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Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae

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Abstract

Among European Neottieae, *Limodorum abortivum* is a common Mediterranean orchid. It forms small populations with a patchy distribution in woodlands, and is characterized by much reduced leaves, suggesting a partial mycoheterotrophy. We have investigated both the photosynthetic abilities of *L. abortivum* adult plants and the diversity of mycorrhizal fungi in *Limodorum* plants growing in different environments and plant communities (coniferous and broadleaf forests) over a wide geographical and altitudinal range. Despite the presence of photosynthetic pigments, CO_2 fixation was found to be insufficient to compensate for respiration in adult plants. Fungal diversity was assessed by morphological and molecular methods in *L. abortivum* as well as in the related rare species *Limodorum trabutianum* and *Limodorum brulloi*. Phylogenetic analyses of the fungal internal transcribed spacer (ITS) sequences, obtained from root samples of about 80 plants, revealed a tendency to associate predominantly with fungal symbionts of the genus *Russula*. Based on sequence similarities with known species, most root endophytes could be ascribed to the species complex encompassing *Russula delica*, *Russula chloroides*, and *Russula brevipes*. Few sequences clustered in separate groups nested within *Russula*, a genus of ectomycorrhizal fungi. The morphotypes of ectomycorrhizal root tips of surrounding trees yielded sequences similar or identical to those obtained from *L. abortivum*. These results demonstrate that *Limodorum* species with inefficient photosynthesis specifically associate with ectomycorrhizal fungi, and appear to have adopted a nutrition strategy similar to that known from achlorophyllous orchids.

Keywords: *Limodorum*, mixotrophy, mycoheterotrophy, orchid mycorrhiza, photosynthetic activity, *Russula*

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Introduction

Among plants, achlorophyllous species represent a fascinating example of nutritional adaptation because, by lacking photosynthetic pigments, they behave as heterotrophs and deploy alternative strategies to acquire organic carbon for growth (Leake 1994). The event of photosynthesis loss has occurred independently several times during plant evolution (dePamphilis 1995). Whereas some achlorophyllous plants are direct epiparasites on photosynthetic species, others acquire organic carbon through mycorrhizal association with fungal mycelia. Leake (1994) introduced the term 'mycoheterotrophy' to describe this peculiar strategy that relies on the ability of fungi to fetch organic compounds from the environment. This strategy arose repeatedly in angiosperm evolution, leading to about 400 mycoheterotrophic plant species (Leake 1994). Recent studies on the identity and diversity of mycorrhizal fungi associated with mycoheterotrophic

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plants belonging to distant taxa have outlined common features and provided key information on their nutritional strategies (see Taylor *et al*. 2002; Leake 2004).

Mycorrhizal symbionts of mycoheterotrophic plants are usually recalcitrant to isolation and growth in axenic culture, and their identification has been greatly aided by molecular methods. A common feature is an unusually high degree of specificity towards mycorrhizal symbionts, irrespective of their taxonomic position and mycorrhizal type. Exclusive associations with a single (or a narrow range of) fungal species have been reported for mycoheterotrophic angiosperms forming fungal associations as diverse as orchid, monotropoid and arbuscular mycorrhizae (Bidartondo *et al*. 2002; Selosse *et al*. 2002a; Taylor *et al*. 2002), as well as for liverworts (Bidartondo *et al*. 2003). Molecular techniques also revealed, as a general feature, that all mycoheterotrophic plants associate with fungi capable of forming mycorrhiza on surrounding autotrophic species.

Orchids form a very large and diverse plant family that comprises approximately 35% of the fully heterotrophic angiosperms (Leake 1994). Robust phylogenetic analyses (Cameron *et al*. 1999; Molvray *et al*. 2000; Bateman *et al*. 2005) revealed that loss of chlorophyll has occurred independently at least 20 times in Orchidaceae. Mycorrhizal fungal diversity has been studied for a few achlorophyllous species in this family. For example, *Cephalanthera austinae* and *Corallorhiza trifida* form mycorrhiza exclusively with fungi belonging to Thelephoraceae (Taylor & Bruns 1997; McKendrick *et al*. 2000a), while *Corallorhiza maculata* and *Corallorhiza mertensiana* associate uniquely with fungi in the Russulaceae (Taylor & Bruns 1999). By contrast, species such as *Neottia nidus-avis* in Europe (Selosse *et al*. 2002a) and *Hexalectris spicata* in North America (Taylor *et al*. 2003) are specialized toward fungi in the Sebacinaceae, which were only recently demonstrated to be ectomycorrhizal on tree species (Selosse *et al*. 2002b). Although most symbionts of mycoheterotrophic orchids are Basidiomycetes, Selosse *et al*. (2004) recently demonstrated that ectomycorrhizal Ascomycetes in the genus *Tuber* (Pezizales) can also associate with these plants. The same rule of specific associations with ectomycorrhizal fungi seems to apply to mycoheterotrophic angiosperms in a subfamily of Ericaceae (Monotropoideae) (Bidartondo & Bruns 2001, 2002) and to mycoheterotrophic liverworts in the Aneuraceae family (Bidartondo *et al*. 2003; Kottke *et al*. 2003).

Identification of the mycorrhizal fungal partners of mycoheterotrophs has prompted the question on the functional role of hyphal links between mycoheterotrophic plants and surrounding ectomycorrhizal trees: these links have been observed morphologically in some of these tripartite symbioses (Björkman 1960; Taylor *et al*. 2002; Bidartondo *et al*. 2003) or suggested by the fact that similar intraspecific polymorphism of fungal DNA occurred in mycoheterotrophic plants and surrounding ectomycorrhizae (Taylor & Bruns 1997; Selosse *et al*. 2002a). Radioisotope tracer studies have actually demonstrated a carbon flow from ectomycorrhizal autotrophic trees to the mycoheterotrophic plants. Severing the mycelium of the shared fungal partner dramatically reduced carbon transfer. Such evidence is available for the orchid *Corallorhiza trifida* (McKendrick *et al*. 2000b), for the monotropoideae *Monotropa hypopitys* (Björkman 1960), and for the liverwort *Cryptothallus mirabilis* (Bidartondo *et al*. 2003). Through the mycorrhizal fungal connection, mycoheterotrophic plants gain access to a large pool of organic carbon and are therefore sometimes considered as cheating parasites towards the fungus and the surrounding photoautotrophs. The significance of plant–fungus specificity in the evolution of this sophisticated nutritional strategy is far from clear, as discussed for example by Gardes (2002), Taylor *et al*. (2002), Leake (2004), and Taylor (2004).

Achlorophyllous plants are necessarily bound to heterotrophy, but also some chlorophyllous plants are likely to obtain part of their carbon heterotrophically and thus not via photosynthesis in their own leaves. Some photosynthetic forest orchids of the Neottieae tribe seem to acquire carbon from their mycorrhizal fungi as suggested by (i) their stable isotopic composition (Gebauer & Meyer 2003; Bidartondo *et al*. 2004; Julou *et al*. 2005) and (ii) the survival of achlorophyllous variants in natural populations (Selosse *et al*. 2004; Julou *et al*. 2005). The term 'mixotrophy' has been proposed by Selosse *et al*. (2004) to indicate this dual (photosynthetic and mycoheterotrophic) strategy, which comes along with the ectomycorrhizal association in the orchid genera *Cephalanthera* and *Epipactis* (Bidartondo *et al*. 2004; Selosse *et al*. 2004; Julou *et al*. 2005). So far, however, direct estimation of *in situ* photosynthesis in green orchids is limited to a single *Cephalanthera damasonium* population, where the orchids' photosynthesis ran near its compensation point due to light limitation in forest (Julou *et al*. 2005).

