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Nutritional regulation in mixotrophic plants: new insights from *Limodorum abortivum*

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Abstract Partially mycoheterotrophic (mixotrophic) plants gain carbon from both photosynthesis and their mycorrhizal fungi. This is considered an ancestral state in the evolution of full mycoheterotrophy, but little is known about this nutrition, and especially about the physiological balance between photosynthesis and fungal C gain. To investigate possible compensation between photosynthesis and mycoheterotrophy in the Mediterranean mixotrophic orchid *Limodorum abortivum*, fungal colonization was experimentally reduced in situ by fungicide treatment. We measured photosynthetic pigments of leaves, stems, and ovaries, as well as the stable C isotope compositions (a proxy for photosynthetic C gain) of seeds and the sizes of ovaries and seeds. We demonstrate that (1) in natural conditions, photosynthetic pigments are most concentrated in ovaries; (2) pigments and photosynthetic C increase in ovaries when fungal C supply is impaired, buffering C limitations and allowing the same development of ovaries and seeds as in natural conditions; and (3) responses to light of pigment and ^{13}C contents in ovaries shift from null responses in natural conditions to responses typical of autotrophic plants in treated *L. abortivum*, demonstrating photoadaptation and enhanced use of light in the latter. *L. abortivum* thus preferentially feeds on fungi in natural conditions, but employs compensatory photosynthesis to buffer fungal C limitations and allow seed development.

Keywords Mycoheterotrophy · Evolution · Photosynthetic pigments · $\delta^{13}C$ · Orchids

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Alessandro Bellino began this research, then Daniela Baldantoni worked alongside him in every phase of this project, from devising the experimental plan to the samplings, analyses, and interpretation of data. Marc-André Selosse proposed additional experiments and measurements, and carried out some of them in his lab. Rossella Guerrieri and Marco Borghetti performed the $\delta^{13}C$ measurements. Anna Alfani coordinated the work. All contributed to writing the paper.

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Introduction

Mycoheterotrophic (MH) plants obtain organic carbon from their mycorrhizal symbionts, which allow C transfer from surrounding autotrophic plants via common mycorrhizal networks (Leake 1994, 2004; Selosse and Cameron 2010) or, for some tropical orchids associated with saprobic fungi, from soil organic matter (Martos et al. 2009; Hynson

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et al. 2013). Mycoheterotrophy evolved independently in many plant lineages, including liverworts, lycopods, ferns, and angiosperms, and represents a common strategy for overcoming the competition for light (Bidartondo 2005; Merckx and Freudenstein 2010). Several relevant features of many MH plants have already been clarified, such as the identity of fungal species involved in mycorrhiza formation, their organic C sources, and some aspects of their physiology and development (Hynson et al. 2013). It was discovered that MH species often evolved within clades of green, partially mycoheterotrophic (PMH) plants, i.e., mixotrophic plants that cumulate photosynthetic C gain with MH nutrition (Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Selosse and Roy 2009; Selosse and Cameron 2010), indicating that PMH nutrition is an intermediate phase in the evolution of MH nutrition (Tedersoo et al. 2007; Selosse and Roy 2009; Hynson et al. 2013).

The shifts to full MH nutrition, however, seem to be rare, and PMH nutrition was proposed to be an evolutionary metastable trait (Selosse and Roy 2009; Roy et al. 2013). The processes and evolutionary paths that led from PMH to MH plants are currently unknown. Studies on the PMH orchid *Cephalanthera damasonium* highlighted that this transition is unlikely to have resulted from a simple loss of photosynthesis, as indicated by the lower fitness of achlorophyllous variants, but requires the joint and progressive evolution of many physiological and morphological traits (Julou et al. 2005; Roy et al. 2013). Clarification of the evolutionary path to MH nutrition and of the metastability of PMH nutrition could arise from deeper investigation of the eco-physiology of PMH species, and especially of the relationship between photosynthesis and mycoheterotrophy.

The results of a few studies investigating plants in different light conditions (Preiss et al. 2010; Matsuda et al. 2012) have suggested that PMH plants in the light incorporate more photosynthetic C in their biomass than those in the shade do, indicating a possible compensation between the two C gains. This was assessed by measuring the natural stable C isotope composition ($^{13}C/^{12}C$), since C arising from photosynthesis is depleted in ^{13}C as compared to fungal C (Selosse and Roy 2009; Hynson et al. 2013). In PMH species, it is possible that either mycoheterotrophy compensates for photosynthesis, which is usually limited by the light conditions in the shade environments colonized, or that photosynthesis compensates for mycoheterotrophy. The two processes, moreover, could be expressed at different stages of the continuum of adaptations from near full autotrophy to near full mycoheterotrophy exhibited by PMH species. In orchids at least, the survival of non-chlorophyllous individuals, although with a lower fitness, shows that fungal C has a limited ability to compensate for photosynthesis (Julou et al. 2005; Abadie et al. 2006; Selosse and Roy 2009; Stöckel et al. 2011). Conversely, the possibility that photosynthesis compensates for fungal C is indirectly suggested by the increase in photosynthetic efficiency and photosynthetic C in the shoots of *C. damasonium* during ovary ripening, when fungi are rare in roots (Roy et al. 2013). However, such compensation has never been experimentally investigated. A compensatory photosynthesis in PMH plants is particularly interesting since it would indicate a preference for fungal C nutrition and an underexpression of photosynthetic capabilities in natural conditions, which would be used as a bet-hedging resource. The establishment of photosynthesis as a secondary C source in PMH species relying mostly on fungal C would have straightforward evolutionary implications. In environmental conditions favoring mycorrhizal association and fungal C supply (such as shadiness and wetness), the balance between the two C gains could constantly tip toward mycoheterotrophy, making photosynthesis less and less required. Such conditions could favor the evolution of MH plants by limiting the impacts on fitness of mutations affecting photosynthetic performance, and allowing successful transitions to MH nutrition. Conversely, PMH nutrition could be preserved in more variable environments, where compensatory photosynthesis could be required to buffer fungal C limitations.

