Can orchid mycorrhizal fungi be persistently harbored by the plant host?

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A B S T R A C T

The environmental distribution of non-obligate orchid mycorrhizal (OM) symbionts belonging to the 'rhizoctonia' complex remains elusive. Some of these fungi, indeed, are undetectable in soil outside the host rhizosphere. A manipulation experiment was performed to assess the importance of neighbouring non-orchid plants and soil as possible reservoirs of OM fungi for *Spiranthes spiralis*, a widespread photosynthetic European terrestrial orchid species. Fungi of *S. spiralis* roots were identified by DNA metabarcoding before and 4 months after the removal of the surrounding vegetation and soil. Although such a treatment significantly affected fungal colonization of newly-formed orchid roots, most OM fungi were consistently associated with the host roots. Frequency patterns in differently aged roots suggest that these fungi colonize new orchid roots from either older roots or other parts of the same plant, which may thus represent an environmental source for the subsequent establishment of the OM symbiosis.

1. Introduction

The spatial distribution of soil micro-organisms, such as root mutualists and pathogens, may act as a driver of spatial patterns of plant species (Ettema and Wardle, 2002). As mycorrhizal fungi play key functions in plant biology (Smith and Read 2008; van der Heijden et al., 2015), the spatial structure of their communities has been addressed in several studies in recent decades (e.g. Lilleskov et al., 2004; Lekberg et al., 2007; Bahram et al., 2015). These studies have mainly focused on the communities of ecto- mycorrhizal and arbuscular mycorrhizal fungi in woodland and agricultural ecosystems. In contrast, spatial patterns of orchid mycorrhizal (OM) fungi in soil remain largely understudied, despite their importance for orchid survival especially in the early growth stages. The relationships between orchids and their mycorrhizal fungi are critical for the conservation of these threatened plants (Swarts et al., 2010; Fay 2018). Dependency on compatible symbiotic fungi is, indeed, extreme in orchids due to their tiny "dust seeds", almost lacking nutritional reserves; seed germination as well as development and survival of the heterotrophic protocorms require colonization by fungi that provide the host plant with organic carbon (Smith and Read, 2008; Rasmussen and Rasmussen, 2014). Orchids retain their mycorrhizal associations also in adult- hood, when they are thought to rely on OM fungi for mineral uptake (Waterman and Bidartondo, 2008) and often for a supplement of organic carbon (Selosse and Roy, 2009; Stöckel et al., 2014; Hynson, 2016; Schweiger et al., 2019).

It has been proposed that the availability of appropriate OM fungi may constrain the establishment and resulting spatial distribution of orchids at a local scale (McCormick and Jacquemyn, 2014) because the patchy distribution of the OM fungal symbionts can affect key processes such as seed germination and plant growth and survival (McCormick et al., 2012, 2018). Patchiness of appropriate conditions favourable to seed germination and plant development, including the presence of compatible OM fungi, would indeed result in the observed patchy and agglomerate distribution of orchid plants (McCormick and Jacquemyn, 2014). Jacquemyn et al. (2012) and Waud et al. (2016), for example, found that local spatial segregation of orchids was determined by the distribution of the distinct mycorrhizal fungi needed by the different species. Sometimes, orchid seed germination can occur at sites where adult orchids are not present (Těšitelová et al., 2012; McCormick and Jacquemyn, 2014), suggesting that recruitment restrictions may operate later after germination stages (Selosse, 2014). In contrast, comparisons of the spatial distribution of both seedlings and adults in several orchids (Batty et al., 2001; Diez, 2007; Jacquemyn et al., 2007, 2012) revealed that seed germination was

restricted to areas where mature orchids already occurred, indicating convergent requirements by juveniles and adult plants. Temporarily appropriate environmental conditions (including the occurrence of OM fungi) may promote seed germination in loca- tions that lack adult orchids, but if such conditions fluctuate in subsequent seasons or years orchids may not survive to maturity in those sites (McCormick et al., 2018). Therefore, the observations reported above suggest that locations where orchids can reach adulthood may be sites with a persistent occurrence of OM fungi, whereas at sites devoid of mature plants environmental conditions (including the presence of fungi) may be ephemeral (McCormick and Jacquemyn, 2014; McCormick et al., 2016). In particular, quantitative real-time PCR analysis on soil-extracted DNA indicated that orchids were more abundant (and less likely to enter dormancy, and more likely to re-emerge) where OM fungi were abundant, pointing to a relationship between OM fungal abundance and orchid density (McCormick et al., 2018).

Our knowledge of the environmental origin of OM fungi (i.e. of their environmental reservoirs when they are not in association with orchid roots) is still limited. Orchids growing in the understory of either temperate deciduous or boreal forests mostly associate with fungi which are also simultaneously ectomycorrhizal on trees, including Cortinariaceae, Sebacinaceae, Russulaceae, and Thele- phoraceae (Jacquemyn et al., 2017). By contrast, much less is known about the lifestyle of the non-ectomycorrhizal OM fungal associates of other orchids (such as species from more open habitats). Photosynthetic orchids in grassland habitats mainly associate, both as seedlings and as adult plants, with fungi previously referred to the 'rhizoctonia' complex (Smith and Read, 2008; Dearnaley et al., 2012; Rasmussen and Rasmussen, 2014). This complex is in fact a polyphyletic assemblage encompassing Agaricomycetes belonging to the Tulasnellaceae and Ceratobasidiaceae, both in the order Cantharellales (Roberts, 1999; Taylor et al., 2002; Weib et al., 2004) and the Sebacinaceae and the Serendipitaceae both belonging to the Sebacinales (Weib et al., 2016). The Tulasnellaceae are often the most frequently retrieved OM fungi in both temperate and tropical orchid roots (Dearnaley et al., 2012; Jacquemyn et al., 2017).