To further elucidate the strategies adopted by putatively mixotrophic orchids, we focused on *Limodorum abortivum* (L.) Swartz, a neottioid species common in the Mediterranean area (Rasmussen 1995). Contrasting data are available concerning its ability to carry out photosynthesis. The small scale-like leaves and the vivid violet colour of the stem of *L. abortivum* has led some authors (see Fitter & Fitter 1985; Flora Europaea 2001) to conclude that *L. abortivum* is achlorophyllous and therefore nonphotosynthetic. Indeed, fully subterranean cleistogamous flowering and fruiting has been described for this species (Bernard 1902; Rasmussen 1995; Selosse & Scappaticci, personal observation). Similarly, Gebauer & Meyer (2003) suggested it to be mycoheterotrophic based on its natural ^{13}C content. However, Blumenfeld (1935) detected chloroplast-containing cells and Griffon (1898) observed a low assimilation of carbon in the light.

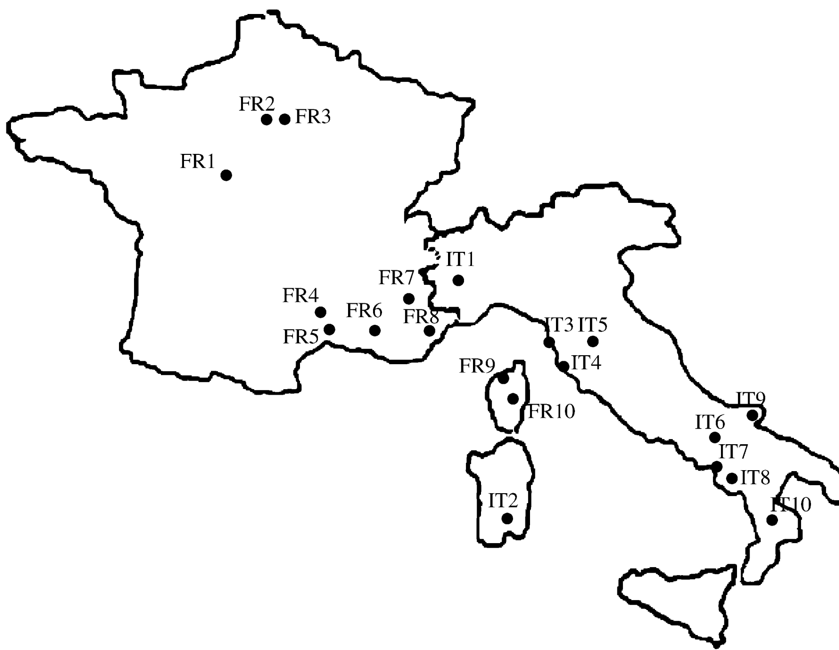

Fig. 1 Sampling sites for roots of *Limodorum* in France and Italy. Roots of the species *L. abortivum* were collected, except when indicated. FR1, Chinon; FR2, Boutigny; FR3, Bouville; FR4, St. Guilhem; FR5, Montpellier; FR6, Jouques; FR7, Gap; FR8, Esterel; FR9, Vivario; FR10, Corte; IT1, Torino; IT2, Cagliari (*L. abortivum* and *Limodorum trabutianum*); IT3, Livorno; IT4, Grosseto; IT5 Siena; IT6, Caserta; IT7, Napoli; IT8, Salerno; IT9, Foggia; IT10 Cosenza (only *Limodorum brulloi*).

To clarify the actual photosynthetic capabilities of *L. abortivum*, we measured the chlorophyll content of adult plants and their photosynthetic activities at different stages of the vegetative and flowering period, both under laboratory and field conditions. Furthermore, we investigated the small- and large-scale diversity of mycorrhizal fungi in *Limodorum* roots, sampled from 36 distinct orchid populations over a wide geographical area in Italy and France. Moreover, presence of shared mycorrhizal fungi was investigated on roots of the surrounding vegetation of *L. abortivum*.

Materials and methods

Study sites and sampling

About 80 samples of *Limodorum abortivum*, *Limodorum trabutianum* Battandier and *Limodorum brulloi* Bartolo & Pulvirenti were collected during early summer in the years 2001–2004. To account for the geographical distribution of fungal symbionts at the large scale, we sampled orchid individuals over a wide area in France and Italy (Fig. 1). Flowering adults were sampled randomly in areas of up to 1 km^2. Site IT10 was at the highest elevation (1420 m above sea level), sites FR9, FR10 and IT6 were at around 1000 m above sea level, whereas all other sites were at lower elevation or near the coast (Table 1). Root samples were collected from one or more individuals in each site, together with a soil core (maximum diameter, 30 cm, and depth, 40 cm).

Within some orchid populations, the distribution of fungal symbionts was investigated in more detail. We tested whether the pattern of occurrence of fungal symbionts was correlated with the type of dominant overstorey trees, using random collections from populations of either conifer- or broadleaf tree-dominated communities (Table 1). Root fragments were rinsed extensively with tap water and brushed gently to remove remaining soil debris. The samples were either processed immediately for fungal isolation and light microscopy, or frozen in liquid nitrogen and stored at –80 °C for subsequent molecular analysis.

Ectomycorrhizal tips of surrounding trees were harvested for a limited number of orchid samples (see Table 1) from the soil core containing *L. abortivum* root material. For the sites FR7, IT7A, IT7B, IT8A and IT8B ectomycorrhizal root tips and/or basidiomes of *Russula delica* and *Russula chloroides* were collected in autumn. All ectomycorrhizal root tips were sorted by morphology under a dissecting microscope, and specimens of each morphotype were frozen for molecular analysis. Basidiomes were identified morphologically based on Sarnari (1998).

Morphological observations

Root infection was evaluated by bright light microscopy. The features of plant–fungus association were further investigated by confocal laser microscopy after staining with wheat germ agglutinin (WGA), a lectin that binds fungal chitin, conjugated with the fluorochrome FITC. For electron microscopy, root samples were processed as described in Selosse *et al*. (2004). Briefly, they were fixed in 2.5% (v/v) glutaraldehyde in 10 mM phosphate buffer (pH 7.2) and post-fixed in 1% (w/v) OsO_4. After dehydration in an ethanol series, samples were infiltrated in LR