In order to investigate the potential for compensatory photosynthesis in PMH plants, we studied in situ, for 2 years, the effects of limited access to fungal C on the photochemical apparatus and the photosynthetic C gain of the Mediterranean PMH orchid *Limodorum abortivum* (L.) Swartz. We expected that these traits would be enhanced in response to reduced access to fungal C, highlighting a contrario their downregulation in natural conditions. Due to its nutritional physiology and phylogenetic position, this species is a particularly useful model for our experiments. First, the photosynthetic rate of *L. abortivum*, measured on stem and scaly leaves, is below the compensation point in full light (Girlanda et al. 2006), making it easier to detect even small changes in photosynthetic pigments and C gain using ^{13}C content as a proxy (Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Selosse and Roy 2009). Second, it is phylogenetically close to the well-studied *Cephalanthera* and *Epipactis* genera (within the *Neottieae* tribe). Specifically, in the first year, we focused on the changes in photosynthetic pigments of leaves, stems, and ovaries; then, in the second year, on the changes in seed ^{13}C content, following fungicide-induced reductions of mycorrhizal colonization. It was hypothesized (Montfort and Küsters 1940) that photosynthesis in *Corallorhiza trifida*, a PMH orchid relying mostly on fungal C, like *L. abortivum* (Zimmer et al. 2008; Cameron et al. 2009), could be preferentially involved in reproduction. To investigate this possibility, we performed the analyses separately on the epigeous organs, expecting a major contribution of ovaries

to the photosynthetic capabilities of *L. abortivum*. This species does indeed have green ovaries, and photosynthesis in such organs may contribute greatly to the C requirements of reproductive structures (see Aschan and Pfanz 2003 for a review). Secondly, in order to further clarify the physiological responses to light of *L. abortivum* following reduced access to fungal C, we took into account the microenvironmental light levels to which plants were exposed in the field. These conditions can influence both photosynthetic pigment production and C isotopic discrimination during photosynthesis (Lichtenthaler et al. 2007a; Farquhar et al. 1989). Autotrophic plants in the shade usually have lower $^{13}C/^{12}C$ ratios than such plants in the light, due to higher isotopic discrimination (Ehleringer et al. 1986). Conversely, PMH plants relying mostly on fungal C are expected to show at most an increase in ^{13}C content in the shade, where enhanced fungal C exploitation may occur. If the photosynthetic activity of *L. abortivum* is enhanced to compensate for reduced mycorrhizal colonization, we therefore expect a shift from responses to light typical of PMH plants to responses typical of autotrophic plants.

Materials and methods

Study area, treatments, and samplings

We studied an Italian population of *Limodorum abortivum* (L.) Swartz (*Neottieae*, *Orchidaceae*) from a thermophile forest of *Pinus halepensis* Miller on the west coast of Campania (40°19′N, 14°56′E, 100 m a.s.l.), monitored for 2 years (2007 and 2008). The pine stand has an uneven spatial distribution (see Online Resource 1 of the Electronic supplementary material, ESM) and age, determining a variable light environment. This is unusual for most Mediterranean PMH orchids, which are commonly found in less variable, darker, and wetter areas (Rasmussen 1995).

To reduce mycorrhizal fungi, and inhibit the translocation of fungal C to orchids, benomyl (Benlate®, Du Pont de Nemours S.p.A., Cavenago di Brianza, Italy), a systemic fungicide, and iprodione (Rovral®, Bayer S.p.A., Milan, Italy), a cytotropic fungicide, both of which are nonphytotoxic, were used during the first and second years, respectively. The use of a different fungicide during the second year was imposed by the lack of a sufficient benomyl stock (it was commercially unavailable at the time). The approach was to reduce the mycoheterotrophic C gain of the treated plants while maintaining the same environmental conditions.

The populations during the 2 years of the study were 43 and 114 specimens, respectively; all of which were considered for the study. Due to high mortality, particularly in the second year, mainly due to accidental (wild boar and cow activities, picking by tourists) factors, the specimens available for the analyses (indicated below; the distribution of them is shown in Online Resource 1 of the ESM) were greatly reduced. Treatments started when the flowering stem had completely developed, but before anthesis, when the ovaries were still covered by floral bracts.

For pigment analyses, 15 randomly chosen specimens were treated during the first year with benomyl, which was applied on two occasions separated by an interval of 7 days, at a dose of 40 mg per plant (using a 0.4 g L^{-1} solution) by injection into the soil at a depth of 10 cm in five holes around each stem. In parallel, 18 specimens were irrigated with the same amount of water and kept as controls. The same methodology and time steps were used during the second year for isotopic analyses, when 21 specimens (different from those treated in the previous year) were treated with iprodione at a dose of 60 mg per plant (using a 0.6 g L^{-1} solution) and 21 specimens were kept as controls. In addition, to evaluate possible C limitations and changes in the reproductive fitness of iprodione-treated plants, ovary length and diameter (related to seed number, Roy et al. 2013) were measured with a vernier calliper on two ovaries per plant 10 days after the last treatment. Moreover, seed sizes were measured on a total of 713 and 717 seeds for control and treated plants, respectively, via image analysis of photographs taken with a Photometrics CoolSnap K4 camera (Roper Scientific, AZ, Tucson, USA), analyzed with the ImageJ 1.44o software (Wayne Rasband, National Institutes of Health, USA).

In both years, mean daily irradiance (MDI) was calculated as the mean of 12 measurements, one per hour starting at sunrise, carried out near each plant on a sunny day during the flowering period with a LI-250A (LI-COR, Lincoln, NE, USA). This experimental setting was chosen due to the short (1-month) epigeous life cycle of *L. abortivum*, the need to obtain relative differences in the light environment among the specimens, and the difficulties involved in performing the measurements.

