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Mixotrophy in Pyroleae (Ericaceae) from Estonian boreal forests does not vary with light or tissue age

Félix Lallemand[1,2,*], Ülle Puttsepp[3], Mait Lang[4,5], Aarne Luud[3], Pierre-Emmanuel Courty[6], Cécile Palancade[1] and Marc-André Selosse[1,7]

[1]Institut de Systématique, Évolution, Biodiversité (ISYEB), UMR 7205 CNRS MNHN UPMC EPHE, Muséum national d'Histoire naturelle, Sorbonne Universités, 57 rue Cuvier, CP39, 75005 Paris, France [2]Master BioSciences, Département de Biologie, École Normale Supérieure de Lyon, Université de Lyon, UCB Lyon1, 46 Allée d'Italie, Lyon, France [3]Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Street Kreutzwaldi 5, 51014, Tartu, Estonia [4]Institute of Forestry and Rural Engineering, Estonian University of Life Sciences, Street Kreutzwaldi 5, 51014 Tartu, Estonia [5]Tartu Observatory, 61602 Tõravere, Tartu County, Estonia [6]Agroécologie, AgroSupDijon, CNRS, INRA, Université de Bourgogne Franche-Comté, 21000 Dijon, France and [7]Department of Plant Taxonomy and Nature Conservation, University of Gdansk, ul. Wita Stwosza 59, 80-308 Gdańsk, Poland
[*]For correspondence. E-mail felix.lallemand@ens-lyon.org

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• **Background and Aims** In temperate forests, some green plants, namely pyroloids (Pyroleae, Ericaceae) and some orchids, independently evolved a mode of nutrition mixing photosynthates and carbon gained from their mycorrhizal fungi (mixotrophy). Fungal carbon is more enriched in ^{13}C than photosynthates, allowing estimation of the proportion of carbon acquired heterotrophically from fungi in plant biomass. Based on ^{13}C enrichment, mixotrophic orchids have previously been shown to increase shoot autotrophy level over the growth season and with environmental light availability. But little is known about the plasticity of use of photosynthetic versus fungal carbon in pyroloids.
• **Methods** Plasticity of mixotrophy with leaf age or light level (estimated from canopy openness) was investigated in pyroloids from three Estonian boreal forests. Bulk leaf ^{13}C enrichment of five pyroloid species was compared with that of control autotrophic plants along temporal series (over one growth season) and environmental light gradients (n=405 samples).
• **Key Results** Mixotrophic ^{13}C enrichment was detected at studied sites for *Pyrola chlorantha* and *Orthilia secunda* (except at one site for the latter), but not for *Chimaphila umbellata*, *Pyrola rotundifolia* and *Moneses uniflora*. Enrichment with ^{13}C did not vary over the growth season or between leaves from current and previous years. Finally, although one co-occurring mixotrophic orchid showed ^{13}C depletion with increasing light availability, as expected for mixotrophs, all pyroloids responded identically to autotrophic control plants along light gradients.
• **Conclusions** A phylogenetic trend previously observed is further supported: mixotrophy is rarely supported by ^{13}C enrichment in the *Chimaphila* + *Moneses* clade, whereas it is frequent in the *Pyrola* + *Orthilia* clade. Moreover, pyroloid mixotrophy does not respond plastically to ageing or to light level. This contrasts with the usual view of a convergent evolution with orchids, and casts doubt on the way pyroloids use the carbon gained from their mycorrhizal fungi, especially to replace photosynthetic carbon.

Key word: *Chimaphila*, Ericaceae, *Moneses*, mixotrophy, mycoheterotrophy, N content, orchids, *Orthilia*, *Pyrola*, response to light, stable isotopes, ^{13}C.

INTRODUCTION

Mycorrhizae are widespread symbioses where the roots of terrestrial plants associate with soil fungi (van der Heijden *et al.*, 2015). Fungi provide mineral elements and protection against soil stresses to plants, which usually reward the fungus with photosynthates: in most cases, partners reciprocally test the other's ability to provide the expected nutrients (Selosse and Rousset, 2011). However, some plants also recover carbon (C) from the fungus (Selosse and Roy, 2009; Hynson *et al.*, 2013*a*); these interactions reverse the usual exchange, question the mutualism of the association, and most importantly open a new chapter of plant nutritional strategies. Nutrition including fungal C has been reported in some fully achlorophyllous plants, the so-called mycoheterotrophic (MH) plants (Leake, 1994; Merckx, 2013), but more recently partial mycoheterotrophy was discovered in some green plants with photosynthetic abilities (Gebauer and Meyer, 2003; Julou *et al.*, 2005; Selosse *et al.*, 2016), a strategy called mixotrophy.

While several taxa of mycorrhizal fungi support MH and likely mixotrophic (MX) nutrition (Martos *et al.*, 2009; Selosse and Roy, 2009; Merckx, 2013; Bolin *et al.*, 2017), most physiological studies have hitherto focused on MX plants associated with Ascomycotas and Basidiomycotas that gain their C

by forming so-called ectomycorrhizae on tree roots (Hynson *et al.*, 2013*a*; Selosse *et al.*, 2016). These fungi fractionate against ^{12}C when recovering C from donating plants (Hobbie *et al.*, 2001; Mayor *et al.*, 2009), a particularity absent from other mycorrhizal fungal taxa (e.g. Courty *et al.*, 2015), and such isotopic abundance makes the fungal contribution to MX nutrition easier to detect in receiving plants. Plants deriving C from ectomycorrhizal fungi are indeed enriched in ^{13}C, especially MH species (Trudell *et al.*, 2003), but also MX ones (Gebauer and Meyer, 2003; Julou *et al.*, 2005). Moreover, the percentage of C coming from mycorrhizal fungi (hereafter, the 'heterotrophy level') can be estimated from ^{13}C enrichment in MX biomass, thanks to a linear mixing model and the use of appropriate enrichment references in fully MH and autotrophic nearby plants (Gebauer and Meyer, 2003; Hynson *et al.*, 2013*a*). Mixotrophic nutrition has mainly been investigated in forest orchids associated with ectomycorrhizal fungi, whose photosynthesis is limited due to either reduced intrinsic photosynthetic capacities (Girlanda *et al.*, 2006; Cameron *et al.*, 2009) or to low understorey light levels (Julou *et al.*, 2005). In some of these species, some variant individuals devoid of chlorophyll, the so-called albinos, survive by mycoheterotrophy over several years (Selosse *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Shefferson *et al.*, 2016), which is further evidence of the ability to obtain significant levels of fungal C. Congruently, fully MH species have often evolved within MX lineages (Selosse and Roy, 2009; Motomura *et al.*, 2010), or from putative MX ancestors (Lallemand *et al.*, 2016). Finally, MX and MH plants are often enriched in total nitrogen compared with autotrophic plants (Abadie *et al.*, 2006; reviewed in Hynson *et al.*, 2013*a*). Mixotrophic and MH nutrition modify the way plants gain nitrogen (N) from fungi (as further suggested by a different ^{15}N enrichment in these plants; Abadie *et al.*, 2006; Hynson *et al.*, 2013*a*), and N content positively correlates with heterotrophy level in MX orchids at least (e.g. Gonneau *et al.*, 2014).

