P*	TS	*P*
Leaf (34,125)	*U* = 311	**<0.001**	*U* = 318	**<0.001**	*U* = 679	**<0.001**
Stem (34,100)	*U* = 382	**<0.001**	*U* = 358	**<0.001**	*U* = 1,679	0.214
Root (25,110)	*U* = 108	**<0.001**	*U* = 541	**<0.001**	*U* = 970	**<0.001**

Note: TS: Test statistic; for $p < 0.05$, Cohen's *d* effect size is always > 1.0, except total N in roots ($d_{Cohen} = 0.7$). Leaf include the lateral shoots and thereon the scale leaves. The fertile individuals of *Equisetum arvense* were excluded as they are achlorophyllous.

(5.2 ± 0.5‰), but least enriched in ^{15}N (0.9 ± 1.0‰); while samples of *E. palustre* were the least enriched in ^{13}C (0.5 ± 0.3‰), in most cases, the most enriched in ^{15}N (7.5 ± 2.9‰). The non-mycorrhizal moss *Polytrichum commune* was significantly enriched in ^{13}C but did not differ in ^{15}N from its site-specific mycorrhizal reference plants (Table S1). *Equisetum sylvaticum* at this additional site mirrored the results already presented in Figure 2A, that is, being significantly enriched in ^{13}C and ^{15}N relative to moss and mycorrhizal plants.

The total N concentrations of the lateral shoots including the scale leaves were highest in *E. palustre*, *E. sylvaticum*, *E. arvense* and *E. fluviatile*, ranging from 1.71 to 3.7 mmol/g_{dw}, and lowest in *E. telmateia*, ranging from 1.22 to 1.57 mmol/g_{dw} when compared to their corresponding reference plants (range = 0.89 to 3.75 mmol/g_{dw}). These findings were mostly congruent also for stems and roots (Table S2). Also, the non-mycorrhizal moss *Polytrichum commune* did not differ in leaf total N concentration from its site-specific mycorrhizal reference plants but had significantly lower leaf total N concentrations than accompanying *Equisetum sylvaticum* individuals in their lateral shoots and thereon the scale leaves (Table S1).

Equisetum sylvaticum and *E. palustre* were significantly ^{2}H-enriched by 14 ± 5.4‰ ($U_{9,20} = 3$, $p = 0.001$) and significantly ^{18}O-enriched by 3.5 ± 1.7‰ ($U_{9,20} = 3$, $p = 0.001$) relative to accompanying reference plants.

Based on two-source linear mixing model calculations, the carbon received from the fungal source covered a range from 9 ± 6% in *Equisetum palustre*, 31 ± 22% in *E. telmateia*, 48 ± 7% in *E. fluviatile* to 62 ± 7% in *E. sylvaticum*, *E. arvense* and E. *hyemale*. On average, the mixing model suggests that 50 ± 22% of C across all *Equisetum* species originated from a fungal source and the remaining from photosynthesis.

3.2 | Cyperaceae

Septate hyphae and intracellular structures, either brown or blue-stained, were found in every investigated species (Figure 1G–I). In *Carex pallescens*, *C. vulpina and Scirpus sylvaticus* very few vesicle-like structures were observed (facultative mycorrhiza); however, eight out of 11 species were classified as non-mycorrhizal but *DSE* colonized. *Carex flacca* was the most pronounced example for very dense aseptate hyphae, that formed mantle-like structures which did not enter the root tissue, and spherical intraradical hyphae (probably, saprotrophic Chytridiomycetes, Terence T. McGonigle *personal communication* cf. Figure 1I). *Eriophorum vaginatum* had to be excluded from the light microscopy investigations, as the fine root structures were unusable after harsh clearing and staining procedure. All Cyperaceae species established dauciform roots, a special form of roots, often found in Cyperaceae which produces shortened club-like root structures.