Pigment analyses

The effects of fungal C shortening on the photochemical apparatus were studied during the first year by comparing the compositions and contents of photosynthetic pigments in fungicide-treated and control plants. The compositions and the relative amounts of photosynthetic pigments were separately analyzed in leaves, including bracts associated with flowers (thereafter collectively referred to as "leaves"), stems, and ovaries, which were sampled 10 days after the last treatment and stored at −80 °C until processing. Plant material was pulverized in liquid nitrogen, and pigments were extracted in absolute acetone for 4 h at 4 °C and then centrifuged at 5,000 rpm for 20 min. Pigment composition

was evaluated in five treated and five control plants by high-performance liquid chromatography (HPLC) separation on a Luna C18(2) 250 × 4.6 5 µm analytical column (Phenomenex, Torrance, CA, USA) with a Summit chromatography system (Dionex Corporation, Titan Way Sunnyvale, CA, USA), according to the protocol of Brotas and Plante-Cuny 2003. Peak identification was based on retention time and diode-array absorption spectra in the range 300–750 nm. Quantification of most of the identified pigments was achieved by Gauss Peak Spectra analysis (Küpper et al. 2007) of the absorption spectra of the extracts, recorded in the range 350–750 nm with a Lambda EZ201 (PerkinElmer, Waltham, MA, USA) dual-beam spectrophotometer. To ensure that iprodione treatment elicited the same responses in *L. abortivum* as benomyl, the same analyses were carried out on six individuals in the subsequent year.

Isotopic analyses

The photosynthetic C gain was studied during the second year by measuring the stable C isotope composition. Seeds from treated and control plants were collected just before dispersion (20 days after the last treatment) from three capsules per plant, pooled, and air-dried until processing. The $^{13}C/^{12}C$ ratio resulting from photosynthetic C (depleted in ^{13}C) and mycoheterotrophic C (enriched in ^{13}C) was measured in seed biomass using a Delta Plus (Finnigan, Bremen, Germany) mass spectrometer connected to a NC2500 (Carlo Erba, Milan, Italy) elemental analyzer. The C isotopic signature was expressed in the δ notation as the relative deviation from the international standard Vienna PeeDee Belemnite (V-PDB). To obtain a reference for the nearby autotrophic plants, ten leaves from five individual plants of *Erica arborea* L., *Phillyrea latifolia* L., *Cistus monspeliensis* L., *Pistacia lentiscus* L., *Pinus halepensis* Miller, *Mirtus communis* L., *Prasium majus* L., and *Olea europaea* L., collected at about the same distance from the soil as the *L. abortivum* fruits, and up to 1.5 m above them for *P. halepensis*, were analyzed. In the same seeds, the C/N ratio—another proxy for the degree of mycoheterotrophy (Abadie et al. 2006)—was calculated from C and N concentrations measured by a Flash EA 1112 CHNS-O analyzer (Thermo Scientific, Waltham, MA, USA).

Effectiveness of fungicides

To test the assumption of reduced mycorrhizal colonization in treated plants, the effectiveness of each of the two fungicides against the fungi colonizing *L. abortivum* roots was later evaluated by (1) in vitro tests on *Russula* ssp., (2) soil respiration measurements, and (3) root mycorrhizal colonization analyses.

In vitro toxicological tests were performed on four species (GenBank accessions KJ482563–KJ482566) of *Russula*, congeneric of the usual *L. abortivum* symbiont (*R. delica*, Girlanda et al. 2006), that were kindly provided by Prof. K. Nara (University of Tokyo). Since we were not able to cultivate *R. delica*, the phylogenetic positions of the four *Russula* species were analyzed in order to evaluate the representativeness of their responses to benomyl and iprodione for other congeneric species. The four *Russula* species were cultivated on modified Melin–Norkrans (MMN) medium (Heinonen-Tanski and Holopainen 1991) with 10 g L^{-1} glucose and 1.3 % agar at 20 °C and in darkness. One control and two dilutions for each fungicide were tested: 0.80 and 0.08 g L^{-1} for benomyl and 1.20 and 0.12 g L^{-1} for iprodione. The dilutions were chosen in order to evaluate the toxicological effects of the fungicides at the cumulative concentrations used in the field and at the expected dilutions in the soil, considering soil volume and porosity. The expected dilution (d) was calculated according to

$$d = \frac{V_i}{V_s \cdot p_t} \qquad with \qquad p_t = \left(1 - \frac{\rho_b}{\rho_s}\right),$$

where V_i is the volume of the fungicide solution injected in the soil (100 mL), V_s is the soil volume (3.14 dm^3), calculated considering a cylinder with $h = 10$ cm and $r = 10$ cm, i.e., twice the distance of each hole from the stem, and p_t is the total soil porosity (~0.33). p_t was calculated from ρ_b and ρ_s, i.e., the bulk density and the effective density of the soil, respectively, measured on three samples taken near *L. abortivum* plants. Such calculations indicate a dilution factor of ~10 %. The fungicides were dissolved in the medium before inoculation, and two replicates per treatment were performed. The growth of each species was evaluated as the measured area of the colonies two weeks after inoculation. DNA was extracted and amplified as in Selosse et al. (2002) to barcode by ITS sequencing (1) the four *Russula* species used for toxicological tests and (2) three root pieces per *L. abortivum* individual treated with benomyl ($n = 7$) or control ($n = 7$), using a 4 mm^3 piece from each root. Edited sequences were deposited in GenBank (accessions KJ482560–KJ482562).

Soil CO_2 evolution was measured in the field near ten benomyl-treated and ten control plants with an EGM-4 (PP Systems, Amesbury, MA, USA) portable CO_2 analyzer before the first treatment and every day for two weeks after it.