Most OM rhizoctonias are regarded as unspecialized soil sap- rotrophs because of their fast growth in axenic *in vitro* conditions (e.g. Smith and Read, 2008; Nurfadilah et al., 2013; van der Heijden et al., 2015). It was, therefore, suggested that the main occurrence of OM rhizoctonias is outside the orchid hosts (Dearnaley et al., 2012; Selosse and Martos, 2014), and experimental burial of orchid seed packets confirmed that many OM rhizoctonias can occur in the environment independently of orchid roots (Těšitelová et al., 2012; McCormick and Jacquemyn, 2014). Indeed, the main argument in support of the idea that OM fungi can easily live in the absence of the orchid host is the observation of seed germination in areas lacking adult plants (Jersáková and Malinová, 2007). However, these germinations could be determined by fungi which do not sustain the adult stage of orchids, as these experiments, indeed, stop at early stages of seed/protocorm development without actually verifying their ability to develop into adult plants. Such a germination could be supported by fungi of minor functional importance colonizing roots of apparent generalist orchid species, that can supply some, but not all, of the functions of the dominant fungal symbiont (Shefferson et al., 2019).

Recent investigations, however, showed that some OM fungi in the Tulasnellaceae could not be detected in the bulk soil outside the orchid rhizosphere (Jacquemyn et al., 2015; Voyron et al., 2017; Egidi et al., 2018). This is in line with a previous attempt to detect the mycorrhizal taxa of interest by using *in situ* 'seed baiting' techniques, which were unable to retrieve the suitable *Tulasnella* spp. in the native site of the orchid *Diuris fragrantissima* (Smith, 2006). There is growing evidence that many fungi have more complex lifestyles than previously thought (Lofgren et al., 2018; Martino et al., 2018; Selosse et al., 2018), and the results of Voyron et al. (2017) and Egidi et al. (2018) suggest that also OM fungi, at least some species, may occur in the roots of neighbouring non- orchid plants that could act as reservoirs for the colonization of orchid seedlings. Indeed, while the ecology of the Tulasnellaceae remains understudied, several reports indicate that Sebacinales and Ceratobasidiaceae may exhibit a variety of ecological and/or nutritional strategies, such as the ability to establish mycorrhizal or non-mycorrhizal endophytic associations with non-orchid plants (Selosse et al., 2002, 2007, 2009; Weib et al., 2004; Oberwinkler et al., 2013; Tedersoo and Smith, 2013; Veldre et al., 2013; Bokati and Craven, 2016). A microcosm experiment was conducted by Ray et al. (2018) to investigate transmission of *Serendipita vermifera*, a fungal isolate from an Australian orchid, from the roots of an inoculated switchgrass (*Panicum virgatum*) to those of three nearby weeds (*Digitaria sanguinalis*, *Panicum texanum*, *Brachiaria platyphylla*). Transmission of *S. vermifera* occurred seemingly via contact between intermingled root systems rather than across root-free bulk soil, since the authors could not report colonization of weeds when the roots were kept separate (Ray et al., 2018).

In this study, the importance of neighbouring non-orchid plants and soil as environmental reservoirs of OM

fungi was explored for *Spiranthes spiralis*, a widespread late-flowering European meadow species, by means of a manipulation experiment aimed at elimi- nating such a possible source. Soil cores containing *S. spiralis* plants surrounded by the natural vegetation were collected in a Medi- terranean grassland at the time of emergence of the new roots, and either left undisturbed (non-manipulated soil cores) or manipu- lated by eliminating either only the surrounding non-orchid plants (sifted plants soil cores), or both the non-orchid plants and the soil, which was replaced with sterile sand (sand cores). We expected elimination of such reservoirs at the time of root emergence to deprive significantly orchid roots of OM fungi.

2. Materials and methods

2.1. Study site, plant species and sampling

The study site is a Mediterranean grassland located in north- western Italy. The area is characterized by meadows assigned to the association *Festuco-Brometalia*; Braun-Blanquet, 1964, typical of Habitat 6210 (*), surrounded by woods and shrubs, at 460 m asl, in a transition zone between Mediterranean and sub-Atlantic climates. As previously recorded, it is a site rich in orchid species (Girlanda et al., 2006, 2011; Ercole et al., 2015).

The study focused on the European terrestrial orchid *Spiranthes spiralis*. *S. spiralis* is a common late-flowering species, characterized by two to six cylindrical-fusiform fleshy roots, leaves forming a basal rosette, and a slender spiral inflorescence that appears in SeptembereOctober, holding about 10e30 small white flowers with a green-yellowish labellum throat (Fig. 1A) (Pridgeon et al., 2003). *Spiranthes spiralis* is a generalist species that can associate with different OM fungal taxa, including *Ceratobasidium* sp. (Tondello et al., 2012). More recently Duffy et al. (2019), who examined OM fungal diversity in 37 populations of this orchid species, occurring in nine regions throughout Europe, confirmed, by NGS sequencing, ceratobasidioid fungi as the main mycobionts, but also detected the presence of serendipitoid, sebacinoid, tulasnelloid, inocyboid, russuloid and thelephoroid fungi, with a higher fungal diversity in the Mediterranean region as compared to Northern Europe.

