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ORIGINAL ARTICLE

Attributing functions to ectomycorrhizal fungal identities in assemblages for nitrogen acquisition under stress

Rodica Pena and Andrea Polle
Forest Botany and Tree Physiology, Georg-August Universität Göttingen, Büsgenweg 2, Göttingen, Germany

Mycorrhizal fungi have a key role in nitrogen (N) cycling, particularly in boreal and temperate ecosystems. However, the significance of ectomycorrhizal fungal (EMF) diversity for this important ecosystem function is unknown. Here, EMF taxon-specific N uptake was analyzed via ^{15}N isotope enrichment in complex root-associated assemblages and non-mycorrhizal root tips in controlled experiments. Specific ^{15}N enrichment in ectomycorrhizas, which represents the N influx and export, as well as the exchange of ^{15}N with the N pool of the root tip, was dependent on the fungal identity. Light or water deprivation revealed interspecific response diversity for N uptake. Partial taxon-specific N fluxes for ectomycorrhizas were assessed, and the benefits of EMF assemblages for plant N nutrition were estimated. We demonstrated that ectomycorrhizal assemblages provide advantages for inorganic N uptake compared with non-mycorrhizal roots under environmental constraints but not for unstressed plants. These benefits were realized via stress activation of distinct EMF taxa, which suggests significant functional diversity within EMF assemblages. We developed and validated a model that predicts net N flux into the plant based on taxon-specific ^{15}N enrichment in ectomycorrhizal root tips. These results open a new avenue to characterize the functional traits of EMF taxa in complex communities.
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Introduction

The roots of most plant species are associated with mycorrhizal fungi that mediate nutrient exchange between the plants and soil and thus have a central role in biogeochemical cycles (Finlay, 2008). In temperate and boreal forests, fungi that form ectomycorrhizas are the dominant symbiotic life form. Ectomycorrhizal fungi (EMF) encase colonized root tips with a dense hyphal net, termed the mantle, and forage the soil for nutrients by extending extraradical hyphae or hyphal cords (Finlay, 2008). As nitrogen (N) is a major limiting nutrient in many forest ecosystems (LeBauer and Treseder, 2008), the role of EMF in the N nutrition of trees has received considerable attention (Hobbie and Hobbie, 2008; Hobbie and Högberg, 2012). In addition to N delivery, recent studies have suggested that EMF may also limit N transfer to host trees under N-limiting conditions (Näsholm *et al.*, 2013).

Correspondence: A Polle, Forest Botany and Tree Physiology, Georg-August Universität Göttingen, Büsgenweg 2, 37077 Göttingen, Germany.
E-mail: apolle@gwdg.de
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Although it has been well established that EMF have key roles in plant nutrition, much less is known about the functions of distinct fungal taxa within complex ectomycorrhizal assemblages for nutrient acquisition and host supply. Ectomycorrhizal communities are usually composed of a diverse flora consisting of several dominant and many infrequent EMF species (Buée *et al.*, 2007; Courty *et al.*, 2010; Pena *et al.*, 2010; Lang *et al.*, 2011; Tedersoo *et al.*, 2012a; Danielsen *et al.*, 2013). EMF community structures are strongly influenced by N deposition (Lilleskov *et al.*, 2011; Kjøller *et al.*, 2012). Stable isotope studies have revealed that EMF species differ in their abilities to exploit different N sources (Hobbie and Högberg, 2012). Furthermore, *in situ* ectomycorrhizal communities exhibit strong temporal differences in the capability of different EMF taxa to access litter-derived N (Pena *et al.*, 2013a). The experimental manipulation of EMF diversity has shown context-dependent effects for fungal mixtures on plant biomass production and N nutrition (Chu-Chou and Grace, 1985; Jonsson *et al.*, 2001). As the mechanistic concepts that explain the interactions between different EMF taxa in complex assemblages are still missing, the functional relevance of EMF identities for tree nutrition remains enigmatic. Elucidating functional diversity

is important for understanding the role of ectomycorrhizal fungi in biogeochemical cycles in a fluctuating environment.