For mycorrhizal colonization analyses, eight plants were treated with benomyl, five were treated with iprodione, and ten were kept as controls. The treatments were carried out in the field on plants different from those treated in the previous years. Mycorrhizal colonization was estimated on two roots for each plant. Roots were collected 10 days after the last treatment, carefully washed in tap water, and fixed in Zirkle's solution (Ruzin 1999). Two transverse freehand

sections per root were taken at the end of the elongation zone (~1.5 cm from the apex), briefly cleared in 10 % KOH, bleached in 2 % HCl, and then observed using dark field microscopy at 40× magnification with a Dialux 20 (Leitz, Wetzlar, Germany) microscope. Two photographs per root were taken with a Photometrics CoolSnap K4 camera (Roper Scientific, Tucson, AZ, USA), analyzed with the ImageJ 1.44o software (Wayne Rasband, National Institutes of Health, USA) and classified into two groups for each treatment, based on the presence of developed or, conversely, collapsed pelotons. In order to perform a very conservative estimation, root sections with developed pelotons in an area of >10 % were classified as containing mycorrhizas unaffected by the fungicide, while all the others were considered to have been affected by the fungicide.

Data analyses

The phylogenetic analysis was performed using the sequences from the four *Russula* species, three sequences from control *L. abortivum* roots, and the sequences from Eberhardt 2002 using the PhyML software package (Guindon et al. 2010) and the R package "phangorn" (Schliep 2011). The tree was built using a generalized time reversible (GTR) substitution model, estimating the equilibrium frequencies and the gamma distribution (with 16 discrete classes). Tree topology was optimized using subtree prune and re-graft (SPR) moves with five random start trees, and branch support was based on the approximate maximum likelihood test described in Anisimova and Gascuel (2006) and Anisimova et al. (2011).

One-way ANCOVA, with treatment as the fixed factor and time as the covariate, was performed to check for differences in soil CO_2 evolution, whereas logistic regression was performed to check for differences in the proportion of roots showing structured/collapsed pelotons. Two-way ANOVA with the treatment and the species as fixed factors was performed in order to check for differences in colony area among the treatments for each cultivated *Russula* species.

Pigment concentrations were subjected to one-way ANOVA with the post hoc test of Tukey HSD (normality was assessed by Jarque–Bera and D'Agostino–Pearson tests, homoscedasticity by the Breusch–Pagan/Cook–Weisberg test) to check for differences among the organs of the control plants. Then, canonical variates analysis (Podani 2007), Hotelling's T^2 test, and a *t* test were applied to check for differences in pigment concentration between treated and control plants. Pearson correlations, considering the normal distribution of the data, were performed to analyze the relationship between pigment concentration and MDI. One-way ANCOVA, with treatment as the fixed factor and MDI as the covariate, was carried out to check for differences in $\delta^{13}C$ and C/N ratio between treated and control plants. Coefficient of variation ($CV = \frac{\sigma}{\mu} \times 100$, where σ and μ are the standard deviation and the mean, respectively) was calculated to evaluate the variations in $\delta^{13}C$ for treated and control *L. abortivum*.

Differences in length and diameter of the ovaries from treated and control *L. abortivum* were studied using *t* tests, according to the normality of the data, whereas differences in seed testa size were studied using a linear mixed model with the treatment as the fixed factor and the plant as the random factor.

The χ^2 test was used to check for differences in PCR success rate and for the frequency of *Russula* DNA sequences in the roots from control and treated *L. abortivum*.

In all tests, the confidence level was set to $\alpha = 0.05$ unless otherwise specified, in order to produce more conservative results. Statistical analyses were performed using the R 2.15.0 programming language (R Core Team 2013), including the stats, nortest, lmtest, ICSNP, and candisc packages.

Results

Sensitivities of the *Russula* species to fungicides

The four tested *Russula* species formed three clades (see Online Resource 2 of the ESM) distributed within the genus *Russula*. All of the species grew well for 2 weeks on control plates (67.04 ± 15.06 mm^2), but did not grow on plates containing benomyl, and formed smaller colonies on plates containing iprodione (3.04 ± 1.22 mm^2 at 1.2 g L^{-1} and 43.46 ± 11.42 mm^2 at 0.12 g L^{-1}). The differences between each treatment and the control were all significant ($P < 0.001$).

Soil CO_2 near benomyl-treated *L. abortivum* was significantly ($P < 0.001$) reduced (~58 % within 4 days) as compared to that measured near control plants.

The proportion of root sections showing more than 90 % collapsed pelotons was significantly ($P < 0.001$) higher in both benomyl-treated (88 %) and iprodione-treated (100 %) plants with respect to the controls (0 %; see Online Resource 3 of the ESM). All fungal ITS sequences from *L. abortivum* roots (GenBank accessions KJ482560–KJ482562) clustered in the *R. delica* clade (see Online Resource 2 of the ESM). Treated roots showed a lower PCR success rate than control roots (63 versus 73%) and a lower *Russula* frequency (37 versus 46 %), but none of these differences was significant.

Pigment content

The pigment pattern in the three organs of control *L. abortivum* encompassed nine major carotenoids and six