Table 1 Sampling sites

Country	Collection location	Code	Population	Altitude*	Dominant over storey trees	Orchid species	No. of plants sequenced	ECM sampling
France								
	Chinon	FR1		100	*Pinus sylvestris*, *Quercus petraea*	*L. abortivum*	2	no
	Boutigny	FR2		140	*P. sylvestris*	*L. abortivum*	3	no
	Bouville	FR3	A	70	*P. sylvestris*, *Q. petraea*	*L. abortivum*	1	no
	Bouville	FR3	B	70	*P. sylvestris*, *Q. petraea*	*L. abortivum*	2	yes
	St Guilhem	FR4	A	470	*P. halepensis*, *Q. pubescens*	*L. abortivum*	1	yes
	St Guilhem	FR4	B	560	*P. halepensis*, *Q. pubescens*	*L. abortivum*	1	no
	St Guilhem	FR4	C	340	*P. halepensis*, *Q. pubescens*	*L. abortivum*	1	no
	Montpellier	FR5	A	150	*P. halepensis*, *Q. pubescens*	*L. abortivum*	1	no
	Montpellier	FR5	B	150	*P. halepensis*	*L. abortivum*	1	no
	Montpellier	FR5	C	170	*Q. pubescens*, *Q. coccifera*	*L. abortivum*	7	yes
	Montpellier	FR5	D	200	*P. halepensis*	*L. abortivum*	6	yes
	Jouques	FR6		400	*P. halepensis*, *Q. ilex*	*L. abortivum*	1	no
	Salles-sous-Bois	FR7		370	*Q. ilex*, *Q. pubescens*	*L. abortivum*	3	no
	Esterel	FR8	A	500	*P. pinaster*	*L. abortivum*	1	no
	Esterel	FR8	B	600	*P. pinaster*	*L. abortivum*	1	no
	Esterel	FR8	C	500	*P. pinaster*	*L. abortivum*	1	no
	Corsica	FR9		1100	*P. pinaster*, *Q. ilex*	*L. abortivum*	2	no
	Corsica	FR10	A	1050	*P. pinaster*	*L. abortivum*	1	no
	Corsica	FR10	B	1050	*P. laricio*	*L. abortivum*	1	
Italy								
	Torino	IT1	A	520	*Q. pubescens*	*L. abortivum*	1	yes
	Torino	IT1	B	380	*Q. robur*	*L. abortivum*	3	yes
	Cagliari	IT2	A	150	*Pinus* sp.	*L. abortivum*	2	no
	Cagliari	IT2	B	300	*Q. ilex*	*L. trabutianum*	1	no
	Livorno	IT3		30	*P. pinea*, *Q. ilex*	*L. abortivum*	1	no
	Grosseto	IT4		50	*Q. ilex*	*L. abortivum*	1	no
	Siena	IT5	A	320	*Q. ilex*	*L. abortivum*	1	no
	Siena	IT5	B	520	*Castanea sativa*	*L. abortivum*	1	no
	Siena	IT5	C	440	*Q. cerris*, *Q. pubescens*	*L. abortivum*	1	no
	Siena	IT5	D	350	*Q. ilex*, *Q. pubescens*	*L. abortivum*	1	no
	Caserta	IT6		1000	*Q. ilex*	*L. abortivum*	1	no
	Napoli	IT7	A	50	*Q. ilex*	*L. abortivum*	6	yes
	Napoli	IT7	B	350	*P. pinaster*, *Q. ilex*	*L. abortivum*	5	yes
	Salerno	IT8	A	650	*P. nigra*	*L. abortivum*	6	yes
	Salerno	IT8	B	600	*C. sativa*	*L. abortivum*	6	yes
	Foggia	IT9		700	*Q. ilex*	*L. abortivum*	2	no
	Cosenza	IT10		1420	*P. laricio*	*L. brulloi*	1	no

*in metres above sea level.

White resin (Polysciences). Semi-thin sections (1 µm) were cut and stained with 1% toluidine blue for morphological observations.

Chlorophyll content and photosynthetic activity

Field samplings for *in vivo*, nondestructive measurements of gas exchange and chlorophyll fluorescence were carried out at the experimental sites IT7B and IT6 (Fig. 1) at the end of July, when the plants were flowering. A green portion of the orchid stem including scaly leaves was enclosed in a plastic cuvette allowing simultaneous measurements of CO_2 and H_2O exchange by infrared gas analysis (LI-COR 6400, LI-COR) and chlorophyll fluorescence (MiniPAM). This system allows control of O_2 and CO_2 concentrations, incident light intensity, air temperature and relative humidity inside the cuvette. A polyfurcated optic fibre was appressed to the stem to measure fluorescence under actinic light (0–1000 µmol photons m^{-2} s^{-1}) and under pulses of saturating light (> 10 000 µmol photons m^{-2} s^{-1}). This

allowed calculations of the photochemical yield, which estimates the quantum yield of photosystem II in dark-adapted and illuminated samples (Van Kooten & Snel 1990). The CO_2 release in the dark (equal to dark respiration) and the release or uptake in the light (the net balance between respiration, photorespiration and photosynthesis, Laisk & Loreto 1996) were measured under ambient air temperature (25 °C to 35 °C), and under ambient (400 µg/g) or enriched (700 µg/g) CO_2 levels to detect dependencies on these environmental variables and the possible effects of future environment on orchid physiology. Measurements were done in triplicate and are presented as average and standard errors. Alternatively, plants were transferred to the laboratory and the same measurements were carried out in a controlled environment.

After *in vivo* measurements, samples were cut and frozen in liquid nitrogen for chlorophyll determination. Samples of the superficial stem layer (2 cm^2) were gently removed and homogenized, and pigments were extracted with 3 mL of methanol (100%) for 4 h at 4 °C. Particulates were removed by centrifugation at 12 000 × *g* and 5 °C for 10 min, and the supernatant was removed and used for pigment determinations. Absorbance was measured at 470, 646.8 and 663.2 nm with a spectrophotometer (PerkinElmer). The extinction coefficients and the equations of Lichtenthaler (1987) were used.

Fungal isolation

Fungal isolation was attempted from all the Italian samples of *L. abortivum* (Table 1). One or two roots from each specimen were surface sterilized with 30% H_2O_2 (Carlo Erba) for 1 min and rinsed three times with sterile water. At least 15 root sections, obtained with a sterile blade, were plated onto malt extract agar (MA) and modified Melin-Norkrans agar (MMN) media, with or without chloramphenicol (50 mg/Kg). Petri dishes were incubated at room temperature for up to 3 months in order to allow the development of slow-growing mycelia.

DNA extraction and PCR amplification

Total DNA from frozen orchid root samples of about 1–2 cm length was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Henrion *et al*. 1992). Total DNA was extracted from ectomycorrhizal root tips using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions.

The fungal ITS sequence (encompassing the ITS1, 5.8S and ITS2 sequences) was amplified using the primers ITS1F and ITS4 (Gardes & Bruns 1993). Polymerase chain reaction (PCR) was carried out in a final volume of 50 µL, including 5 µL of DNA at the appropriate dilution, 30.5 µL of sterile water, 5 µL of 10 × buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 11 mM $MgCl_2$, 0.1% gelatin), 3 µL of each primer (0.6 µM ITS1F and ITS4), and 1.5 U of RED *Taq*TM DNA polymerase (Sigma). PCR amplifications were run in a PerkinElmer/Cetus DNA thermal cycler, using the following temperature profile: 95 °C for 5 min (1 cycle); 94 °C for 40 s, 55 °C for 45 s, 72 °C for 40 s (30 cycles); 72 °C for 7 min (1 cycle). To further confirm the absence of usual orchid symbionts with highly derived rDNA sequences, i.e. tulasnelloid and sebacinoid basidiomycetes, additional amplifications were carried out using the specific PCR primers ITS4-*tul* (Bidartondo *et al*. 2003) and ITS3S (Selosse *et al*. 2004), respectively. PCR products were separated on 1% agarose gel and purified with the QIAEX II Gel Extraction Kit (QIAGEN) following the manufacturer's instructions.

Cloning and ITS-RFLP analysis

The purified ITS fragments were either sequenced directly (most French samples), or cloned into pGEM-T (Promega) vectors (all Italian samples). XL-2 Blue ultracompetent cells (Stratagene) were transformed following the manufacturer's instructions. After transformation, white colonies randomly taken for each orchid sample were transferred to a fresh LB plate and the bacterial cells lysed at 95 °C for 10 min. Plasmid inserts were amplified using the ITS1F and ITS4 primers under the following conditions: 94 °C for 5 min (1 cycle); 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min (25 cycles); 72 °C for 7 min (1 cycle). The ITS fragments amplified from 20 to 100 clones per plant were digested with the restriction enzymes *Alu*I and *Hha*I and fragments separated on 1.6% agarose gels for restriction fragment length polymorphism (RFLP) profiling.

DNA sequencing and sequence analysis

Purified PCR amplicons were sequenced using the primer pair used for amplification. Cloned ITS inserts of extracted plasmids (Plasmid Purification Kit, QIAGEN), representative of the different RFLP types, were sequenced using the forward and reverse M13 plasmid primers. Dye sequencing was performed on a 310 ABI DNA Sequencer (Applied Biosystems).