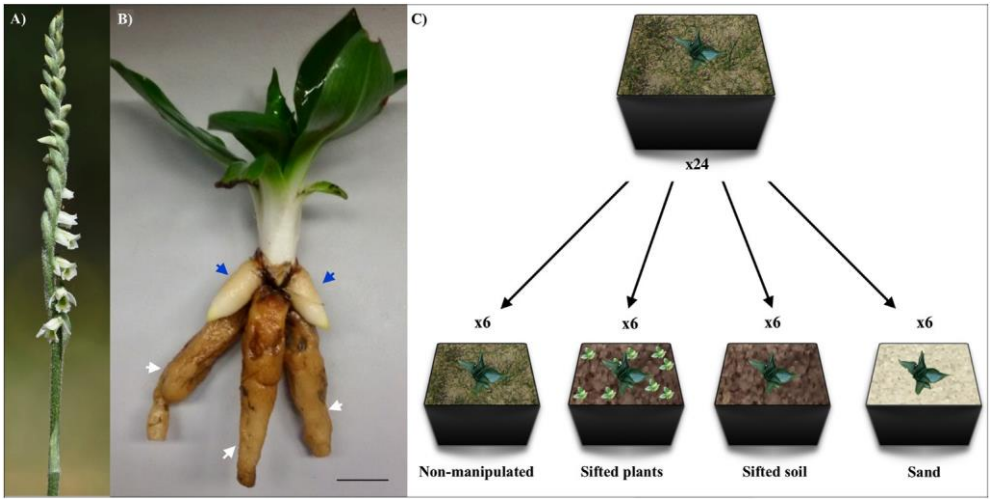

Fig. 1. (A) Inflorescence of *Spiranthes spiralis*, a widespread late-flowering European orchid. (B) Plant at first collection time (time zero) with old/mature roots of the previous year (white arrows) and newly emerged roots (blue arrows). Bar, 1 cm. (C) Experimental design of the manipulation experiment. The 24 collected soil cores containing one *S. spiralis* plant were divided into four groups (six cores each): (1) non-manipulated soil cores; (2) cores where soil was sifted and both the orchid and the non-orchid neighbouring plants were put back in their original positions (sifted plants); (3) cores where soil was sifted and the orchid was put back while the neighbouring non-orchid plants were removed (sifted soil); and (4) cores where both the soil and the neighbouring non-orchid plants were removed and the orchid was put in double-sterilized sand after surface sterilization (sand).

In this study, a total number of 24 square soil cores (20 cm x 20 cm x 15 cm depth) were collected in October-November 2016, around single *S. spiralis* plants, about 1 m apart from each other, at the above mentioned site. Each core was placed in a 20 20 cm pot. Identification of the non-orchid species surrounding the central S. spiralis plant was performed for each core (Supporting Information Fig. S1). Pots were divided into four homogeneous groups based on non-orchid neighbouring plant species. At collection time (time 0), all S. spiralis plants exhibited both two-three newly emerged roots (approx. 1 cm in length), and old, longer roots produced the previous year (Fig. 1B). Root samples were collected both from old (OR) and new (NR) roots from four pots for each group (as a precautionary measure, to account for the possible subsequent death of some plants resulting from removal of one out of the two-three roots, since the orchid plants had to survive for four further months at least), and preserved at 20°C until use. The four groups of pots were then either left undisturbed or manipulated as follows: the first group of six pots was maintained as a non-manipulated control (non-manipulated soil cores); in the second group, all plants were removed, the soil was sifted (2 mm mesh size) to eliminate all plant roots, and then both orchids and all the neighbouring non-orchid plants were put back to their original position (sifted plants cores); in the third group, soil was sifted as previously described but then only the orchid plants were put back in the centre of the pots (sifted soil cores); in the last group, orchids were removed from the cores, washed under tap water for 30 min, their roots were surface sterilized with a 1.25% solution of sodium hypochlorite for 20 min (followed by three washes in sterile water), and then put in previously double sterilized silica sand (sand cores) (Fig. 1C). Six replicates per treatment were set up. All 24 pots, randomly arranged, were then placed in a stone basin, protected by grids and plexiglass, under natural climatic conditions at the Botanic Garden of the University of Turin (45°3′ 21″ N, 7° 41′ 9″ E).

Inside the basin, the pots were put over a double plastic sheet to avoid contamination from the ground. Pots were checked every week and newborn non-orchid seedlings were periodically removed from the pots of the sifted soil cores.

After 4 months (to allow elongation of orchid and non-orchid roots), in April 2017 (time 1), a second root sampling (ER, elon- gated roots) was performed on four-six replicates per treatment (as some orchid plants had died during the incubation). At this time, the old roots observed at time 0 were completely withered and detached from the respective orchid.

2.2. DNA extraction, PCR amplification and amplicon sequencing

One root per plant per time point was sampled. Prior to DNA extraction, the roots were surface sterilized with a 1.25% solution of sodium hypochlorite for 20 min (followed by three washes in sterile water) and checked for mycorrhizal colonization under a light microscope (cross sections). Total DNA was extracted from a segment about 0.5 cm in length (approx. 100 mg) from the intermediate, mycorrhizal part of the root (thus excluding both the most proximal part of the root and the non-mycorrhizal apex). Extraction was performed with the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The quality and quantity of DNA samples were assessed by spectrophotometry (ND-1000 Spectro- photometer NanoDropH; Thermo Scientific, Wilmington, Ger- many). The nuclear ribosomal ITS2 region was amplified from all DNA extracts by means of a semi-nested PCR approach. In the first PCR, the entire ITS (ITS1-5.8S-ITS2) region was amplified with the ITS1-OFa, ITS1-OFb and ITS4-OF primers, specifically designed for OM fungi (Taylor and McCormick, 2008). For the nested PCR, ITS3mod and ITS4 (White et al., 1990) tagged primers were used to amplify the ITS2 region. ITS3mod is a modified version of ITS3 (Voyron et al., 2017). The first PCR was carried out in a final volume of 20 mL, including 0.4U of Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific, Courtaboeuf, France), 1x Phusion HF buffer, 0.5 lM of ITS OF primers, 0.2 mM of each dNTPs and 1 □L of genomic DNA (6-46 ng/□L). PCR conditions used were: 96 °C (2 min), 94 °C (30 s, 35 cycles), 58 °C (40 s), 72 °C (45 s), 72 °C (10 min).