Relative to reference plants composed of species forming AM, ECM, ErM or being NM and clustering around zero, with a standard deviation of ±1.0‰ in ^{13}C and of ±1.3‰ in ^{15}N (Figure 3), most species of the Cyperaceae plant family were significantly ^{15}N-enriched by 3.3 ± 2.1‰ ($U_{209,499} = 8{,}714$, $p = 0.001$, $d_{Cohen} = 1.7$), whereby ^{13}C was in the range of their references (0.4 ± 1.1‰; $U_{209,499} = 40{,}102$, $p = 0.001$, $d_{Cohen} = 0.4$; Figure 2). The effect size represented by Cohen's *d* value supports the significance in ^{15}N enrichment ($d \geq 0.8$), but not for ^{13}C ($d < 0.8$). The ^{15}N enrichment ranged from −0.2 ± 0.1‰ in *Carex siderostricta*, to 5.0 ± 1.7‰ and 5.1 ± 4.0‰ represented by *Carex vesicaria* and *T. cespitosum* respectively.

Leaf total N concentrations for Cyperaceae ranged from 0.1 to 3.0 mmol/g_{dw}, with a mean of 1.2 ± 0.4‰ mmol/g_{dw}, being slightly lower than this of the reference plants with a mean of 1.4 ± 0.5‰ mmol/g_{dw} (range = 0.5–3.0 mmol/g_{dw}; $U_{194,469} = 37{,}521$, $p < 0.001$, $d_{Cohen} = 0.3$).

Cyperaceae did not show significant enrichment in ^{2}H, when compared to their references (2.2 ± 9.3‰ vs. 0 ± 7.9‰, respectively; $U_{82,195} = 6{,}885$, $p = 0.068$, $d_{Cohen} = 0.2$). In contrast, Cyperaceae showed a significant depletion in ^{18}O relative to the reference plants (−0.5 ± 2.4‰ vs. 0 ± 1.8‰, respectively; $U_{82,194} = 6{,}600$, $p = 0.022$). However, with a Cohen's *d* value of <0.3, these differences are quite small.

3.3 | Caryophyllaceae

Colonization rates of *DSE* in the investigated Caryophyllaceae were more than twice as high when compared to their accompanying reference plants (mean 0.66 vs. 0.27 *DSE* per field of view, respectively; cf. Figure 1K–M). In contrast, aseptate hyphae, vesicles and