Sequences were edited and assembled using SEQUENCHER 4.1 for MacOS9 and sequence identity determined using the BLASTN algorithm available through the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/BLAST/index.html). The sequences were aligned using CLUSTAL_X (Thompson *et al*. 1997), adjusted manually in GENEDOC (Nicholas *et al*. 1997) and used for neighbour-joining analyses in PAUP 4.0. About 480 bp of the ITS region were considered in the alignment because the 3′ end of ITS2 was too variable among species to be aligned with confidence.

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Table 2 Chlorophyll content, photochemical yield as assessed by chlorophyll fluorescence, and exchange of CO_2 of darkened (20 min) and illuminated stems of *Limodorum abortivum*

Treatment	Chlorophyll content (μg cm^{-2})	Fv/Fm (arbitrary units)	CO_2 exchange (μmol CO_2 m^{-2} s^{-1})
Darkened stems	34.8 ± 2.4a	0.55 ± 0.7a	−0.9 ± 0.3a
Illuminated stems			
400 μmol photons m^{-2} s^{-1} 25 °C, 400 μg/g CO_2	35.3 ± 1.8a	0.25 ± 0.4b	−0.2 ± 0.2b
400 μmol photons m^{-2} s^{-1} 25 °C, 700 μg/g CO_2	36.6 ± 2.7a	0.33 ± 0.7b	0.5 ± 0.3c
400 μmol photons m^{-2} s^{-1} 35 °C, 400 μg/g CO_2	33.9 ± 3.1a	0.24 ± 0.5b	2.2 ± 0.7d

Negative values of CO_2 exchange indicate emitted CO_2, while positive values indicate uptake of CO_2. In the dark, CO_2 emission is attributed to respiration. In the light, CO_2 uptake is net photosynthesis (the balance between photosynthesis, photorespiration and respiration). CO_2 exchange of illuminated stems was assayed under the temperature and CO_2 conditions recorded at the experimental site (first row), and under increasing CO_2 concentration and temperature (last two rows, respectively). The light intensity was set to a level saturating net photosynthesis and similar to the growth light intensity (250–400 μmol photons m^{-2} s^{-1} during field measurements). Means ± standard errors (n = 3) are shown. Means statistically different within the same column ($P > 0.05$, Tukey's test) are followed by different letters.

Results

Photosynthesis in Limodorum abortivum

Biochemical analyses demonstrate that *Limodorum abortivum*, in contrast to mycoheterotrophic orchids such as *Neottia nidus-avis*, does produce and accumulate chlorophyll in both stems and leaves (Table 2). We also demonstrate that chlorophyll-dependent photochemical processes are active in *L. abortivum,* and in particular that photosystem II is competent to carry on linear electron transport, although the low value of the ratio between variable and maximal fluorescence in dark-adapted leaves (Fv/Fm) as compared to leaf levels (van Kooten & Snel 1990) indicates a relatively inefficient use of the light and low quantum yield of photosystem II.

The actual capability of *L. abortivum* stems and leaves to fix CO_2 through photosynthesis during vegetative growth and flowering was assessed in field experiments. The results show a reduced CO_2 production when leaves are illuminated with moderate light (Table 2). This may be due to the inhibition of dark respiration in the light but it may also be due to the onset of photochemical CO_2 fixation by photosynthesis. When CO_2 concentration was artificially elevated to 700 μg/g, a small CO_2 uptake (the positive value of Table 2 denotes CO_2 fixation) could even be observed, which may be consequent to a decrease of photorespiration. Consistent with this interpretation, the quantum yield of photosystem II in illuminated stems was not significantly affected at raising CO_2. This indicates that the electron transport rate requested to drive photosynthesis is balanced by the suppression of photorespiratory-dependent electron transport (Laisk & Loreto 1996). The uptake of CO_2 was particularly high also when monitored at elevated air temperature (Table 2). Leaf sections did not exhibit any Kranz anatomy (not shown).

Morphology of Limodorum *mycorrhizal roots and fungal isolation*

Plants were sampled over a wide geographical area in France and Italy (Fig. 1), in woodlands either dominated by conifers species (*Pinus halepensis*, *Pinus laricio*, *Pinus nigra*, *Pinus pinaster*, *Pinus pinea*, *Pinus sylvestris*) or broadleaf trees (*Castanea sativa*, *Quercus cerris*, *Quercus coccifera*, *Quercus ilex*, *Quercus petraea*, *Quercus pubescens*, *Quercus robur*) (Table 1).

Limodorum abortivum and its close relatives *Limodorum trabutianum* and *Limodorum brulloi* display a rhizome densely covered with thick roots. The cortical cells of plants collected in the different environments were heavily colonized by fungal hyphae forming dense intracellular coils (Fig. 2). Starch was abundant in the uncolonized root cortical cells, but completely absent in cells colonized by the mycorrhizal fungi (Fig. 2). A dominant hyphal morphology was usually observed in the root sections, most pelotons being formed by narrow unclamped hyaline hyphae (2.5–5.5 μm diameter). Much wider septate and unclamped hyphae (8–14 μm diameter) were sporadically observed (Fig. 2), but they were consistently found in samples collected from sites IT1B and IT2B.

Attempts to isolate *in vitro* the mycorrhizal *Limodorum* endophytes were in most cases unsuccessful. However, all plants collected in sites IT1B (*L. abortivum*) and IT2B (*L. trabutianum*) yielded fungal mycelia that could be assigned by morphological criteria to the form-genus *Rhizoctonia*. This finding agrees with our above-mentioned morphological observations, which recorded abundant intracellular coils formed by large septate hyphae (Fig. 2). In the IT1B samples, a small proportion of ITS sequences (about 6%) amplified from *L. abortivum* root samples could be actually identified as *Rhizoctonia* (*Ceratobasidiales*, accession no. DQ061931).

