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RESEARCH ARTICLE

Variation in mycorrhizal communities and the level of mycoheterotrophy in grassland and Forest populations of *Neottia ovata* (Orchidaceae)

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Abstract

1. Orchid mycorrhiza forms unique symbiotic associations between members of the Orchidaceae and multiple ecological guilds of fungi. Because orchids associate with a wide variety of fungi with different ecological functions, they represent an ideal study system to address fundamental questions about the evolution and ecophysiology of mycorrhizal symbiosis. Although it is well established that shifts in mycorrhizal associations are linked to transitions in plant trophic mode, it remains unclear what ecological drivers promote these evolutionary changes.
2. Here, we investigated mycorrhizal communities and isotope signatures across six populations of the terrestrial orchid *Neottia ovata* growing under contrasting light conditions in temperate Europe. We hypothesized that plants growing in forests would associate with different mycorrhizal fungi than plants occurring in grasslands and that the limited light availability in forests leads to a higher contribution of fungi to the carbon budget of orchids.
3. Our results showed that *N. ovata* predominantly associated with rhizoctonia fungi of the family Serendipitaceae in both habitats, but plants in forests also recruited ectomycorrhizal fungi. Root communities highly resembled soil communities and variation in root communities was significantly related to habitat type and edaphic factors. In contrast, isotope signatures (^{13}C, ^{15}N, ^{2}H and ^{18}O) and N concentration showed no significant relationship with habitat type. In addition, both ^{13}C and ^{2}H were not significantly correlated to habitat's light availability.
4. Although it has been suggested that the presence of a wide variety of ectomycorrhizal fungi in root communities of orchids can serve as a precursor for evolutionary shifts to partial mycoheterotrophy (mixotrophy), the presence or absence of

ectomycorrhizal fungi did not substantially influence the isotope signatures of *N. ovata*. These results indicate that rhizoctonia fungi played the major functional role in C and nutrient supply and that ectomycorrhizal fungi did not substantially contribute to the carbon budget of the plants.

KEYWORDS
ecophysiology, evolutionary ecology, metabarcoding, mixotrophy, mycorrhizal symbiosis, orchid mycorrhiza, partial mycoheterotrophy, stable isotope signatures

1 | INTRODUCTION

Orchid mycorrhiza is exclusively formed in the highly diversified plant family Orchidaceae and represents a unique study system to address fundamental questions about the evolutionary trajectory of plant trophic mode and mycorrhizal associations (Jacquemyn & Merckx, 2019; Rasmussen et al., 2015; van der Heijden et al., 2015). All orchid species are mycoheterotrophic at the early stages of development when their minute seeds and achlorophyllous protocorms have an obligate demand for fungal-derived C (Dearnaley et al., 2016; Merckx, 2013; Rasmussen, 1995). Although most adult orchids are assumed to become autotrophic (being capable of performing photosynthesis), fully or partially mycoheterotrophic orchids continue to depend on fungal-derived C throughout their entire life cycle or in combination with photosynthesis, respectively (Merckx, 2013; Selosse & Roy, 2009; Těšitel et al., 2018). Partially mycoheterotrophic orchids usually show intermediate enrichment in stable isotopes (^{13}C, ^{15}N and ^{2}H) between autotrophic and fully mycoheterotrophic species (Gebauer et al., 2016; Gebauer & Meyer, 2003; Hynson et al., 2013; Jacquemyn et al., 2021). Recent measurements of ^{2}H in combination with the frequently used ^{13}C and ^{15}N stable isotope abundance have suggested that partial mycoheterotrophy may be much more widespread among orchids than previously acknowledged (Gebauer et al., 2016; Gebauer & Schweiger, 2021; Schiebold et al., 2018). Mycoheterotrophy has been hypothesized to be an adaptation to survival in low-light habitats (Gomes, van Bodegom, Merckx, et al., 2019; Merckx, 2013; Selosse & Roy, 2009) and the degree of mycoheterotrophy can be variable and depend on the light availability of different habitats (Bidartondo et al., 2004; Preiss et al., 2010; Schweiger et al., 2019). Mycoheterotrophic orchids often rely on different mycorrhizal fungi compared to their autotrophic relatives, suggesting that a shift from autotrophy to mycoheterotrophy is accompanied by a shift in fungal symbionts (Jacquemyn & Merckx, 2019; Selosse et al., 2022; Wang et al., 2021). However, we still do not know the ecological drivers behind these different fungal associations.

The evolutionary transition in trophic mode of orchids from autotrophy, via partial to full mycoheterotrophy, is accompanied by shifts in mycorrhizal partners, from associations with typical rhizoctonia fungi (members of three families Ceratobasidiaceae, Tulasnellaceae and Serendipitaceae; Dearnaley et al., 2012) to non-rhizoctonia ectomycorrhizal fungi or wood/litter saprobes in the phyla Basidiomycota and Ascomycota (Bidartondo et al., 2004; Motomura et al., 2010; Ogura-Tsujita et al., 2012, 2021; Selosse et al., 2022; Selosse & Roy, 2009; Wang et al., 2021; Yagame et al., 2016). For instance, in the tribe Neottieae, one of the scarce lineages comprising a broad range of putatively autotrophic, partially and fully mycoheterotrophic species (Feng et al., 2016; Lallemand et al., 2019; Zhou & Jin, 2018), mycorrhizal associations switch from rhizoctonia fungi in putatively autotrophic species to ectomycorrhizal fungi in fully mycoheterotrophic species (e.g. Sebacinaceae, Russulaceae, Tuberaceae, Pyronemataceae) (Bidartondo et al., 2004; Girlanda et al., 2005; Roy et al., 2009; Selosse et al., 2002; Suetsugu et al., 2017; Těšitelová et al., 2012). Yet, the environmental conditions under which this increase in mycorrhizal partner breadth occurs, remain unclear.

Neottia ovata is a chlorophyllous terrestrial orchid species that is sister to partially and fully mycoheterotrophic species in the genus *Neottia* (Feng et al., 2016; Lallemand et al., 2019; Zhou & Jin, 2018). It therefore represents a good study species to investigate variation in symbiotic associations and the level of heterotrophy and how they are linked to environmental conditions. First, within the genus *Neottia*, symbiotic switches most likely occurred from rhizoctonia Serendipitaceae fungi to ectomycorrhizal Sebacinaceae fungi towards the evolutionary end point of full mycoheterotrophy (characterized by the fully mycoheterotrophic species *N. nidus-avis*) (Bidartondo et al., 2004; Jacquemyn et al., 2015, 2021; McKendrick et al., 2002; Oja et al., 2015; Selosse et al., 2002; Těšitelová et al., 2015; Yagame et al., 2016), showing that mycorrhizal shifts can occur within the fungal order Sebacinales (Weiß et al., 2016). Second, despite its inconsistent enrichment in ^{13}C across different habitats (Gebauer et al., 2016; Schweiger et al., 2019; Těšitelová et al., 2015), *N. ovata* has been reported to be significantly enriched in ^{2}H (Gebauer et al., 2016; Schweiger et al., 2019), possibly indicating a C gain from mycorrhizal fungi. Third, several studies using molecular identification techniques have shown that *N. ovata* mainly associated with rhizoctonia fungi of the family Serendipitaceae, while occasional associations with ectomycorrhizal fungi were observed as well in forest habitats (Jacquemyn et al., 2015; Oja et al., 2015; Těšitelová et al., 2015; Vogt-Schilb et al., 2020). These results suggest that *N. ovata* may have high plasticity in fungal associations and show increased levels of mycoheterotrophy in particular habitats (Selosse et al., 2022; Wang et al., 2021). This plasticity of associating with multiple fungal species of different functional groups

potentially places *N. ovata* at the early stages of the evolutionary shift from autotrophy to mycoheterotrophy which repeatedly occurred within *Neottia* (Figure 1). A more detailed and simultaneous investigation of mycorrhizal communities and ecophysiology over contrasting habitats may therefore shed light on the ecological factors that influence these variables.