The nested PCR was performed with 1U of Phusion High Fidelity polymerase, 1x HF buffer, 0.5 lM of the primers ITS3-mod and ITS4 (White et al., 1990) with barcodes, 0.2 lM of each dNTPs and 2 mL of PCR product, in a total volume of 25 □L.

All the PCR amplifications were run in triplicate in a T3000 thermal cycler (Biometra GmbH, Göttingen, Germany); for nested PCR each amplification was performed in three replicates and the following temperature profile was adopted: 98 °C (30 s), 98 °C (10 s, 30 cycles), 64 °C (30 s), 72 °C (20 s), 72 °C (10 min). All PCR products were checked on 1% agarose gel, replicates pooled together and then purified with the Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions. After quan- tification with Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA), by using the Qubit™ dsDNA HS Assay Kit, sequencing li-

braries were prepared by mixing equimolar amounts of purified PCR products. The libraries were paired-end sequenced using the Illumina MiSeq (2 250 bp) by IGA Technology Services Srl (Udine, Italy).

2.3. *Bioinformatic analyses*

Paired-end reads from each library were initially merged using PEAR v.0.9.2 (Zang et al., 2014), with the quality score threshold for trimming the low-quality part of a read set at 28 and the minimum length of reads after trimming set at 200 bp.

Assembled reads were then processed using the Quantitative Insights into Microbial Ecology (QIIME) v.1.8 software package (Caporaso et al., 2010). Initial sequence processing and sample assignment were performed with a minimum sequence length cutoff of 200 bp, minimum Phred quality score of 28, calculated over a sliding window of 50 bp, and allowing a maximum mismatch of 3 bp over the forward and reverse primers. Sequences were reorientated when necessary to 5′ to 3', and demultiplexed based on the tags and primers. Chimeric sequences were identified and removed performing a *de novo* (abundance based) detection using USEARCH61 (Edgar, 2010), as implemented in the QIIME pipeline. Operational taxonomic units (OTUs) were determined using an open reference-based clustering strategy, with the USEARCH61 method, at 98% similarity; only clusters encompassing at least 10 sequences were retained. The Full "UNITE INSD" dataset database version 7.2 for QIIME was used as a reference for OTU picking and taxonomy assignment (Abarenkov et al., 2010; Koljalg et al., 2013; http://unite.ut.ee, last accessed July 31, 2019); the BLAST algorithm (Altschul et al., 1990) was used as taxonomy assignment method, using an e-value of $1e^{-5}$ as threshold. The OTU representative sequences generated in this study (i.e. the most abundant sequence within each OTU) were submitted to GenBank and recorded under the following strings of accession numbers: MN602715- MN602738, MW577335-MW577346.

2.4. *Phylogenetic analyses*

Maximum likelihood (ML) analyses were carried out with the representative sequences of the tulasnelloid, ceratobasidioid, sebacinoid, serendipitoid and thelephoroid OTUs. Sequences included in the ML analyses comprised best BLAST hits as well as fungal sequences from a variety of terrestrial orchids, including the target species, from different continents and environments, as well as from non-orchid plants, fungal mycelia and fruiting bodies. Se- quences were aligned using the program MUSCLE (Edgar, 2004) with default conditions for gap opening and gap extension penalty. Alignments were then imported into MEGA v.7.0.26 (Kumar et al., 2016) for manual adjustment. ML estimation was performed with RAxML v.8 (Stamatakis, 2014) through 1000 bootstrap replicates (Felsenstein, 1985) using the GTR GAMMA algorithm to perform a tree inference and search for a good topology on CIPRES Science Gateway (Miller et al., 2010). Support values from bootstrapping runs were mapped on the globally best tree using the ef option of RAxML and -x 12345 as a random seed. Nodes receiving a bootstrap support <70% were not considered as well supported. Alignments and tree topologies are archived in the database TreeBASE (http://www.treebase.org; submission ID 25484).

2.5. *Statistical analyses*

To allow for comparisons among datasets obtained from the different root samples, subsampling at even sequencing depth from each sample (5632 sequences per sample) was performed by means of the *rarefy_even_depth* function in the R (v 3.6.0) package phyloseq (v 1.22.3) (McMurdie and Holmes, 2013).

Chi-squared tests (Tallarida and Murray, 1987) were carried out to compare proportions of OTUs recovered from the different root groups.

The effects of root age and treatment on the composition of OM fungal assemblages were evaluated using permutational multivar- iate analysis of variance (PERMANOVA, 999 permutations), as implemented in the *adonis* routine of the *vegan* package (v 2.5e4) of R (Oksanen et al., 2013; R Core Team, 2013). The multivariate homogeneity of group dispersions was first assessed by means of the *betadisper* and *permutest* (with 999 permutations) functions in the R package *vegan* (Oksanen et al., 2013). Indicator species anal- ysis, a classification-based method to measure associations be- tween species and groups of sites (Dufrene and Legendre, 1997), was carried out using the *multipatt* function in the *indicspecies* (v. 1.7.6) R package, with 999 permutations (De Cáceres and Legendre, 2009), in order to assess whether fungi (and, if so, which ones) were significantly associated with a particular root group. All analyses were based on the Jaccard similarity index. Differences in single OTU frequency among different root groups were tested for significance by means of Kruskal-Wallis tests.

