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Why do mixotrophic plants stay green? A comparison between green and achlorophyllous orchid individuals in situ

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Abstract. Some forest plants adapt to shade by mixotrophy, i.e., they obtain carbon both from photosynthesis and from their root mycorrhizal fungi. Fully achlorophyllous species using exclusively fungal carbon (the so-called mycoheterotrophic plants) have repeatedly evolved from such mixotrophic ancestors. However, adaptations for this evolutionary transition, and the reasons why it has happened a limited number of times, remain unknown. We investigated this using achlorophyllous variants (i.e., albinos) spontaneously occurring in *Cephalanthera damasonium*, a mixotrophic orchid. In two populations, we compared albinos with co-occurring green individuals in situ. We investigated vegetative traits, namely, shoot phenology, dormancy, CO₂ and H₂O leaf exchange, mycorrhizal colonization, degree of mycoheterotrophy (using ¹³C abundance as a proxy), and susceptibility to pathogens and herbivores. We monitored seed production (in natural or experimental crosses) and seed germination. Albinos displayed (1) more frequent shoot drying at fruiting, possibly due to stomatal dysfunctions, (2) lower basal metabolism, (3) increased sensitivity to pathogens and herbivores, (4) higher dormancy and maladapted sprouting, and, probably due to the previous differences, (5) fewer seeds, with lower germination capacity. Over the growing season, green shoots shifted from using fungal carbon to an increasingly efficient photosynthesis at time of fruiting, when fungal colonization reached its minimum. Conversely, the lack of photosynthesis in fruiting albinos may contribute to carbon limitation, and to the above-mentioned trends. With a 10³× fitness reduction, albinos failed a successful transition to mycoheterotrophy because some traits inherited from their green ancestors are maladaptive. Conversely, mycoheterotrophy requires at least degeneration of leaves and stomata, optimization of the temporal pattern of fungal colonization and shoot sprouting, and new defenses against pathogens and herbivores. Transition to mycoheterotrophy likely requires progressive, joint evolution of these traits, while a sudden loss of photosynthesis leads to unfit plants. We provide explanations for the evolutionary stability of mixotrophic nutrition and for the rarity of emergence of carbon sinks in mycorrhizal networks. More broadly, this may explain what prevents the emergence of fully heterotrophic taxa in the numerous other mixotrophic plant or algal lineages recently described.

Key words: autotrophy; dormancy; fruit set; herbivory; mycorrhizae; Neottieae; orchids; parasitic load; partial mycoheterotrophy; plant phenology; seed germination.

INTRODUCTION

Eukaryotes repetitively evolved photosynthesis by acquisition of plastids through endosymbiosis with symbiotic algae (Keeling 2010). In further evolution, several lineages reverted to heterotrophy: Full plastid loss occurred in oomycetes, ciliates (Reyes-Prieto et al. 2008), and trypanosomids (Hannaert et al. 2003), while non-photosynthetic plastids were retained in the para-

sitic Apicomplexa (Lim and McFadden 2010) and some free-living euglenids (Gockel and Hachtel 2000). Reversion to heterotrophy also occurred in terrestrial plants, which retain non-photosynthetic plastids too, thanks to two strategies (Krause 2008). Some achlorophyllous plants are directly parasitic on other plants: Such heterotrophy evolved at least 11 times in angiosperms, representing 1% of the species diversity in this taxon (Westwood et al. 2010). Some other heterotrophic plants receive carbon from fungi colonizing their roots, with which they form mycorrhizal symbioses: The so-called mycoheterotrophic (MH) species also arose repeatedly, with 400 MH species in eight plant families (Björkman 1960, Leake 1994, Rasmussen 1995). MH nutrition is especially frequent among orchids, one of the biggest

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angiosperm families, where it arose at least 30 times (Freudenstein and Barrett 2010), and the current work focuses on the evolutionary transition to MH nutrition in orchids.

Although the steps for the acquisition of plastids are well studied (Keeling 2010), with some known intermediate steps in this process (e.g., Okamoto and Inouye 2005), steps for the loss of photosynthesis are less well known, perhaps because fewer models are available. Some euglenid species have been reported to form achlorophyllous clones, e.g., in darkness or on high-sugar medium (Rosati et al. 1996), with deleted plastidial genomes (Heizmann et al. 1981). Such variants may be the source of achlorophyllous euglenid species (Gockel and Hachtel 2000), and of the trypanosomids, a parasitic taxon phylogenetically close to euglenoids that lost plastids (Hannaert et al. 2003). Few other species display both autotrophic and fully heterotrophic individuals suggestive of a transition step to heterotrophy. Among terrestrial plants at least, mutations leading to achlorophyllous individuals (i.e., albinos) are frequent, due to the numerous nuclear and plastidial genes required for proper assembly of the photosynthetic apparatus (Barkan 2011). For example, a mutagenesis experiment on *Arabidopsis thaliana* Feldmann (1991) produced 1.2% albino mutants. Nevertheless, except under specific laboratory conditions, such mutants do not survive.

As a remarkable exception, some perennial orchid species possess two permanent phenotypes: The most frequent one is chlorophyllous, but some rare individuals are achlorophyllous (albino), surviving by MH nutrition (Appendix A). This mainly occurs in the Neottieae orchid tribe, in perennial *Epipactis* species (Rener 1938, Salmia 1986, Selosse et al. 2004, Stöckel et al. 2011) and *Cephalanthera* species (Rener 1938, Maiold and Weber 1950, Julou et al. 2005, Abadie et al. 2006). As compared with green plants, albinos have an elevated $^{13}\text{C}:^{12}\text{C}$ ratio (Julou et al. 2005, Abadie et al. 2006), exactly as MH plants (Gebauer and Meyer 2003, Trudell et al. 2003, Roy et al. 2009a), reflecting the high $^{13}\text{C}:^{12}\text{C}$ ratio of the mycorrhizal fungi providing carbon. Moreover, albinos have lower C:N and higher $^{15}\text{N}:^{14}\text{N}$ ratios than green plants and fungi, reflecting a position in the trophic chain above fungi, exactly as MH species (Trudell et al. 2003, Julou et al. 2005, Abadie et al. 2006). These albinos are relevant models for the evolutionary transition to heterotrophy for two additional reasons. First, they are phylogenetically close to fully MH species within Neottieae (Roy et al. 2009a), which occur in the genera *Cephalanthera* (Pedersen et al. 2009), *Aphyllorchis* (Roy et al. 2009a), and *Neottia* (Selosse et al. 2002). Second, they belong to species where green individuals are themselves partly MH, as shown by their $^{13}\text{C}:^{12}\text{C}$ and $^{15}\text{N}:^{14}\text{N}$ ratios, which are intermediate between those of full autotrophs and MH (or albino) plants (Gebauer and Meyer 2003, Bidartondo et al. 2004, Julou et al. 2005, Abadie et al. 2006).