In this study, we examined whether mycorrhizal communities of *N. ovata* in specific microhabitats and whether variation in fungal community composition relates to changes in the degree of mycoheterotrophy. Mycorrhizal communities of *N. ovata* were investigated using high-throughput sequencing across six sites ranging from open grassland and shady forests in Europe. The full set of ^{13}C, ^{15}N, ^{2}H and ^{18}O stable isotope signatures was measured, as well as microhabitat conditions (light intensity and edaphic factors). Specifically, we hypothesized that orchid root mycorrhizal fungal communities (hereafter 'root communities') differ between forest and grassland populations (#H1). We further expected that the composition of root communities is determined by fungal availability in the soil and influenced by edaphic factors (#H2). Lastly, we expected that the level of mycoheterotrophy (represented by ^{13}C and ^{2}H signatures) is significantly related to light availability (#H3).

2 | MATERIALS AND METHODS

2.1 | Study species and sampling procedures

A total of 30 individuals of *N. ovata* were sampled at six sites (forest sites: BE, VP and VS; grassland sites: KA, ZI and KL) in North-East Bavaria (Germany) and the Netherlands in June 2021 (Table 1). Within each site, five 1 m^2 plots containing *N. ovata* were randomly selected as soon as the vegetative parts of *N. ovata* could be identified. Within each plot, a light sensor (silicon photodiode BPW 21, Infineon, Germany) was installed beside the targeted orchid individual at a height of approximately 15 cm to record the irradiance level till sample collection (2 weeks to 1 month later) using a data logger (HOBO H8; ONSET, USA). Following the calibration procedure (Preiss et al., 2010), the light intensity received by each orchid individual during the measuring period was calculated (Appendix S1). We collected leaf, root and soil samples within each plot when the targeted orchid individual was at a budding or early flowering stage. One or two pieces of leaf of *N. ovata* ($n = 30$) and autotrophic reference plants ($n = 90$) were collected for stable isotope measurements. Reference plants were always growing in close spatial proximity and therefore under identical light conditions as the respective orchid individuals. For each orchid individual, at least five root pieces (3–5 cm) were randomly chosen for DNA extraction and microscopic observation of fungal pelotons. Within each plot, five topsoil cores were randomly taken with a 2.5-cm-diameter soil auger to a depth of 5 cm below the litter layer and mixed as one homogenized bulk-soil sample for soil nutrient analysis. All soil samples were preserved in a cooling box immediately after collection and stored in a fridge (4°C) in the laboratory until further processing.

2.2 | Fungal barcoding

Root pieces were surface sterilized using 1% sodium hypochlorite and rinsed in sterile distilled water. Total DNA from five root sections (1 mm thickness) was extracted using the CTAB method (Doyle, 1987). DNA from bulk soil (0.25 g) was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN). The forward primer fITS7 (5′-GTGARTCATCGAATCTTTG-3′; Ihrmark et al., 2012) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al., 1990) were used to amplify the fungal nuclear ITS2 region for both root and soil samples. According to our pilot study (Appendix S1; Table S1),

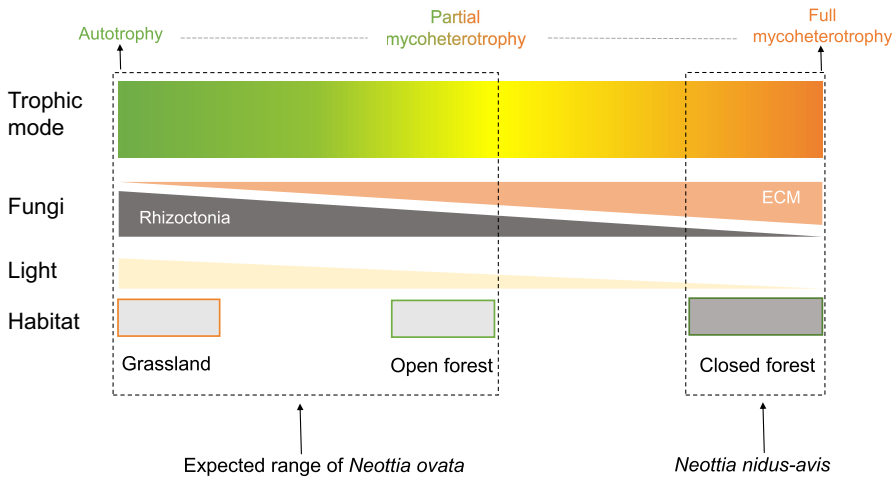

FIGURE 1 Expected spectra of trophic mode, fungal associations and light availability for *Neottia ovata*. Compared with the fully mycoheterotrohic *N. nidus-avis* (Jersáková et al., 2022) that exclusively associates with ectomycorrhizal fungi (ECM), *N. ovata* (Kotilínek et al., 2015) is mainly associated with rhizoctonia fungi and hypothesized to be at an early stage of partial mycoheterotrophy (also broadly termed 'mixotrophy', Selosse & Roy, 2009). The level of partial mycoheterotrophy is a continuum between autotrophy and full mycoheterotrophy and hypothesized to relate to habitat's light availability.

TABLE 1 Overview of all sampling sites of *Neottia ovata* used in this study.

Country	Site	Site ID	Habitat description	Collection date
Germany	Betzenstein (49.40°N/11.23°E)	BE	Mixed beech forest (*Fagus sylvatica*)	10 June 2021
Germany	Velden (plain site) (49.37°N/11.34°E)	VP	Mixed pine forest *Pinus sylvestris* with spruce *Picea abies* and beech *Fagus sylvatica*	9 June 2021
Germany	Velden (slope site) (49.40°N/11.23°E)	VS	Monodominant pine forest (*Pinus sylvestris*)	9 June 2021
Germany	Kalkberg (50.04°N/11.14°E)	KA	Dry calcareous grassland	7 June 2021
Germany	Zips (49.37°N/11.34°E)	ZI	Wet grassland	10 June 2021
The Netherlands	Klip (52.15°N/4.38°E)	KL	Humid dune slack	8 June 2021

the primer pair fITS7 and ITS4 amplified a wider range of orchid mycorrhizal fungi than the primer pair ITS3 and ITS4OF specifically targeting orchid mycorrhizal fungi (Taylor & McCormick, 2008). Fungi from the family Tulasnellaceae forming typical orchid mycorrhizal fungi were not captured by both sets of primers in our pilot study (Table S1). Since this is potentially due to the high variability of the ITS2 region of Tulasnellaceae (Li et al., 2021; Oja et al., 2015; Waud et al., 2014), we designed new forward primers to detect Tulasnellaceae fungi: Tul1F (5′-CGTYGGATCCCTYGGC-3′) and Tul2F (5′-TGGATCCCTTGGCACGTC-3′) positioning at the 5.8S region to match with reverse primer ITS4Tul2 (5′-TTCTTTTCCTC CGCTGAWTA-3′; Oja et al., 2015) based on sequence alignment (Appendix S1; Table S2). Thus, each sample was amplified using the fungal general primer pair and the newly designed primer pair. PCRs were duplicated for each DNA template to reduce PCR stochasticity and to increase the diversity of detected fungi (Alberdi et al., 2018). After checking the amplification products by gel electrophoresis, duplicates of amplicons were pooled after the first PCR step and attached with identical Nextera indexes (Illumina) in the second PCR according to the manufacturer's guidelines. The end pool was sequenced to generate 300-bp paired-end reads using the Illumina MiSeq platform (BaseClear).