Co-occurrence network analysis was also carried out to compare the general orchid root fungal communities

in the different soil cores at time 1. The non-random co-occurrence hypothesis be-tween taxa was tested using the checkerboard score (C-score) under a null model preserving samples frequencies (Stone and Roberts, 1990). The non-random co-occurrence hypothesis was tested in R (v 3.6.0) using the *oecosimu* function of the *vegan* package. Networks were created using fungal OTUs as nodes and co-occurrence relationships between the OTUs as edges (either including or excluding OTUs shared by all treatments). Co-occurrence was evaluated calculating all possible Spearman's rank correlations, with the R script published by Williams et al. (2014). Co-occurrence relationships were considered only if Spearman's correlation coefficient (r) was greater than 0.6 and if the associated *p*-value was lower than 0.01 (Junker and Schreiber, 2008). To assess possible impact on network structure, we considered network topological parameters which likely indicate the stability of the community (Assenov et al., 2008; Steele et al., 2011): average node degree, clustering coefficient and average edge betweenness. Node degree corresponds to the number of connections each OTU has with other nodes (OTUs with a high degree value being central in the network). The clustering coefficient describes the ability of the network nodes to cluster in well separated modules, which may be unaffected by environmental factors acting on other modules (networks featuring a high clustering coefficient are therefore considered as being resistant to environmental disturbances). Edge-betweenness describes instead how nodes of a network, just like hubs, play a central role in the co-occurrence patterns/interactions: the higher this value, the more fragile the community coping with environmental perturbations, due to the presence of few bottleneck OTUs with a likely strongly central role in biological interactions, the missing of which has strong impacts on network stability (Finn et al., 2020). Network editing and parameters calculation were carried out with the interactive platform Cytoscape 3.5.1 (Shannon et al., 2003). Average node degree, clustering coefficient and average edge betweenness values distributions were statistically compared with the Kruskall-Wallis test followed by a Dunn's post-hoc test. Statistical tests were performed in R with the function *dunn.test* of the *dunn.test* package (v 1.3.5) (Dinno et al., 2015). Barcharts were created for the significantly different distributions by means of the *ggplot* function of the *ggplot2* package (v 3.1.1) (Wickham, 2016) in R environment.

3. Results

At collection time (time 0), the *S. spiralis* plants exhibited newly emerged roots (hereinafter referred to as "new roots") approx. 1 cm in length, alongside with the old, longer roots ("old roots") produced in the previous year (Fig. 1B). Although already colonized by fungi, colonization of new roots by OM fungi was erratic (i.e. absent in some roots). After 4 months (time 1), the new roots had grown to approx. 2-3 cm in all treatments (developing into "elongated roots"), whereas the old roots observed at time 0 were completely withered and detached from the respective orchid plants.

3.1. *Fungal diversity in S. spiralis roots*

After filtering and cleaning of the Illumina MiSeq reads obtained by fungal metabarcoding of all root samples, 258.072 high-quality sequences were obtained, which were clustered in 459 (98% sequence identity) OTUs.

S. spiralis roots hosted a diverse array of tulasnelloid, ceratobasidioid, sebacinoid, serendipitoid and thelephoroid fungi (two, eight, three, five and four OTUs, respectively). Only OTU 5 and OTU 486 could be identified at the species level (*Tulasnella helicospora* and *Sebacina incrustans*, respectively). Most putative OM OTUs were closely related to fungi retrieved from orchid plants. In particular, the tulasnelloid OTU 5 and the ceratobasidioid OTU 23 exhibited 100% and 98.8e100% sequence identity with fungi already retrieved in *S. spiralis* populations (Tondello et al., 2012; Duffy et al., 2019). Other OTUs were instead closely related to previously obtained fungal sequences from either non-orchid plant roots or soil, including soil of the study area (Voyron et al., 2017) (Supporting Information Figs S2-S5).

As expected, *S. spiralis* roots also hosted other fungi of Basidiomycota (101 OTUs, 21.8% of total sequences), as well as a number of Ascomycota (200 OTUs, 43.5% tot. seq.), other fungal groups (25 OTUs, 7.7% tot. seq., of which 13 OTUs assigned to Mortierellomycota, 7.6% tot. seq.), as well as unidentified fungi (98 OTUs, 11.1% tot. seq.). In particular, *Fusarium* spp. (OTUs 373, 552 and 811), unidentified Ascomycota spp. (OTUs 679 and 686), *Pleosporales* sp. (OTU 719), *Alternaria* sp. (OTU 476), *Sordariomycetes* sp. (OTU 316), *Mortierella* sp. (OTU 43), *Clonostachys* sp. (OTU 372) and one unidentified fungus (OTU 21) occurred in at least 65% of the examined roots (Supporting Information Table S1).

3.2. *Time zero*

In new roots, fungal metabarcoding identified mostly *Inocybe* sp. (OTU 135, Fig. 2) and *Mortierella* spp. (occurring in 80% and approx. 70% plants, respectively). Tulasnelloid, ceratobasidioid, serendip- itoid, sebacinoid, thelephoroid and russuloid fungi occurred in <20% plants. *Inocybe* sp. (OTU 135) was significantly associated with these roots (Supporting Information Table S1).

All old roots were colonized by OM fungi, including ceratobasidioid fungi (Fig. 2, Supporting Information Fig. S6). Several of the latter (such as OTUs 14, 23, 25 and 151) were more frequent in these roots than in new roots (Supporting Information Table S1).

3.3. Time 1

At time 1 orchid roots in the four parallel experiments (i.e. non-manipulated, sifted plants, sifted soil and sand cores) hosted in total 184 fungal OTUs, 42.9% of which were shared by all treatments (Supporting Information Fig. S7). The core OTUs included 10 potential OM OTUs (corresponding to 58.8% of OM OTUs from elongated roots), which encompassed tulasnelloid, ceratobasidioid, sebacinoid, serendipitoid and thelephoroid fungi found in these plant groups (Supporting Information Fig. S7, Table S1).

All non-manipulated elongated roots were colonized by tulasnelloid and sebacinoid fungi. Indeed the tulasnelloid OTU 5 (*T. helicospora*) and the sebacinoid OTU 423 were significantly associated with these roots (Supporting Information Table S1).