Moreover, these green individuals show reduced photosynthesis, due to low light environments (Julou et al. 2005) and/or reduced efficiency of their photosynthetic apparatus (Girlanda et al. 2006, Cameron et al. 2009). Thus, part of their biomass is derived from mycorrhizal fungi, in a nutrition called partial mycoheterotrophy or mixotrophy (MX; see Selosse and Roy 2009 for review). MX species also occur out of Neottieae, and interestingly, MH species are often phylogenetically nested within them, e.g., in the orchid genera *Platanthera* (Yagame et al. 2012) and *Cymbidium* orchids (Moto-mura et al. 2010), and in Ericaceae (Tedesoo et al. 2007). Thus, MX nutrition is often considered as a predisposition to MH evolution (Selosse and Roy 2009), and albinos furnish relevant candidates for a further step during transition to full heterotrophy.

However, the biology of albinos remains poorly documented, and most data derive from few European populations. Salmia (1986, 1988, 1989a, b) investigated an *Epipactis helleborine* population in Finland where albinos sometimes undergo greening (and vice versa; Salmia 1989b): Albinos showed a reduction of shoot height, leaf size, and stem thickness, together with a lower number of flowers and fruits, which were produced later, although albino fruits and seeds are similar to green ones, but little statistical support is available for these trends (Salmia 1989b). In *Cephalanthera* species, the phenotype remains stable (Tranchida-Lombardo et al. 2010) for up to 14 years (Abadie et al. 2006), and in some populations variegated individuals with intermediate autotrophy levels have been found (Stöckel et al. 2011). In three investigated *Cephalanthera damasonium* populations, albinos did not aggregate spatially and were not genetically closer to other albinos than to surrounding green individuals (Tranchida-Lombardo et al. 2010). Thus, they represented neither a unique clone, nor a localized phenotype. No significant morphological difference between albinos and green individuals was found in a French *C. damasonium* population (except that albino leaves tended to be smaller; Julou et al. 2005) or in an Estonian *C. longifolia* population (except that albinos had shorter shoot height one year; Abadie et al. 2006), but the low albino number again limited statistical analyses. However, albino shoots appeared later and often dried earlier in the summer, before fruit ripening, and in *C. damasonium*, albinos displayed higher stomatal conductance and lower CO_2 gas production in the dark (Julou et al. 2005). Fungal mycorrhizal associates did not differ in both phenotypes for species of *Epipactis* (Salmia 1989a, Selosse et al. 2004) and *Cephalanthera* (Julou et al. 2005, Abadie et al. 2006), but fungal colonization was denser in albino roots. Lastly, albinos less often produced shoots in two consecutive years as compared with green individuals (Salmia 1986, Tranchida-Lombardo et al. 2010), suggesting a shorter life span and/or a higher frequency of “dormancy” (a stage of underground life without aboveground shoots for one or more years;

Rasmussen 1995, Shefferson 2009). Moreover, albinos occur in few populations where they remain rare and increase in number more slowly than green individuals (Salmia 1986, Abadie et al. 2006, Tranchida-Lombardo et al. 2010). Albino individuals, therefore, look like a somewhat imperfect transition to MH nutrition: They can be seen as ecological equivalents to mutants in genetics, i.e., their dysfunctions may suggest what would make MH nutrition successful. Albinos offer fascinating models for comparing the physiology of MX and MH individuals within similar genetic and environmental backgrounds.

The reasons for albino rarity in populations and lower survival remain unclear, although previous studies have pointed to some possible factors. We therefore undertook a multidisciplinary comparative analysis of albino and green phenotypes in *C. damasonium* to obtain a global view of the albino syndrome in natura for this model species. We investigated a newly discovered population with numerous phenotypically stable albinos (up to $n = 101$ in 2007) and no variegated individuals, at Montferrier, near Montpellier, in southern France (Tranchida-Lombardo et al. 2010). Whenever possible, studies were duplicated in the smaller northern French population previously analyzed by Julou et al. (2005) at Boigneville near Paris. We report here experimental data and observations obtained over up to three years, allowing a comparative analysis of vegetative and reproductive features between phenotypes. Concerning plant vegetative life, we considered below- and above-ground traits such as shoot development and survival, susceptibility to pathogens, herbivory and drying, carbon nutrition throughout the growing season (fungal vs. photosynthetic origin), as well as mycorrhizal colonization. Concerning reproduction, we investigated flowering, fruiting (in natural or experimental crosses), and early seed germination. Finally, we discuss the diverse factors explaining the lower albino fitness and assemble them in a general view to suggest what could a contrario make an emerging MH plant successful, unraveling important candidate adaptations to MH life.