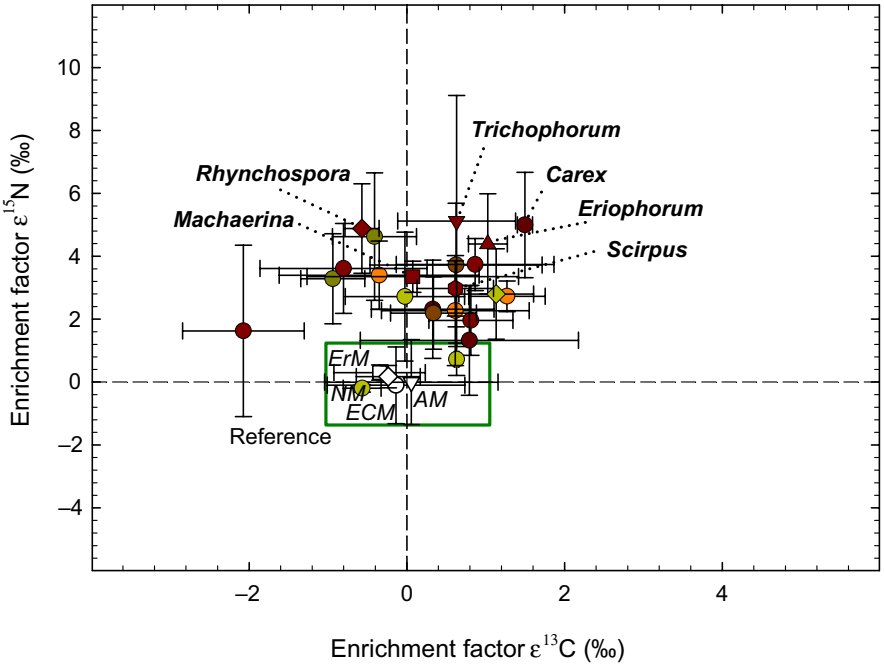

FIGURE 3 Carbon and nitrogen stable isotope enrichment factors ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) in leaves of 23 plant species of the Cyperaceae ($n = 209$; coloured symbols) and of reference plants comprising arbuscular mycorrhizal (AM, white triangle), ectomycorrhizal (ECM, white circle), ericoid mycorrhizal (ErM white square) and non-mycorrhizal (NM white diamond) plant species ($n = 499$). The green frame represents the standard deviation of all reference plants. Each coloured symbol represents a single species belonging to the Cyperaceae. Identical symbols represent affiliation to identical genera as indicated. All data are shown with mean values and standard deviations

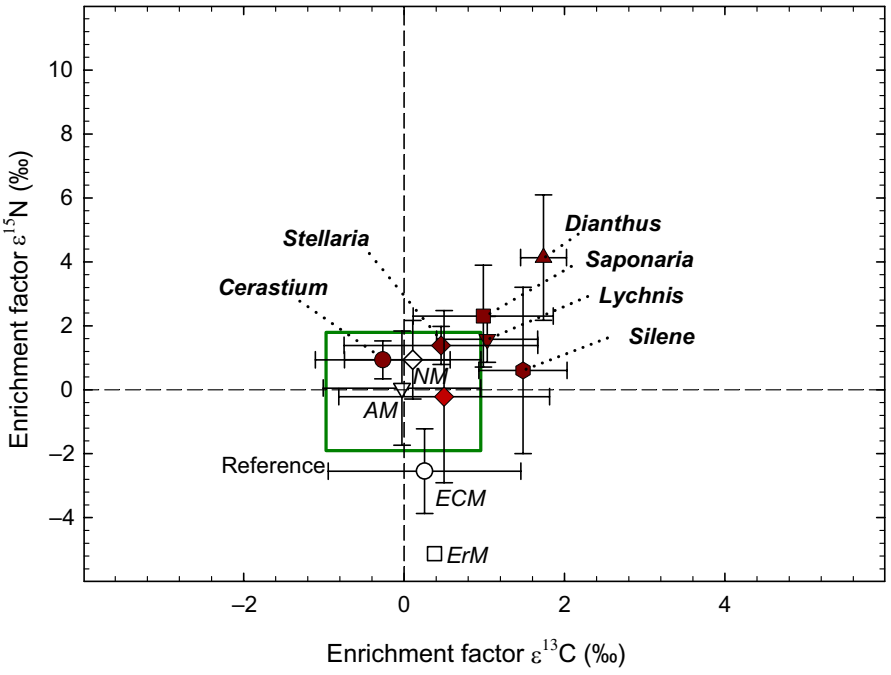

FIGURE 4 Carbon and nitrogen stable isotope enrichment factors ($\varepsilon^{13}C$ and $\varepsilon^{15}N$) in leaves of seven plant species of the Caryophyllaceae ($n = 48$) and of reference plants comprising arbuscular mycorrhizal (AM, white triangle), ectomycorrhizal (ECM, white circle), ericoid mycorrhizal (ErM white square) and non-mycorrhizal (NM white diamond) plant species ($n = 165$). The green frame represents the standard deviation of all reference plants. Each coloured symbol represents a single species belonging to the Caryophyllaceae. Identical symbols represent affiliation to identical genera as indicated. All data are shown with mean values and standard deviations

arbuscules, structures indicative for AM fungi, were more prominent in the accompanying reference plants and only in a neglectable amount in Caryophyllaceae (0.3 AM in reference plants vs. <0.05 AM per field of view in Caryophyllaceae).