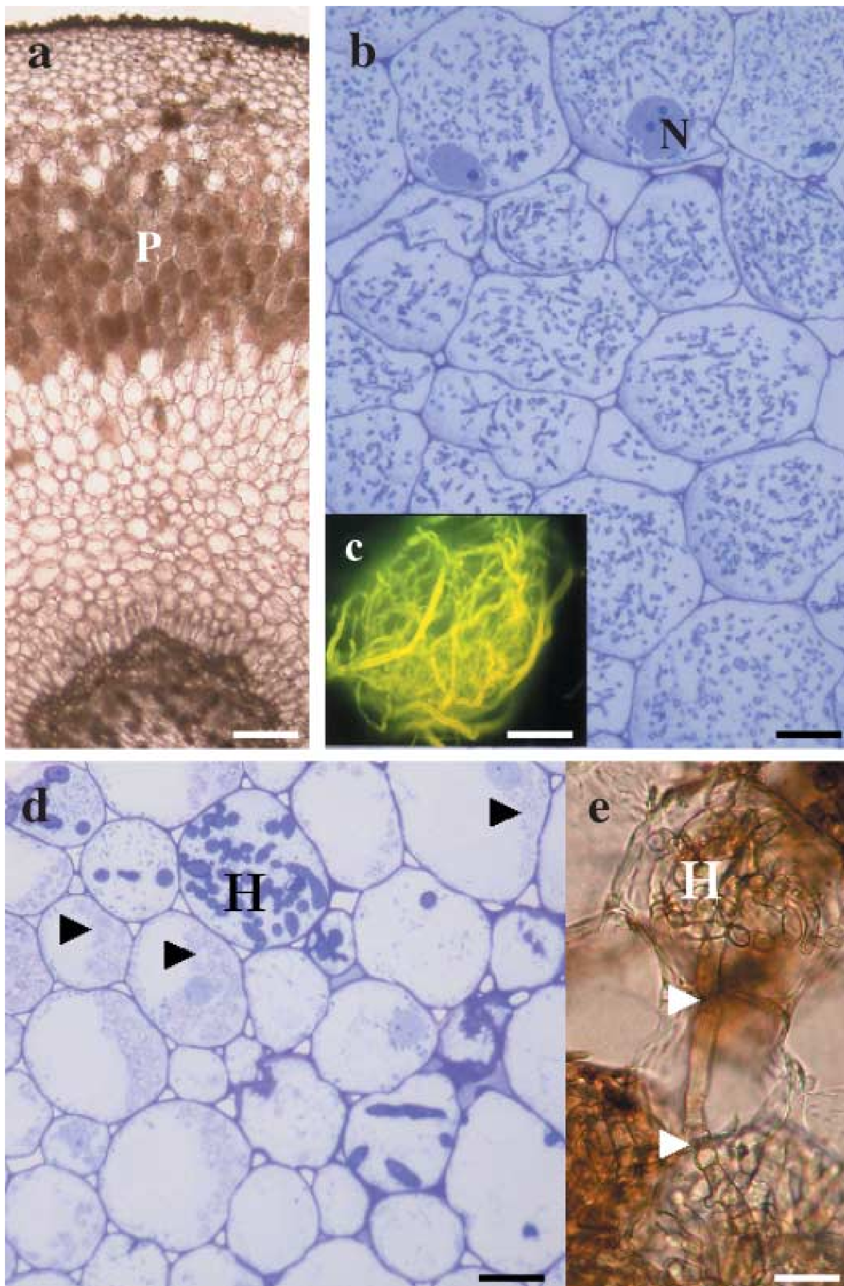

Fig. 2 Morphological features of mycorrhizal *Limodorum abortivum* roots. (a) Cross-section of a mycorrhizal root showing fungal colonization in the outer cortical parenchyma (P). Bar, 380 µm. (b) Semi-thin section from a fixed and resin embedded root, showing intense colonization by thin fungal hyphae in cells of the root parenchyma. N, plant nucleus. Bar, 65 µm. (c) A single fungal peloton visualized by immunofluorescent staining (FITC-labelled wheat germ agglutinin). Bar, 45 µm. (d) Semi-thin section from a fixed and resin embedded root sampled at site IT1B, showing colonization by thick fungal hyphae (H). Cells colonized by the fungal hyphae are deprived of starch, otherwise abundant in the surrounding cells (arrowheads). Bar, 80 µm. (e) Pigmented thick hyphae of fungal pelotons in fresh root material (H). Transcellular hyphae are indicated by arrowheads. Bar, 55 µm.

Ribosomal gene sequence analysis of Limodorum *fungal associates*

Given the poor success in the isolation of mycorrhizal fungi from *Limodorum* roots, a molecular approach was applied to assess fungal diversity in these orchids. Sequences produced from amplicons of total root DNA, using the fungal specific primer pair ITSF/ITS4, were used as queries in BLAST searches. Irrespective of the site of origin, the sequences obtained from *L. abortivum*, *L. trabutianum* and *L. brulloi* identified the dominant fungal symbiont as belonging to the Russulaceae (Table 3). For about 80% of the plant samples, the closest match was either with *Russula delica* or *Russula chloroides*, two species with very high ITS sequence similarities that formed a single cluster by neighbour-joining analysis (Fig. 3). The remaining sequences matched most closely to other GenBank accession sequences of *Russula* species. For two *L. abortivum* plants from Sardinia (IT2A-3, accession no. DQ061928; IT2A-5, accession no. DQ061929), the amplified fungal sequences yielded closest matches to accessions of *Macowanites vinaceodorus*, and for one plant (out of six investigated) from site IT8B (accession no. DQ061930), the closest match was *Gymnomyces fallax*. Both fungal species are gasteroid

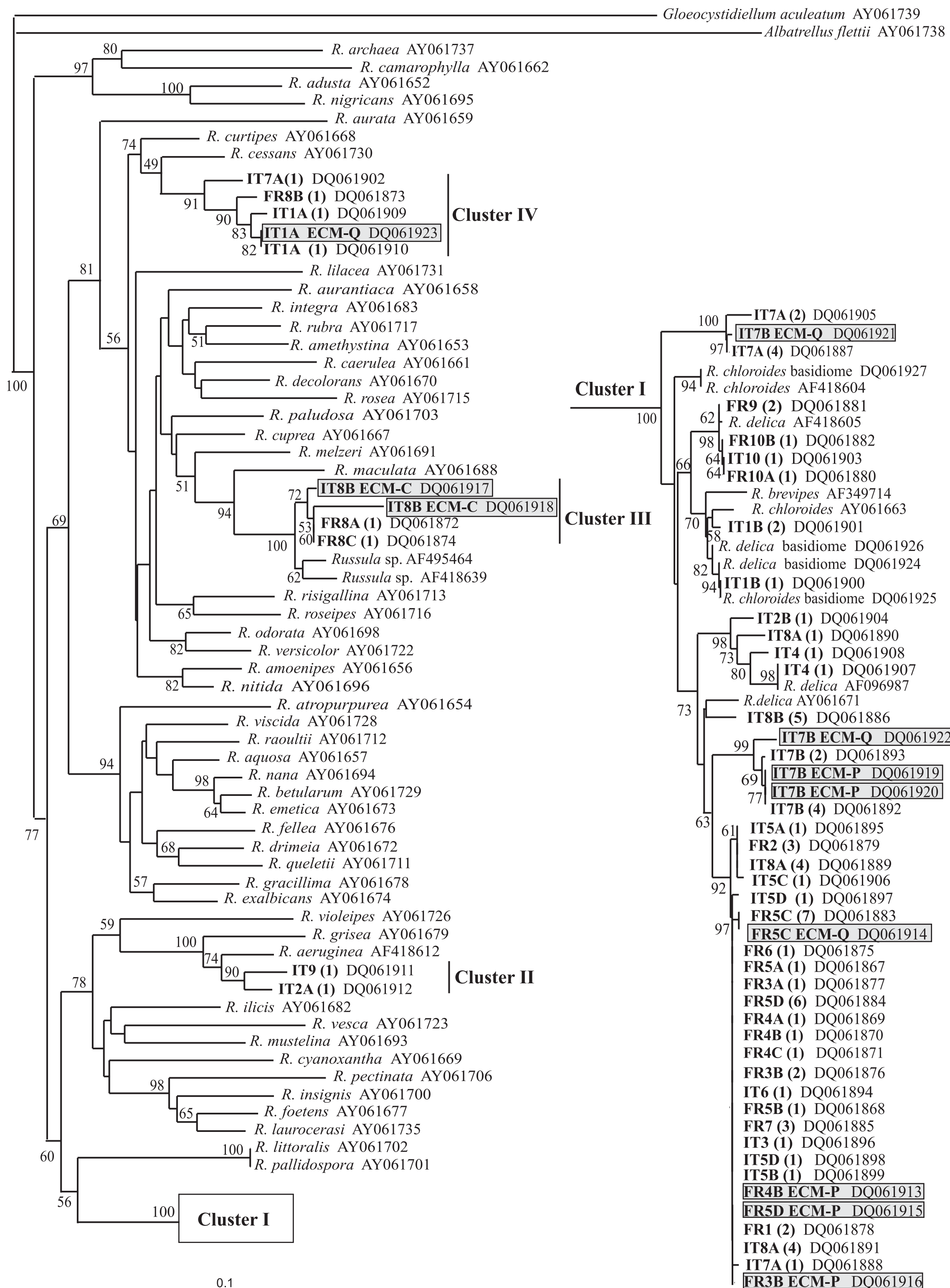

Fig. 3 Rooted neighbour-joining tree obtained from ITS sequences of fungal root symbionts of *Limodorum*. GenBank sequences of reference species were chosen to represent all subgroups of *Russula* according to Miller & Buyck (2002). Kimura 2-parameter distances were used. Bootstrap support ≥ 50% is indicated (1000 replicates). *Albatrellus flettii* and *Gleocystidiellum aculeatum* were used as outgroup. For site codes refer to Table 1. The number of individuals sharing the same ITS sequence within each population is indicated in parenthesis. In some cases, more than one sequence were identified in the same root sample. In boxes, DNA sequences from ectomycorrhizal tips (ECM) of surrounding trees are encoded by site code and plant genus (Q, *Quercus*; P, *Pinus*; C, *Castanea*).