METHODS

Study species and sampling sites

Cephalanthera damasonium is an orchid species with MX nutrition (Julou et al. 2005, Cameron et al. 2009, Preiss et al. 2010, Stöckel et al. 2011) and autogamous mating (Scacchi et al. 1991, Micheneau et al. 2010) that sprouts one or more shoots per year from perennial rhizomes, also bearing the mycorrhizal roots (Rasmussen 1995). The study was performed at Montferrier (Mon) near Montpellier (43°39' N, 3°51' E; Tranchida-Lombardo et al. 2010), and partly replicated in another *C. damasonium* population at Boigneville (Boi) near Paris (2°21' E, 48°21' N; Julou et al. 2005). Mon is a 15-year-old poplar plantation on a former vineyard where *Populus nigra* is planted every 7 m, with young *Quercus pubescens* growing inbetween, and a very homogeneous

light environment (Appendix A), limiting the physiological variations reported for MX plants when the light environment varies (Preiss et al. 2010). The Mon understory is covered by grasses, *Clematis vitalba*, *Rubia peregrina*, and *Hedera helix*, and is sometimes flooded by the Lez, a nearby river, in spring and fall. The young, but denser *Quercus robur* L. and *Corylus avellana* L. forest at Boi is described in Julou et al. (2005). At both sites, the soil is formed on limestone, but thick and rich in clay, covered by a calcic mull. The Mediterranean climate at Mon (mean annual temperature measured at the nearest meteorological station to Montpellier was 14.2°C; coldest month, 5.9°C; warmest month, 28.4°C) is hotter than the temperate oceanic climate at Boi (at Bretigny-sur-Orge, 10.8°C, 6.4°C, 15.2°C, mean, coldest, and warmest month, respectively) and drier (the precipitations are 750 vs. 940 mm/yr at Mon and Boi, respectively, but spread throughout the year at Boi vs. over 1–2 weeks in spring and fall at Mon). Destructive samplings were limited to minimize the impact of this study on the two populations.

Phenological and vegetative morphological traits

In 2007, a detailed phenological survey was conducted over the entire growing season at Mon by visiting the site every week from the beginning of March till the end of June (fruit opening). For each shoot we recorded the time of appearance, first flower opening (flowering), first fruit development (fruiting), and first drying of leaves and shoots if any; missing shoots were noted as well. The same survey was carried out every two weeks in 2006 and 2008 at Mon, as well as in 2007 and 2008 at Boi, and all individuals were marked with an aluminum label. For all these years, morphological parameters were recorded in mid-June, related to the shoots (height and number per individual, assuming that shoots growing <5 cm away issued from the same rhizome; our own observations at Mon), leaves (leaf number, length, and width of first [lower] developed leaf), flowers (number of flowers), and fruits (number of fruit present or aborted; length, width, fresh and dry mass at ripening). To avoid excessive destruction, only leaves collected for isotope analysis at Mon (see *Carbon (C) and nitrogen (N) isotope compositions* section) were used to measure the specific leaf area (SLA, m²/kg), by measuring their area at harvesting and weighing them after 3 d at 65°C. To investigate stomatal density, leaves ($n = 10$ per phenotype) were collected in 2006 at Mon and their cuticle was separated with thin tweezers. For each leaf, 10 counts of stomata number were performed at 400× magnification, randomly on the leaf. To measure leaf and cuticle thickness, 1 cm² from each of these leaves was collected at the edge, fixed in 2.5% (volume by volume [v/v]) glutaraldehyde in a 10 mmol/L Na-phosphate buffer (pH 7.2), and embedded as in Roy et al. (2009b). Then, transverse sections (10 μm) were cut with a microtome, differentially stained with a mixture of safranin and Fast Green FCF dye, rinsed with distilled water, and

observed under the light microscope at a 400× magnification. For transmittance measurements, whole shoots were transferred within 20 min to the laboratory in May 2009. We used an AvaSpec-2048 with a halogen light source HL-2000 (Avantes, Vanves, France) to measure light reflected by the leaf (l), as well as by dark (d) and white references (w) from the manufacturer. Measurements were performed every 0.3 nm between 380 nm and 780 nm, on $n = 8$ leaves from $n = 4$ shoots of each phenotype. Leaf reflectance was established as $(l - d)/(w - d)$, and mean values were computed over the whole wavelength range for each leaf, and then among leaves.

Leaf pigments

Leaves from green and albino individuals were collected in 2006 from different plants at Mon ($n = 5$ per phenotype) and Boi ($n = 4$ per phenotype). They were measured, weighed, transferred to carbonic ice for transport to the laboratory, and kept frozen at -80°C until use. Entire leaves were ground in liquid nitrogen and pigments were extracted in 80% (v/v) acetone in water on ice. Absorbance spectra were obtained with an Aminco spectrophotometer (Olis, Bogart, Georgia, USA) to measure quantities of carotenoids and chlorophyll a and b , as in Julou et al. (2005). Carotenoids were analyzed by high-performance liquid chromatography as in Everroad et al. (2006), on two leaves per phenotype at Mon and one at Boi.

Leaf gas exchange and temperature

Instantaneous measurements of CO_2 and water gas exchanges were performed in 2006, 2007, and 2008 at Mon at photosynthetic photon flux density (PPFD) = 0 and PPFD = $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Gas exchange responses to continuous PPFD and vapor pressure deficit (VPD) variations were measured in 2007 and 2008 (May and June) at Mon, and in June 2006 and 2007 at Boi. Measurements used an infrared gas analyzer (LI 6400, LI-COR, Lincoln, Nebraska, USA) between 08:00 and 10:00 hours (Local Solar Time), to avoid high temperature and stomata closure. The CO_2 concentration entering the leaf chamber was maintained constantly close to the atmospheric CO_2 concentration ($\sim 380 \mu\text{mol} \text{CO}_2/\text{mol}$) by using a 50 L buffer recipient located 2 m away and connected to the reference inlet of the apparatus.