An important challenge is to understand the physiology of MX plants (Selosse *et al.*, 2017) and their use of fungal and photosynthetic C. These questions have hitherto been mainly investigated in MX orchids, using ^{13}C enrichment to estimate the heterotrophy level in various organs, and albinos as references for MH ^{13}C enrichment. First, the heterotrophy level is variable between species and populations (Hynson *et al.*, 2013*a*), and depends especially on the light level: the heterotrophy level increases with decreasing light level or after experimental shading (Preiss *et al.*, 2010; Gonneau *et al.*, 2014). Second, different organs rely differently on fungal C versus photosynthetic resources: while photosynthates do not detectably contribute to underground parts such as rhizomes (Gonneau *et al.*, 2014), they form a major part of flowers and fruit biomass (Roy *et al.*, 2013; Bellino *et al.*, 2014). Shoot nutrition and leaf nutrition vary over the growing season, shifting from using mostly fungal C at aboveground emergence to being mostly photosynthetic at fruiting time (Roy *et al.*, 2013; Gonneau *et al.*, 2014). Thus, MX nutrition is environmentally flexible and physiologically dynamic, in MX orchids at least.

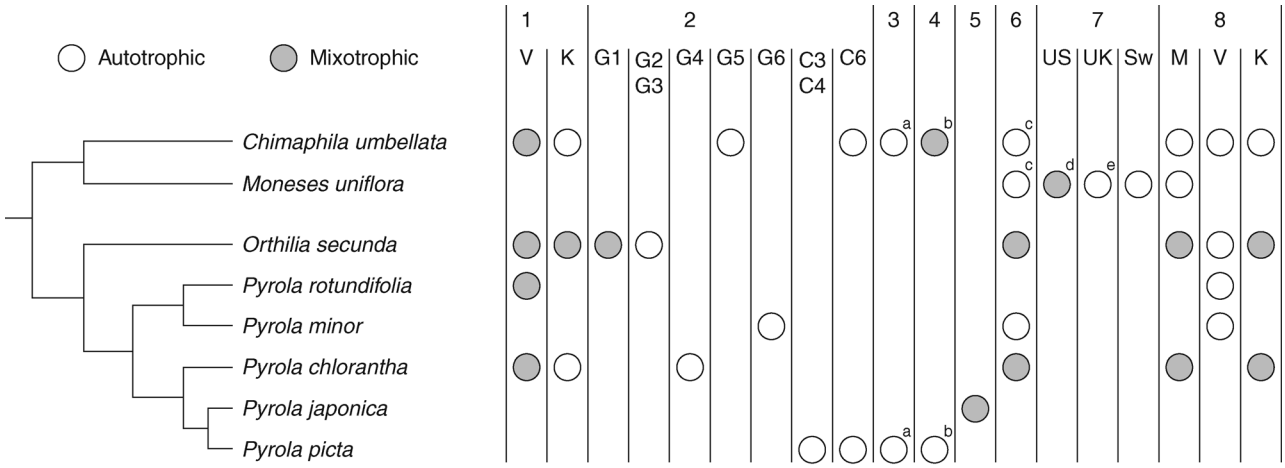
Mixotrophic nutrition supported by ectomycorrhizal fungi also occurs in Ericaceae of the tribe Pyroleae (hereafter, the pyroloids; Hynson and Bruns, 2009; Liu *et al.*, 2011; Lallemand *et al.*, 2016) which live in the forest understorey. To our best knowledge, photosynthetic abilities have not been assessed in pyroloids. However, ^{13}C enrichment provides evidence for MX nutrition in some species, although the heterotrophy level fluctuates more than in MX orchids, ranging for a given species from null to significant depending on the sampled populations (Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007; Hynson *et al.*, 2015; Johansson *et al.*, 2015) (Fig. 1). There is limited and debated evidence that pyroloid MX nutrition follows the flexibility and dynamic patterns found in MX orchids. First, *Pyrola japonica* from a Japanese forest has ^{13}C enrichment inversely correlated with light availability (Matsuda *et al.*, 2012), meaning higher heterotrophy levels in shadier conditions. Congruently, plants in the darkest conditions were more densely colonized by the *Russula* fungal symbiont preferred by *P. japonica*. A similar scenario was supported by observations of a higher heterotrophy level in populations at lower irradiance in *Orthilia secunda* (Zimmer *et al.*, 2007), *Moneses uniflora* (Hynson *et al.*, 2015) and *Pyrola chlorantha* (Tedersoo *et al.*, 2007); however, the opposite was found for *O. secunda* and *Chimaphila umbellata* (Tedersoo *et al.*, 2007). Comparison between distant sites can be biased by local abiotic characteristics or fungal communities, so that comparisons should be made within a similar forest site, or after experimental shading. Indeed, no increase in leaf heterotrophy was detected in an experimental 50 % irradiance decrease on Californian *Pyrola picta* and *C. umbellata* (Hynson *et al.*, 2012); however, when examining only leaf sugars to avoid isotopic signals from polymers synthesized before the experiment, ^{13}C enrichment suggested that heterotrophy decreased in *C. umbellata* after shading, but paradoxically increased in *P. picta* (Hynson *et al.*, 2012). Moreover, a decrease in heterotrophy in shoots and leaves over the growing season, as observed in MX orchids, has rarely been seen in pyroloids: Hynson *et al.* (2012) did not detect significant variations in ^{13}C enrichment, and thus heterotrophy level, of bulk leaf tissues and sugars of *P. picta* and *C. umbellata* during their 44-days monitoring. Changes in heterotrophy level during shoot lifespan (which covers at least 2 years in these evergreen species) remain to be assessed more broadly in pyroloids.

In this study, we investigated MX nutrition in pyroloids at three Estonian circumscribed sites, to allow comparisons in relatively homogeneous environments, plant populations and fungal communities. We investigated how leaf age and light level impact heterotrophy level in five pyroloid species, using natural local gradients of light and seasonal monitoring over 1 year.

MATERIALS AND METHODS

Study sites

We investigated three sites in Estonia, each harbouring at least four evergreen pyroloid species (Table 1) and the MH species *Hypopitys monotropa*. In all, five pyroloid species were considered: *Chimaphila umbellata*, *Orthilia secunda*, *Moneses uniflora*, *Pyrola chlorantha* and *Pyrola rotundifolia*. The sites included three boreal *Pinus sylvestris* forests (Mändjala, 58°12′44″ N, 22°19′13″ E; Värska, 57°56′34″ N, 27°39′58″E;



[a]Based on the pooled data from four different Californian sites (*C. umbellata*) and six different Californian sites plus one from Oregon (*P. picta*).