Our study aimed to attribute functions for N acquisition to ectomycorrhizal species identities in root-associated assemblages and uncover taxon-specific responses to environmental stress factors. We used young beech (*Fagus sylvatica* L.) trees, which are the major tree species of the natural vegetation in Central European temperate forests (Ellenberg and Strutt, 2009). The current beech forest distribution range is endangered as a result of drought stress because of climate change (Weber *et al.*, 2013). Beech trees are tolerant of deep shade in the youth phase (Ellenberg and Strutt, 2009); however, shade-induced carbon limitations have a negative impact on EMF colonization (Druebert *et al.*, 2009). Here, we conducted controlled experiments with beech seedlings cultivated in natural forest soil to develop characteristic EMF communities. The mycorrhizal trees were subsequently grown in sand to permit analysis of intact root systems and supplied with ammonium (NH_4^+) concentrations similar to those found in beech forest soils (median 0.5 mmol NH_4^+ kg^{-1} soil; range: 0.05–2 mmol NH_4^+ kg^{-1} soil; Gessler *et al.*, 2005; Göransson *et al.*, 2006; Dannenmann *et al.*, 2009; Andreasson *et al.*, 2012). Subsets of the plants were exposed to full light or shade according to the characteristic light climate in beech forests (median: 150 μmol PAR $m^{-2}s^{-1}$, range 25–255 μmol PAR $m^{-2}s^{-1}$; Kreuzwieser *et al.*, 1997; Lemoine *et al.*, 2002; Mayer *et al.*, 2002; Fotelli *et al.*, 2003; Gessler *et al.*, 2005; Hertl *et al.*, 2012). Light and shade treatments were combined with sufficient irrigation or water shortage to mimic typical environmental stresses. N acquisition was measured after the application of ^{15}N in root tips associated with distinct EMF species and non-mycorrhizal root tips. We tested the hypotheses that (i) ectomycorrhizal assemblages show taxon-specific differences for NH_4^+ acquisition and (ii) environmental stress results in functional shifts in EMF species for N acquisition. Partial fluxes for EMF-associated root tips were assessed with whole-plant N uptake for mycorrhizal and non-mycorrhizal plants. We provide evidence that the taxon-specific ^{15}N enrichments in EMF-associated root tips can be used to predict net N flux into the host plant. These results provide a basis for testing functional redundancy and response diversity of EMF assemblages in future field studies.

Materials and methods

Plant cultivation and experimental treatments

Fungicide-treated beech nuts (*Fagus sylvatica* L., provenance: Forstsaatgutstelle Oerrel, Niedersachsen, Germany) were grown in sterilized or untreated forest soil in individual pots as described previously (Pena *et al.*, 2013b). Ah horizon soil (20 cm depth) collected in the Tuttlingen beech forest (latitude 47°59′N, longitude 8°45′E) was used. The germinated seedlings were maintained in a greenhouse under ambient conditions (20 °C, 55% air humidity) with additional light to achieve a 16-h photoperiod with 200 μmol PAR $m^{-2}s^{-1}$ at plant height (lamps series 3071, Schuch, Worms, Germany). After 4 months, eight seedlings per treatment were evaluated for their mycorrhizal status showing that roots of seedlings in untreated soil were 40 ± 4% colonized by EMF, whereas seedlings in sterilized soil were non-mycorrhizal. A total of 120 mycorrhizal and 120 non-mycorrhizal beech seedlings were transplanted without adherent soil individually into 660 ml pots with a sand-peat mixture and supplied daily with 56 ml of nutrient solution containing 0.4 mM NH_4^+ as the sole N source. This concentration was chosen because it was similar to that in forest soil of the Tuttlingen site (Dannenmann *et al.*, 2009), well above K_m values for NH_4^+ uptake of various EMF (0.005–0.25 mM; Jongbloed *et al.*, 1991; Eltrop and Marschner, 1996) and because preceding analysis with attached beech roots at the Tuttlingen site showed saturation of NH_4^+ uptake at concentrations above 0.05 mM (Gessler *et al.*, 2005). The seedlings were grown either in full light (200 μmol PAR $m^{-2}s^{-1}$) or in the shade (35–40 μmol PAR $m^{-2}s^{-1}$ at plant height). The light climate was chosen according to the conditions in thinned and unthinned beech plots in the Tuttlingen forest with mean seasonal light levels of 176 and 25 μmol PAR $m^{-2}s^{-1}$, respectively (Gessler *et al.*, 2005). After 2 months, the irrigation solution was reduced to 37% for half of the plants grown under each light regime. After 16 days, when the well-irrigated plants had a predawn leaf water potential of −0.36 ± 0.02 MPa and those subjected to a limited water supply had a predawn leaf potential of −1.34 ± 0.06 MPa, the plants were harvested (Pena *et al.*, 2013b). During the last 3 days before harvest, each beech seedling received a total of 1.864 mg of N in a solution of either non-labeled NH_4Cl or $^{15}NH_4Cl$ (99 atom %, Cambridge Isotope Laboratories, Inc., Hampshire, UK). Biomass and whole plant ^{15}N data were determined (Pena *et al.*, 2013b). Ten ^{15}N labeled and three non-labeled plants per treatment were randomly selected and used for the analyses.