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Table 3 Fungal root endophytes in *Limodorum*

		Limodorum plants
Fungal groups		
Basidiomycota	*Russula delica/R. chloroides*	67 (7)
	Other *Russula* species	7
	Macowanites	2
	Gymnomyces	1
	Rhizoctonia	0 (3)
Ascomycota	*Tuber*	0 (2)
	Chaetothyriales	0 (2)

Fungal groups represent closest-related taxa from NCBI BLAST results. Each row lists the corresponding numbers of plants associated with each fungal group. Numbers of plants yielding multiple fungi are listed in parentheses. Only fungal groups detected at a frequency > 10% (as ITS clones) in at least one *Limodorum* plant were considered.

taxa grouping within *Russula* clades in molecular phylogeny of the Russulales based on LSU rDNA (Miller *et al*. 2001). PCRs using primers pairs specifically designed to detect tulasnelloid and sebacinoid basidiomycetes failed to detect any of these fungi in *L. abortivum*.

A neighbour-joining tree revealed the phylogenetic relationship of *Limodorum* symbionts within the genus *Russula* (Fig. 3). The great majority, as already indicated by the BLAST search, fall in a single cluster (Cluster I) including GenBank sequences from three known species: *R. delica, R. chloroides* and *Russula brevipes* and sequences from basidiomes growing in the same areas and identified morphologically as either *R. delica* or *R. chloroides*. Sequences forming three additional clusters within the genus *Russula* were found two to three times, each. Noteworthy, Cluster IV included sequences from three distant geographical areas.

Intrapopulational symbiont diversity

In parallel to a random sampling over a wide geographical area, fungal diversity in *Limodorum* plants was investigated at finer scale, within individuals and populations, in distinct environments. Four populations of *L. abortivum* in southern Italy and one in southern France were selected because they grew within ectomycorrhizal plant communities dominated by either conifers or broadleaf trees. In particular, three populations (FR5C, IT7A, IT8B) were growing under a canopy of broadleaf trees (*Quercus pubescens*, *Q. ilex* and *Castanea sativa*, respectively), whereas the other two (IT7B, IT8A) were growing in sites dominated by *Pinus pinaster* and *P. nigra*, respectively (Table 1).

To investigate fungal diversity within these selected populations, a cloning step of the amplified fungal ITS and an RFLP analysis of the individual clones (20–100 for each plant) was routinely used for each investigated root fragment. Figure 4 reports, for four to six plants belonging to these different populations, the percentage of clones with distinct RFLP profiles. Although the frequency of RFLP types may not reflect strictly their relative abundance in the mycorrhizal roots, the results clearly show that a largely dominant RFLP type was amplified from all five *L. abortivum* populations, irrespective of the dominant tree species. The corresponding sequences matched *R. delica* and/or *R. chloroides* in GenBank. In populations IT7A and IT8B, both growing under broadleaf trees, unique dominant RFLP profiles were found, which correspond to ITS sequences matching other species (Cluster IV in Fig. 3 and *Gymnomyces* sp., respectively).

When considering individual plants, commonly all RFLP profiles were identical, suggesting the presence of a single or dominant fungal symbiont (Fig. 4). Only for 7 out of the 27 plants investigated, a small proportion of clones had an RFLP profile different from the dominant type. In the plant sample IT8A-12 (associated with *P. nigra*), the less abundant RFLP profile (10% of clones) still corresponded to *R. delica/R. chloroides*. By contrast, the minor RFLP types identified in samples 3, 5 and 6 from the IT7A population (associated with *Q. ilex*) gave high sequence similarities with ascomycetous fungi, namely *Tuber melanosporum* (6% of clones), *Epicoccum nigrum* (1% of clones) and *Fusarium oxysporum* (10% of clones). In population FR5C, an unknown *Chaetothyriales* accounted for a limited number of clones in two individuals (12% and 3% of clones, respectively), whereas up to 30% of clones of a single individual found as nearest match a *Tuber* sequence obtained from *Epipactis* roots (Bidartondo *et al*. 2004). This was the only plant where we could identify such a high percentage of non-*Russula* sequences, but, as already mentioned, abundances after cloning may not exactly reflect their abundances in roots.

Molecular analysis of ectomycorrhizal roots

Because members of Russulaceae all form ectomycorrhizae (ECM), an obvious question was whether ECM plants surrounding *L. abortivum* shared the same mycobionts. ECM root tips formed by the surrounding tree species were therefore collected in some of the orchid sampling sites. Fungal sequences obtained from ectomycorrhizal root tips often showed highest similarities to *Russula* species also found in *Limodorum*.

Additional *Russula* species were identified exclusively as symbionts of ectomycorrhizal trees, not associated with *Limodorum* plants (data not shown). An abundant ECM morphotype in sites IT7A and IT8A was formed by *Sebacina* species, known to associate symbiotically with the mycoheterotrophic orchid *Neottia nidus-avis* (Selosse *et al*. 2002a). However, this sequence was never amplified from *Limodorum* roots in these sites.

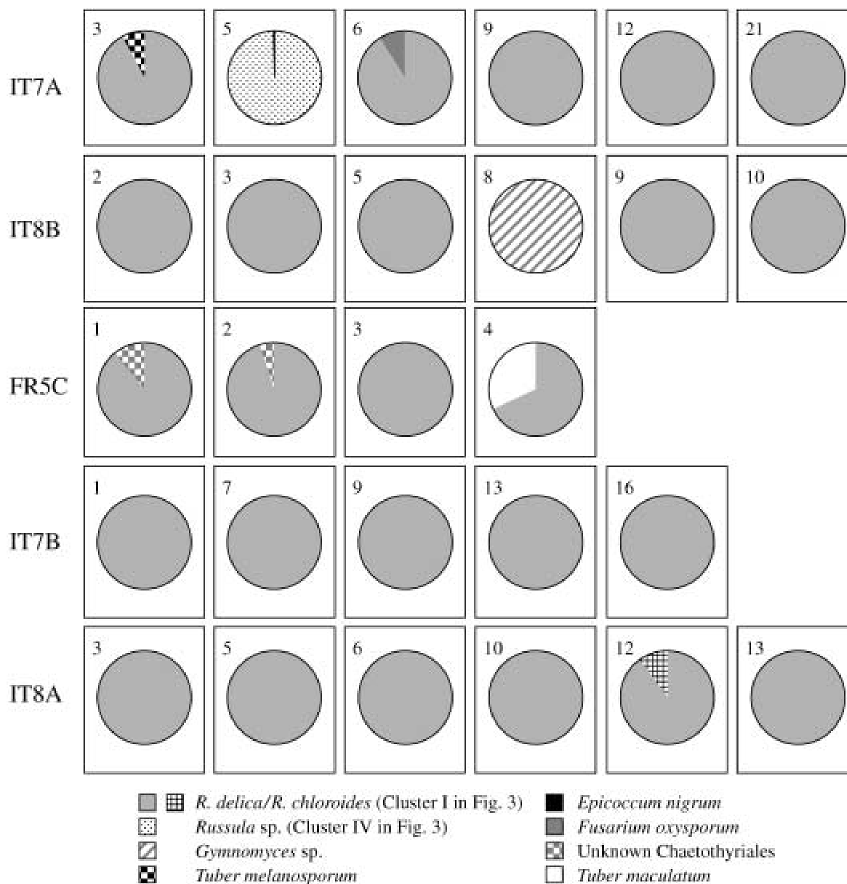

Fig. 4 Fine-scale analysis of fungal symbionts in *Limodorum abortivum* associated with different ectomycorrhizal trees. Frequency of restriction fragment length polymorphism (RFLP) profiles per plant obtained by restriction digesting 20–100 ITS inserts with *Alu*I and *Hha*I. The sites IT7A, IT8B and FR5C were stands of broadleaf trees (*Quercus ilex*, *Castanea sativa* and *Quercus* spp., respectively) and the sites IT7B and IT8A were stands of conifers (*Pinus pinaster* and *Pinus nigra*, respectively). Shading patterns indicate the ITS-sequence-based species affiliations of RFLP types, as identified by BLAST (*E*-values 0.0).