After demultiplexing, paired reads of each sample were merged and low-quality sequences (error rate >0.5) were filtered out by VSEARCH (Rognes et al., 2016). Merged sequences were separated by primer pair and subsequently trimmed from primers using CUTADAPT 1.0 (Martin, 2011). Chimeric sequences were trimmed by the UCHIME chimera detection program (de novo algorithm) (Edgar et al., 2011). After quality filtering and chimera removal, fungal operational taxonomic units (OTUs) were clustered based on a 97% similarity threshold using VSEARCH. Global singletons were removed because they may reduce the accuracy of diversity estimates (Ihrmark et al., 2012; Waud et al., 2014). The remaining OTUs were assigned with taxonomic identities to the best match by USEARCH (Edgar, 2010) using the Unite reference dataset (utax_reference_dataset_10.05.2021.fasta, https://unite.ut.ee/repository.php) as annotation resources. Finally, OTUs were manually screened for possible orchid mycorrhizal fungal families summarized in the literature (Dearnaley et al., 2012; Wang et al., 2021) and reported in previous studies on *N. ovata* (Jacquemyn et al., 2015; Oja et al., 2015; Těšitelová et al., 2015). The quality-filtered sequencing data generated a total of 3342 OTUs (1,514,336 sequences) for 30 root and 60 soil samples, of which 543 OTUs (693,154 sequences—45.8% of all filtered sequences) were assigned to putative orchid mycorrhizal fungi belonging to 15 fungal families (Table S3; Appendix S1). Because the specific Tulasnellaceae primer pair did not yield considerably more Tulasnellaceae OTUs than the general primer pair, only orchid mycorrhizal fungi captured by the general primer were retained for further analyses.

2.3 | Stable isotope signatures

The leaf material of all sampled plants was oven-dried at 105°C, ground to fine powder in a ball mill (Retsch Schwingmühle MM2) and stored in a desiccator before measuring stable isotope abundances of C, N, H, O and total N concentrations. All measurements of stable isotope signatures were conducted in the BayCEER Keylab of Isotope Biogeochemistry. Relative natural abundance analysis of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopes as well as N concentrations were determined simultaneously using an EA-IRMS coupling combining an elemental analyser (EA IsoLink CN; Thermo Fisher Scientific) with a continuous flow isotope ratio mass spectrometer (delta V advantage; Thermo Fisher Scientific). Relative natural abundances of hydrogen ($^{2}H/^{1}H$) and oxygen isotopes ($^{18}O/^{16}O$) were measured separately using a TC-IRMS coupling, which links a thermal conversion through pyrolysis unit (HTO; HEKAtech) to a continuous flow isotope ratio mass spectrometer (delta V advantage; Thermo Fisher Scientific). Device coupling was always via a ConFlo IV open-split interface (Thermo Fisher Scientific). For H isotope abundance, each sample was measured four times in a row with the first three measures being neglected and we analysed target orchid samples and respective reference plant samples together in identical sample batches (Gebauer et al., 2016). The oxygen isotope abundances were measured to assess a potential transpiration effect on the relative enrichment in ^{13}C and ^{2}H caused by differences in stomata regulation and transpiration between orchids and the non-orchid reference plants. Transpiration affects C, H and O isotope abundance in plant tissues simultaneously (Cernusak et al., 2004; da Silveira Lobo Sternberg, 1989; Ziegler, 1989). A transpiration effect

can only be excluded if the ^{18}O isotope abundance of the target species is similarly distributed as the ^{18}O isotope signature of the non-orchid reference plants (Gebauer et al., 2016).

Measured relative isotope abundances (δ values) were calculated according to the following equation: $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1000$ [‰], where R_{sample} and $R_{standard}$ are the ratios of heavy to light isotope of the samples and the respective standard. To enable comparisons of stable isotope abundances across sampling sites, we used isotope enrichment factors (ε) to normalize δ values using the equation $\varepsilon = \delta_S - \delta_{REF,}$ where δ_S is a single $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ or $\delta^{2}H$ value of an orchid individual and δ_{REF} is the mean value of all autotrophic reference plants within the same plot (Preiss & Gebauer, 2008). The δ and ε values and total N concentrations of all orchid samples and their autotrophic references are available in Table S4.

2.4 | Data analyses

2.4.1 | Fungal diversity

Because of the variation in sequencing reads among samples, the abundance of OTUs of all samples was rarefied to the sample with the fewest reads. Unless stated otherwise, the 'PICANTE' R package (Kembel et al., 2010) was used for all community analyses. To investigate whether alpha diversity (α-diversity) of orchid mycorrhizal fungi differed between sites and habitats, we calculated for each sample OTU richness (SR) and phylogenetic α-diversity, Faith's (1992) PD. The phylogeny of fungal OTUs was reconstructed using the Maximum Likelihood method (see details about phylogenetic analyses in Appendix S1) and used for the calculation of PD. Analysis of variance (ANOVA) or Kruskal–Wallis rank-sum tests was used to evaluate differences in mean SR and PD of root and soil samples among sites, respectively. If the null hypothesis was rejected, post hoc Tukey HSD or Dunn's test was performed to analyse pairwise differences. To test the hypothesis (#H1) that fungal α-diversity of root or soil samples differ between habitats, we compared measures of SR and PD between habitats by fitting linear mixed models with 'Habitat' as fixed effect and 'Site' as random effect using the R package 'LME4' (Bates et al., 2015).

2.4.2 | Fungal community structure

To assess differences in mycorrhizal community structure between sampling sites and habitat types (β-diversity), we calculated Bray–Curtis (Bray & Curtis, 1957) and UniFrac dissimilarity metrics (Lozupone & Knight, 2005) based on the presence–absence information of OTUs. The Bray–Curtis index is widely used for assessing community dissimilarity in community ecology (Anderson et al., 2006), while the UniFrac distance considers the phylogenetic relatedness of fungal OTUs by measuring the unique fraction of the phylogenetic distances not shared between two samples. Variation in mycorrhizal community structure of root or soil samples among sites was visualized using non-metric multidimensional scaling (NMDS) using the 'VEGAN' R package (Oksanen et al., 2019). After checking for homogeneity of variances with the function 'betadisper' from 'VEGAN', we performed a PERMANOVA (Anderson, 2001) using 'adonis2' to test whether mycorrhizal communities differed between sites. To test the hypothesis that mycorrhizal communities differed between habitats, a nested PERMANOVA was performed and 'strata = Site' was specified to constrain random permutations between sites. In addition, we ran indicator species analyses on root samples to identify fungal OTUs that were characteristic for a given site using the 'indval' function from the 'LABDSV' R package (Roberts, 2019). Indicator species are species that preferentially occur in a community, and the indicator value of a species reflects species importance in the community (Dufrêne & Legendre, 1997).

2.4.3 | Effect of soil properties and soil fungi on root communities

To test the hypothesis (#H2) that root fungal communities are determined by the availability of fungal OTUs in the soil, we first related fungal alpha diversity (SR and PD) of soil samples to that of root samples using linear regressions. Second, Mantel tests with 10,000 permutations were used to examine the similarity between the Bray–Curtis metrics of root communities and bulk-soil samples. Furthermore, we calculated Bray–Curtis metrics for both root samples and the corresponding bulk-soil samples and examined the dissimilarity in fungal composition among sample types by PERMANOVA. In addition, to investigate whether particular fungal families are preferentially recruited in orchid roots from soil, the relative sequence abundance (MiSeq reads) of each fungal family was compared between root and soil sample within each site.