EMF identification and quantification

For each growth regimen, the whole root system of 10 beech seedlings per treatment was inspected using a binocular microscope (Leica M205 FA, Leica Microsystems, Wetzlar, Germany). Within each sample, the root tips were assigned to one of the following fractions: vital ectomycorrhizal (EM), vital non-ectomycorrhizal (NM), dead ectomycorrhizal (DM) and dead non-ectomycorrhizal (DR). Live and dead EM or NM root tips were distinguished as

previously described (Downes *et al.*, 1992; Winkler *et al.*, 2010). Vital EM root tips were classified using the morphotyping key of Agerer (1987–2012). The abundance of each morphotype was quantified in the whole root system. The morphotypes were photographed (Leica DFC 420 C, Leica Microsystems), and a scale bar was used to determine ectomycorrhizal lengths. For anatomical analysis, the morphotypes were embedded in styrene-methacrylate (Ducic *et al.*, 2008). Cross-sections with a thickness of 1 μm were cut with an autocut microtome (Ultracut E, Reichert-Jung, Vienna, Austria) and used to measure mantle thickness (Pena *et al.*, 2013a).

Approximately 20 root tips from each morphotype were collected, and aliquots were used for the molecular identification of fungal species by ITS sequencing as previously described (Lang *et al.*, 2011). The sequences obtained were assigned to fungal taxa by BLAST searches carried out against public databases (National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/ and UNITE, http://unite.ut.ee). The sequences were deposited in NCBI GenBank under the accession numbers HM748636–HM748643 (Supplementary Figure S1).

C, N and ^{15}N measurements in EMF-colonized root tips

Among the fungal species colonizing the beech roots, five were sufficiently abundant for each treatment to be collected for isotope analysis. Depending on fungal morphology, 20 (*Tomentella punicea*) to 60 root tips (*Cenococcum geophilum*) were required for a suitable sample for isotope analysis. One replicate comprised the root tips obtained from one individual plant. EM morphotypes, NM, DM and DR root tips of each plant were cut under the binocular microscope. Two different sets of instruments were used to handle non-labeled and labeled plants to avoid cross contamination. EM tips were excised at the last lateral root ramification ensheathed by the hyphal mantle; NM tips were sampled at the youngest and active 'white' zone (Evert and Eichhorn, 2007). Dead root tips with a shrunken and dry appearance were cut at the same position as the NM tips. The samples were freeze-dried.

Freeze-dried root tips were weighed (0.2–1.1 mg) using a super-micro balance (S4, Sartorius, Göttingen, Germany). Total C, N and ^{15}N concentrations in root tips were determined with an isotope ratio mass spectrometer (IRMS Delta^{plus}, Thermo Finnigan Mat, Bremen, Germany) coupled to an elemental analyzer (EA 1108, Fisons, Rodano, Italy). The relative ^{15}N abundance was expressed as the following ratio: $^{15}N(\text{atom } \%) = \frac{^{15}N}{^{14}N + ^{15}N} \times 100$

^{15}N content per root tip was calculated as follows:

^{15}N content (ng) = (biomass (g) × N concentration (ng g^{-1}) × ^{15}N atom % excess)/100,

where the biomass represents the mean dry mass of one root tip as determined for each EM fungal species, and ^{15}N atom % excess = ^{15}N atom % labeled − ^{15}N atom % unlabeled, where labeled and unlabeled refer to samples obtained from plants exposed to ^{15}N and unlabeled nutrient solutions, respectively.

The calculation of partial fluxes and prediction of total N flux is described in the Supplementary information SI1.

Statistical analysis

Statistical analysis was performed using Statgraphics Plus 3.0 (StatPoint, Inc., St Louis, MO, USA). When necessary, data were logarithmically or square root transformed to satisfy the criteria of normal distribution and homogeneity of variance. When transformation of the data did not meet these requirements, the Kruskal–Wallis and Mann–Whitney *U*-tests were applied instead of analysis of variance. Means or medians were considered to be significantly different from each other when $P \leqslant 0.05$.

The Shannon–Wiener index of diversity (H′) was calculated with the following equation (Shannon and Weaver, 1949):

$$H' = \sum_{i=1}^{s} p_i \ln p_i$$

where S is the number of species in the sample and p_i is the proportion of species *i* in the sample.

Evenness was calculated as follows:

$$E = H'/H(_{max})$$

with $H_{max} = \ln (S)$.

The effects of stress treatments on EMF community composition were analyzed by principal component analysis using the free PAST software package 2.17c (http://folk.uio.no/ohammer/past/, Hammer *et al.*, 2001). A variance–covariance matrix, which centers the data and in which the treatments were incorporated as categorical nominal variables, was used to perform principal component analysis (Jolliffe, 1986). The analysis was followed by a multiple analysis of variance to detect differences between species.