At PPFD = 0 and $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (temperature = 25°C), measurements were made after 20 min of leaf adaptation to the chamber conditions by checking for H_2O and CO_2 reference stability. Each measurement corresponded to a mean of 10 records on the same leaf in a single minute. For response curves to VPD and PPFD, each value was recorded after equilibration for 20 min before the first measurements, and then for 5 to 10 min between the following measurements to allow leaf adaptation to the new conditions. VPD variations were artificially obtained by increasing ambient air dryness over CaCl_2 , and light variations were obtained by

modulating the intensity of the LED light source (LI-6400-02B; LI-COR). Natural variations of leaf temperature during the day were followed with a Minisight Plus infrared thermometer (Optris, Berlin, Germany) for two green leaves and two albino leaves enclosed in a LI-COR measurement chamber from 9:00 to 15:00 hours on 24 and 25 June 2007; outside air temperature was simultaneously measured, but not experimentally manipulated.

Carbon (C) and nitrogen (N) isotope compositions

Seasonal variations of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in green *C. damasonium* leaves were monitored at Mon by collecting leaves in 2007 at the end of March (shoot appearance), the end of April (full shoot vegetative development), mid-May (flowering time), the end of May (fruit formation), mid-June (one-month-old fruits), and the end of June (beginning of seed dispersal). At each time, we sampled five leaves of green *C. damasonium*, as well as of *Clematis vitalba* and *Rubia peregrina* as reference for autotrophic perennials (only leaves of the year were sampled), all from the same location. To avoid destroying too many albinos, only $n = 5$ leaves were collected at the end of April and mid-June as an MH reference. All leaves were collected at same light level and <30 cm from the ground, to avoid $\delta^{13}\text{C}$ variations in green tissues due to higher photosynthetic rate or to CO_2 resulting from soil respiration, respectively. Samples were dried at 65°C for 72 h and handled as in Tedersoo et al. (2007) at the Technical Platform of Functional Ecology (OC081, INRA, Nancy, France) to measure total N, C:N, and abundances of ^{13}C and ^{15}N that were expressed as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in conventional δ notation (in ‰), respectively on the VPDB (Vienna Pee Dee Belemnite) or atmospheric N_2 scale. The standard deviation of the replicated standard samples ($n = 13$) was 0.032‰ for ^{13}C and 0.177‰ for ^{15}N . Heterotrophy rate was calculated using a two-source mixing model (Gebauer and Meyer 2003), with mean $\delta^{13}\text{C}$ values of autotrophic species and albino plants as respective references for autotrophic and MH (100% fungus-derived) biomass.

Assessment of colonization by mycorrhizal fungi, pathogens, and herbivores

To assess mycorrhizal colonization over the growing season, two plants per phenotype were harvested on 15 January, 15 March, 15 May, and 15 July in 2008. The repetition number was limited to preserve the population, although this limits the statistical analysis. Roots were washed and thin hand-cut sections were investigated every centimeter, starting from the rhizome, under a 500× magnification light microscope, to look for intracellular hyphal coils (pelotons). Sections were divided into four colonization categories defined as follows: c0, no pelotons in the section's cells; c1, pelotons in 1–30% of the cells; c2 pelotons in 31–60% of the cells; c3, pelotons in $>60\%$ of the cells (the whole

cortex was often colonized). Thus, mycorrhizal sections were included in categories c1 to c3. Mean percentage of area of root section colonized per phenotype and per sampling time was calculated by averaging the percentage colonization of all investigated sections ($n = 25\text{--}110$) from the two corresponding plant individuals.

Every week in June 2008, we recorded the presence of herbivores feeding on Mon plants; namely crickets, caterpillars, mites, and aphids (the two latter taxa were scored on flowers or fruits, respectively, where they mostly occur), as well as Thomisidae (spiders hunting in inflorescences where they eat pollinators). Herbivore damage (i.e., missing part of the organ with irregular border, not attributable to necrosis) was observed and counted on leaves, flowers, and fruits. Leaf fungal infections were identified and counted as black spots (Appendix B). The two latter analyses were repeated in 2007 and 2008 at Boi. Polymerase chain reaction (PCR) amplification and sequencing of the internal transcribed spacer of the fungal ribosomal DNA (as in Roy et al. 2009a) from black spots at Mon suggest that the causative agent was a *Pseudocercospora* sp. (GenBank accession number HQ395393), a taxon parasitic on many plants including orchids (da Silva and Liparini Pereira 2011), but we did not do an inoculation experiment to verify cause.

Survival and reproductive traits

Dormancy at Mon and Boi was noted when individuals were not recorded in 2007, but formed shoots in 2006 and 2008. To test whether fruit size is a good proxy for seed content, 20 green and 20 albino fruits were collected in June 2008 from different plants just before fruit opening and seed dispersal. Fruit length was measured as the distance between the insertion on the shoot and the sepal scar. Their diameter was measured at mid-distance between shoot and sepal insertion. They were weighed (wet and dry), and after drying, their whole seeds were extracted and weighed. Then, 1 mg of seeds was spread on a Petri dish, and the seeds were counted under a binocular microscope; the number of seeds was extrapolated for the whole fruit and compared with the other fruit parameters.

To assess seed quality, fruits were collected at the end of July 2006 and 2008 at Mon from albino and green individuals (at least $n = 20$ each, from different individuals) for observation of embryo presence (viable seeds). Additionally, 41 albino and 50 green fruits were collected in mid-July 2006 from different individuals to build two seed pools (from albino and green individuals) for sowing. For each pool, 90 mesh bags, each containing ~ 200 seeds enclosed between two 4×4 cm pieces of nylon cloth (50- μm pore size) were prepared as in Van den Kinderen (1995) and dried on silica gel for one month to mimic natural conditions of fruit drying. Mesh bags were buried in mid-August 2006 at a depth of 5–7 cm as in Van den Kinderen (1995), 10 cm apart, within the mother population. Seed packets were recovered 18 months later, in January 2008, washed

with sterile water, and observed under a dissecting microscope (60 \times magnification) on the same day. Seeds were assigned to four states: A, no pro-embryo formed with decayed testa; B, intact testa containing a pro-embryo; C, swollen pro-embryo with cracked testa; and D protocorm with trichomes. Successful germination was defined by stages C and D.