The sequences from ECM root tips were aligned together with those obtained for *L. abortivum* mycorrhizal endophytes, and only the *Russula* sequences falling in clusters comprising *L. abortivum* endophytes are shown (Fig. 3, boxed). Although sampling was limited, the results indicate that orchid roots and ectomycorrhiza collected in the same soil core usually harbour fungi with identical (FR3B and FR5C) or similar (IT7B and IT1A) ITS sequences (percentage of similarity equal or higher than 92.5%). In rare cases, there were high similarities between ECM and *L. abortivum* mycobionts, which had a distant origin. For example, in Cluster III in Fig. 3, sequences from ECM root tips collected from *Castanea sativa* in southern Italy (IT8B) were found to cluster with sequences from *L. abortivum* roots collected in southern France (FR8A and FR8C). This may be due to the limited number of samples investigated.

Discussion

The CO_2 budget of Limodorum abortivum

The results demonstrate that synthesis of chlorophyll and photosynthesis do occur in *Limodorum abortivum*, although the photosynthetic efficiency is very low in field conditions as indicated by the net loss of CO_2 even under light conditions that saturated photosynthetic CO_2 uptake. The sensitivity of CO_2 uptake to CO_2 concentration suggests that *L. abortivum* carbon fixation is carried out by a C3 mechanism, as C4 plants suppress photorespiration and do not increase photosynthesis in response to elevated CO_2. Congruently, leaf sections do not exhibit any Kranz anatomy.

Under present field conditions, photosynthesis does not compensate for the respiratory processes causing CO_2 release in the atmosphere. This biochemical evidence for a functional photosynthesis in both leaves and stems of *L. abortivum* agrees with the presence of stomata (M. A. Selosse, personal observation), which are usually absent in wholly mycoheterotrophic species (Leake 1994). An ongoing analysis of plastid genome organization within the Neottieae provides further support to the observation that photosynthetic abilities are retained in *L. abortivum*. No difference in gene order or the sequences of photosynthetic genes were found between *Limodorum* and the close relative and fully autotrophic *Listera ovata*. On the contrary, numerous indels were detected in the same plastid regions in the nonphotosynthetic *Neottia* (Cafasso *et al*. in prep.).

Thus, *L. abortivum* is not fully heterotrophic, contradicting some published expectations (e.g. Gebauer & Meyer 2003), but it undoubtedly requires an additional carbon source. Unlike *Cephalanthera damasonium*, a related fully autotrophic neottioid species that has normal photosynthetic abilities but is limited by *in situ* light availability (Julou *et al*. 2005), *L. abortivum* photosynthesis was found to be inefficient also under saturating light conditions.

Mycorrhizal symbionts of Limodorum

Identity and diversity of mycorrhizal fungi associated with *L. abortivum* fit well with the insufficient photosynthesis of this plant species, and demonstrate that *L. abortivum* shares the same fungal preferences as wholly mycoheterotrophic species. All roots of our analysed plants were colonized by ectomycorrhizal fungi related to the genus *Russula*. Some root tips from neighbouring trees showed ectomycorrhizal morphotypes which yielded sequences of the same *Russula* species after PCR amplification. This finding supports earlier speculation that *L. abortivum* may share common fungal symbionts with trees in the near surrounding (Wood 1993). Molecular markers more sensitive to genetic polymorphisms are required to assess whether the same fungal individual is actually shared between orchid and ectomycorrhizal plants (Selosse *et al*. 2002a).

Sequences of the dominant symbionts of *L. abortivum* across Italy and France, occurring in almost 80% of plant samples, clustered in the *Russula delica*/*Russula chloroides*/*Russula brevipes* species complex. These three species belong to the same section *Lactarioides* (Miller & Buyck 2002), and their ITS sequences do not show sufficient divergence to identify separate clusters. Thus, it remains to resolve whether the *Limodorum* symbionts belong to a single or to a group of related species. Other more variable DNA regions may be more helpful for this purpose; that is, however, beyond the scope of this work.

Occasionally, other fungal partners colonized the root cortical cells in these orchids. *Rhizoctonia* was identified by isolation in a limited number of samples, and accounted for the two morphologically distinct pelotons observed in root sections (Fig. 2). Although only sporadically found, multiple colonization deserves further attention because it may have been underestimated by the limited sampling in most sites. However, it was found consistently within some of the orchid populations (e.g. IT1B and IT2B) and was already reported by Riess & Scrugli (1987). Coexistence of *Rhizoctonia* with ectomycorrhizal partners was already described in the mycoheterotrophic orchid *Hexalectris spicata* (Taylor *et al*. 2003) and in the mixotrophic *Epipactis microphylla* (Selosse *et al*. 2004). The rare and sporadic association of *Limodorum* with *Tuber* species also needs to be further investigated, as this ectomycorrhizal Ascomycete has been found to be a symbiont of other orchids from the Neottieae tribe (Bidartondo *et al*. 2004; Selosse *et al*. 2004; Julou *et al*. 2005). Some fungal sequences found during the molecular analysis may reflect fungal contaminants from the root surface or nonmycorrhizal root endophytes (e.g. *Fusarium, Chaetothyriales*) in the *Limodorum* samples.

The only other orchids reported to be specifically associated with fungi of the genus *Russula* are *Corallorhiza maculata* and *Corallorhiza mertensiana*, two mycoheterotrophic species widely distributed in North America (Taylor & Bruns 1997, 1999; Taylor *et al*. 2004). Based on ITS-RFLP profiles and sequences, it was estimated that *C. maculata* associates with 22 different *Russula* species (Taylor & Bruns 1999; Taylor *et al*. 2004). This number is well above the six or seven mycorrhizal fungal species identified in our study for *L. abortivum*, despite a similar number of plants and plant populations sampled. A neighbour-joining tree constructed with sequence data from our own work and from Taylor *et al*. (2004) shows that only one symbiont of *C. maculata* (named type O) clustered together with the *Limodorum* symbionts, namely in the *R. delica*/*R. chloroides*/*R. brevipes* species complex (data not shown). However, type O was found only rarely in *C. maculata* roots, thus suggesting that these two orchids have either specialized on different fungal species, or that these fungi occur at different abundance in the two geographical areas. No sequence data are available for the fungal associates of *C. mertensiana* (Taylor & Bruns 1999).

Our analysis on the level of *L. abortivum* populations showed that individual plants usually associated with symbionts from a single, dominant phylogenetic *Russula* cluster (Cluster I). A few *L. abortivum* were usually colonized by a different fungal symbiont (see populations IT7A and IT8B, Fig. 4). In the IT7A population, the alternative mycobiont was still a *Russula* species, whereas in the IT8B population the alternative mycobiont was *Gymnomyces*, a gasteroid genus phylogenetically deriving from *Russula* (Miller *et al*. 2001). Similar situations were found in populations IT1, IT2A and IT9 where, despite the low number of plants tested, individuals were found to associate with distinct mycobionts (Fig. 3).

Such associations with alternative symbiotic partners may be explained by within host plant genotypic variation in specificity, as it was reported for *C. maculata* (Taylor *et al*. 2004). In *C. maculata* different plant genotypes rarely shared the same *Russula* species, despite co-occurrence of both symbiotic partners.