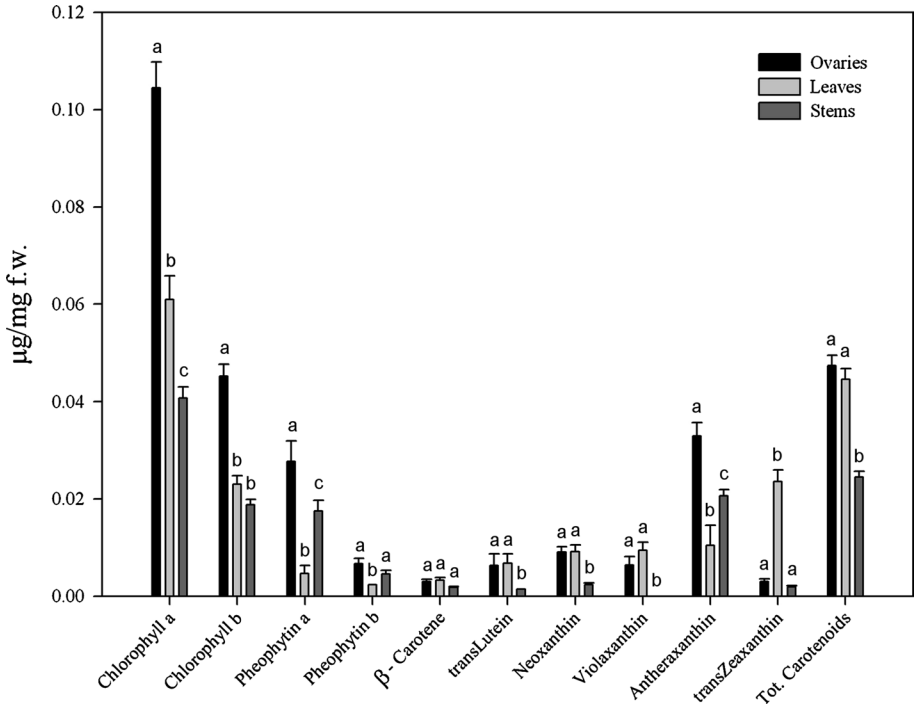

Fig. 1 Pigment concentrations in ovaries, leaves, and stems of *L. abortivum* control plants. Data are means of $n = 18$ samples ± s.e.m. The significance (one-way ANOVA with $\alpha = 0.05$) of the differences among the organs is also reported, with different letters indicating significant differences. Additional information on the HPLC results is available in Online Resource 4 of the ESM

chlorophylls. While pigment composition was the same in leaves, stems, and ovaries, the relative amount of pigments varied considerably among the three organs (Fig. 1, Online Resource 4 of the ESM). Leaves showed a prevalence of zeaxanthin relative to violaxanthin and antheraxanthin and, together with the ovaries, the highest concentration of neoxanthin. In contrast, a prevalence of antheraxanthin was found in the ovaries and stems. Ovaries showed a total chlorophyll concentration twice that in leaves and almost triple that in stems (Fig. 1; Online Resource 4 of the ESM). The chlorophyll *a*/*b* ratio was significantly higher in leaves (2.9 ± 0.1) than in both stems (2.2 ± 0.1; $P = 0.001$) and ovaries (2.3 ± 0.1; $P < 0.001$), which did not differ in chlorophyll *a*/*b* ratio. In all organs, chlorophylls mostly occurred in their common conformations, with traces of their C-10 epimers (see Online Resource 4 of the ESM).

Canonical variates analysis showed changes in pigment composition in ovaries but not in leaves or stems due to the benomyl treatment (Fig. 2). All of the variables (chlorophyll *a*, chlorophyll *b*, and total carotenoids) were mainly correlated with the first canonical axis ($r = 0.982$, $r = 0.997$, and $r = 0.869$, respectively) and provided a clear-cut division of ovary groups ($P = 0.011$ according to Hotelling's T^2 test). Benomyl treatment caused a significant increase in both chlorophyll *a* and *b* ($P = 0.001$ and $P = 0.005$, respectively) and in total carotenoid ($P = 0.009$) concentrations in ovaries (Fig. 3). On average, chlorophyll *a* was 1.3× higher, chlorophyll *b* was 1.2× higher, and total carotenoids were 1.2× higher in treated *L. abortivum* with respect to the controls. The chlorophyll *a*/*b* ratio, on

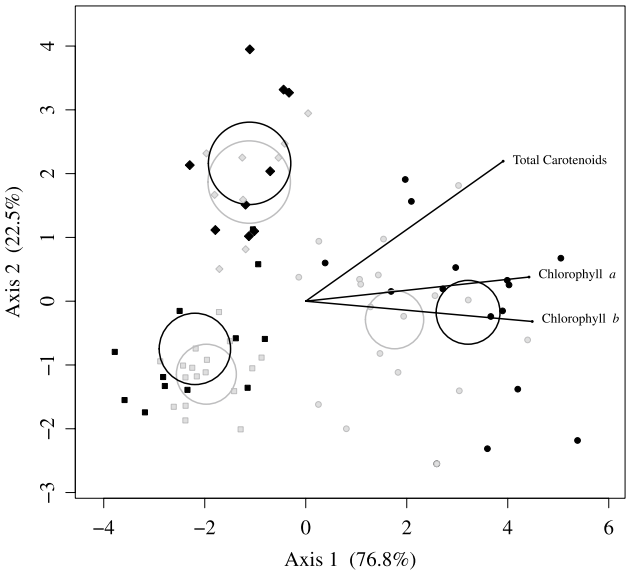

Fig. 2 Biplot of canonical variates analysis of groups of leaves (*gray diamonds* $n = 9$; *black diamonds* $n = 9$), stems (*gray squares* $n = 17$; *black squares* $n = 12$), and ovaries (*gray circles* $n = 18$; *black circles* $n = 15$) of treated (*black*) and control (*gray*) *L. abortivum*. Confidence circles (for $\alpha = 0.05$) and the percentage of the variance explained by each axis are shown

the contrary, did not vary significantly between treated and control *L. abortivum* ovaries. Analyses of iprodione-treated *L. abortivum* gave similar results for pigment contents (data not shown due to the limited sample size, $n = 3$). Chlorophyll *a* ($r = 0.526$, $P < 0.05$), chlorophyll *b* ($r = 0.453$,