To test the effect of edaphic factors on root community composition, we conducted a distance-based redundancy analysis (db-RDA) in 'vegan'. Explanatory variables tested in the db-RDA model were soil organic content (SOC), extractable N (including nitrate concentration—NO_3^- and ammonium—NH_4^+), extractable P, pH and soil moisture content (see detailed measurements of soil properties in Appendix S1). To test the proportion of variation in root fungal communities explained by edaphic factors after controlling for differences by habitat type (forest vs. grassland), we performed a partial db-RDA model that used habitat type as a covariate. Edaphic factors that best fit the db-RDA model were selected using the 'ordiR2step' function from 'VEGAN'. The significance of the selected variables was assessed by the function 'anova.test' in 'VEGAN'.

2.4.4 | Phylogenetic analyses of Sebacinaceae fungi associated with *Neottia* species

To explore the phylogenetic relationship of Sebacinaceae fungi between *N. ovata* and its fully mycoheterotrophic relatives (*N.*

nidus-avis, *N. camtschatea* and *N. accuminata*), we download the fungal ITS sequences reported in the literature (Chen et al., 2019; McKendrick et al., 2002; Selosse et al., 2002; Těšitelová et al., 2015) from NCBI GenBank database. Those downloaded sequences were aligned with Sebacinaceae OTUs of *N. ovata* in this study and used to reconstruct maximum likelihood phylogenetic trees using RAxML in Geneious Prime v. 2019. The three Sebacinaceae sequences (KJ188478, KJ188509 and KJ188545) of *N. ovata* reported by Těšitelová et al. (2015) were also included for phylogenetic reconstruction, and the Serendipitaceace OTUs detected by this study were used as outgroup sequences.

2.4.5 | Orchid stable isotope signatures and correlation with light intensity

For each site, isotope compositions ($\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{18}O$, $\varepsilon^{2}H$ and total N) of orchid and reference plant leaves were compared by fitting linear mixed models. All models included 'plot' as random effect due to our plot-wise sampling scheme. Differences in isotope compositions of orchid samples between sites were assessed using ANOVA or Kruskal–Wallis rank-sum tests. Linear mixed models were performed to test the effect of habitat on isotope composition using 'Site' as random effect. In addition, linear mixed models using 'Site' as random effect were used to assess the relationship between light intensity and $\varepsilon^{13}C$ and $\varepsilon^{2}H$, individually (#H3).

3 | RESULTS

3.1 | Mycorrhizal fungal community of *N. ovata* encompasses rhizoctonia symbionts and site-dependent ectomycorrhizal fungi

Within root communities, rhizoctonia fungi of the Serendipitaceae were present in all samples regardless of site (Figure S1A,B), while the presence of ectomycorrhizal fungi was highly site dependent. Apart from associations with members of the Serendipitaceae, forest root communities comprised several ectomycorrhizal fungi of Sebacinaceae (sites BE, VP and VS), Inocybaceae (sites BE, VP and VS) and Thelephoraceae (sites VP and VS), whereas grassland root communities comprised rhizoctonia fungi of Tulasnellaceae (sites KA and ZI) and Ceratobasidiaceae (sites KA, KL and ZI) (Figure S1A,B). Besides these, other fungal families occasionally occurred in root communities of *N. ovata* with <1% sequence abundance, including Hymenogastraceae, Tuberaceae, Cortinariaceae, Pyronemataceae, Pezizaceae, Psathyrellaceae and Tricholomataceae (Figure S1A,B). The preference of forest and grassland individuals for ectomycorrhizal and rhizoctonia fungi was also shown by phylogenetic mapping of fungal OTUs and indicator species analyses (Figure 2; Table S5). Each site (except site KL) included different Serendipitaceae OTUs as indicator species. In addition, forest sites had indicator species belonging to ectomycorrhizal families (BE—Sebacinaceae; VP—Inocybaceae, VS—Sebacinaceae, Thelephoraceae, Inocybaceae, Atheliaceae and Russulaceae), whereas grassland sites had rhizoctonia indicator species (KA—Tulasnellaceae and Ceratobasidiaceae; KL—Ceratobasidiaceae).

Root communities of *N. ovata* differed significantly in α-diversity (SR and PD) between sites (Table S6), with forest sites VP and VS showing significantly higher α-diversity than grassland sites KA and KL (Figure 3A,B; Table S6). Significant variation in α-diversity was also found between habitat types (SR: $R^2=0.424$, $p<0.001$; PD: $R^2=0.324$, $P<0.001$; Table S7). Root community structure differed significantly between sites (Bray–Curtis metric: $R^2=0.305$, pseudo-$F=3.164$, $p<0.001$; UniFrac metric: $R^2=0.217$, pseudo-$F=2.065$, $p<0.001$) (Figure 3C,D) and between habitat types (Bray–Curtis metric: $R^2=0.142$, pseudo-$F=4.463$, $p<0.001$; UniFrac metric: $R^2=0.179$, pseudo-$F=5.884$, $p<0.001$).

3.2 | Roots recruit available fungi from the soil

Bulk soil samples (416 OTUs) had higher diversity of orchid mycorrhizal fungi than root samples (253 OTUs) (Figure S2). Similar to the variation in root communities, soil fungal communities varied substantially between sites and habitats in composition (Figure S1C,D) and α-diversity (Figure S3; Tables S6 and S7). Soil fungal community structure was significantly different between sites (Bray–Curtis metric: $R^2=0.444$, pseudo-$F=3.669$, $p<0.001$; UniFrac metric: $R^2=0.473$, pseudo-$F=4.131$, $p<0.001$) and between habitat types (Bray–Curtis metric: $R^2=0.110$, pseudo-$F=3.321$, $p<0.001$; UniFrac metric: $R^2=0.208$, pseudo-$F=7.100$, $p<0.001$). There was a significant positive correlation between α-diversity indices of soil and root samples (Figure S4). Mantel tests also revealed a high similarity in fungal community composition between root and soil samples ($R=0.504$, $p<0.001$). Furthermore, strong similarity in fungal community structure was found between root and soil samples (Figure 4A) (Bray–Curtis metric: $R^2=0.086$, pseudo-$F=0.703$, $p=0.866$).

Root fungal community composition was significantly related to edaphic variables (pseudo-$F=1.721$, $p=0.001$, $R^2_{adj}=0.134$) (Figure 4B; Table S8). NO_3^-, SOC, P and pH significantly explained differences in root fungal communities between sites (RDA1: $F=3.767$, $p=0.001$; Table S9). Edaphic variables remained significant after controlling for the effect of habitat type (partial db-RDA, $R^2_{adj}=0.064$, pseudo-$F=1.6$, $p=0.002$).

Despite the high similarity in fungal community composition between root and soil samples, the relative abundance of one fungal family in the roots did not always respond to its relative abundance in the soil (Figure 4C). Rhizoctonia fungi, especially those belonging to the family Serendipitaceae, exhibited relatively higher abundances in the roots than in soil, indicating that *N. ovata* preferably recruits these fungi from the soil even though they were not dominant in the soil community.