Results

The EM community structure in response to irradiance and water availability

The vital root tips of beech seedlings were 42 ± 4% colonized with 10 different EMF species, regardless of light ($F = 2.22$, $P = 0.144$) or drought treatment ($F = 2.16$, $P = 0.150$). The most abundant species (*T. punicea*, *Cenococcum geophilum*, *Tuber rufum*, *Tuber* sp.1 and *Tuber* sp.2) were associated with approximately 95% of colonized root tips, whereas the other detected fungal species (*Tomentella badia*, unknown EMF (Telephoraceae), *Sebacina* sp., *Cortinarius* sp., *Tomentella viridula*) colonized <5% of the EM root tips (Supplementary Figure S2). All fungal species were previously identified in natural assemblages of beech EMF in the same forest from

which the present soil inoculum was used (Pena *et al.*, 2010).

Principal component analysis revealed shifts in the EMF community structure under light or shade with sufficient or limited water availability (Figure 1). The first two components of the principal component analysis represented 96% of the variation in EMF species abundance in response to drought and shade (Supplementary Table S1a). All variables (LC, LD, SC and SD) were highly loaded onto PC1 ($0.850 < r < 0.951$), with vectors of similar lengths and directions suggesting that all sets of variables were correlated and equally important in explaining variation in EMF abundance. LC treatment was the main source of variation for PC2 ($r = 0.472$). The minimum hulls revealed the contrasting influence of light and shade on fungal abundance. Multiple analysis of variance confirmed differences between fungal species ($F = 60.5$, $P_{species} < 0.001$) and revealed significant interaction between light and EMF species ($F = 2.7$, $P_{(species \times light)} = 0.005$, Supplementary Table S1b); *T. rufum* increased in full light (LC), whereas *T. punicea* and *Tuber* sp. 1 increased in response to shade (Figure 1). The abundance shifts in EM fungal species composition did not affect the Shannon diversity index H′ or Evenness of the assemblages (Supplementary Table S2).

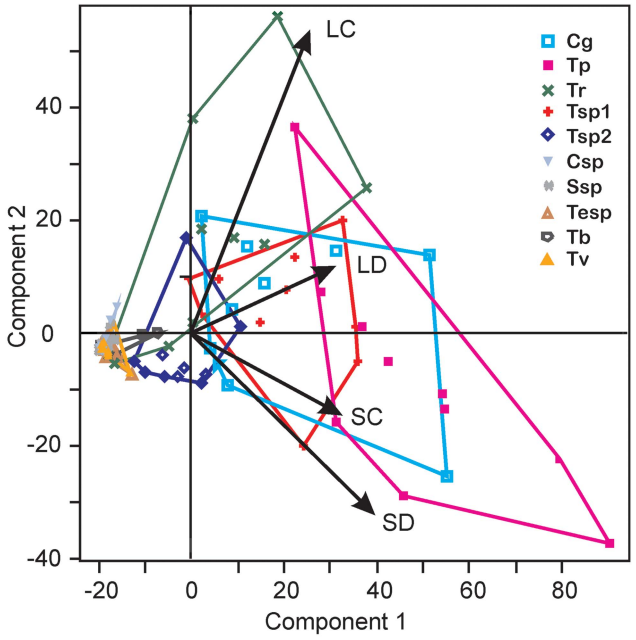

Figure 1 Biplot of the principal component analysis (PCA) ordination for similarities in the relative abundances of ectomycorrhizal fungal species associated with the roots of young beech trees (*Fagus sylvatica*). LC = full light, well-irrigated; LD = full light, drought; SC = shade, well-irrigated; SD = shade, drought. The abbreviations refer to the following fungi: Cg, *Cenococcum geophilum*; Tp, *Tomentella punicea*; Tr, *Tuber rufum*; Tsp1, *Tuber* sp.1; Tsp2, *Tuber* sp.2; Csp, *Cortinarius* sp.; Ssp, *Sebacina* sp.; Tesp, Telephoraceae; Tb, *Tomentella badia*; Tv, *Tomentella viridula*. Species clusters are indicated by minimum hulls.