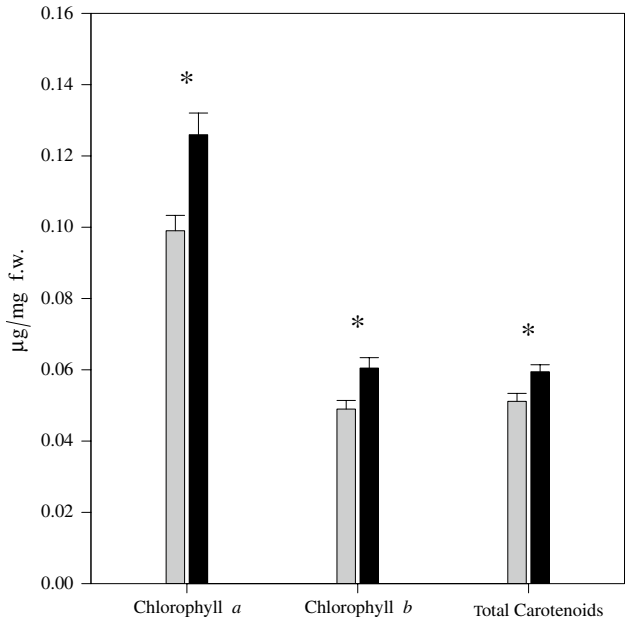

Fig. 3 Concentrations of chlorophyll *a*, chlorophyll *b*, and total carotenoids in the ovaries of treated (*black*) and control (*gray*) *L. abortivum*. Data are means ± s.e.m. of $n = 15$ and $n = 18$ samples for treated and control plants, respectively. *Asterisks* indicate significant differences between treatments according to *t* tests with $\alpha = 0.01$

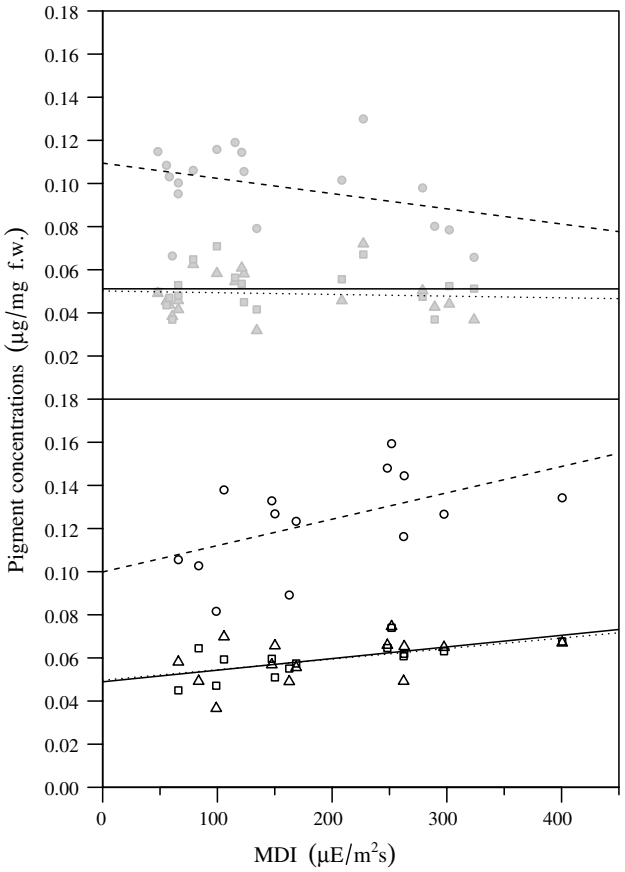

Fig. 4 Relationships between the mean daily irradiance and chlorophyll *a* (*circles*), chlorophyll *b* (*triangles*), and total carotenoid (*squares*) concentrations in ovaries of control (*upper panel*, *gray symbols*) and treated (*lower panel*, *white symbols*) *L. abortivum*. Linear regression lines are shown (*dashed*, *dotted*, and *continuous* for chlorophyll *a*, chlorophyll *b*, and total carotenoids, respectively). Statistical details are given in the text

$P = 0.05$), and total carotenoids ($r = 0.658$, $P < 0.01$) in ovaries of treated plants were positively and linearly correlated with the MDI of the microsite in which they were growing (Fig. 4), whereas no correlation was observed in control plants. On average, the mean photon flux did not vary significantly (for $\alpha = 0.1$) between treated and control *L. abortivum* in either the first or the second year of the research.

Isotopic signature

Seeds of iprodione-treated *L. abortivum* had significantly ($P < 0.05$) lower $\delta^{13}C$ and a wider range of values (CV = 3.34) than controls (CV = 1.53) (Table 1) and, at the same time, higher ($P < 0.001$) $\delta^{13}C$ than leaves of autotrophic references (-28.92 ± 0.39). Iprodione treatment caused a marked variation in the relationship between $\delta^{13}C$ and MDI with respect to that observed for control *L. abortivum* (Fig. 5). Notably, $\delta^{13}C$ of seeds from control plants slightly decreased linearly, but not significantly, with increasing MDI, while the $\delta^{13}C$ of seeds from treated plants varied according to the following exponential linear function ($r = 0.805$, $P < 0.001$):

$$\delta^{13}C = -24.431 - 3.337 \cdot e^{-0.018 \cdot i} - 0.002 \cdot i \qquad (1)$$

This model held true even after the removal of the observations associated with photon fluxes below 40 µE m^{-2} s^{-1},

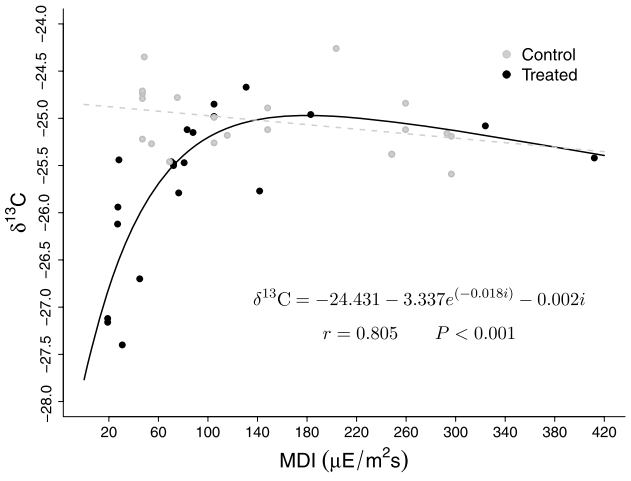

Fig. 5 Relationship between the $\delta^{13}C$ (‰) of seeds from treated and control *L. abortivum* and the mean daily irradiance