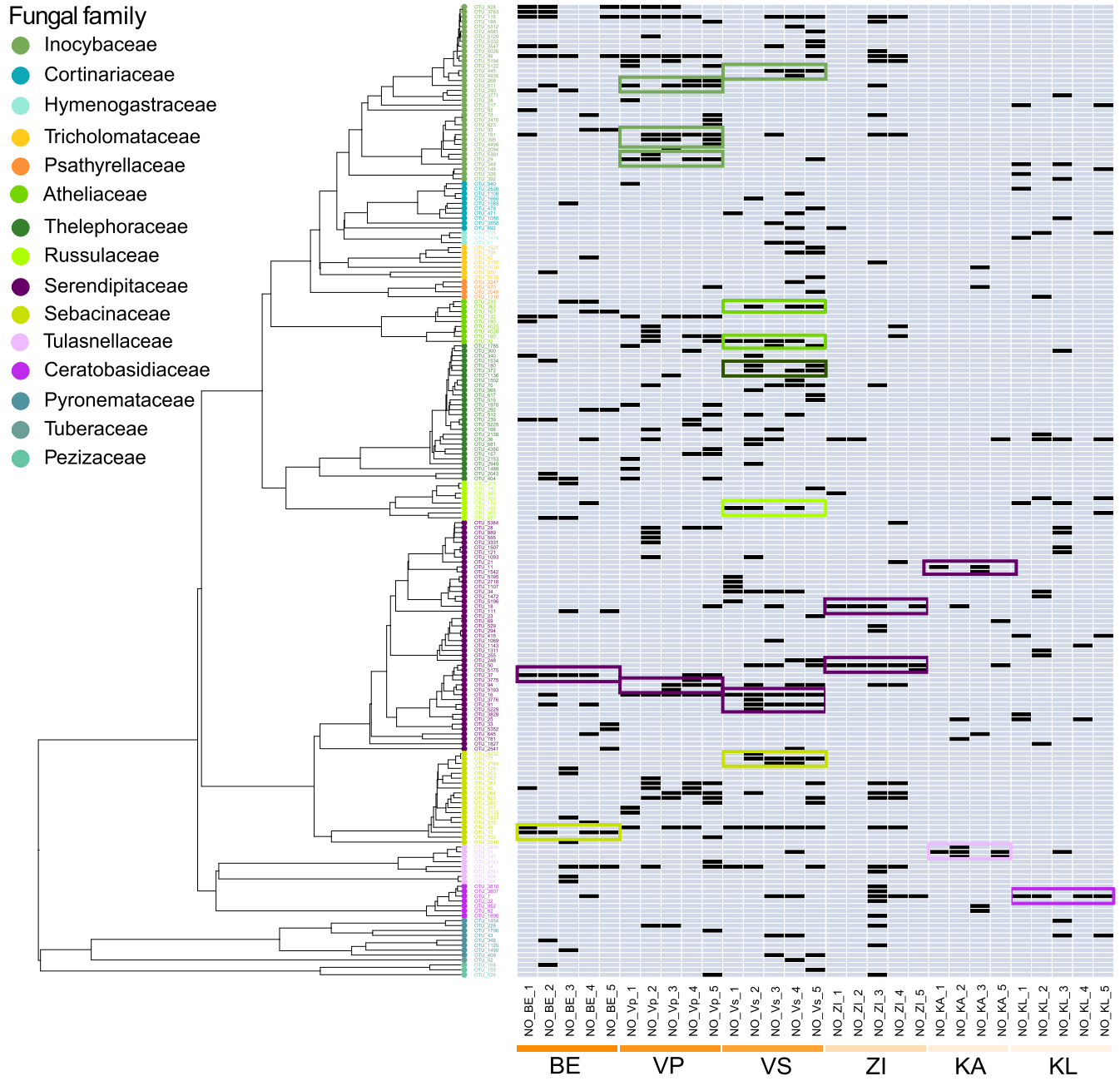

FIGURE 2 Phylogenetic mapping of orchid mycorrhizal operational taxonomic units (OTUs) with indication of indicator species for each site. Tips of the tree are coloured by fungal family. Rhizoctonia, ectomycorrhizal and saprotrophic families (in tips of the tree) are in shades of purple, green and yellow, respectively. Indicator species for each site are marked with rectangular boxes and show identical colours with tree tips. The light availability of orchid individuals among sites is displayed by the opacity of orange colour bar at the bottom of the plot. The forest sites BE, VP and VS exhibited lower light intensity: 69, 84 and 99 μmol photons $m^{-2} s^{-1}$ on average, while grassland site KA, KL and ZI exhibited higher light intensity: 474, 593, 650 μmol photons $m^{-2} s^{-1}$ on average.

3.3 | The Sebacinaceae fungi detected in *N. ovata* are phylogenetically clustered with those in mycoheterotrophic relatives

Our phylogenetic analysis showed that the Sebacinaceae OTUs of *N. ovata* were highly clustered with the Sebacinaceae sequences of fully mycoheterotrophic relatives (*N. nidus-avis*, *N. accuminata* and *N. camtschatea*) (Figure S5). Conversely, the three Sebacinaceae sequences detected by Těšitelová et al. (2015) and one sequence of *N. accuminata* was placed at the basal and close to the Serendipitaceae OTUs (Figure S5).

3.4 | The level of mycoheterotrophy does not vary substantially between habitats of *N. ovata*

At each site, leaf samples of *N. ovata* were significantly enriched in ^{15}N (except for the individuals from the forest site BE) and ^{2}H,

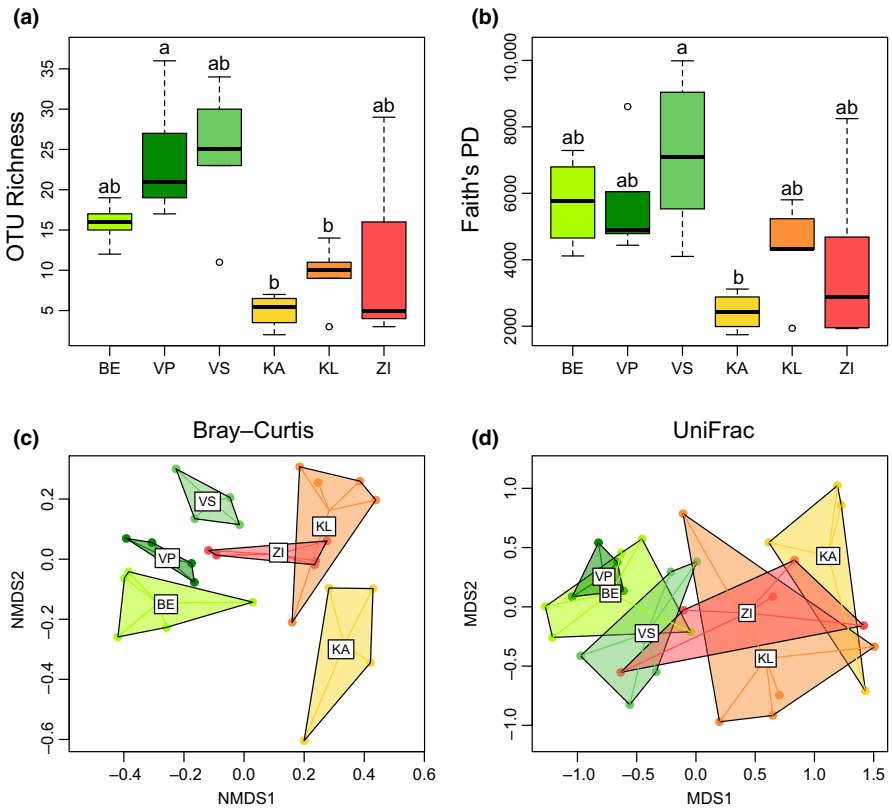

FIGURE 3 Diversity and community structure of fungal communities in *Neottia ovata* roots sampled at different grassland and forest sites. (A) Operational taxonomic unit (OTU) richness (SR) and (B) Faith's PD of root communities. Letters above the bars indicate the outcomes of post hoc analysis conducted to compare the difference between sites. Root community structure is visualized by NMDS plots based on (C) Bray–Curtis and (D) UniFrac distance metrics. Forest sites (BE, VP and VS) and grassland sites (KA, KL and ZI) are distinguished by colour.

but not in ^{13}C, compared with autotrophic references (Table 2; Table S10). Notably, negative enrichment in ^{18}O was found for *N. ovata* at the three forest sites (BE, VP and VS) and grassland site ZI (Table S10), suggesting that a higher transpiration rate of orchids at these sites might have underestimated the heterotrophic C gain indicated by the ^{2}H enrichment.