Reference plants were again composed of species forming AM, ECM, ErM or were NM. Their enrichment factor ε clustered around zero with a standard deviation of ±1.0‰ in ^{13}C and of ±1.9‰ in ^{15}N (Figure 4). The NM plant species *Brassica napus* and *Capsella bursa-pastoris* (Brassicaceae) were less ^{15}N-enriched than Caryophyllaceae plant species. Most of the Caryophyllaceae species investigated in the present study were significantly enriched in ^{13}C by 0.7 ± 1.1‰ ($U_{48,165} = 2{,}394.5$, $p = 0.001$, $d_{Cohen} = 0.6$) and ^{15}N by 1.2 ± 2.0‰ ($U_{48,165} = 2055$, $p = 0.001$, $d_{Cohen} = 0.8$). While some species were considerably high ^{15}N-enriched, for example, *Dianthus arenarius* (4.1 ± 1.9‰), *S. officinalis* (2.3 ± 1.6‰), *S. media* (1.4 ± 0.6‰), few species did not significantly differ from their reference plants (Table S7). In ^{13}C, the here investigated Caryophyllaceae ranged from −0.2 ± 2.7‰ in *S. holostea* to 1.7 ± 0.3‰ in *Dianthus arenarius*.

Across all investigated Caryophyllaceae, leaf total N concentration ranged from 0.6 to 3.0 mmol/g_{dw} (mean = 1.6 ± 0.6 mmol/g_{dw}), which was in the range of the reference plants (mean = 1.7 ± 0.8 mmol/g_{dw}; range = 0.5–5.5 mmol/g_{dw}; $U_{48,165} = 3{,}485$, $p = 0.207$, $d_{Cohen} = 0.2$).

3.4 | Synopsis

In total, we investigated 36 plant species of the families Equisetaceae, Cyperaceae and Caryophyllaceae. These families are traditionally considered as non-mycorrhizal. However, we could demonstrate that these families across species are consistently colonized by fungi belonging to the *DSE*. In addition, for the Equisetaceae, we could identify microscopic structures closely resembling AM fungi forming *Paris*-type coils and vesicles; colonization by AM fungi was also confirmed by molecular data. Moreover, plants of all three families were significantly enriched in ^{15}N when compared to local reference plants, irrespective of whether these references were AM, ECM, ErM or NM. The ^{15}N enrichment was highest for Equisetaceae followed by Cyperaceae and Caryophyllaceae (Figure 5; Table 2). In addition, the plants turned out as enriched in ^{13}C, with Equisetaceae showing highest enrichment, followed by Caryophyllaceae and Cyperaceae (Figure 5; Table 2). However, effect sizes confirmed a meaningful ^{13}C enrichment only for the species belonging to the Equisetaceae. Achlorophyllous fertile stems of *E. arvense* were more enriched in ^{13}C than all other plant species here investigated. Equisetaceae