Effects of ecological and geographical factors on fungal partners preference in Limodorum

Limited data are so far available on the influence that habitats impose on the specificity of mycoheterotrophic hosts. Taylor & Bruns (1999) showed that forest composition strongly correlates with the occurrence of particular fungal

species as orchid symbionts, as there was no overlap in *Russula* species between *C. maculata* populations in conifer and oak forests. There was also a perfect association between fungal species composition and elevation, a pattern that may also be related to forest composition. A very different situation was apparent for *L. abortivum*, despite the limited sampling of individuals in most populations. Plants associated with the dominant *R. delica*/*R. chloroides*/*R. brevipes* fungal symbionts throughout the area, irrespective of the dominant tree species and the elevation. In Europe, *R. delica* and *R. chloroides* are ubiquitous species and associate, like *Limodorum*, with both conifers and broadleaf trees (Romagnesi 1985; Sarnari 1998).

Some investigations have shown that mycoheterotrophic plants maintain tight specificity also when surrounded by numerous fungal species, contrary to the hypothesis that specificity may be simply due to an absence of alternative symbionts. For example, individuals of *C. maculata* sampled in an area of several hundred square metres were associated with a single *Russula* species which was never found fruiting on the plot, while mushrooms of six other *Russula* species were collected throughout the plot (Taylor & Bruns 1999). Similarly several ectomycorrhizal morphotypes were identified near *Limodorum* roots, some formed by other *Russula* species as well as by fungal symbionts of other orchid species (e.g. *Sebacina*; Selosse *et al*. 2002a).

Mycorrhizal specificity in photosynthetic orchids

Specificity in orchid mycorrhiza has been controversial for many years (Taylor *et al*. 2002) and is still contentious for green orchids. In earlier studies based on *in vitro* determination of the range of fungi which support seed germination, Hadley (1970) indicated low specificity towards a range of *Rhizoctonia* species. The bias towards culturable fungi and poor taxonomic resolution of the form-genus *Rhizoctonia* by means of vegetative morphology have sustained this view, although field studies demonstrated variable degrees of specificity in terrestrial (Taylor *et al*. 2002; Ma *et al*. 2003) as well as epiphytic orchids (Otero *et al*. 2002).

By contrast, patterns of specificity appear to be much clearer for fully mycoheterotrophic plants. This might be due to enhanced resolution of the molecular tools applied to identify the symbionts in this group of plants. Although it remains to be elucidated whether mycobionts of mycoheterotrophic plants are simply exploited by the heterotrophic plant, or whether they obtain any benefit from the association (e.g. see Bidartondo *et al*. 2000), the high specificity of mycoheterotrophic species towards their fungal partners has been related to the evolution of a specific nutritional strategy as cheating parasites (Taylor & Bruns 1997; Taylor 2004). Parasitism tends to favour specificity by selection for resistance and evolutionary arms-races (Taylor & Bruns 1997; Taylor 2004). These arguments would imply that fully mycoheterotrophic orchids are more specific than photoautotrophic orchids, and that the degree of specificity is correlated with the degree of heterotrophy. Heterotrophy may depend either on peculiar life strategies, or on ecological conditions. For instance, some green orchids show prolonged stages of adult dormancy. Adult dormancy consists of periods of one or more years where no sprouts are produced and no photosynthesis occurs. A recent study on the genus *Cypripedium*, which exhibits this phenomenon, indicates that mycorrhizal specificity towards Tulasnellaceae is fairly high (Shefferson *et al*. 2005).

Green forest orchids, which likely experience reduced photosynthetic efficiency in a shaded environment, may also represent an excellent system to study the evolution of mycoheterotrophy (Bidartondo *et al*. 2004; Selosse *et al*. 2004), and to investigate whether the degree of specificity to a given mycobiont may mirror the degree of heterotrophy and nutritional dependency for carbon. Here, we document that the mixotrophic species *L. abortivum* is highly specific and conclude that this might be explained by a strong dependence on fungal carbohydrates.

However, other recent studies have reported contrasting data concerning specificity in green forest orchids (Bidartondo *et al*. 2004; McCormick *et al*. 2004; Julou *et al*. 2005). For example, high specificity was observed by McCormick *et al*. (2004) in *Goodyera pubescens* and *Liparis lilifolia*, as all fungi isolated belonged to the single genus *Tulasnella*. Since *L. lilifolia* grows on the dusky floors of dense forests and produces only one or two green leaves in spring, it may depend heavily on carbon supplied by the fungus (McCormick *et al*. 2004), as does *L. abortivum*. Similarly, specific associations with ectomycorrhizal fungal taxa were found for other mixotrophic forest Neottieae, such as *Cephalanthera* and *Epipactis* species, although other green forest orchids may associate with a much broader range of mycobionts. For example, adult plants of *Tipularia discolor* were found to associate with at least four distinctly different groups of tulasnelloid fungi (McCormick *et al*. 2004), and *C. damasonium* was reported to harbour very diverse mycobionts (Bidartondo *et al*. 2004; Julou *et al*. 2005). The amount of carbon obtained from fungi was estimated for *C. damasonium* by variation in stable isotope abundance, but the range was quite wide, from *c*. 85% (Gebauer & Meyer 2003) to 30–50% (Bidartondo *et al*. 2004; Julou *et al*. 2005). These data suggest a strong influence of the environment on the relative contribution of autotrophic photosynthesis and heterotrophism to the carbon metabolism of mixotrophic orchids, and may at least in part explain the contrasting results in mycobiont specificity. *L. abortivum* is to our knowledge the only green forest orchid for which photosynthetic abilities have been measured under field

conditions and found to be limiting irrespective of the local conditions. The degree of heterotrophy of other 'putatively photosynthetic' orchids remains to be established in future studies.

Conclusions

An insufficient photosynthesis and a predominant association with ectomycorrhizal fungi of the genus *Russula* in *Limodorum abortivum* and its close relatives *Limodorum trabutianum* and *Limodorum brulloi* confirmed their suspected mycoheterotrophy. The finding of a functional, although not sufficiently effective, photosynthesis lets us conclude that *L. abortivum* is a partially mycoheterotrophic (= mixotrophic) plant. Leake *et al*. (2004) have provided strong evidence that the distribution of a single mycorrhizal fungus can forcefully constrain the establishment and resulting distribution of a mycoheterotrophic angiosperm (*Monotropa hypopitys*). Our findings suggest that distribution of *Limodorum* may also be potentially constrained by the occurrence of its fungal symbionts, which connect the orchid plants to ectomycorrrhizal trees for the acquisition of carbohydrates. Knowledge of this mycorrhizal strategy has important implications for the conservation and management of these threatened plants.

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The work reported here is the result of a collaborative study on the mycorrhizal stategies of orchids in the genus *Limodorum*. At the University of Turin, Mariangela Girlanda and Silvia Perotto share a long standing interest in the genetic and functional diversity of mycorrhizal fungi. Stefano Ghignone is interested in bioinformatics and sequence analyses. Raffaella Fabbian was an undergraduate student at the time this study was conducted, and part of the work is Rossana Segreto's PhD research subject. At the University of Montpellier II, Marc-André Selosse is interested in ecology and evolution of temperate mycorrhizal associations, and the work is part of his programme on mixotrophic orchids and their fungal associates. At the University of Naples, Salvatore Cozzolino and Donata Cafasso employ molecular markers to understand evolutionary factors that shape patterns of genetic and phenotypic variation in natural orchid populations. At the University of Molise, Sebastiano Delfine and Paola Pinelli are crop ecophysiologists focusing on the impact of environmental stress on plant functions. At the CNR in Rome, Federico Brilli and Francesco Loreto are plant physiologists studying biosphere-atmosphere interactions and the impact of global change on plants.