Table 1 Mean ± s.e.m. values of the MDI and the parameters measured on seeds and ovaries of control and iprodione-treated *L. abortivum*; different letters indicate significant differences

	Control	Treated
$\delta^{13}C$ (‰)	−25.065 ± 0.080 a ($n = 21$)	−25.506 ± 0.174 b ($n = 21$)
C/N	22.62 ± 0.27 a ($n = 21$)	22.32 ± 0.22 a ($n = 21$)
Seed size (mm^2)	0.266 ± 0.061 a ($n = 713$)	0.283 ± 0.069 a ($n = 717$)
Ovary length (mm)	25.68 ± 2.27 a ($n = 42$)	26.67 ± 3.44 a ($n = 42$)
Ovary diameter (mm)	8.44 ± 0.28 a ($n = 42$)	8.97 ± 0.49 a ($n = 42$)
Mean daily irradiance ($\mu E\ m^{-2}\ s^{-1}$)	145.14 ± 20.05 a ($n = 21$)	105.67 ± 22.46 a ($n = 21$)

available only for treated *L. abortivum* (data not shown). In addition, one-way ANCOVA highlighted significant differences for both the treatment ($P < 0.001$) and the interaction between the treatment and the MDI ($P < 0.01$). Seed C/N ratio, on the contrary, did not differ significantly between treated and control plants (Table 1), nor in relation to MDI. Similarly, the sizes of ovaries and seeds did not vary significantly between control and treated *L. abortivum* (Table 1).

Discussion

Despite the lack of direct fungicide tests on *L. abortivum* symbionts (since, to our knowledge, no strain of the *R. delica* clade is available in pure culture), the responses to benomyl and iprodione of the four *Russula* species (GenBank accessions KJ482563–KJ482566) can be assumed to be representative of the *Russula* genus, considering their diverse phylogenetic positions. Benomyl and iprodione, used in our trials at concentrations higher than those reported by Diaz et al. (2003) to be effective against basidiomycetes, efficiently reduced in vitro growth and mycorrhizal colonization of *L. abortivum*. Iprodione is less effective in vitro, but also enhances peloton degradation. The functional status of collapsed pelotons is currently unknown and debated: they could represent a recycling of old symbiotic interfaces (with no direct role in nutrient transfer) or a stage at which nutrients arising from lysis are transferred to the host (Rasmussen 1995; Yukari et al. 2014). In the first explanation, collapsed pelotons are evidence for reduction of the functional symbiotic interface; in the second, the reduced frequency of freshly colonized cells displaying intact pelotons also indicates a reduced access to fungal C. We ignore the impact of the fungicides on *Ceratobasidiaceae* spp. sporadically colonizing *L. abortivum* roots (Girlanda et al. 2006; Paduano et al. 2011); however, they are considered marginal symbionts, unlikely to be the main C provider. Indeed, the two *Ceratobasidium* ever detected in *L. abortivum* (GenBank accessions DQ061931 and HM117643) belong to orchid mycorrhizal clades among *Ceratobasidiaceae*, and do not form ectomycorrhizas (Veldre et al. 2013), unlike the usual C providers of temperate MH orchids (Hynson et al. 2013). Although we cannot ensure the full extirpation of *Russula* spp. from roots, since they remain detectable by PCR and microscopy, the observed changes in the morphological and physiological status of the fungi and the impact on soil respiration allow us to interpret the observed changes in pigment composition and ^{13}C abundance as a result of functional inhibition of fungal activity.

However, non-target effects of the employed fungicides must also be considered. First, these fungicides target other fungi apart from *Russula* spp., such as pathogens in soil or tissues and endophytes. Bayman et al. (2002), for example, suggested that benomyl could restrict the growth of deleterious fungi more than that of beneficial fungi, but how this would affect pigment content and $\delta^{13}C$ values remains unclear. Organ-specific increases in pigment concentrations have never been described as a non-target effect of benomyl to our knowledge. On the contrary, we are only aware of reports of pigment decreases after fungicide application (Ahmed et al. 1983). Some parasitic fungi enhance stomatal opening (Grimmer et al. 2012), a situation that is expected to decrease $\delta^{13}C$ values (Farquhar et al. 1989), but then fungicide treatment would increase $\delta^{13}C$ rather than decrease it as observed here. Second, plant physiology can be modified, although we did not observe phytotoxicity or changes in the development and life duration of treated *L. abortivum* (data not shown). Interestingly, iprodione can reduce transpiration rates in grasses (Kaufmann and Williamson 1981), and thus decrease $\delta^{13}C$. In this case, however, the effect would be arguably similar for all of the samples and not related to the light conditions, as observed, so we can safely exclude this possibility.

The different concentrations of photosynthetic and photoprotective pigments in leaves, stems, and ovaries of *L. abortivum* suggest a differentiation of functions among epigeous organs toward photoprotection or photosynthesis. Leaves and bracts play a photoprotective role, as indicated by the equilibrium among the carotenoids of the xanthophyll cycle, which is shifted toward zeaxanthin formation, and by their preferential accumulation of anthocyanins (Leake 1994). Bracts, in particular, physically protect ovaries from excess light. A protective role of scales and bracts against herbivory has also been experimentally demonstrated in MH plants (Klooster et al.

2009), making a good raison d'être for their persistence in PMH species. The highest chlorophyll content of ovaries, on the other hand, suggests a higher photosynthetic potential that may have a role in seed nutrition. Indeed, chlorophyll content is usually correlated with photosynthetic activity (Šesták and Čatský 1962). Unfortunately, the finding that *L. abortivum* photosynthesizes below the compensation point in full light was previously established using stems and leaves (Girlanda et al. 2006). Measurements of gas exchange on the ovaries are now awaited, since they can be expected to photosynthesize above the compensation point. Anyway, our isotopic data are congruent with a contribution of photosynthesis to the C nutrition of *L. abortivum* seeds, which are less enriched in ^{13}C than expected for MH orchids (Gebauer and Meyer 2003; Selosse and Roy 2009).