Significant differences among sites were found for leaf enrichment factors $\varepsilon^{2}H$ and $\varepsilon^{15}N$, but not for $\varepsilon^{13}C$ and total nitrogen concentration (Figure 5; Table S11). However, no consistent, significant difference in $\varepsilon^{2}H$ and $\varepsilon^{15}N$ of *N. ovata* was found between sites according to post hoc pairwise comparisons (Figure 5; Table S11). Specifically, a significantly higher $\varepsilon^{15}N$ was found for *N. ovata* collected from grassland sites (KA and KL) compared with individuals collected from forest sites (BE and VP). A significant difference in $\varepsilon^{2}H$ was only found between the grassland site KL and the forest site VS. Our linear mixed models showed that $\varepsilon^{15}N$ ($R^2=0.436$, $p<0.001$) and total nitrogen concentration ($R^2=0.149$, $p<0.05$) differed significantly between forest and grassland sites, but no such differences were found for $\varepsilon^{13}C$ ($R^2=0.001$, $p=0.838$) and $\varepsilon^{2}H$ ($R^2=0.137$, $p=0.177$) (Table S12). While light intensity differed greatly between forest and grassland sites (Appendix S1), no significant relationship between light intensity and $\varepsilon^{13}C$ ($R^2=0.002$, $p=0.823$) or $\varepsilon^{2}H$ ($R^2=0.014$, $p=0.633$) was observed (Table S13).

4 | DISCUSSION

In line with previous studies (Jacquemyn et al., 2015; Oja et al., 2015; Těšitelová et al., 2015; Vogt-Schilb et al., 2020), we found that *N. ovata* predominantly associated with rhizoctonia fungi from the Serendipitaceae, while recruiting a wide range of site-dependent ectomycorrhizal fungi (Figure 2)—this is common if not general in rhizoctonia-associated orchids (Selosse et al., 2022). Our phylogenetic analyses showed that *N. ovata* has already recruited the set of ectomycorrhizal Sebacinaceae fungi that tightly associated with its fully mycoheterotrophic relatives (Figure S5). In support of our first hypothesis (#H1), the diversity and composition of root communities varied greatly between sites, especially between forests and grasslands (Figure 3; Figure S1). Moreover, variation in root communities was highly correlated with variation in soil communities (Figure 4A; Figures S3 and S4) and significantly related to edaphic factors (Figure 4B), supporting our second hypothesis (#H2). In general, we found significant enrichment in ^{15}N and ^{2}H, but not in ^{13}C (Table 2; Figure 5), confirming the partially mycoheterotrophic mode of *N. ovata* and the usual trend for rhizoctonia-associated orchids (Gebauer et al., 2016; Schiebold et al., 2018; Schweiger et al., 2018). However, stable isotope signatures did not significantly differ between sites and habitat types (Figure 5; Tables S11 and S12) and no correlation was found between ^{13}C or ^{2}H and light availability (Table S13), rejecting our third hypothesis (#H3).

4.1 | Recruitment of ectomycorrhizal fungi precedes the evolutionary transition to heterotrophy in *Neottia*

The trend of symbiotic shifts from rhizoctonia Serendipitaceae to ectomycorrhizal Sebacinaceae fungi in parallel with an increased

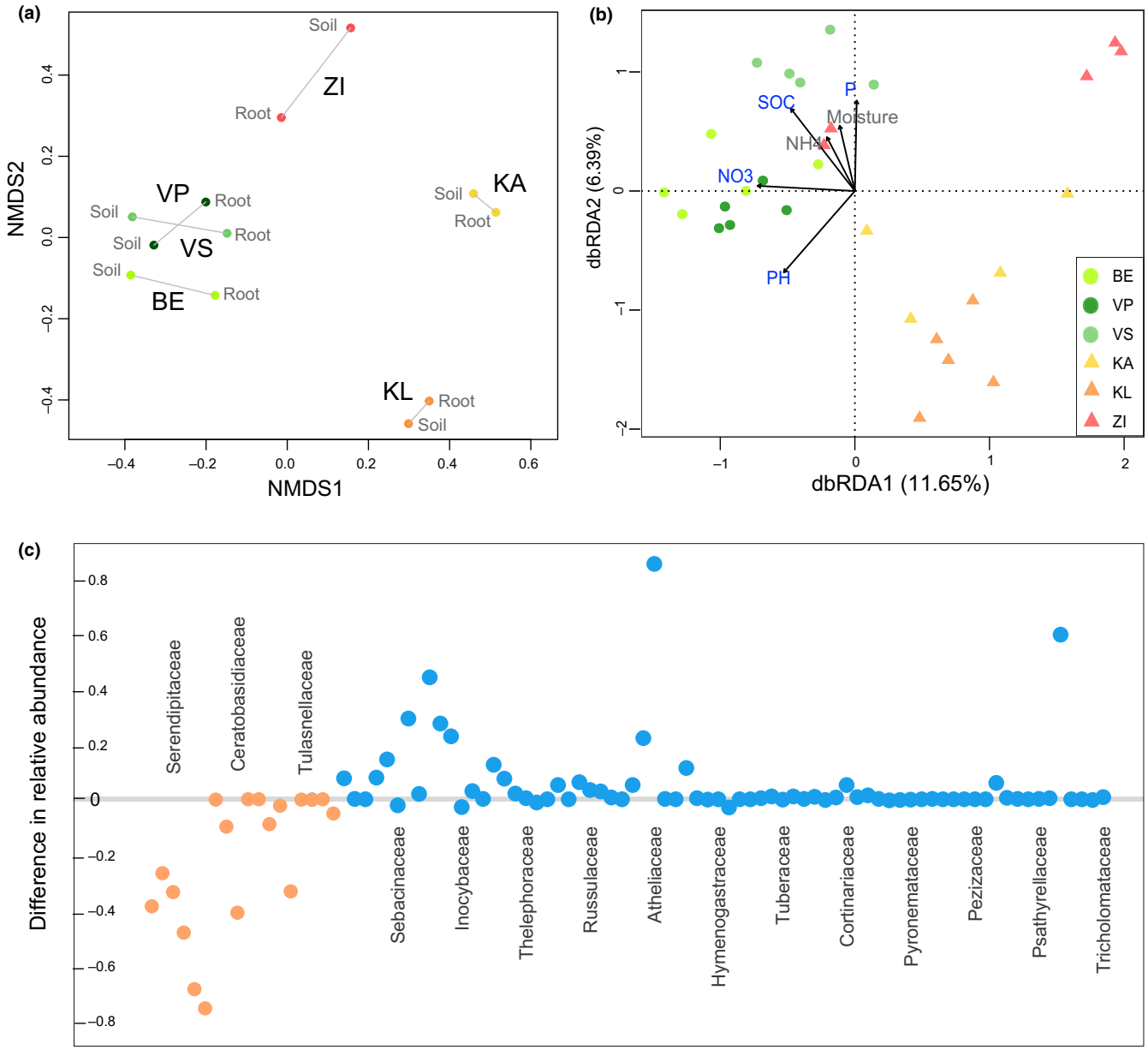

FIGURE 4 Relationship between soil fungal community and soil properties and root communities. (A) NMDS graph displaying the similarity in fungal community structure among root and soil samples across sites. (B) dbRDA model shows the effect of soil properties on root communities. Significant soil variables are coloured in blue. SOC: soil organic content; NO_3^-: nitrate concentration; NH_4^+: ammonium concentration; Moisture: soil moisture content. (C) Differences in the relative abundance of fungal families found in soil and root samples. For each site, the relative abundance (MiSeq reads) of each fungal family is calculated for soil and root samples separately and subsequently the difference in relative abundance is calculated as the relative abundance in soil—relative abundance in roots. Points below or above the zero line are interpreted as being more represented in root or soil sample, respectively. Rhizoctonia and non-rhizoctonia fungal families are shown in orange and blue, respectively.