Fungal species identities determine ectomycorrhizal N acquisition in response to environmental stress

We reasoned that specific N enrichment (measured as ^{15}N atom–% above natural abundance) is the result of N influx and export, as well as the exchange of ^{15}N with the N pool of the root tip. Therefore, specific N enrichment is an indicator of the physiological activity of N metabolism in a given type of root tip if it exceeds the ^{15}N enrichment of dead root tips (DR and DM) and natural abundance of ^{15}N. In DM and DR, the ^{15}N signature was significantly higher than the natural ^{15}N abundance (0.3661 ± 0.0002; Figure 2). This unexpected enrichment in DR or DM might have been caused by soaking with the nutrient solution, by very low physiological activity of root tips or root tips that were initially active but died during the labeling phase or by activities of microfungi and bacteria colonizing the mycorrhizosphere (Heinonsalo *et al.*, 2001; Calvaruso *et al.*, 2007; Izumi and Finlay, 2011). Only ^{15}N enrichment that was above the DR and DM threshold was considered to reflect active ^{15}N uptake.

The specific ^{15}N enrichment of vital NM root tips of non-mycorrhizal beech trees was similar to that of NM root tips of inoculated beeches ($P = 0.228$, means shown in Figure 2) and well above that of dead root tips (Figure 2). Specific ^{15}N enrichment in NM root tips decreased in response to drought and shade compared with well-irrigated and irradiated

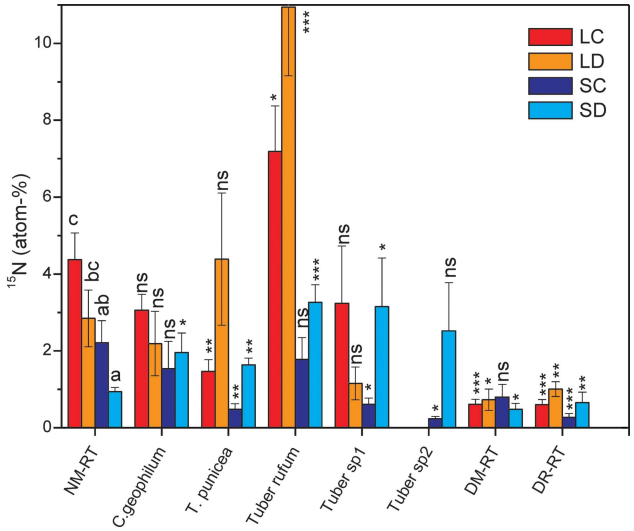

Figure 2 The specific ^{15}N enrichment of young beech root tips (RT) colonized by different ectomycorrhizal fungal species (*Cenococcum geophilum*, *Tuber rufum*, *Tomentella punicea*, *Tuber* sp 1 and *Tuber* sp 2). Data show the means ($n = 5 \pm$ s.e., except $n = 4$ for *T. rufum* and *Tuber* sp.2 under SC, $n = 3$ for *T. rufum* under SD and $n = 10$ for NM and DR). Different letters for NM-RT indicate significant differences at $P \leqslant 0.05$ between treatments. The asterisks indicate significant differences ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) compared with NM-RT. DM, dry ectomycorrhizal; DR, dry non-mycorrhizal; NM, non-mycorrhizal; LC, full light, well-irrigated; LD, full light, drought; SC, shade, well-irrigated; SD, shade, drought; NS, not significant.

plants (Figure 2), indicating that the physiological activity of NM root tips is highly sensitive to environmental stress.

The specific ^{15}N enrichment of root tips colonized with *C. geophilum* was similar to that of NM root tips under LC conditions but was unresponsive to stress and therefore exceeded the ^{15}N enrichment of NM root tips under low light and low water availability (Figure 2).

Notably, the specific ^{15}N enrichment of root tips from well-irrigated beech plants colonized with *T. punicea* was even lower than that of NM root tips, regardless of the light level, and almost as low as in DR (Figure 2). This finding suggests that EMF formed with *T. punicea* did not significantly participate in active N acquisition under LC and SC conditions (Figure 2). However, in drought-stressed beech plants, ^{15}N accumulation in *T. punicea*-colonized root tips was increased moderately in LD root tips and strongly in SD root tips and therefore significantly exceeded the ^{15}N enrichment of dead or NM root tips (Figure 2).

Root tips colonized with *Tuber* sp. 1 and *Tuber* sp. 2 behaved similarly to those colonized with *T. punicea* (Figure 2). In shaded, well-irrigated plants, the specific ^{15}N enrichment of the *Tuber*-colonized root tips was similar to that of DR but was significantly enriched when the shaded plants were drought stressed (Figure 2).

The strongest specific ^{15}N enrichment was found in root tips of light-exposed beech plants colonized with *T. rufum*, particularly when the plants were exposed to drought stress (Figure 2). However, in contrast with the other two *Tube*r species, the physiological activity of *T. rufum*-colonized root tips was very sensitive to shade ($P = 0.03$).