[b]Based on th $\delta^{13}C$ of leaf soluble sugars. MX was not detectable from bulk tissues.

[c]Samples were depleted in ^{13}C compared to autotrophic references.

[d]Based on the pooled data from seven different sites.

[e]Based on the pooled data from two different sites. Samples were depleted in ^{13}C compared to autotrophic references.

FIG. 1. Trophic status of different pyroloid species including the five studied here, from current and previously published data, as deduced from bulk leaf ^{13}C enrichment. Open circles, autotrophic; grey circles, mixotrophic. The cladogram is based on Lallemand *et al.* (2016) for genera relationships and Liu *et al.* (2014) for *Pyrola* species relationships. References are as follows (with sites indicated below the reference number): 1, Tedersoo *et al.*, 2007 (V, Värska; K, Kärla); 2, Zimmer *et al.*, 2007 (same site names as in the referenced article); 3, Hynson *et al.*, 2009; 4, Hynson *et al.*, 2012; 5, Matsuda *et al.*, 2012; 6, Johansson *et al.*, 2015; 7, Hynson *et al.*, 2015 (Sw, Sweden); 8, this study (M, Mändjala; V, Värska; K, Kärla).

TABLE 1. *Species sampled for isotopic study at each of the three sampling sites, with number of individuals sampled. To investigate nutrition type by isotopic analysis, autotrophs, mixotrophs or mycoheterotrophs were sampled for reference species in addition to the pyroloid species studied. Young and old leaves were harvested for pyroloids, noted as 'y' and 'o', respectively. See Supplementary Data Table S1 for the seasonal sampling*

Site	Nutrition	Species (number of samples)
Mändjala (23 July 2009)	Pyroloid	*Orthilia secunda* (o: 14, y: 11), *Pyrola chlorantha* (o: 13, y: 13), *Chimaphila umbellata* (o: 14, y: 13), *Moneses uniflora* (o: 6, y: 6)
	Mycoheterotroph	*Hypopitys monotropa* (6)
	Autotroph	*Berberis vulgaris* (1), *Dianthus arenarius* (3), *Fragaria vesca* (1), *Frangula alnus* (1), *Galium album* (2), *Galium boreale* (1), *Galium pomeranicum* (1), *Galium verum* (7), *Hieracium umbellatum* (6), *Luzula pilosa* (5), *Quercus robur* (5), *Sorbus aucuparia* (3), *Thymus serpyllum* (3), *Vaccinium vitis-idaea* (1), *Viola rupestris* (1)
Värska (14 June 2009)	Pyroloid	*Orthilia secunda* (o: 10, y: 9), *Pyrola rotundifolia* (9), *Chimaphila umbellata* (7)
	Mycoheterotroph	*Hypopitys monotropa* (3)
	Autotroph	*Festuca rubra* (7), *Luzula pilosa* (10), *Lycopodium clavatum* (9)
Kärla (16 June 2009)	Pyroloid	*Orthilia secunda* (11), *Pyrola chlorantha* (9), *Chimaphila umbellata* (o: 9, y: 9)
	Mixotroph	*Epipactis atrorubens* (9)
	Mycoheterotroph	*Hypopitys monotropa* (9)
	Autotroph	*Hieracium umbellatum* (2), *Luzula pilosa* (9), *Pulsatilla pratensis* (3)

and Kärla, 58°20′36″ N, 22°17′59″ E). The last two were previously described by Tedersoo *et al.* (2007). The Mändjala and Kärla sites were of the *Cladina* type (type group of boreal heath forests; Paal, 1997) on poor sandy soils, covered by a Scots pine (*P. sylvestris*) forest [average age 100 (60–160) years] with sparse Norway spruce (*Picea abies*) undergrowth. The Värska site was of the *Vaccinium myrtillus* type (type group of dry boreal forests; Paal, 1997) on sandy soil, covered by a 70- to 90-year-old Scots pine and silver birch (*Betula pendula*) forest. The Mändjala site was on the Baltic seashore (<200 m). All soils were haplic podzols on sand. The sites experienced mean annual temperatures of +7.8 °C (Mändjala, Kärla) and +6.8 °C (Värska), and ~12 °C during vegetation periods (April to October inclusive). They received rainfall of 570 mm $year^{-1}$ (Mändjala, Kärla) and 783 mm $year^{-1}$ (Värska) during 2008–2009 (all measurements from the closest weather stations, 12 and 18 km from Mändjala and Kärla, respectively, and 40 km from Värska).

Sampling

Samples for isotopic studies were collected with records of light (see below) at Värska on 16 June 16, at Kärla on 18 June 2009 and at Mändjala on 23 July 2009 (Table 1 and Supplementary Data Table S1). At each site, some ten to 13 plots measuring 20 cm ×20 cm were identified that (1) displayed contrasting light levels between plots; (2) had similar light exposure over the plot itself; and (3) were situated >5 m from each other. In each plot, we sampled one leaf from up to two individuals from each available pyroloid species. Moreover, whenever available, in each plot we harvested one inflorescence of *H. monotropa*, one leaf of the MX orchid *Epipactis atrorubens* in Kärla plots only and one leaf from each available autotrophic species, as references for MH, MX and autotrophic nutrition, respectively (see Table 1 and Table S1 for samples actually harvested for each plot and raw data).

The effects of age on leaf isotopic abundance and N content were estimated (1) between years and (2) over a growth season. First, the difference between leaves of different years was addressed during the main sampling at each site (above), by collecting young and old leaves on each individual of *O. secunda* at Värska and *C. umbellata* at Kärla, as well as *O. secunda*, *P. chlorantha C. umbellata* and *M. uniflora* at Mändjala (Table 1): 'young' leaves were recently terminal leaves of the rosette, expanded during the spring (<2 months), while 'old' leaves were basal-most leaves from previous year(s) that did not show any sign of senescence. Second, seasonal isotopic variations of plant leaves were addressed in 2009 by obtaining additional plant samples in the same year on 24 April, 20 August and 4 November at Värska, and on 13 May, 25 July and 2 November at Kärla (see samplings in Table S1; unfortunately, the phenology of *E. atrorubens* at Kärla did not allow its use as a control in this seasonal monitoring).

Light analysis

Light conditions in each sampling plot for each sampling date were inferred from canopy openness to yield a value representative of the mean exposure to sunlight during the day. Digital hemispherical photographs were taken at ground level in the nadir view direction at sampling time using a Canon EOS 5D camera with a Sigma F3·5 EX DG lens. The camera was oriented so that the north–south direction could be determined later for each image. All image data were stored as sensor raw readings (raw data) so that we could use the linear ratio method (Cescatti, 2007; Lang *et al.*, 2010, 2013) to estimate the canopy gap fraction. Radiance measurements were corrected for sensor dark current. Corrections of lens projection distortion and vignetting were applied and a sky radiance reference image was constructed as in Lang *et al.* (2010). The procedure was carried out using Hemispherical Project Manager software (Lang, 2014). Canopy gap fraction images were then imported into Gap Light Analyzer (GLA) version 2.0 (Frazer *et al.*, 1999) to obtain values of gap light transmission. Input variables for GLA calculations included site coordinates, elevation, vegetation period length (15 April to 15 October), an assumed cloudiness index of 0·5, a spectral fraction of 0·5, a solar constant of 1367 W m^{-2} and a clear-sky transmission coefficient of 0·65. For diffuse radiation flux, the model of uniform overcast sky was selected.