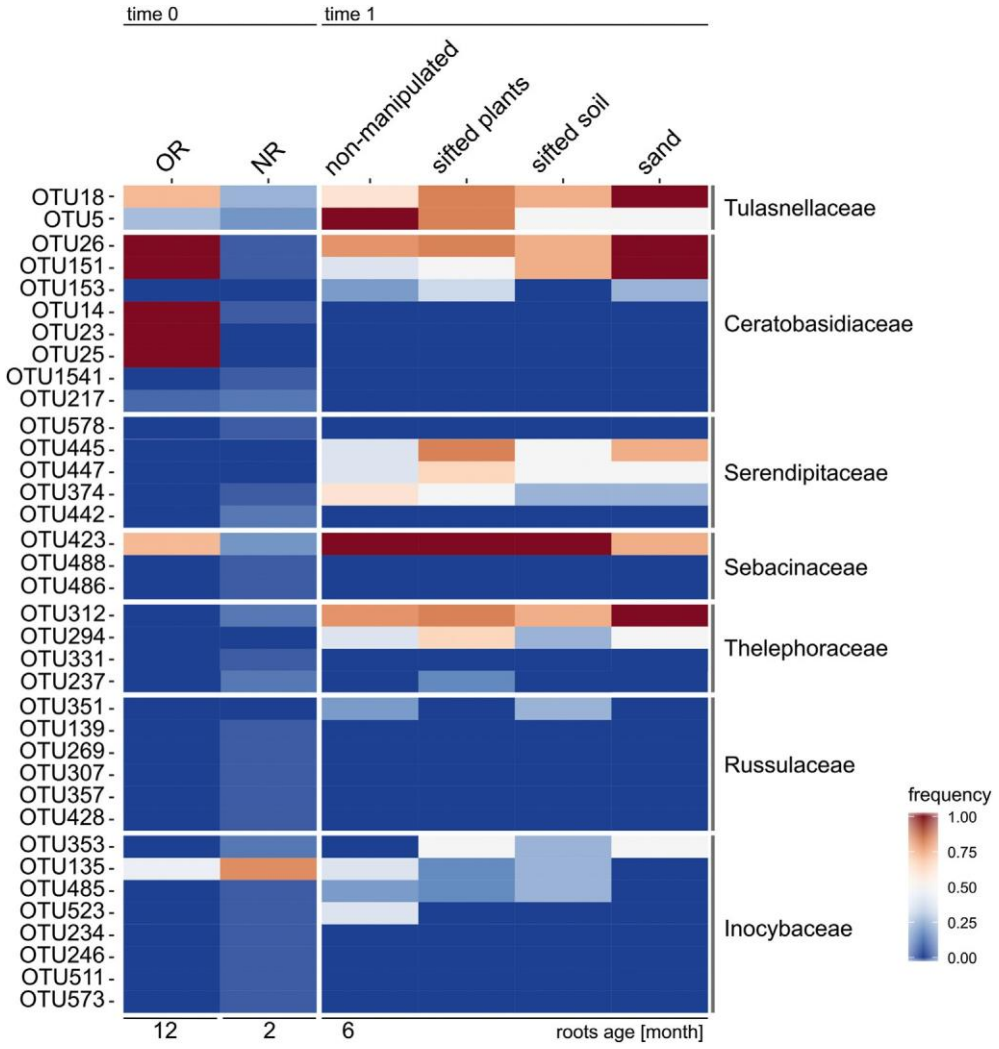

Fig. 2. Frequency of the OM OTUs in *Spiranthes* spiralis roots collected at time 0 (OR, old roots; NR, new roots) and elongated roots collected at time 1 (all treatments). Frequency is expressed on a 0-1 scale (1 indicates occurrence in all the examined plants).

Elongated roots in the sand cores were colonized by a significantly lower number of OTUs than in the other

treatments (chi- square tests, $p < 0.001$ for all comparisons).

Only a few fungi were significantly associated with a single or several treatments, such as OTUs 393 (an unidentified Agaricales) and 523 (*Inocybe* sp.), which were significantly associated with the non-manipulated elongated roots (Supporting Information Table S1).

Fungal co-occurrence network structures differed among treatments. When ubiquitous fungi (fungi shared by all treatments) were excluded, the networks of the non-manipulated soil cores featured both a significantly higher node degree than networks of the sifted plants and sifted soil cores, as well as a higher clustering coefficient than the networks in the sifted soil cores (Kruskal-Wallis test, $p < 0.05$ for all comparisons, Fig. 3A and B), two parameters associated with network resistance to environmental disturbances (the sand core network was not included in these analyses due to the extreme poverty in edges). When all fungi were included, networks in the non-manipulated soil cores exhibited a lower edge betweenness than networks in the sifted plants, sifted soil and sand cores (Kruskal-Wallis test, $p < 0.05$ for all comparisons, Fig. 3C).

3.4. Comparison among the differently aged orchid roots

Overall, at time 0 fungal colonization of the old roots was less diverse (i.e. lower number of OTUs) than for the new roots (chi- squared tests, $p < 0.0001$), and differed significantly (PERMANOVA, p 0.0030). The fungal spectrum in the non-manipulated elongated roots at time 1 differed significantly (PERMANOVA, p 0.030) from the new roots (time 0), being less diverse (lower number of OTUs; chi-squared test, $p < 0.0001$). As compared to the old roots at time 0, the elongated roots featured a more diverse fungal colonization (i.e. a higher number of OTUs; chi-squared tests, $p < 0.0001$), and differed significantly (PERMANOVA, $p = 0.003$).