We repeated the experiment under stronger drought stress and confirmed the behavior of the EMF species with regard to the ^{15}N enrichment in response to the stress treatments (Supplementary Figure S3).

To test whether the ^{15}N enrichment of ectomycorrhizas was related to fungal biomass, the fungal mantle volumes of distinct EMF species were calculated and related to the total amount of ^{15}N in colonized root tips. A negative relationship was found between the amount of ^{15}N in root tips and the fungal mantle volume, suggesting that thicker fungal mantle structures led to a dilution rather than an enrichment of the ^{15}N label (Supplementary Figure S4). Greater ^{15}N enrichment is therefore not a result of greater ectomycorrhizal biomass.

Modeling plant N uptake by partial N flux assessment for different ectomycorrhizal taxa

As the beech plants received ^{15}N-labeled ammonium as the sole N source during the last 3 days before harvest, the ^{15}N content of the entire plant was the result of the net N flux through all active root tips during this time period. We assumed that the formation of new root tips and death of old root tips were negligible within our 3-day labeling period. Based on the total number of vital root tips and whole plant ^{15}N content, we calculated mean fluxes of 3.3 and 1.6 ng N h^{-1} root tip^{-1} for well-irradiated and shaded plants, respectively (Supplementary Table S3). However, the previous analyses of specific ^{15}N enrichment (Figure 2) implied that not all vital root tips were actively involved in N metabolism to the same extent under different experimental treatments. We reasoned that it should be possible to predict total plant ^{15}N content by assessing the contributions of different categories of root tips to total uptake if the specific ^{15}N enrichment of a given root tip category was proportional to the N flux through this type of root tip. Based on this assumption, we introduced activity coefficients for each root tip category, which were normalized relative to the ^{15}N enrichment of the root tips of NM plants (Supplementary Information SI1). With the NM plants, independent flux measurements were obtained and used to predict the fluxes of EM plants. Assessment of the relative contributions of the different root tip categories to the total flux clearly revealed that EMF contributed less to N flux than NM root tips in well-irrigated beeches, but their relative importance increased strongly under drought conditions (Figure 3a). The relative contribution of EMF to N flux was highest under SD conditions (Figure 3a).

The ^{15}N content in EM plants was predicted by the sum of the partial fluxes for each experimental treatment (LC, LD, SC and SD) (Supplementary Information SI1). Using a complementary approach, the ^{15}N of NM plants was predicted based on the data for EM plants (Supplementary Information SI1). As the predicted ^{15}N in EM plants was based on measured ^{15}N values in NM plants and vice versa, the two data sets yielded independent predictions of ^{15}N and therefore could be used to validate each other. A curvilinear relationship established for the N uptake of NM plants predicted the calculated N uptake of EM plants with a highly significant correlation ($P = 0.006$, $R_{(\text{adjusted})} = 92\%$). The measured values for the ^{15}N content of the plants were plotted against the calculated values for total ^{15}N uptake (Figure 3b). A linear regression model with a slope of 1 was highly significant (Figure 3b). This result supports the notion that the specific enrichment of ^{15}N in root tips is an indicator of flux through the root tip.

Discussion

EMF assemblages show interspecific differences for N acquisition and response diversity to stress

We documented clear differences in the N acquisition by ectomycorrhizas of different identities and provided evidence that uptake through ectomycorrhizas increased under drought stress and