Experimental pollen crosses

Experimental crosses were performed to compare the fruiting of green individuals and albinos in controlled conditions. On 18 May 2008 at Mon, when flowers opened, pollinia from both phenotypes were harvested to pollinate either a flower from the same inflorescence (geitonogamy) or a flower from a different individual. In the latter case, the second individual had either the same phenotype (homogamy) or the opposite phenotype (heterogamy). Inflorescences were protected from further pollinations by a mesh bag closed with cotton and wool strings, and held by a wood stick to avoid shoot injury. Each treatment was performed on five plants, with up to five experimental crosses per plant. Five naturally pollinated inflorescences were also enclosed in mesh bags, as controls for non-biased mating without herbivores. Naturally pollinated plants provided more fruits (8–9 per plant) than experimental crosses (up to four crossed fruits per plant). On 3 July 2008, fruits were collected on remaining shoots and their length and width were measured. Due to unequal maturity at end of this experiment, some fruits had already lost some seeds, making any seed weighing unreliable; nevertheless, since no significant difference was detected in length and width between open and closed fruits in preliminary measurements at Mon in 2007 (not shown), an estimated fruit volume ($\text{length} \times [\text{maximum radius}]^2 \times 3.14$) was used as conservative proxy of the seed content (see *Results*).

Statistical analyses

All statistical tests were performed using R software v. 2.10.1 (<http://www.r-project.org/>). When data were collected on several sites and/or years, data distribution was analyzed by fitting a general linear model (GLM) including year and/or site as a fixed effect. Percentages were logit-transformed before any test. Binomial data were tested against a binomial distribution. For all other data, a Gaussian distribution was used to test our model. For all GLM tests, an ANOVA was performed on the result of the GLM, to extract the significance of the model. Multivariate analyses were not done since data on morphology and germination were not recorded from the same individuals or at the same time. Proportions of herbivory damage, reproductive individuals, and drying symptoms were compared to a binomial distribution using a GLM. Parameters obtained from a few individuals only (e.g., total N, C:N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ studied throughout the season, mean transmittance, absorbance, reflectance, and pigment concentrations) were compared among phenotypes by Mann-Whitney tests, while my-

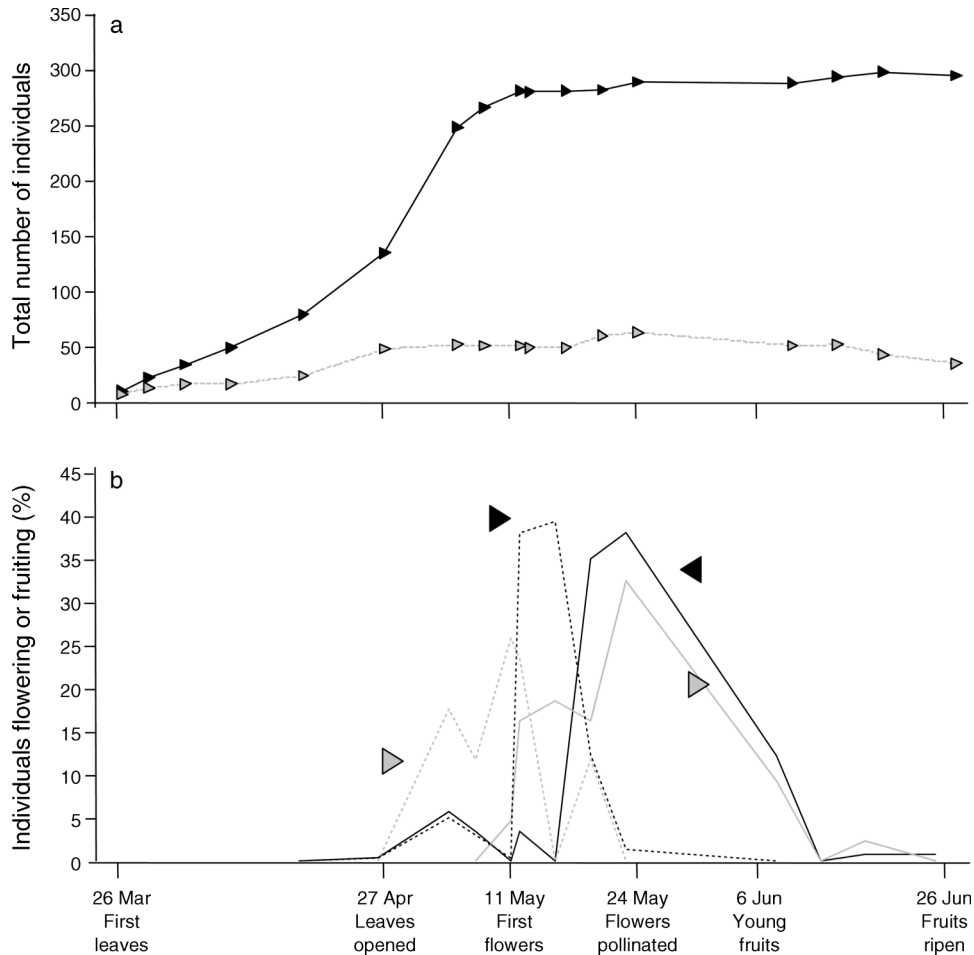


FIG. 1. Aboveground population dynamics of albino *Cephalanthera damasonium* (gray triangles) and green *C. damasonium* (black triangles) at Montferrier (Mon), near Montpellier, France, in 2007 (some individuals show more than one shoot; Table 2). (a) Total number of individuals showing at least one aboveground shoot, and the (b) percentage of individuals flowering (dotted lines) or fruiting (solid lines), with average phenological stage.

corrhizal colonization was compared by date and by phenotype by Kruskal-Wallis tests. Physiological data were compared either for each measuring point between phenotypes by Student's *t* test or for each phenotype between points by ANOVA. Correlations between physiological responses (CO_2 assimilation, stomatal conductance, leaf temperature) and change in environmental parameters (VPD, PPFD, outside air temperature) were tested by a linear regression for each phenotype, and the Pearson *r* value of both correlations were compared by a Fisher *r*-to-*z* test after normalization. Results of artificial crosses measured by fruit volume were compared between phenotypes and treatments by fitting a GLM, and performing pairwise Student's tests. The proportion of mature fruits (not fallen, not aborted) produced from artificial crossings and natural pollination were compared between phenotypes and treatments by fitting a GLM (binomial distribution). Bars on all diagrams and deviations in all tables are standard deviations; all times are given in universal time.