Isotopic analyses, N content and mixotrophy assessment

Leaf and inflorescence samples were ground in 1.5-mL Eppendorf tubes using two 2-mm diameter glass balls in a Retsch MM301 vortexer (Retsch). Abundances of ^{13}C and ^{15}N were measured using an online continuous flow CN analyser (NA 1500; Carlo Erba) coupled with an isotope ratio mass spectrometer (Delta S; Finnigan). This measurement also quantified N content. Relative isotope abundances are denoted as δ values, which were calculated according to the following equation:

$$\delta^{13}C \text{ or } \delta^{15}N = (R_{sample} / R_{standard} - 1) \times 1000 [‰]$$

where R_{sample} and $R_{standard}$ are the ratios of heavy isotope to light isotope of the sample and the respective standard (Vienna Pee-Dee Belemnite or atmospheric N_2 for C and N, respectively). When appropriate, the enrichment factor ε (*sensu* Hynson *et al.*, 2013*a*) was used, following the equation:

$$\varepsilon^{13}C \text{ or } \varepsilon^{15}N = \delta_{sample} - \delta_{ref} [‰]$$

where δ_{sample} is the $\delta^{13}C$ or $\delta^{15}N$ of the sample and δ_{ref} is the mean $\delta^{13}C$ or $\delta^{15}N$ of all autotrophic reference plants occurring in the same plot. The standard deviations of the replicated standard samples were 0·021 ‰ for ^{13}C and 0·222 ‰ for ^{15}N. Mycoheteretrophic nutrition for MX species was quantified from $\delta^{13}C$ values based on a linear mixing model using autotrophic and MH references as in Gebauer and Meyer (2003; see also Hynson *et al.*, 2013*a*). Throughout this study, a population was considered MX exclusively if its average $\delta^{13}C$ value differed significantly from that of autotrophic references.

Statistical analysis

All statistical analyses were performed using the R environment for statistical computing (R Development Core Team, 2007). Analysis of variance (ANOVA) was performed to evaluate differences in mean $\delta^{13}C$ and $\delta^{15}N$ among species (function aov). Tukey's honestly significant difference (HSD) test was then used to make pairwise comparisons of the means (function TukeyHSD). For N content, given the heteroscedasticity of the data, the Games–Howell test was used for pairwise comparisons of the means using the source code of the function posthocTGH as implemented in the package userfriendlyscience v0.5-2 (Gjalt-Jorn, 2016). Differences between young and old leaves were assessed by pairing leaves according to their sampling plot. Paired Student's *t*-tests were thus used to compare $\delta^{13}C$, $\delta^{15}N$ and N content means (function t.test). We tested $\delta^{13}C$ for linear relationship with canopy openness using the lm function. The α type-I error threshold was set at 0·05.

RESULTS

Variation of MH level with species at each site

The following analyses were made using old leaves only for all species. In all three sites, the MH species *H. monotropa* was

significantly enriched in $\delta^{13}C$ compared with all other species and in $\delta^{15}N$ compared with all autotrophic references (Fig. 2). The five pyroloids investigated differed in isotopic signatures (Fig. 2). *Orthilia secunda* showed higher $\delta^{13}C$ than autotrophic references at Mändjala and Kärla, suggesting 41 and 34 % MH nutrition, respectively, based on a two-source linear mixing model, but was autotrophic at Värska; it showed higher $\delta^{15}N$ at Mändjala only. *Pyrola chlorantha*, studied at Mändjala and Kärla only, always had higher $\delta^{13}C$ and $\delta^{15}N$ than autotrophic references, suggesting 40 and 30 % MH nutrition, respectively. *Chimaphila umbellata* did not differ from autotrophic references at the three study sites, together with *P. rotundifolia* and *M. uniflora* (at Mändjala and Värska, respectively). The MX orchid *E. atrorubens* showed higher $\delta^{13}C$ and $\delta^{15}N$ values than autotrophs at Kärla, suggesting 39 % MH nutrition. Finally, considering total N content (Supplementary Data Fig. S1), only *E. atrorubens* and young *C. umbellata* leaves at Kärla and *C. umbellata* at Värska showed significant enrichment compared with autotrophic references. Thus, among pyroloids, only *O. secunda* and *P. chlorantha* were MX at Mändjala and Kärla based on $\delta^{13}C$ values.

Variation of mycoheterotrophy level with age

The canopy tended to open over the 2009 growth season at Kärla and Värska (Fig. 3A). The $\delta^{13}C$ values did not vary over the growth season at Kärla (Fig. 3C), but tended to decrease for all investigated species at Värska (significantly for only *C. umbellata* and *O. secunda*; Fig. 3B). For $\varepsilon^{13}C$, no variations were observed for any pyroloid at any site over the growth season (Supplementary Data Fig. S2). No differences were observed in N content or $\delta^{15}N$ over the growth season (not shown; Table S1). Furthermore, old and young leaves sampled at a given time in June or July 2009, depending on the site (Table 1), did not differ in $\delta^{13}C$ and $\delta^{15}N$ values for *O. secunda* at Värska and *C. umbellata* at Kärla, nor for these species, *P. chlorantha* and *M. uniflora* at Mändjala (Supplementary Data Fig. S3a, b), suggesting that the mixotrophy level did not change over leaf lifespan. However, in all species and sites, young leaves were richer in nitrogen than old ones (Supplementary Data Figs S2 and S3c).

Variation of mycoheterotrophy level with light availability

Mändjala was the site with the greatest gradient of light availability (median canopy openness 20 %, minimum 5 %, maximum 39 %), while Värska (14 %, 6–19 %) and Kärla (17 %, 12–25 %) were more shaded and less variable (Supplementary Data Fig. S4 and Table S1). In all, over the three sites we observed an order of magnitude of canopy openness (Supplementary Data Fig. S4).