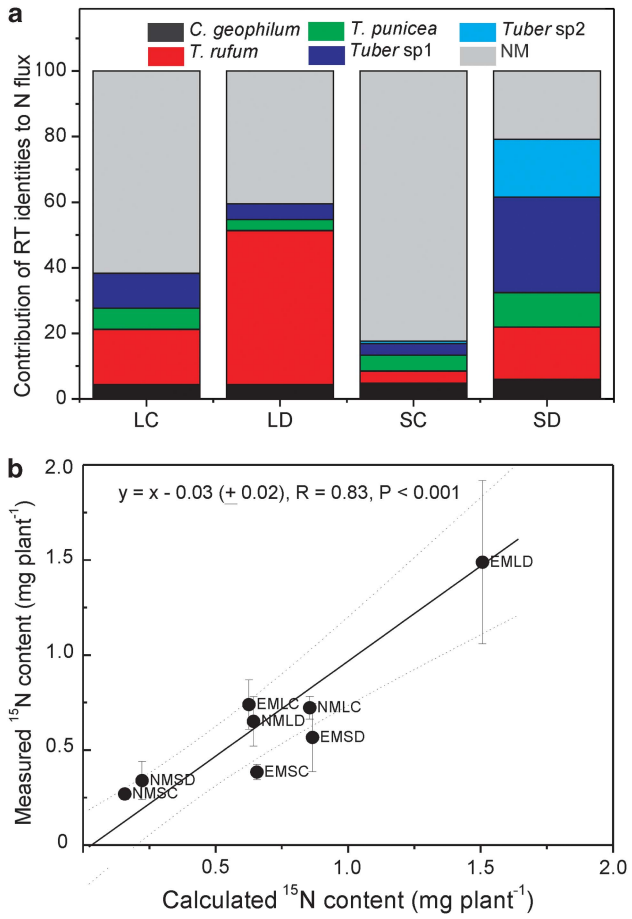

Figure 3 Relative contribution of different root tip categories to the total N flux (**a**) and correlation of the predicted and measured N uptake of young beech (*Fagus sylvatica*) trees (**b**). EM, ectomycorrhizal plants; NM, non-mycorrhizal plants; LC, full light, well-irrigated; LD, full light, drought; SC, shade, well-irrigated; SD, shade, drought. Data indicate means ($n = 5$).

decreased in strongly shaded plants, with pronounced interspecific differences. These results support our initial hypotheses and may have wider ecological implications for ecosystem functions when anthropogenic disturbances lead to species loss. For example, strong dominance of the universal species *C. geophilum* in drought-stressed environments may be less beneficial with regard to N nutrition than associations with *T. punicea*.

Previous analyses of natural carbon and N isotope discrimination have mainly focused on functional differences of EMF species for the utilization of different resources (Zeller *et al.*, 2007; Högberg *et al.*, 2008; Tedersoo *et al.*, 2012b). In contrast, studies investigating the utilization of the same resource by distinct taxa in the same environment are scarce, but this information is important for our understanding of the functional redundancy of EMF as contributors to ecosystem resilience. For example, in a forest community, most ectomycorrhizas, regardless of the fungal species, showed early enrichment of litter-derived N, likely from released solutes (Pena *et al.*, 2013a). However, EMF with emanating hyphae and known saprotrophic capacities, such as *Cortinarius* sp. and *Tomentella viridis*, ultimately accumulated more N from degrading leaf litter compared with EMF with short extraradical mycelium, indicating spatiotemporal differentiation for access to a complex organic N source (Pena *et al.*, 2013a). In our study, the aforementioned species were also present but were rare, suggesting that beech trees may foster fungal species without immediate benefits because the supply with NH_4^+ does not require the degradation of organic matter. Furthermore, long distance transport of N via fungal rhizomorphs is carbohydrate demanding (Ekblad *et al.*, 2013). Therefore, long distance EMF are probably less favored by small plants with limited light resources than short distance EMF.

In temperate forests, NH_4^+ is an important soluble inorganic N source, rapidly taken up by beech trees (Gessler *et al.*, 1998). In our experiment, we used an NH_4^+ concentration similar to that found in the Tuttlingen forest (Dannenmann *et al.*, 2009), where the soil for beech mycorrhizal inoculation was sampled. The beech trees in the Tuttlingen forest are colonized by a characteristic EMF flora, including all species present in this study (Buée *et al.*, 2005; Pena *et al.*, 2010; Lang *et al.*, 2011). Intact roots of these trees showed saturation of NH_4^+ uptake above a threshold of 50 μM NH_4^+ when exposed to feeding solutions with increasing NH_4^+ concentrations (Gessler *et al.*, 2005). As the plants in our experiment were acclimated to NH_4^+ concentrations well above this threshold, the pronounced interspecific differences in EMF NH_4^+ accumulation were unexpected. *T. rufum* and *T. punicea* exhibited the greatest contrast in N acquisition. *T. punicea* forms rhizomorphs (Agerer, 2001), which have typically been associated with water transport and improvement of host water status (Duddridge *et al.*, 1980; Brownlee *et al.*, 1983; Plamboeck *et al.*, 2007). Here, we showed that in addition to possible functions in water transport, *T. punicea* is important for nutrient supply under stress in shaded plants. In contrast, *T. rufum*, which displayed the highest specific N enrichment under full light, was highly sensitive to shade. Similar to our results, this species was frequently found on the root tips of sun-exposed beech trees and completely lost after long-term shade exposure (Druebert *et al.*, 2009). As *T. rufum* has a thin mantle, it is unlikely that carbohydrate demand for colonization and maintenance is responsible for this behavior (Markkola *et al.*, 2004).

The ascomycetes, *C. geophilium* and three truffle species actively participated in NH_4^+ uptake but showed divergent behavior for N acquisition in response to drought and shade. Although the small number of species precludes numerical analyses, our results support the notion that lineage-specific classification is unsuitable as a proxy for functional traits (Tedersoo *et al.*, 2012b). In ecological terms,

our findings reflect functional redundancy and response diversity (Mori *et al.*, 2013). However, more extensive analyses, particularly under field conditions and along environmental gradients, are required to characterize distinct EMF traits in *in situ* assemblages and their adaptive importance for plant nutrition.