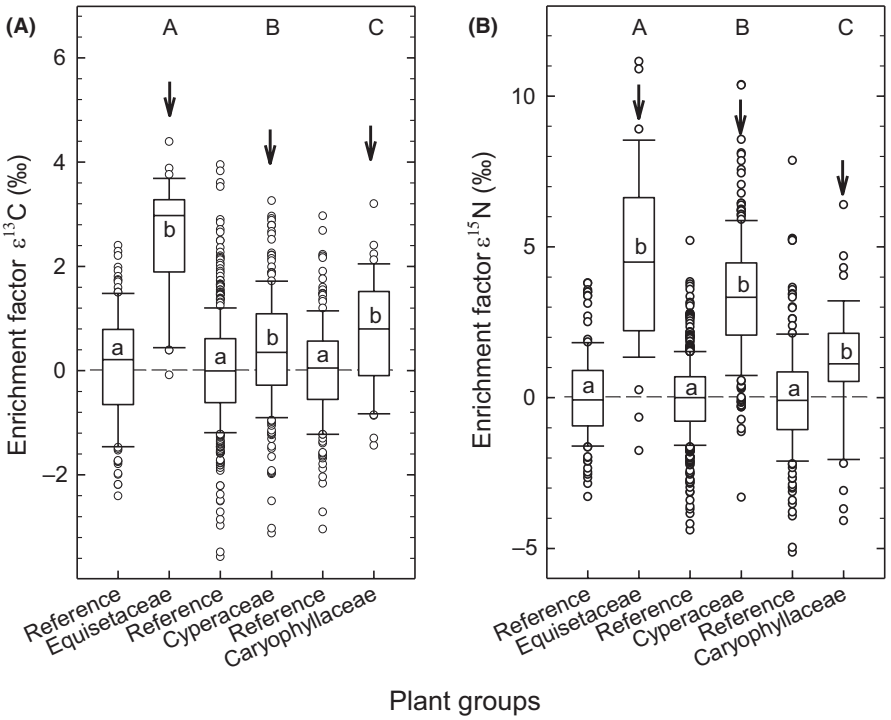

FIGURE 5 Box-and-whisker plots for stable carbon (A) and nitrogen (B) isotope enrichment factors (ε^{13}C and ε^{15}N) of lateral shoots including the scale leaves of six plant species belonging to the Equisetaceae ($n = 34$), leaves of 23 plant species belonging to the Cyperaceae ($n = 209$) and leaves of seven plant species belonging to the Caryophyllaceae ($n = 48$) and their respective reference plants ($n = 789$, in total). The dashed line represents the mean of the reference plants. The capital letters illustrate significance of difference between the TP species belonging to Equisetaceae, Cyperaceae and Caryophyllaceae (arrow). The lower-case letters indicate significance of difference between the TP family and their respective reference plants. The reference plants were not significantly distinguished in ε^{13}C and ^{15}N while significance in total N is not counted as relevant (Cohen's $d = 0.4$). The dashed line marks 0‰ enrichment. The range of the boxes illustrate the first and third quartile, the horizontal solid lines represent the medians, the whiskers enclose data within the 1.5× interquartile range, white circles are data extremes

TABLE 2 Pairwise comparison for Equisetaceae (Equi), Cyperaceae (Cype) and Caryophyllaceae (Cary) in enrichment factors ε of ^{13}C (‰), ^{15}N (‰) and of total N concentrations (mmol/g_{dw}) for leaf samples. Dunn's post hoc (Z) test including Bonferroni-Holm correction. Significances are highlighted in bold

	ε^{13}C		ε^{15}N		Total N	
Species (N)	**TS**	***p***	**TS**	***p***	**TS**	***p***
Equi (34) versus Cary (48)	$Z = 4.8$	**<0.001**	$Z = 6.2$	**<0.001**	$Z = 2.5$	**0.005**
Equi (34) versus Cype (209)	$Z = 7.5$	**<0.001**	$Z = 2.4$	**0.007**	$Z = 5.9$	**<0.001**
Cary (48) versus Cype (209)	$Z = 2.1$	**0.020**	$Z = -5.9$	**<0.001**	$Z = 3.3$	**0.001**

Note: TS: test statistic; Kruskal–Wallis test: ε^{13}C $H(2) = 57.5$, $p = 0.001$; ε^{15}N $H(2) = 46.5$, $p = 0.001$; Total N $H(2) = 40.3$, $p = 0.001$. *Equisetum* leaf include the lateral shoots and thereon the scale leaves.

also had significantly higher total N concentrations in their lateral shoots including the scale leaves when compared to their reference plants as well as to Cyperaceae and Caryophyllaceae (Figure S3), and *Equisetum* species were significantly enriched in ^{2}H in comparison to their reference plants.

The ^{13}C and ^{15}N enrichment factors and N concentrations as means and standard deviations for all investigated species of the Equisetaceae, Cyperaceae and Caryophyllaceae as well as their respective reference plants are available from the supplement (Tables S2, S4, S6) which includes also the statistics for each plot (Tables S3, S5, S7).