RESULTS

Shoot phenology over the growing season

At Mon in 2007, the first albino and green shoots emerged on 26 March, and new individuals continuously appeared until June (Fig. 1a). Average time of shoot appearance did not differ between phenotypes (Table 1). Since some shoots also disappeared, the net result was a plateau for green individuals and a regression for albinos from May onwards (Fig. 1a). Flowers began to open in May, significantly earlier for albinos that flowered and fruited younger than green individuals (Table 1, Fig. 1b). In some individuals, mostly albinos, leaf drying started in late April (Table 1; Appendix B). Drying symptoms amplified in June and sometimes extended to the whole shoot; shoot drying explained 96% of lack of shoots at the end of June. Albinos entered the process earlier than green individuals (Table 1) and were more affected: Half of albinos were dry before seed dispersal (vs. 7.6% of

TABLE 1. Shoot emergence and reproductive cycle of albinos and green individuals of *Cephalanthera damasonium* at Montferrier (Mon), near Montpellier, France, in 2007.

Plant stage	Green	Albino	Test
Shoot appearance			
First appearance of a shoot (<i>J</i>)	26 March	26 March	
Mean date of shoot appearance	$J + 46 \pm 11$ (26 April)	$J + 31 \pm 8$ (11 May)	A ns
Number of individuals recorded	419	87	
Flowering			
Mean date of first flower opening	$J + 49 \pm 6$ (14 May)	$J + 43 \pm 4$ (8 May)	A*
Mean age at flower opening (d)	24.6 \pm 6.9	15.7 \pm 7.2	A*
Fruiting			
Mean date of first fruit formation	$J + 58 \pm 2$ (11 May)	$J + 54 \pm 5$ (26 April)	A****
Mean age at fruit formation (d)	40.8 \pm 10.1 (23 May)	25.0 \pm 8.4 (19 May)	A****
Drying			
First date of shoot drying	24 April	2 April	
Mean date of shoot drying	$J + 79 \pm 4$ (13 June)	$J + 69 \pm 8$ (3 June)	A***
Age at shoot drying (d)	50.0 \pm 12.4	45.9 \pm 9.1	A ns
Mean leaf surface dried (%)†	11.5	76.2	GLM bin. logit****
Individuals dried (%)‡	7.6	51.3	GLM bin.****

Notes: *J* is the day when the first shoot was seen. Abbreviations are: A, ANOVA; GLM bin., general linear model fitted on a binomial distribution; and GLM bin. logit, GLM binomial using logit-transformed data for proportions.

† Percentage calculated at the end of June for surviving individuals.

‡ Percentage calculated at the end of June.

* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant.

green individuals; Table 1). Identical phenology and drying trends were observed at Mon in 2006 and 2008, as well as Boi in 2007 and 2008, except that drying was only 1.2–2.1× more frequent for albinos than for green individuals at Boi (not shown).

Vegetative morphological traits and leaf pigments

At Mon, albino shoots tended to be significantly smaller than green ones, while shoot number per individual and leaf number per shoot did not differ significantly in 2006 to 2008 (Table 2). The same trends

TABLE 2. Morpho-anatomical and biochemical comparison between albino and green individuals of *C. damasonium* at Mon.

Organ and parameter	Year	Green		Albino		Test
		Mean \pm SD	<i>n</i>	Mean \pm SD	<i>n</i>	
Shoot						
Shoot height (cm)	2006–2008†,‡	120.0 \pm 4.9	1098	14.8 \pm 3.7	137	GLM****
Shoot number per individual	2006–2008†,‡	1.4 \pm 0.6	339	1.3 \pm 0.3	104	GLM ns
Leaf						
Leaf number per shoot	2006–2007†,‡	3.2 \pm 1.0	840	3.4 \pm 1.0	76	GLM ns
Surface at flowering (cm ²)§	2006–2007†	7.4 \pm 2.7	79	3.0 \pm 2.5	79	GLM*
Surface at fruiting (cm ²)§	2007	11.4 \pm 2.2	20	8.7 \pm 0.8	20	GLM****
SLA (m ² /kg)	2007	3.1 \pm 0.2	15	13.4 \pm 2.6	7	MW***
Leaf thickness (µm)	2006	390 \pm 02	5	220 \pm 40	5	MW***
Cuticle thickness (µm)	2006	12 \pm 2	5	7 \pm 1	5	MW***
Stomata density (nb/mm ²)	2006	10.9 \pm 1.2	26	9.3 \pm 0.8	12	GLM*
Chlorophyll (ng/g FM)	2006¶	3.52 \pm 0.86	5	0.05 \pm 0.02	5	MW*
Total carotenoids (µg/g FM)	2006¶	0.054 \pm 0.024	2	0.010 \pm 0.005	2	...
Zeaxanthin (%)	2006¶	not detected	2	not detected	2	...
Carotenoids in total pigments (%)#	2006¶	33.0 \pm 12.2	2	58.9 \pm 23.2	2	...
Reflectance	2007	0.41 \pm 0.02	8	0.58 \pm 0.03	8	MW****
Root						
Root surface colonized (%)	2007	59.5 \pm 9.1	4	63.3 \pm 10.2	6	MW ns

Notes: Abbreviations are: FM, fresh mass (g); *n*, number of individuals; MW, Mann Whitney test; GLM, general linear model. For detailed pigment composition, the small number of samples did not allow any statistical test (shown with ellipses).