All autotrophic and pyroloid species tended to increase in $\delta^{13}C$ with light availability at all sites, with the exceptions of *L. pilosa* and *O. secunda* at Kärla (no variation), while the MX species *E. atrorubens* underwent a significant decrease (Table 2). The MH species *H. monotropa* did not vary in $\delta^{13}C$ with light availability. The pyroloids *O. secunda* at Mändjala and Värska, *P. chlorantha* at Mändjala and *P. rotundifolia* at Värska all displayed a significant increase in $\delta^{13}C$ with light

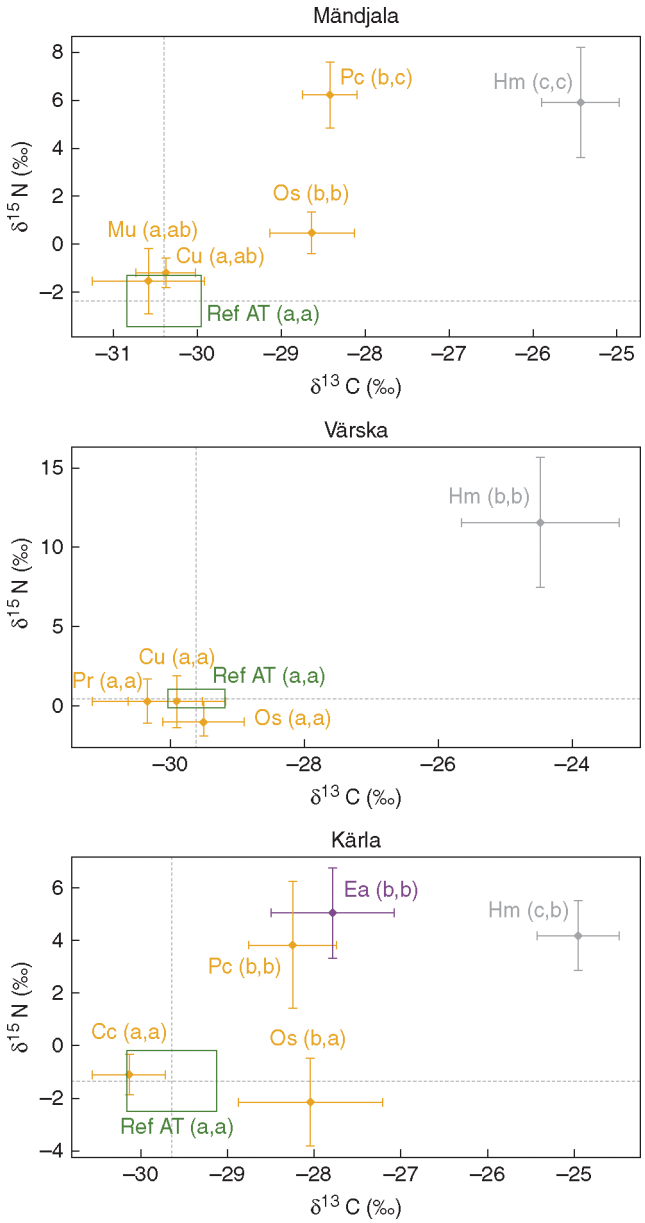


FIG. 2. Mean $\delta^{13}C$ and $\delta^{15}N$ values of bulk leaf tissues of the species investigated at the three sites of this study. Ref AT, reference for autotrophs pooling all autotrophic samples (see Table 1 for a list for each site); Mu, *Moneses uniflora*; Cu, *Chimaphila umbellata*; Os, *Orthilia secunda*; Pc, *Pyrola chlorantha*; Pr, *Pyrola rotundifolia*; Hm, *Hypopitys monotropa*; Ea, *Epipactis atrorubens*. Different letters in parentheses denote different means for, successively, $\delta^{13}C$ and $\delta^{15}N$, according to Tukey's HSD test; bars (or rectangle sides for Ref AT) represent 95 % confidence intervals of the mean.

level, with substantial R^2 values (0·27, 0·29, 0·47 and 0·47, respectively; Table 2), as expected for autotrophic, but not MX, species. In order to contrast the response in MH nutrition of pyroloids to that of autotrophic references, we calculated the $\varepsilon^{13}C$ (*sensu* Hynson *et al.*, 2013*a*). Whatever the study site, the $\varepsilon^{13}C$ of pyroloids did not respond to light level (Supplementary Data Table S2), while the MX species *E. atrorubens* at Kärla showed a significant decrease in $\varepsilon^{13}C$ with increasing light (R^2=0·46, P=0·044; Table S2). Considering pyroloids found at