4 | DISCUSSION

Our investigation of natural abundances in stable N isotopes in 24 plant species belonging to the families Equisetaceae, Cyperaceae and Caryophyllaceae confirm N isotope abundances from our database for another 12 plant species of these three plant families (Tables S2, S4, S6) and earlier investigations by Michelsen, Schmidt, Jonasson, Quarmby, and Sleep (1996), Michelsen, Quarmby, Sleep, and Jonasson (1998). We show consistent ^{15}N enrichments relative to accompanying putatively autotrophic plant species forming AM, ECM, ErM or being NM. In their studies, Michelsen et al. (1996), Michelsen et al. (1998) grouped ^{15}N abundances for species belonging to the Equisetaceae, Cyperaceae and Caryophyllaceae together with species obviously capable of forming AM (e.g. *Festuca ovina*, *Geranium sylvaticum*, *Juniperus communis*, *Sorbus aucuparia* and *Trientalis europaea*), and classified them as a mixed group of NM/AM. When comparing them to co-occurring plant species forming ECM and ErM, they found that this mixed group of NM/AM plants showed enrichments in ^{15}N. Our study sheds a closer view on Michelsen's et al's data of the group classified as NM/AM and demonstrates that this group should be better separated according to two categories: the ^{15}N-enriched species belonging to the Equisetaceae, Cyperaceae and Caryophyllaceae and the plant species belonging to, for example, Apiaceae, Asteraceae, Cupressaceae, Geraniaceae, Primulaceae, Rosaceae usually forming AM (Brundrett & Tedersoo, 2019; Wang & Qiu, 2006). Thereby, the ^{15}N isotope enrichment of the majority of Michelsen's et al.'s plant species, most likely forming AM, falls back into the range of most ECM and some ErM plant species. This isotopic distinction between AM-forming plant families on the one hand and Equisetaceae, Cyperaceae and Caryophyllaceae on the other hand raises the question as to why species of the latter group are enriched in ^{15}N?

A shared feature of species belonging to Equisetaceae, Cyperaceae and Caryophyllaceae is their constant and dense colonization by *DSE* fungi as reported in the literature (Jumpponen & Trappe, 1998) and confirmed by our own microscopic and molecular findings. We therefore suggest a functional role of *DSE* in N acquisition and probably also acquisition of other mineral nutrients in analogy to the recent finding for plants colonized by *FRE* (Field et al., 2019; Hoysted et al., 2019). The site of nutrient transfer might be the fungal hyphae itself as previously demonstrated by a nanoSIMS application by Hill et al. (2019). Plants colonized by *FRE* and forming a nutritional mutualism were also enriched in ^{15}N (Hoysted et al., 2019). Our suggestion of a functional role of *DSE* fungi in nutrient acquisition is supported by early experiments by Haselwandter and Read (1982), recent findings on *DSE*-colonized *Deschampsia antarctica* (Poaceae) and *Colobanthus quitensis* (Caryophyllaceae) from Antarctica (Hill et al., 2019) and meta-analyses (Mandyam & Jumpponen, 2005; Newsham, 2011). Haselwandter and Read (1982) inoculated *Carex* species with *DSE* fungi and found a significant increase in dry weight of roots, shoots and whole plants as well as an increase in shoot phosphorous content; this effect was even higher when organic N was provided (Jumpponen & Trappe, 1998; Mandyam & Jumpponen, 2005; Upson, Read, & Newsham, 2009). The findings by Haselwandter and Read (1982) and our observation of ^{15}N enrichment are in agreement with the enzyme repertoire of *DSE* (Caldwell, Jumpponen, & Trappe, 2000) presumably allowing them to access ^{15}N-enriched soil organic compounds. Thus, *DSE* fungi may serve as providers of ^{15}N-enriched organic N compounds in Equisetaceae, Cyperaceae, Caryophyllaceae and probably other plants families and, in turn, may be rewarded by organic C compounds from plant photosynthesis. This suggestion is supported by the absence of ^{15}N enrichments found for the achlorophyllous fertile stems of *E. arvense*. These fertile stems are heterotrophic and therefore presumably cannot serve as providers of organic C and thus, there seems to be no more any mutualistic exchange in nutrients between host and fungus.