The response of ovaries—but not of leaves and stems—to fungicide treatment further suggests that pigments in ovaries have a metabolic role, and compensate for decreased fungal supplies by increasing photosynthesis. In control *L. abortivum*, the photosynthetic rate does not seem to adapt to the light level, at least considering the photosynthetic pigments, whose concentrations do not vary along a light gradient. This response indicates that, in natural conditions, photosynthesis is a secondary C source, and its optimization is not required to support plant development or reproduction. This is congruent with a mixotrophic behavior where C is mostly supplied by the fungi. Conversely, fungicide-treated plants show clear photoacclimation responses, indicating that the development of the photochemical apparatus is modulated to optimize the photosynthetic rate when fungal C is limiting. This is expected in autotrophic plants (Lichtenthaler et al. 2007a, b), and indicates an increase in both light utilization by treated *L. abortivum* and the relative importance of this process in contributing to the C nutrition of the seeds. The heterogeneous light environment in which the plants grew, however, also led to high variance and small differences in $\delta^{13}C$ between control and fungicide-treated plants. The lower $\delta^{13}C$ values in fungicide-treated ovaries—closer to the values of autotrophic plants—can be nonexclusively interpreted as either a reduction in the fungal C gain or an enhancement of photosynthesis, which would be congruent with the increases in pigment content. Indeed, both of these processes result in a reduction in the $\delta^{13}C$ and their relative contribution cannot be addressed from $\delta^{13}C$ only (as discussed by Matsuda et al. 2012 for ^{13}C enrichment in shaded versus light-exposed *Pyrola japonica*). In this context, the absence of an impact of treatment on fruit and seed sizes indicates that C was not limiting and that compensation occurred. Since reserves are usually enriched in ^{13}C as compared to photosynthetic tissues (Cernusak et al. 2009), we can rule out the possibility that C reserves have been mobilized, and instead conclude that photosynthesis actively compensates for fungal C. The relationship between $\delta^{13}C$ and irradiance observed in treated plants further demonstrates a shift toward an autotrophic behavior in ovaries in response to a reduced access to fungal C. The exponential–linear model that fits the variation in $\delta^{13}C$ along the irradiance gradient is similar to that observed for autotrophic plants (Ehleringer et al. 1986). Light conditions have a direct effect on the ratio between the leaf intercellular and environmental CO_2 concentrations, and therefore on C isotopic discrimination (Farquhar et al. 1989). A decrease in this ratio, due to higher photosynthetic rates or reduced stomatal conductance, limits the photosynthetic discrimination against ^{13}C and increases the $\delta^{13}C$. The increase in $\delta^{13}C$ with increasing irradiance in treated *L. abortivum* thus demonstrates an increase in C fixation, since a reduction in stomatal conductance can be ruled out considering the irradiance range (below 150 $\mu E/m^2s$). Control plants, on the contrary, deviate strikingly from this behavior, and their null response to the light conditions in terms of $\delta^{13}C$ and photosynthetic pigments indicates that photosynthesis marginally contributes to the C nutrition of the seeds. A similar response has already been described for mixotrophic plants along light gradients (Preiss et al. 2010; Matsuda et al. 2012), which incorporate more fungus-derived C in low light.

To summarize, the reduction in mycorrhizal colonization in *L. abortivum* fulfilled our two main predictions. First, photosynthesis compensated in fruits for reduced availability of fungal C through enhanced photosynthetic apparatus development in ovaries. Second, the physiological response to the micro-environmental light level after fungicide treatment followed the response expected for autotrophic plants, demonstrating a higher dependence on photosynthesis.

Ecological implications and evolutionary perspectives

The evidence presented here demonstrates the potential of *L. abortivum* to employ a compensatory photosynthesis in the ovaries to buffer fungal C limitations and support seed development. In natural conditions, therefore, this species preferentially feeds on fungi and downregulates the development of its photochemical apparatus. This is not due to environmental constraints, but rather to a cost-limiting strategy that allows *L. abortivum* to reduce the costs of the photochemical apparatus and photoacclimation when fungal C supply is sufficient (see also Beyrle and Smith 1993).

Compensatory photosynthesis in the ovaries may have deep ecological implications, since it can reasonably have positive effects on both the main components of fitness in PMH species, i.e., reproduction and rhizome (persistence structures) survival. As long as *L. abortivum* has access to

sufficient fungal C, it supports both seed development and the rhizome. If, for any reason, fungal C becomes limiting, photosynthesis will allow seed development and, at the same time, relax the demand of fungal C for this process, allocating it to rhizome survival, as suggested by Leake (1994). It has been suggested that photosynthesis also supports seed production in other PMH species (Montfort and Küsters 1940; Roy et al. 2013), and this idea deserves further investigation. These features should be analyzed in the framework of the evolution to full MH nutrition in the *Neottieae* tribe, e.g., in the closely related *Aphyllorchis* spp. (Roy et al. 2009; Selosse and Roy 2009). On the one hand, the ecological plasticity of these strategies likely contributes to the evolutionary stability of PMH nutrition. On the other hand, they can allow the evolution of MH nutrition by tightly binding photosynthesis use to the availability of fungal C. Indeed, in PMH species colonizing light-limiting environments, fungal C exploitation could be selected positively while, under the mechanisms above, photosynthesis would be concurrently repressed, driving a progressive evolution toward mycoheterotrophy. In such conditions, mutations affecting autotrophic nutrition would negligibly affect fitness, and so could be fixed during evolution. In this view, PMH nutrition can be considered a pre-adaptation to MH nutrition (see Roy et al. 2013), with this final stage reached on the rare occasions that environmental constraints are stable across evolutionary timescales. Conversely, partial mycoheterotrophy could be evolutionarily stable, and could occur in a continuum from near full autotrophy to near full mycoheterotrophy in variable environments where fungal C supply is relatively unpredictable and photosynthesis is required to buffer fungal C limitations.

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