No OTU was retrieved in all age groups (Supporting Information Table S1). Several OM fungi occurring at time 0 in either old or new roots were not retrieved at time 1; most notably, the ceratobasidioid OTUs 14, 23 and 25, which occurred in all examined old roots, were not retrieved in any root examined at time 1 (Supporting Information Table S1).

In some plants (16 plants, four per treatment), both old and new roots at time 0 and elongated roots at time 1 were examined (Supporting Information Fig. S8, Fig. 2).

In these plants, some OTUs (e.g. the thelephoroid OTU 312 and the serendipitoid OTU 445) were retrieved from 75% elongated roots (including 75% plants in the sand cores), while they were not retrieved either from old or new roots (Kruskal-Wallis tests, $p < 0.05$). The ceratobasidioid OTU 26 was instead retrieved in all elongated and old roots, but not in new roots (Kruskal-Wallis tests, $p < 0.05$).

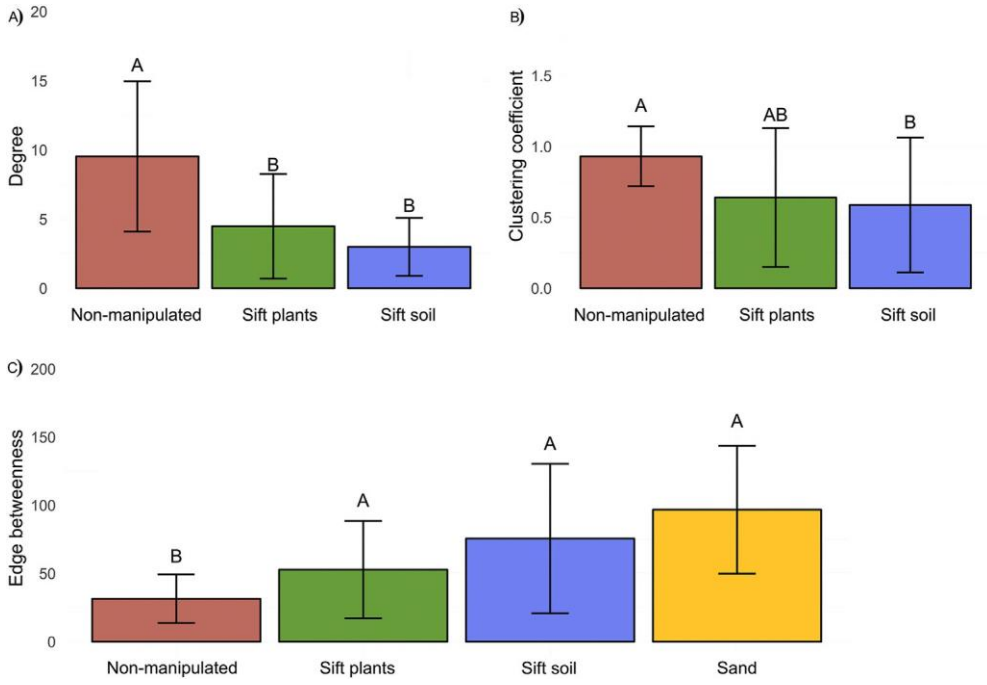

Fig. 3. Topological parameters of fungal co-occurrence networks. (A) Node Degree; (B) Clustering Coefficient; (C) Edge Betweenness. Networks in (A) and (B) were created excluding OTUs shared by all treatments (the sand core network was not included in these analyses due to extreme poverty in edges), while networks in (C) included all fungal OTUs.

Other OTUs, which were instead retrieved from all orchid root groups, exhibited nevertheless varying frequency in the differently aged root groups. *Tulasnella helicospora* (OTU 5) exhibited higher frequency in elongated than in new roots (Kruskal-Wallis test, $p < 0.05$). The tulasnelloid OTU 18, the ceratobasidioid OTU 151 and the sebacinoid OTU 423 were instead more frequent in both elongated and old than in new roots (Kruskal-Wallis tests, $p < 0.05$).

The same frequency patterns were observed for common non-OM OTUs (Supporting Information Fig. S9). For instance, OTU 7 (*Strobilurus* sp., Basidiomycota) was retrieved in 75% plants examined at time 1 (including 100% plants in the sand cores), while being undetected either in old or new roots (Kruskal-Wallis test, $p < 0.05$). The Ascomycota OTUs 316 (*Sordariomycetes* sp.), 476 (*Alternaria* sp.), 740 (*Pleosporales* sp.) and 812 (*Pleosporales* sp.) as well as the Basidiomycota OTU 362 (*Laetiporus* sp.) were instead more frequent in both elongated and old than in new roots (Kruskal-Wallis test, $p < 0.05$).

4. Discussion

In this study, a diverse spectrum of putative OM (tulasnelloid, ceratobasidioid, sebacinoid, serendipitoid, thelephoroid, inocyboid, russuloid) fungi was found to be associated with differently aged *Spiranthes spiralis* roots from plants in non-manipulated soil cores. As expected, OM fungal communities varied in the differently aged root groups (the number of fungal OTUs decreasing with increasing root age), and no single OM OTU was found in all age groups, consistently with the temporal dynamics of orchid fungal associations observed in previous studies (e.g. Ercole et al., 2015; Oja et al., 2015). In particular, *S. spiralis* new roots hosted only a few OM fungi, whereas tulasnelloid and sebacinoid fungi, and ceratobasidioid fungi were frequently retrieved in approx. 6 month-old roots ("elongated roots") and one year-old roots ("old roots"), respectively. Duffy et al. (2019) examined OM fungal diversity in 37 populations of the same orchid species, occurring in nine regions throughout Europe that represent the range of ecological conditions experienced by *S. spiralis*, across a 3000 km latitudinal gradient. Notably, the ceratobasidioid OTU 23 in our study (occurring only in the old roots) is most likely conspecific with OTU 7 in Duffy et al. (2019), which they found in all the sampled populations (Supporting Information Fig. S2), consistently with the age of the roots examined by the latter authors (corresponding to the old root condition in our study). It remains unclear whether, for *S. spiralis* as well as for other previously studied orchid species, the temporal shifts observed in OM root fungal partners are a consequence of intrinsic (changes in orchid nutritional demands across the life cycle) or extrinsic (seasonal variation in the colonization of orchid roots by environmental fungi) factors, or a combination of both (Cevallos et al., 2018).