Simultaneously, the fertile stems of *E. arvense* turned out as most enriched in ^{13}C among all investigated plant samples. The enrichment in ^{13}C by the achlorophyllous fertile stems of *E. arvense* is in the typical range of ^{13}C enrichments found for fully mycoheterotrophic plants associated with AM fungi (Courty et al., 2011; Gomes et al., 2020; Merckx et al., 2010). Based on this ^{13}C enrichment found most pronounced for the achlorophyllous fertile stems of *E. arvense* and to a lower extent for all samples of chlorophyllous *Equisetum* species, we propose also an additional functional role of AM fungi for the here investigated Equisetaceae next to the function of *DSE* fungi—being aware of the fact that AM fungi were only sporadically found in our microscopic and molecular survey and that they are also only occasionally reported in the literature (Dhillion, 1993; Dickson et al., 2007; Fernández et al., 2008; Hodson et al., 2009; Koske et al., 1985). Equisetaceae are known to produce rather extensive rooting systems (Hauke, 1979). Thus, even weak fungal colonization per unit of *Equisetum* root biomass may be of significant relevance for the fungus–plant nutrient exchange on an entire plant level (van der Heijden, 2001). Importantly, AM fungal colonization by Equisetaceae always followed the *Paris*-morphotype. Hitherto, all investigated fully mycoheterotrophic plants associated with AM fungi (Imhof, Massicotte, Melville, & Peterson, 2013) and also the recently as partially mycoheterotrophic identified *Paris quadrifolia*, *Anemone nemorosa* (Giesemann, Rasmussen, et al., 2020) and *Pterygocalyx volubilis* (Suetsugu et al., 2020) always shared the *Paris*-morphotype. Based on this pattern, we suggest that the C isotope positioning between autotrophic reference plants and the achlorophyllous fertile

stems of *E. arvense* found here for six chlorophyllous *Equisetum* species indicates a partially mycoheterotrophic C gain (i.e. a simultaneous C gain from two sources, own photosynthesis and AM fungi). Based on linear two-source mixing model calculations, the proportional C gain from the fungal source by the six chlorophyllous *Equisetum* species studied here ranges in the order of 50 ± 22% with species-specific peculiarities. Similar proportional C gains from fungal sources were reported for partially mycoheterotrophic orchids and Ericaceae associated with ECM fungi (Hynson et al., 2013) and for the partially mycoheterotrophic and AM mycorrhizal *Bartonia virginica*, *Obolaria virginica*, *Pterygocalys volubilis* (Gentianaceae: Cameron & Bolin, 2010; Suetsugu et al., 2020), *Burmannia coelestris* (Burmanniaceae: Bolin, Tennakoon, Majid, & Cameron, 2017) and for *Paris quadrifolia* (Melanthiaceae) and *Anemone nemorosa* (Ranuculaceae; Giesemann, Rasmussen, et al., 2020).