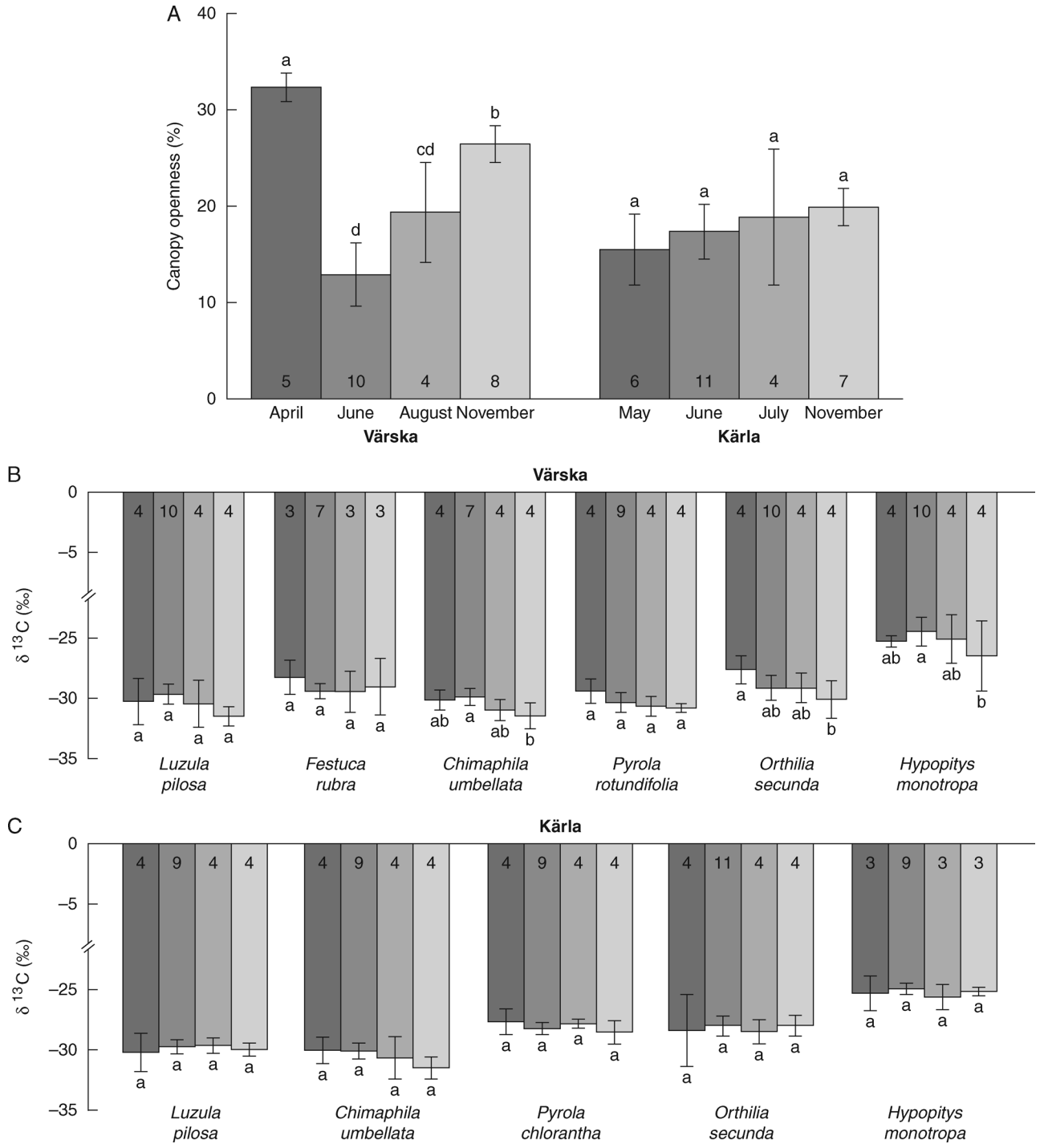


FIG. 3. Canopy openness (A) and changes in $\delta^{13}C$ mean values over the 2009 growth season at Värska (B) and Kärla (C). Successive samplings were in April, June, August and November at Värska and in May, June, July and November at Kärla (for exact dates see Materials and methods section; numbers in columns are sample numbers). For a given species at a given site, # indicates MX nutrition based on its isotopic signature (see Fig. 1). Different letters denote different means according to Tukey's HSD test; bars represent 95 % confidence intervals of the mean.

more than one site (*C. umbellata*, *O. secunda* and *P. chlorantha*) and pooling data from all plots at all sites, $\varepsilon^{13}C$ again did not show any response to light level (not shown). Finally, $\delta^{15}N$ did not respond to light in any species (Supplementary Data Table S3). Thus, pyroloids did not differ in light response from autotrophic species.

DISCUSSION

Our results, based on an analysis of bulk leaf ^{13}C enrichment of 405 samples, show that mixotrophy in pyroloids does not respond to age or light level. Our approach was very conservative, since we did not make any correction in statistical tests for multiple comparisons (any such correction would lower all

TABLE 2. *Summary of linear regressions for bulk leaf $\delta^{13}C$ values against light level at the three studied sites. When P<0·05, the values of R^2 (left) and P (right) are given. + and − indicate a positive and negative relationship, respectively. When P>0·05, an indication of R^2 is given as follows: +, R^2>0·1 with a positive correlation; −, R^2>0·1 with a negative correlation; 0, R^2<0·1. The P values are not corrected for multiple comparisons. H. monotropa at Värska was too rare to test for linear regression*

Nutrition	Species	Mändjala	Värska	Kärla
Autotroph	*Galium verum*	+0·57; 0·05		
	Hieracium umbellatum	+0·79; 0·018		
	Luzula pilosa		+0·47; 0·03	0
	Festuca rubra		+	
	Lycopodium clavatum		+	
Pyroloid	*Moneses uniflora*	+		
	Chimaphila umbellata	+	+	+
	Pyrola chlorantha	+0·47; < 0·001 #		+ #
	Pyrola rotundifolia		+0·47; 0·041	
	Orthilia secunda	+0·27; 0·01#	+0·29; 0·017	0#
Mixotroph	*Epipactis atrorubens*			−0·5; 0·034
Mycoheterotroph	*Hypopitys monotropa*	0		0

#Species for which isotopic signature denotes mixotrophic nutrition at this site (see Fig. 1)

probabilities and reveal even less signal). We discuss these findings below in the complex framework of evolution of mixotrophy in pyroloids (Lallemand *et al.*, 2016).

A phylogenetic signal for mixotrophy in pyroloids

Only *O. secunda* and *P. chlorantha* displayed MX nutrition, with estimated heterotrophy levels around 30–40 %. Yet the former species was not MX at Värska, joining the list of studies supporting MX nutrition at one, but not all sites for a given pyroloid (e.g. Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007; Hynson *et al.*, 2015; Fig. 1 and our unpublished data). The reasons for this are unclear, but may fall into three non-exclusive categories. First, some populations may simply have different genetic backgrounds, entailing variable MX abilities. Second, different environments may entail different heterotrophy levels, although our results discussed here do not support any impact of light conditions at least. Similarly, we investigated the impacts of soil C, N and phosphorus (P) contents in all three plots studied, but again this did not correlate with heterotrophy level (data not reported here). Finally, all associated fungi, even if mostly ectomycorrhizal (Tedersoo *et al.*, 2007), may not provide organic resources enriched in ^{13}C. A statement similar to the latter point was recently made in orchids, where fungal associates with $\delta^{13}C$ similar (or close) to that of photosynthetic resources are suspected to lead to hidden C inflow in orchids (Selosse and Martos, 2014). Furthermore, our results at Värska, where *C. umbellata*, *O. secunda* and *P. rotundifolia* were not MX, are somewhat at odds with previous analyses of populations from the same forest (Tedersoo *et al.*, 2007), where these species were MX (Fig. 1). Two factors may explain this: first, the sampling at slightly different subsites may drive these differences, but we disfavour this explanation since subsites were distributed over the same area in both years; second, unknown factors (biotic or fungal) may have changed over 6 years in this population, driving different heterotrophy levels. The latter explanation also strongly argues against the first reason (different genetic backgrounds) proposed above for variability in mixotrophy level between populations, since the samples in our two successive studies likely belong to the same genetic population for the three investigated species.

Our results further support the previous report suggesting a phylogenetic trend (Matsuda *et al.*, 2012). The genera *Orthilia* and *Pyrola*, on the one hand, and *Chimaphila* and *Moneses*, on the other hand, cluster together in pyroloid phylogeny (Freudenstein, 1999; Braukmann and Stefanović, 2012; Matsuda *et al.*, 2012; Lallemand *et al.*, 2016). While $\delta^{13}C$ analyses often reveal MX nutrition in the first cluster, the second less often displays such trends (although one can claim that this isotopic clue alone does not fully reject MX nutrition; see e.g. Hynson *et al.*, 2015). Yet exceptions to this pattern (Fig. 1) suggest complex evolutionary patterns (Lallemand *et al.*, 2016) and/or ecological variations. All these genera start their lifecycle through MH germination (Hashimoto *et al.*, 2012; Hynson *et al.*, 2013*b*), with MH seedlings enriched in ^{13}C (Johansson *et al.*, 2015), so that, assuming that fungal partners are the same at all ages, $\delta^{13}C$ values in adults suggest that *Chimaphila* and *Moneses* revert to autotrophy (or to undetectable levels of MH nutrition).