gain of fungal C is conceivable in the *Neottia* genus (Yagame et al., 2016). Such evolutionary changes have been supported by both stable isotope analyses and molecular identification of mycorrhizal associates of leafy and leafless *Neottia* species displaying different degrees of mycoheterotrophy. It has been well reported that *Neottia nidus-avis*, positioning at the evolutionary endpoint of full mycoheterotrophy, is significantly enriched in ^{13}C, ^{15}N and ^{2}H (Bidartondo et al., 2004; Gebauer et al., 2016; Gebauer & Meyer, 2003; Jacquemyn et al., 2021; Preiss et al., 2010; Schiebold et al., 2018) and specializes on ectomycorrhizal Sebacinaceae fungi (Jacquemyn et al., 2021; McKendrick et al., 2002; Selosse et al., 2002; Yagame et al., 2016). Previous studies have showed that ectomycorrhizal Sebacinaceae fungi occurred in root communities of *N. ovata* (Jacquemyn et al., 2015; Oja et al., 2015; Těšitelová et al., 2015) and several other leafy *Neottia* species (Těšitelová et al., 2015; Yagame et al., 2016). The presence of Sebacinaceae in root communities of *N. ovata* in forest sites is confirmed by this current study (Figure 2). In disagreement with

TABLE 2 Mean enrichment factors $\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$, $\varepsilon^{18}O$ and total nitrogen concentration of *Neottia ovata* sampled at three forest and three grassland sites. Mean values in bold represent significant differences ($p < 0.05$) in leaf isotope composition between *N. ovata* and autotrophic references within each site using linear mixed models. All models include 'plot' as random effect and *p* values were computed using a Wald *t*-distribution approximation (see detailed results in Table S10).

		$\varepsilon^{13}C$		$\varepsilon^{15}N$		$\varepsilon^{2}H$		$\varepsilon^{18}O$		Total N	
Site	Habitat	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BE	Forest	0.66	1.53	−0.09	1.03	**15.70**	5.73	**−3.05**	0.91	**2.56**	0.59
VP	Forest	−0.72	1.33	**0.81**	0.23	**19.30**	0.91	**−1.99**	0.63	**2.61**	0.38
VS	Forest	0.01	1.67	**1.90**	1.13	**9.56**	4.11	**−3.65**	0.99	**2.29**	0.35
KA	Grassland	0.24	0.74	**4.55**	1.25	**16.40**	7.85	−0.98	0.86	**2.02**	0.31
KL	Grassland	−0.39	1.51	**3.99**	2.91	**22.50**	3.07	−0.10	1.10	**2.21**	0.40
ZI	Grassland	0.39	0.67	**2.97**	1.87	**19.60**	3.92	**−1.62**	0.80	**2.27**	0.23

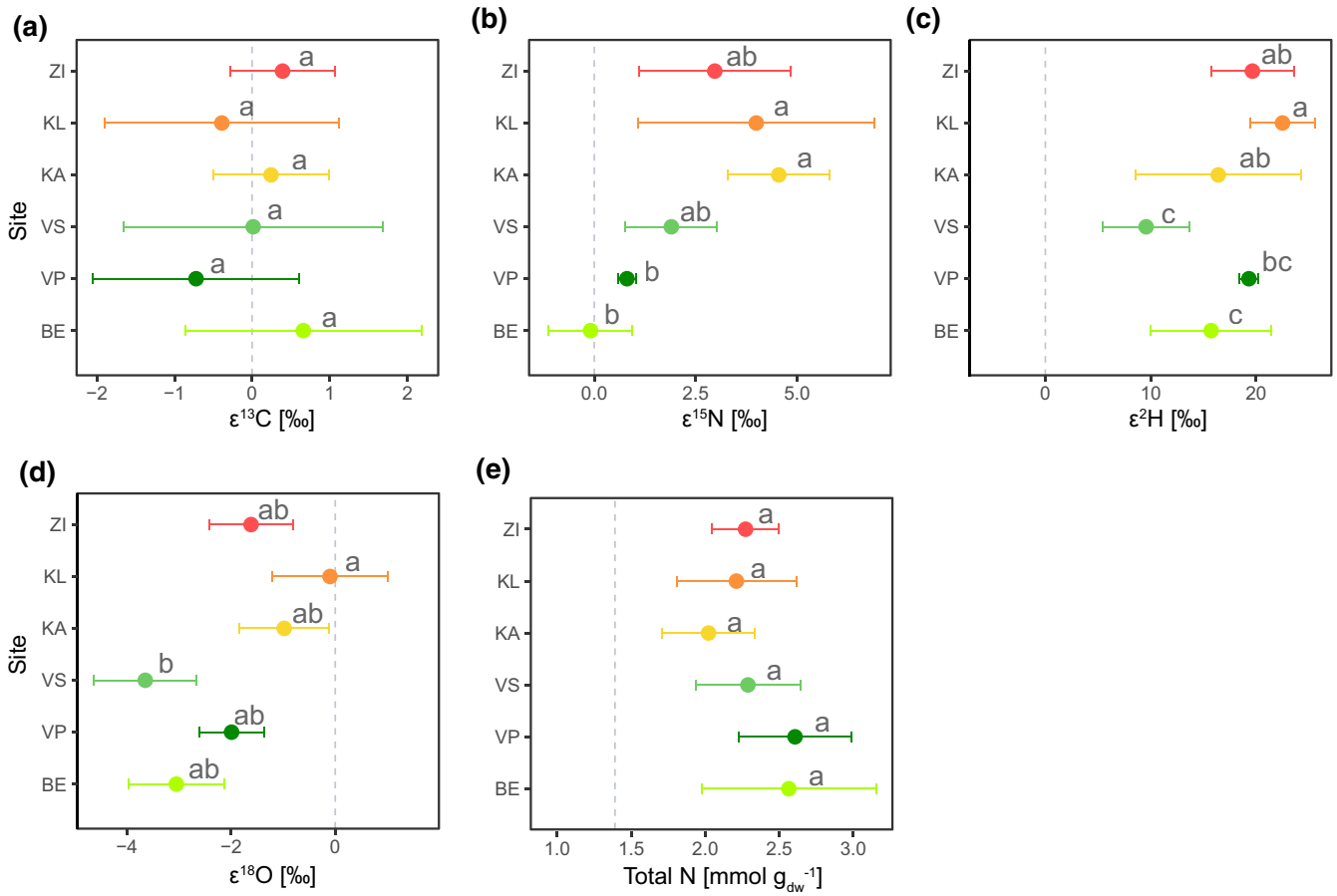

FIGURE 5 Stable isotope signatures ($\varepsilon^{13}C$, $\varepsilon^{15}N$, $\varepsilon^{2}H$, $\varepsilon^{18}O$ and total N) of *Neottia ovata* sampled at six different sites in Europe. Forest sites include BE, VP and VS, whereas grassland sites include KA, KL and ZI. Sites with different letters are significantly ($p < 0.05$) different from each other.

previous findings of phylogenetic separation of Sebacinaceae symbionts between *N. ovata* and the fully mycoheterotrophic *N. nidus-avis* (Těšitelová et al., 2015), our phylogenetic analyses further showed that Sebacinaceae fungi detected in *N. ovata* are closely related to mycorrhizal symbionts of its fully mycoheterotrophic relatives (*N. nidus-avis*, *N. accuminata* and *N. camtschatae*) (Figure S5). Our findings indicate that the predisposition of Sebacinaceae fungi in *Neottia* may have opened the door for symbiotic shifts at the fully mycoheterotrophic stage, which is in line with the results of broad-scale evolutionary inferences in Orchidaceae (Wang et al., 2021) and is predicted under the 'waiting room hypothesis' (Selosse et al., 2022).