† The effect of year was not significant according to a GLM, and data were thus pooled for all investigated years.

‡ In a GLM including data from Boigneville (Boi), near Paris, France (see Appendix C), the effect of site was not significant, and similar trends occurred in Boi.

§ Surface was calculated on the first (=lower) developed leaf.

¶ Similar trends were observed at Boi on the same year (see Appendix C).

Carotenoids were calculated on a mass basis.

|| Mean value was between 380 and 780 nm, measured in May.

* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant.

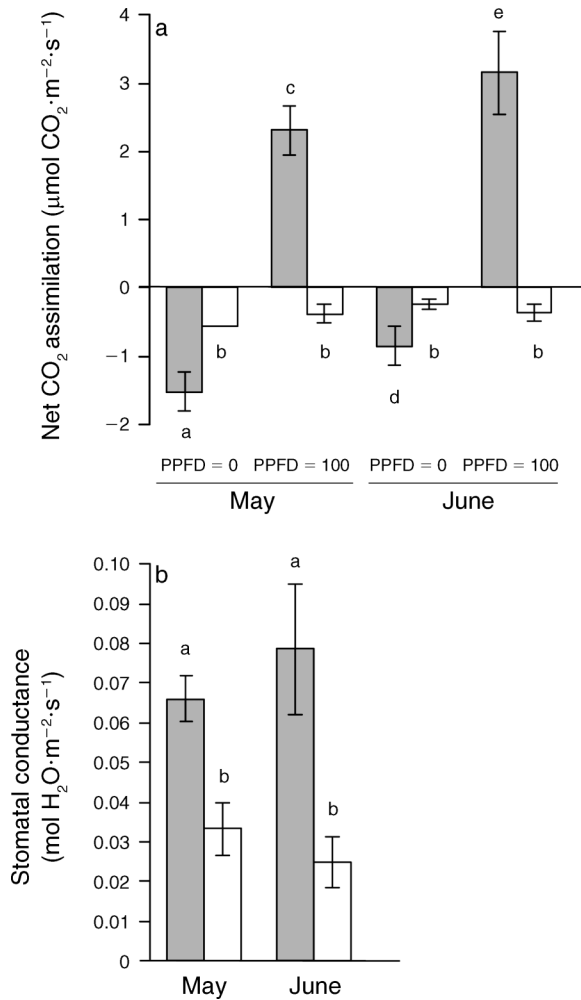


FIG. 2. Instantaneous measurements of leaf gas exchanges at Mon for albino (white bars) and green *C. damasonium* (gray bars), on leaves of flowering individuals in May 2007 and fruiting individuals in June 2007 ($n = 10$ individuals per phenotype; temperature fixed at 25°C). (a) CO₂ exchanges at photosynthetic photon flux density (PPFD) = 0 and PPFD = 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; (b) water vapor stomatal conductance at PPFD = 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For each panel, values with different letters significantly differ according to pairwise ANOVA ($P < 0.001$).

were observed at Boi in 2007 and 2008 (Appendix C). Albino leaves at Mon were significantly smaller and thinner, with a 4× higher SLA, and had significantly thinner cuticle and lower stomatal density (Table 2). Chlorophylls *a* and *b* were 70× less concentrated in albino leaves, and the total quantity of carotenoids was 5× lower (Table 2). HPLC revealed that carotenoid composition and relative abundances did not differ between phenotypes (data not shown); especially, zeaxanthin, a light stress-indicative carotenoid, was not detected in any phenotype (Table 2). Similarly, albino leaves at Boi contained 63× less chlorophyll, 4× less carotenoids, and no detectable zeaxanthin (Appendix C). Leaf reflectance, measured at Mon in 2009, was 1.5×

lower in green than in albino leaves (Table 2). Thus, albinos were likely heterotrophic and did not exhibit evidence of higher light stress than green individuals.

Leaf gas exchange and temperature

Since observed responses were similar between years (2006 to 2007 at Mon, and 2007 to 2008 at Boi), we selected data from one year for each site only for illustration. Instantaneous measurements of leaf CO₂ exchanges at Mon revealed that basal metabolism, as established by dark respiration, was 2–4× higher in green leaves at Mon (Fig. 2a) and 3.0–3.3× higher at Boi (data not shown). Dark respiration was always similar in May (flowering individuals) and June (fruiting individuals), except in 2007 at PPFD = 0 at Mon (Fig. 2a). Positive net CO₂ assimilation was never detected in albinos at Mon and Boi, while it occurred in green individuals (Fig. 2a). As expected, photosynthesis in green individuals positively responded to PPFD increase (Fig. 3a), and net CO₂ assimilation was significantly lower in flowering individuals (in May) than in fruiting individuals (June) at all light intensities (see Fig. 2a for PPFD = 100 at Mon). The light compensation point for green individuals diminished about twofold between May and June (e.g., it decreased from $20.0 \pm 0.8 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to $9.0 \pm 0.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in 2007 at Mon; $n = 12$; significant difference according to the Mann-Whitney test, $P < 0.001$).

Water vapor stomatal conductances were 2.5–5× significantly lower in albinos at Mon (Fig. 2b) and Boi (data not shown). Flowering individuals (in May) had nonsignificantly higher conductances than fruiting individuals (in June; Fig. 2b); as a result, water use efficiency did not significantly vary (43.52 ± 5.22 and $48.47 \pm 11.42 \mu\text{mol CO}_2/\text{mol H}_2\text{O}$, respectively; Mann-Whitney test, $P = 0.07$). Over 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, PPFD increase entailed a slight increase of stomatal conductance in green individuals, but albinos had a lower stomatal conductance that was not regulated by light in the same range of PPFD (Fig. 3b). As a consequence of these differences in stomatal conductance, transpiration was higher in green individuals, and the difference between phenotypes increased with VPD (Appendix D). When measuring leaf temperatures for a whole day at Mon, both phenotypes behaved similarly for air temperatures below 35°C ($F_{39,39} = 0.0$, $P = 0.5$), while above 35°C, albino leaves were warmer (Fisher Z test on Pearson r^2 , $F_{39,39} = 5.52$, $P < 0.0001$; Fig. 4).