No evidence for change in mixotrophy over pyroloid leaf lifespan

Our default expectations based on MX orchids (Roy *et al.*, 2013; Gonneau *et al.*, 2014) were that (1) young leaves formed at the beginning of the growth season display a strong contribution of fungal C enriched in ^{13}C, and (2) with the rise in photosynthetic activity, a higher contribution of photosynthetic C reduces ^{13}C enrichment later in the growth season. However, we found no evidence for a change in heterotrophy level over leaf lifespan in bulk leaf tissues of MX pyroloids. Over the growth season, N content did not vary, but $\delta^{13}C$ values decreased somewhat in some pyroloids, suggesting a possible shift to higher autotrophy (because photosynthates are more ^{13}C-depleted than fungal resources). Variations in canopy openness are unlikely to explain this, since small increases should normally increase $\delta^{13}C$ (Farquhar *et al.*, 1989; see below effect of light on $\delta^{13}C$). However, the $\delta^{13}C$ decrease was rarely supported (i.e. *O. secunda* and *C. umbellata* at Värska only), and $\varepsilon^{13}C$ values

did not show a similar decrease, meaning that autotrophic references displayed similar behaviours. Thus, this more likely reflects the usual shift from using plant reserves (which are somewhat ^{13}C-enriched; Cernusak *et al.*, 2009; Gonneau *et al.*, 2014) during leaf ontogenesis to locally produced photosynthates, which is the common trend over a growth season even in autotrophs (Roy *et al.*, 2013). Indeed, the observed δ^{13}C shift over the growth season was of much lower amplitude than that of MX orchids (compare with Roy *et al.*, 2013; Gonneau *et al.*, 2014).

In the longer term, our comparison of young (<2 months) and old (>12 months) leaves did not reveal any shift in δ^{13}C values related to age, but in this case N content was lower in ageing leaves (no surrounding autotrophic species provided a relevant comparison as they do not have evergreen, sufficiently lasting leaves). Since we did not observe such a decrease in N content over the growth season, the decrease seems to occur at the beginning of the second year. However, given the stability of δ^{13}C values, this decrease in N content is not related to a variation in heterotrophy level. Indeed, such a diminution in N is reported in evergreen species, where leaves reach their highest N (and mineral) content and activity at beginning of their first year (e.g. Jonasson and Chapin, 1985; Hunter and Leehowiez, 1992), before a decrease starts, which can be partly explained by a progressive senescence process. Most importantly, even if unrelated to MX nutrition, the variations we observed in leaf N content demonstrate that our sampling can reveal developmental changes in evergreen leaves.

The absence of developmental shift in heterotrophy level for MX pyroloids suggests a constant contribution of fungal C to bulk leaf resources throughout the leaf lifespan. An alternative explanation is a bias due to the fact that abundant polymers with low turnover that are synthesized early in development, namely lignin and cellulose from the cell wall, buffer any further ^{13}C variation (a masking effect that does not apply to N content because they do not contain N). Indeed, compared with the forest orchid leaves analysed by Roy *et al.* (2013) and Gonneau *et al.* (2014), pyroloid leaves are much thicker and richer in sclerenchyma and cuticles. Such a possibility had already been considered by Hynson *et al.* (2012), who restricted their analyses to leaf sugars to avoid such bias, and revealed trends not observed in bulk tissues. However, in the latter study, sugars and bulk leaf δ^{13}C did not vary over the 44 d of their control condition (in which no experimental treatment was applied). Thus, our results are congruent with those of Hynson *et al.* (2012), although our monitoring was five times longer. This suggests a stable contribution of fungal and photosynthetic C to bulk leaf biomass over the growth season, and that leaf age is not an important parameter when sampling pyroloid leaves for isotopic studies.

No response of mixotrophy to light level in pyroloids

We followed an order of magnitude of canopy openness (Supplementary Data Fig. S4) over the different sites, where the monodominance of evergreen *Pinus* in the canopy limits its seasonal light changes, especially in the weeks preceding our sampling. Measuring light availability by canopy opening takes into account the variations in solar positions and light flecks that can occur in the forest, more than instantaneous measurements of light values, but it limits comparisons with other studies dealing with spontaneous variations in the light environment (e.g. Preiss *et al.*, 2010; Matsuda *et al.*, 2012; Gonneau *et al.*, 2014; Liebel *et al.*, 2010) or experimental shading (Hynson *et al.*, 2012). Most autotrophic plants responded in the expected way, i.e. light and δ^{13}C decreased jointly, thanks to two mechanisms. Firstly, shade favours stomata opening and thereby improves equilibration of [$^{13}CO_2$] and [$^{12}CO_2$] in the stomatal chamber; secondly, low photosynthesis in the shade depletes $^{12}CO_2$ in the stomatal chamber in a slow process that can be compensated for by diffusion of external $^{12}CO_2$. In all, better fractionation produces photosynthates more depleted in ^{13}C (Farquhar *et al.*, 1989; Preiss *et al.*, 2010; Liebel *et al.*, 2010). In Kärla, the MX orchid *E. atrorubens* showed the opposite behaviour, as expected in MX plants: δ^{13}C increased with canopy closure since shaded plants contain relatively more ^{13}C-enriched fungal C, as reported in MX orchids (Preiss *et al.*, 2010; Gonneau *et al.*, 2014) and in the Japanese MX species *P. japonica* (Matsuda *et al.*, 2012).

Strikingly, all pyroloids responded to light like autotrophic plants, whatever their nutritional type as inferred from δ^{13}C, i.e. considered MX or autotrophic. The absence of dynamic response to light variation in MX pyroloids can be interpreted in several ways. First, the light reduction entailed by canopy closure may be insufficient to affect photosynthesis in these dark-adapted plants, but the observed response of the MX species *E. atrorubens*, which is also adapted to similar conditions, rejects this possibility (and, at least, emphasizes the difference between orchids and pyroloids). Second, *O. secunda* and *C. umbellata* have metric rhizomes (Henderson, 1919), and long-distance C exchange within the plant may homogenize the photosynthates over distances larger than the light patches. On the one hand, source organs such as leaves are unlikely to receive carbon, but on the other hand this could explain the discrepancy with *P. japonica*, a non-rhizomatous species that responds to light as expected for MX plants (Matsuda *et al.*, 2012). Third, Matsuda *et al.* (2012) suggested that pyroloids may react to shaded conditions by changing mycorrhizal partners: if such a change involves fungi whose biomass is less ^{13}C-enriched, then any higher input of fungal resources would go undetected by ^{13}C enrichment. As mentioned above, increasing indirect evidence suggests that some plants rely on fungal C that does not change δ^{13}C (Selosse and Martos, 2014), such as some green temperate orchids (Gebauer *et al.*, 2016). The fact that mycorrhizal fungi may provide resources with low δ^{13}C in some sites would also explain (1) the variable δ^{13}C for a given pyroloid species among sites, here (for *O. secunda*) and in other studies (Tedersoo *et al.*, 2007; Hynson *et al.*, 2015), and (2) the fact that δ^{13}C values of some pyroloids are even below these of surrounding autotrophic species (Hynson *et al.*, 2015; Johansson *et al.*, 2015). A last possibility is that pyroloids present a fixed, non-dynamic mixing ratio of fungal and photosynthetic C, a static mixotrophy we explore in the conclusion below.