Our suggestion of a partially mycoheterotrophic nutrition by chlorophyllous *Equisetum* species is supported by two other findings: (1) Significantly higher total N concentrations in all plant compartments in comparison to autotrophic reference plants. Increased total N concentrations are known as a wide-spread feature of many fully and partially mycoheterotrophic plants irrespective whether associated with fungi forming ECM (Gebauer & Meyer, 2003; Hynson et al., 2013; Stöckel, Meyer, & Gebauer, 2011) or AM (Gomes et al., 2020). (2) Significant ^{2}H enrichments in the lateral shoots including the scale leaves of the three investigated *Equisetum* species in comparison to leaves of accompanying putatively autotrophic reference plants. ^{2}H enrichment is a hallmark for heterotrophic nutrition of plants (Cormier, Werner, Leuenberger, & Kahmen, 2019; Cormier et al., 2018; Ziegler, 1994) and has been reported for fully, partially and initially mycoheterotrophic orchids, irrespective of whether associated with ECM or saprotrophic fungi of the rhizoctonia group (Gebauer et al., 2016; Schiebold, Bidartondo, Lenhard, Makiola, & Gebauer, 2018; Schweiger, Bidartondo, & Gebauer, 2018) as well as for fully and partially mycoheterotrophic plants associated with AM fungi (Giesemann, Rasmussen, et al., 2020; Gomes et al., 2020). Furthermore, our suggestion of a partially mycoheterotrophic nutrition by chlorophyllous *Equisetum* species due to significant ^{13}C and ^{2}H enrichments is supported by two previous investigations. Niu, Jiang, Gao, Li, and Liu (2003) reported on comparatively low net photosynthetic rates, high transpiration and low water use efficiency in Equisetaceae. All these factors are known to drive plants towards decreasing ^{13}C and ^{2}H abundances (Farquhar, Ehleringer, & Hubick, 1989; Ziegler, 1989) instead of the ^{13}C and ^{2}H enrichments as found here. And, in fact, Porter, Yiotis, Montañez, and McElwain (2017) found in chamber experiments under controlled conditions more negative δ^{13}C values in *Equisetum telmateia* and a couple of other ancient sporophytes in comparison to Gymnosperms and Angiosperms. Thus, our finding of ^{13}C and ^{2}H enrichments in Equisetaceae growing under natural field conditions can also not be explained by a deviating ecophysiology in photosynthesis and transpiration of these phylogenetically ancient plants.

In conclusion, plant species of the families Equisetaceae, Cyperaceae and Caryophyllaceae, traditionally considered as non-mycorrhizal turned out as conspicuous in their ^{15}N stable isotope natural abundance. Collective colonization by *DSE* and by this way access to ^{15}N-enriched organic N compounds in exchange for organic C compounds is assumed as most likely reason for this ^{15}N enrichment. This conclusion is further on supported by the absence of ^{15}N enrichments in representatives of the NM Brassicaceae. The additional enrichment in ^{13}C and ^{2}H found for green *Equisetum* species suggests them to act simultaneously as partial mycoheterotrophs on AM fungi of the *Paris*-morphotype (cf. Giesemann, Rasmussen, et al., 2020) while the achlorophyllous, fertile stems of *E. arvense* resemble a stable isotope pattern as known for fully mycoheterotrophic plants associated with AM fungi. Thus, so far, mostly underappreciated fungi classified as *DSE* are suggested to occupy an ecologically relevant role similar to mycorrhizae and the occurrence of simultaneous functions of *DSE* and AM fungi in Equisetaceae is proposed.

Our suggestion of an ecologically relevant function of *DSE* fungi should be tested in further laboratory tracer experiments as performed recently by Field et al. (2019) and Hoysted et al. (2019) when elucidating the functional role of *FRE* fungi.

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AUTHORS' CONTRIBUTIONS

P.G. comprised the data of three fieldwork campaigns, analysed and treated the results and wrote the first manuscript draft; D.E. conducted the sampling of Cyperaceae, M.S. and P.G. sampled Equisetaceae, L.F.S. and P.G. sampled Caryophyllaceae; Microscopic and isotope analyses were performed by P.G., D.E., M.S. and L.F.S. S.I.F.G. and V.S.F.T.M. were responsible for DNA analysis; G.G. initiated the project and coordinated the research design, supervised the isotope abundance survey and supported data treatment. All authors contributed to the manuscript.

DATA AVAILABILITY STATEMENT

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.brv15dv7m (Giesemann, Eichenberg, et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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