4.2 | Functional switches to ectomycorrhizal fungi were not detected for *N. ovata* in the studied habitats

Ectomycorrhizal fungi tend to have different types of access to nutrient resources compared with soil-dwelling rhizoctonia fungi and may therefore explain the distinct stable isotope signatures

observed in orchids predominantly associating with ectomycorrhizal fungi (Bidartondo et al., 2004; Gebauer & Meyer, 2003; Gebauer & Schweiger, 2021; Schiebold et al., 2018). It is well supported that ectomycorrhizal-dominant mycoheterotrophic orchid species are more enriched in ^{13}C, ^{15}N and ^{2}H than rhizoctonia-associated orchid species. Here, we expected that populations associating with a higher proportion of ectomycorrhizal associations would have a higher ^{13}C enrichment of orchid leaves. However, we found no significant variations in stable isotope signatures between forest and grassland individuals of *N. ovata* (Figure 5). Considering the predominant association with rhizoctonia fungi of the Serendipitaceae (Figures 2 and 3) and the non-negligible host preference towards those fungi (Figure 4C), we speculate that ectomycorrhizal fungi contribute little to the nutritional budget of *N. ovata* in the studied habitats when rhizoctonia fungi still take the major functional role in external C and nutrient supply from fungal resources. Thus, our results hint towards an early stage of partial mycoheterotrophy for *N. ovata* in line with the waiting room hypothesis (Selosse et al., 2022), a stage when mycorrhizal partners for a later stage have already been recruited (as endophytes) but the functional symbiotic switch to new partners probably has not yet been achieved.

4.3 | Does the heterotrophic level of *N. ovata* vary with the irradiance level?

Light availability was hypothesized to be negatively correlated with the level mycoheterotrophy. Orchid individuals in low-light habitats were therefore hypothesized to gain a higher amount of fungal C to compensate for their reduced photosynthetic C than individuals exposed to high irradiance levels (Gonneau et al., 2014; Preiss et al., 2010; Schweiger et al., 2019). However, we found no correlation between enrichment factor ^{13}C of *N. ovata* and light intensity (Table S13). Our results are not in line with previous findings for partially mycoheterotrophic *Cephalanthera* species that associate with ectomycorrhizal fungi (Preiss et al., 2010) and *Ophrys insectifera* that associates with rhizoctonia fungi (Schweiger et al., 2019), but in agreement with previous reports on *N. ovata* (Onipchenko et al., 2023; Schweiger et al., 2019) and other rhizoctonia-associated orchids, such as *Cypripedium calceolus* (Preiss et al., 2010), *Goodyera repens* (Liebel et al., 2015) and *Epipactis palustris* (Lallemand et al., 2018). Particularly, *E. palustris* and *N. ovata* reside in different genera consisting of mycoheterotrophic species within in the same tribe Neottieae. ^{2}H enrichment was considered as suitable nutrition indicator for rhizoctonia-associated orchids because of their usually inconspicuous ^{13}C enrichment (Gebauer et al., 2016). Because *N. ovata* was not enriched in ^{13}C (Figure 5), we further expected to find a correlation between ^{2}H and light intensity. However, this expectation was also not met in the current study (Table S13).

Several factors may influence the stable isotope signatures of *N. ovata* and hamper our understanding of its heterotrophic nutrition. Those factors include the transpiration effect (Cernusak et al., 2004; Onipchenko et al., 2023; Ziegler, 1989), the sampling range of light availability (Schweiger et al., 2019), the plants' alternative strategies of adaption (Liebel et al., 2015) and soil nutrient availability or stoichiometry (Gomes, Van Bodegom, & Soudzilovskaia, 2019). Detailed elaborations on those factors can be found in Appendix S1. Nonetheless, understanding the exact factors influencing the mycoheterotrophic nutrition of orchid species awaits future in situ and ex situ studies in which environmental factors can be manipulated.

5 | CONCLUSIONS

Our comprehensive investigations showed that *N. ovata* is capable of dual associations with rhizoctonia symbionts and supplementary ectomycorrhizal fungi in relatively low-light forest habitats. However, the presence of ectomycorrhizal fungi did not substantially influence the isotope signatures of *N. ovata* in forests. Although we cannot rule out the possibility that the C and H enrichments were masked by higher transpiration rates of forest individuals, our findings indicate that the contribution of ectomycorrhizal fungi to the C and nutrition budget of *N. ovata* is probably minimal in the studied habitats, and that rhizoctonia fungi play the major functional role in its nutritional supply from fungal resources.

AUTHOR CONTRIBUTIONS

Vincent S. F. T. Merckx, Deyi Wang, Gerhard Gebauer and Hans Jacquemyn designed the experiment. Gerhard Gebauer, Franziska E. Zahn, Hans Jacquemyn and HVDH contributed to the selection of the sampling site. Deyi Wang, Vincent S. F. T. Merckx, Hans Jacquemyn, Franziska E. Zahn and Johanna Lorenz collected and processed the samples. Deyi Wang analysed data with input from Vincent S. F. T. Merckx, Gerhard Gebauer, Hans Jacquemyn, Franziska E. Zahn and Sofia I. F. Gomes. Deyi Wang wrote the first manuscript draft. All authors commented and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Additional Supporting Information will be found online in the Supporting Information section at the end of the article. Illumina sequencing data have been deposited with links to BioProject accession number PRJNA952620 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1: Methodological details and supplementary results.

Figure S1: Fungal community composition of root and soil samples across site.

Figure S2: Orchid mycorrhizal fungal OTU abundance of root and soil samples.

Figure S3: Alpha diversity of mycorrhizal community in bulk soil.

Figure S4: Linear regression of SR and PD values between root and soil samples.

Figure S5: Phylogenetic relationship of the Serendipitaceae fungi detected in *Neottia ovata* and its fully mycoheterotrophic relatives.

Table S1: A pilot study to test the efficiency of two commonly used primer pairs for capturing orchid mycorrhizal fungi.

Table S2: Sequence list and alignment of Tulasnellaceae for primer design.

Table S3: Taxonomic assignments of fungal OTUs of root and soil samples.

Table S4: Stable isotope signatures of orchid individuals and reference plants, habitat type, light intensity and soil properties.

Table S5: Indicator species with indicator value larger than 0.5 and significant *P*-value.

Table S6: ANOVA, Kruskal–Wallis tests and post hoc pairwise comparisons of alpha diversity index (SR and PD) of root and soil samples across sites.

Table S7: Habitat effect on SR and PD of root and soil samples using linear mixed models. 'Site' was used as a random effect.

Table S8: Soil properties of sampling sites.

Table S9: Results of the dbRDA model.

Table S10: Model difference between leaves of orchid and reference plants within each site, plot as random effect, for example, lmer ($\varepsilon^{13}C \sim Category + [1|Plot]$, data = BE).

Table S11: ANOVA, Kruskal–Wallis tests and post hoc pairwise comparisons of leaf isotope signatures and total N concentration across sites.

Table S12: Habitat effect on stable isotope signatures and total N concentration of *N. ovata* leaves. 'Site' was used as random effect, for example, lmer ($\varepsilon^{13}C \sim Habitat + (1|Site)$).

Table S13: Test the effect of light on ^{13}C and ^{2}H enrichment of *N. ovata* leaves. 'Site' was used as random effect, for example, lmer ($\varepsilon^{13}C \sim Light + (1|Site)$).

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