Conclusions – a 'static' mixotrophy in pyroloids?

We cannot exclude the possibility that the gradients analysed or our sampling effort may have been too low for any effect to

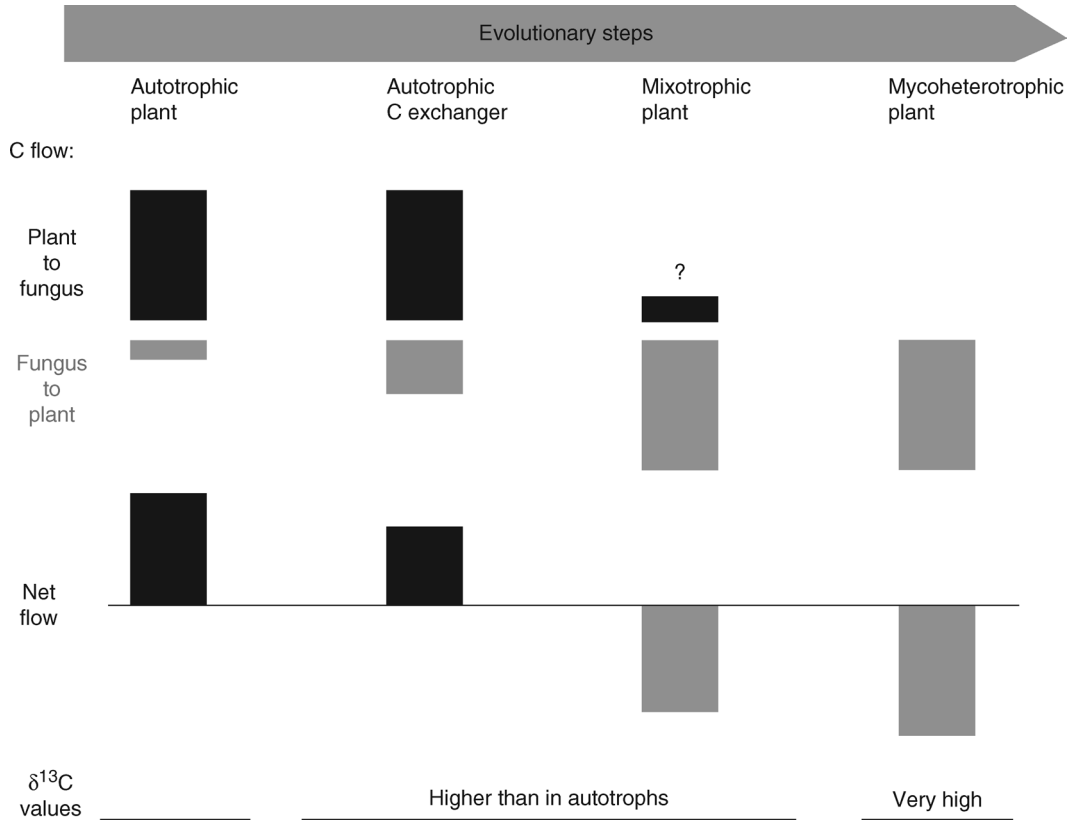


FIG. 4. A model of evolution to mixotrophy by way of an overlooked 'C-exchanger' autotrophic status that may explain the absence of dynamic response to light and ageing in pyroloids with elevated $\delta^{13}C$.

be seen. Yet the fact that *E. atrorubens* responds to the investigated light gradient suggests that MX pyroloids differ from MX orchids in light response. We faced a static response of pyroloids for the two investigated parameters (ageing and light level), although changes observed over 6 years at Värska or for a given species between sites (see above) suggest that some unknown parameters may affect heterotrophy level in pyroloids. The difference from MX orchids, which change heterotrophy level with ageing or light level, suggests a different meaning of MX/mixotrophy in pyroloids compared with orchids.

It is often overlooked that an elevated bulk $\delta^{13}C$ does not mean that there is a *net* flow of C from fungus to plant: it simply means that there is a *large raw flow*, so that a large part of the plant's C is of fungal origin. Yet some species may gain large quantities of fungal C as a by-product of a specific function of their mycorrhizal exchange, which they reward by even more photosynthetic C: thus, despite ^{13}C enrichment, such 'C-exchangers' would stay globally autotrophic, and not really MX (Fig. 4). It is clear in MX orchids (Hynson *et al.*, 2013*a*; Selosse *et al.*, 2016) that a net flow exists: this is demonstrated by albinos, i.e. achlorophyllous variants found in some MX orchid populations (Selosse *et al.*, 2004; Abadie *et al.*, 2006; Roy *et al.*, 2013; Gonneau *et al.*, 2014). The survival of such albinos (Shefferson *et al.*, 2016) obviously demonstrates a net C flow from fungus to plant. Strikingly, albinos were never reported in pyroloids, and it may be that pyroloids are such C-exchangers, where fungal C contributes poorly to the plant C budget.

Such C-exchangers may have a constant contribution of fungal C to their biomass that at least does not respond to light or phenology. Putting them in an evolutionary framework, C-exchangers are obviously predisposed to evolve into true MX species, with a net inflow from the fungus (like e.g. *P. japonica*; Matsuda *et al.*, 2012), or even into full MH species (like *P. aphylla*; Hynson and Bruns, 2009; Fig. 4). But their high level of C trade with fungal partners, which affects their $\delta^{13}C$ values, is not meaningful in terms of C nutrition and thus not responsive to ontogenetic time or environmental changes. This reasoning at least emphasizes that (1) multiple strategies can affect $\delta^{13}C$ values, and (2) ^{13}C enrichment does not necessarily mean net exploitation of fungal C.

More physiological analyses in pyroloids, focusing especially on circulating carbon to take into account the polymers with low turnover (Hynson *et al.*, 2012), are now required for further understanding of the fungal C dynamics within these plants. Fungal partners and their isotopic signatures also deserve further investigation to test whether any hidden C flow occurs in pyroloids displaying 'autotrophic' $\delta^{13}C$ values. Subsequent studies may clarify how, despite some convergences with MX orchids in physiology (Tedersoo *et al.*, 2007) and development (Hashimoto *et al.*, 2012; Hynson *et al.*, 2013*b*), pyroloids represent a quite distinct way of using fungal C, and do not detectably use C gained from their mycorrhizal fungi to replace photosynthetic C.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: mean values of total N content in the three sites studied. Figure S2: changes in $\varepsilon^{13}C$ mean values over the 2009 growth season for the pyroloids found at Värska (a) and Kärla (b). Figure S3: average difference between old and young leaves from the same plot for (a) $\delta^{13}C$, (b) $\delta^{15}N$ and (c) N content. Figure S4: variability of light conditions in the three studied sites. Table S1: raw dataset used in this study. Table S2: summary of linear regressions of bulk leaf $\varepsilon^{13}C$ against light level in the three studied sites. Table S3: summary of linear regressions for bulk leaf $\delta^{15}N$ values against light level in the three studied sites.

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