As the newly emerged roots were almost uncolonized by OM fungi, we expected our manipulation experiment, aimed at eliminating possible environmental reservoirs of orchid root fungi, to impact on subsequent fungal colonization of *S. spiralis* roots.

Indeed, significant differences were found among treatments, such as a reduced fungal diversity in colonized roots in the sand treatment, which hosted the lowest number of fungal OTUs. The general root fungal co-occurrence network in the non-manipulated soil cores featured a lower edge betweenness than networks in the sifted plants and sifted soil cores, as well as a higher clustering coefficient than the sifted soil cores, two parameters which are interpreted in terms of high ecological resistance of the fungal community to perturbations.

As for single taxa, a specific frequency pattern could indicate colonization from the surrounding non-orchid plants. Such a pattern would be a significant higher frequency/occurrence in the elongated orchid roots in non-manipulated cores, concurrent with lower frequency/undetection in both the elongated roots of the manipulated soil cores (where contact between orchid roots and roots of the neighbouring, non-orchid plants had been disrupted) and the new roots at collection time (which had been exposed, for some time, to the soil environment). In our experiment, this was only the case for the non-OM OTU 393 (an unidentified Agaricales). By contrast, several fungi, including the ceratobasidioid OTUs 14, 23 and 25, occurred in all old roots while being undetected both in the new roots and in any root examined at time 1 (elongated roots). As old roots were withered and detached from the plant at time 1, the occurrence of these fungi in old roots (concurrent with undetection in new roots) is consistent with a status of late colonizers arriving from an external source (either soil or neighbouring non-orchid plants). Interestingly, the ceratobasidioid fungus OTU 14 had been previously amplified from soil of the study area (Voyron et al., 2017).

Other putative OM fungi (such as the ceratobasidioid OTUs 26 and 151, the sebacinoid OTU 423, the two tulasnelloid OTUs, the thelephoroid OTU 312) were instead detected frequently in the elongated roots in both the manipulated and non-manipulated soil cores, while being undetected/infrequent in the new roots, indi- cating

they are not early colonizers of orchid roots (i.e. they colonized after time 0). As these fungi were frequent in the sand cores, it is tempting to speculate an internal origin, i.e. they colonize the elongated roots either from old roots (previous year) (as it may be the case for the ceratobasidioid OTUs 26 and 151, and the tulasnelloid OTU 18, which were frequent in old roots) or other orchid tissues (e.g. the thelephoroid OTU 312 or OTU 5, *Tulasnella helicospora*, which were instead undetected or infrequent, respectively, in old roots), thus qualifying as orchid endophytes.

In this study, we did not test the surrounding soil or the roots of neighbouring non-orchid plants for the occurrence of *S. spiralis* OM fungi, and therefore further environmental reservoirs cannot be ruled out for these putative orchid endophytic fungi, which were frequent both in the sand cores and the other controls/treatments. However, the OM fungi exhibiting higher frequency in the sand cores (where orchid roots hosted depauperate fungal communities) than in other conditions, may exhibit poor competitive ability in rich fungal communities (such as the soil community). This would lend further credit to the hypothesis that OM fungi could use their host plants as a 'refuge' for survival and persistence in the environment (Selosse, 2014; Selosse and Martos, 2014; Oja et al., 2015), and that soil is not their main environmental reservoir (Voyron et al., 2017; Egidi et al., 2018). This implies that the mutualistic nature of the OM association is not necessarily restricted to a nutritional perspective. Interestingly, such a frequency pattern was found in all the main OM fungal groups (i.e. the ceratobasidioid OTUs 26 and 151, the tulasnelloid OTU 18, the serendipitoid OTU 445, the thelephoroid OTU 312), suggesting a convergent strategy in different OM taxa.

The idea of OM fungi as endophytes within the orchid host has implications for the interpretation of orchid distribution, providing a possible new explanation for the pattern of spatial clustering of seedlings and adults that has been observed in several European (Diez, 2007; Jacquemyn et al., 2007, 2012) and Australian (Batty et al., 2001) terrestrial orchid species. For either mycoheterotrophic (*Corallorhiza odontorhiza*; McCormick et al., 2009) and autotrophic, photosynthetic (*Galearis spectabilis*; McCormick et al., 2018) orchids, soil patches where OM fungi were abundant were small and closely tied to the locations of individual orchids (McCormick et al., 2018). Regardless of the nutritional strategy of the orchid, plots where orchid adult plants offer a 'refuge' to OM endophytes would be enriched in the latter and would therefore represent the most suitable sites for orchid seed germination and recruitment.

In conclusion, this study provides evidence that the removal of the neighbouring, non-orchid plants and soil impacts the root fungal community in *S. spiralis*. The OM fungal frequencies in differently aged roots in manipulated and non-manipulated soil cores suggest that some OM fungi may colonize mature orchid roots from either older roots or other parts of the same host plant, thus indicating an additional or alternative route to colonization. Future investigations should address the relative importance of different environmental reservoirs of OM fungi (soil, neighbouring non-orchid plants, orchid tissues), to assess whether these fungi spend most of their lifetime outside orchids or inside the host (in the latter case qualifying as "ecologically obligate" orchid symbionts). The possible occurrence of OM fungi in orchid tissues other than roots should also be explored.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2021.101071.

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