^{15}N-specific enrichment in root tips as a proxy for N flux and the buffering functions of ectomycorrhizas in response to environmental stress

The high concordance of measured and predicted uptake with activity coefficients lends support to the suggestion that specific N enrichment is a proxy for N transport activities at the level of the root tip. This is an important result because it implies that stable isotope applications under field conditions can be used to assess the relative taxon-specific contributions of EMF in assemblages to plant N nutrition after correction for unspecific N enrichment. Whether this model is also valid for other N sources, such as nitrate, amino acids or complex compounds, must be further investigated.

Ectomycorrhizas did not increase N uptake or biomass compared with non-mycorrhizal plants under propitious environmental conditions (Pena *et al.*, 2013b). The EMF colonization rate and fungal species richness of the beech trees in our study were similar to those reported in other studies with young trees (Bledsoe *et al.*, 1982; Wilson and Harley, 1983; Högberg, 1989; Dahlberg, 2001; Izzo *et al.*, 2006) but were much lower than in old-growth forests, where usually 95% to 99% of the vital root tips are colonized by EMF (Pena *et al.*, 2010; Lang and Polle, 2011; Näsholm *et al.*, 2013). Therefore, the mean N fluxes, which are similar to those reported by others (for example, Högberg, 1989: approximately 4 ng N h^{-1} RT^{-1}), may be confounded by NM root tips. Our data suggest that fully mycorrhizal, well-irrigated and irradiated trees would even exhibit lower N uptake than that observed because of the low activity coefficients for NH_4^+ of the prevailing EMF. This reasoning contrasts with the widely accepted beneficial implications of EMF for plant nutrition but supports results obtained in a boreal forest (Näsholm *et al.*, 2013). The authors concluded that when N but not carbon was the limiting factor, EMF could aggravate N limitation in trees (Näsholm *et al.*, 2013). EMF control of the host N supply may also be a reason for the decreased N/C ratios commonly found in EM compared with NM plants (Ducic *et al.*, 2008; Druebert *et al.*, 2009; Jones *et al.*, 2009; Pena *et al.*, 2013b).

Notably, the impact of EMF on host N supply is mitigated by moderate drought. Episodes during which roots are exposed to water limitations frequently occur in the upper soil layer (Holst *et al.*, 2009), where the majority of root tips reside (Meinen *et al.*, 2009). EMF colonization delays the drought-induced decrease of the plant water potential (Beniwal *et al.*, 2010; Pena *et al.*, 2013b). Although the high N flux rates of unstressed NM plants imply that EMF is not necessary for plant N nutrition, the susceptibility of NM root tips to water limitation underlines the ecological significance of EMF in buffering plant nutrition against fluctuating environmental conditions. The underlying physiological or molecular mechanisms of this advantage are unclear but may be related to improved water retention by hyphae (Lehto and Zwiazek, 2011) or fungal-induced activation of osmotic solutes in roots (Luo *et al.*, 2009a, b). Furthermore, fungal stress tolerance is important. Among the fungi in our study, *C. geophilum* is known as a drought-tolerant species (Coleman *et al.*, 1989; Di Pietro *et al.*, 2007). It is abundant on beech roots in dry habitats (Jany *et al.*, 2003; Pena *et al.*, 2010), but its function for forest tree N nutrition has been disputed (Herzog *et al.*, 2012; Kipfer *et al.*, 2012). The current results indicate a buffering function for *C. geophilum* against varying environmental conditions but also suggest that other EMF species provide greater host benefits under water-limiting conditions.

In conclusion, this study shows that the analyzed ectomycorrhizal taxa use NH_4^+ in *in situ* assemblages to strongly diverging degrees. As NH_4^+ is an important N source across temperate forest ecosystems (Gobert and Plassard, 2008), its uptake must be granted under different environmental conditions. We provide evidence that the benefits of the ectomycorrhizal assemblage for N uptake and host transfer are mainly realized under environmental constraints. The physiological and molecular bases for the diverging responses within and between fungal species are currently unknown. With the advent of mycorrhizal genome projects (Marmeisse *et al.*, 2013), novel opportunities to uncover the molecular processes that are involved in response diversity are expected. The use of molecular tools together with stable isotope incorporation will unravel the functions of EMF assemblages, and we expect great progress in expanding our understanding of ecosystem resilience, which depends on both diversity and functional redundancy.

Conflict of Interest

The authors declare no conflict of interest.

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