Seasonal variations in carbon nutrition

The contribution of fungal carbon to aerial biomass was monitored during 2007 at Mon, using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (usually higher in MH than in autotrophic plants) as proxies. The $\delta^{13}\text{C}$ values of albinos did not differ between the end of April and mid-June, nor did C:N and $\delta^{15}\text{N}$ values (not shown; Mann-Whitney tests, $P < 0.01$). These values, which, as expected, significantly differed from these in autotrophs, suggesting a perma-

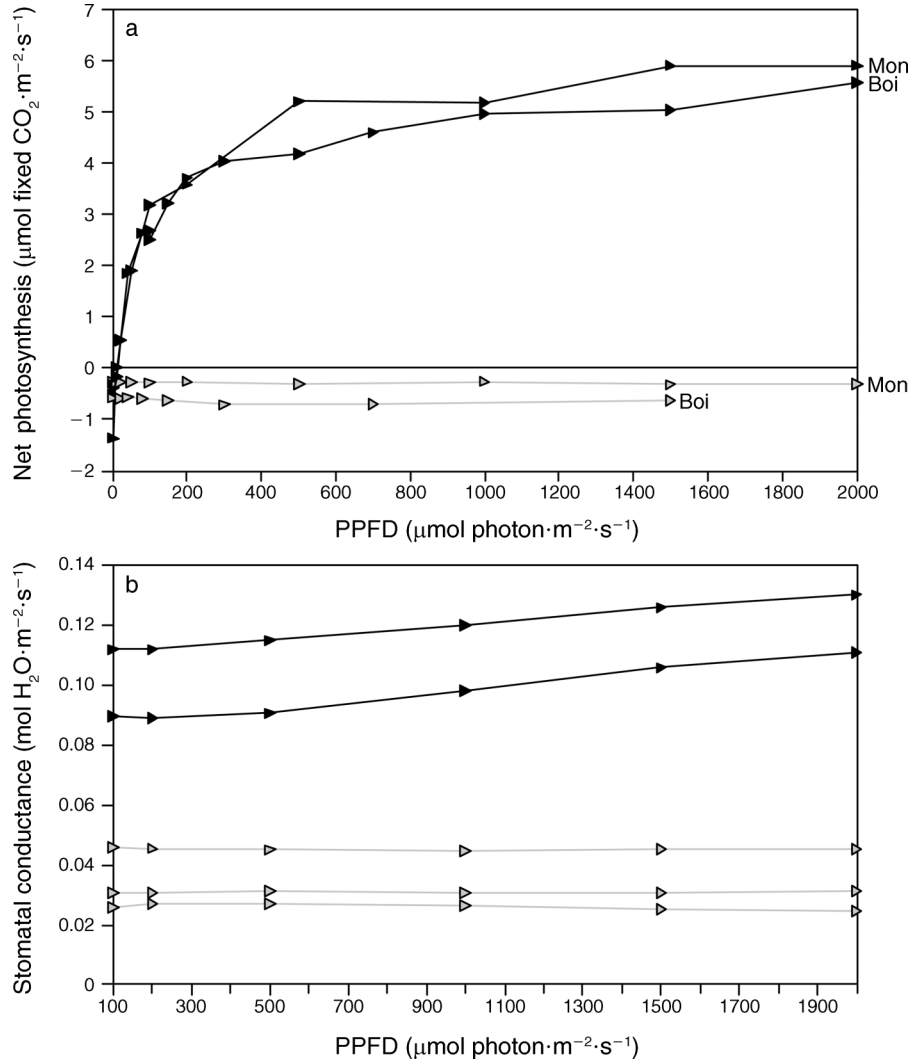


FIG. 3. Response of gas exchange to PPFD of albinos (gray symbols) and green *C. damasonium* (black symbols), with temperature fixed at 25°C. (a) Photosynthesis light response curve, mean of $n=3$ individuals from each phenotype at Mon on June 2008 and Boigneville (Boi), near Paris, France, on June 2006; (b) water vapor stomatal conductance from $n=2$ green individuals and $n=3$ albinos at Mon on June 2008. For each individual in panels (a) and (b), one point is a mean of 10 replicate measurements on a single leaf.

ment heterotrophy and were averaged to establish an MH baseline (Fig. 5a; Appendix E).

Considering $\delta^{13}\text{C}$, the autotrophic *Rubia peregriana* and *Clematis vitalba* never significantly differed, either among them at each date (except in June) or between sampling dates for each species (ANOVA, $P > 0.09$). Values for green *C. damasonium* were always significantly higher than those of autotrophs, except for *R. peregriana* at the last sampling, and lower than those of albinos, except at the first sampling (Fig. 5a). Using a two-source mixing model, the heterotrophy rate thus continuously and significantly decreased between dates (ANOVA, $F_{4,4} = 21.97$, $P < 0.0001$; Fig. 5b), from $\sim 80\%$ in March to $\sim 20\%$ in June. Fruits did not differ from the leaves in $\delta^{13}\text{C}$ for albinos and green individuals

(Fig. 5a), suggesting similar carbon sources in both organs.

As expected, $\delta^{15}\text{N}$ values were always significantly higher in green individuals than in autotrophs, but lower than in albinos (Appendix E). No clear trend was seen in green leaves, except a significant increase between the two first samplings, so that $\delta^{15}\text{N}$ values did not reflect the previous decrease in heterotrophy. As expected again, C:N values (usually lower in MH than in autotrophic plants) were significantly lower in green *C. damasonium* leaves than in autotrophs (except *R. peregriana* in the first sampling), but was always $3\times$ higher than in albinos (Appendix E). C:N increased slightly but significantly in green leaves over the season (ANOVA, $F_{4,4} = 2.22$, $P = 0.097$; Appendix E) as expected if autotrophy increases.

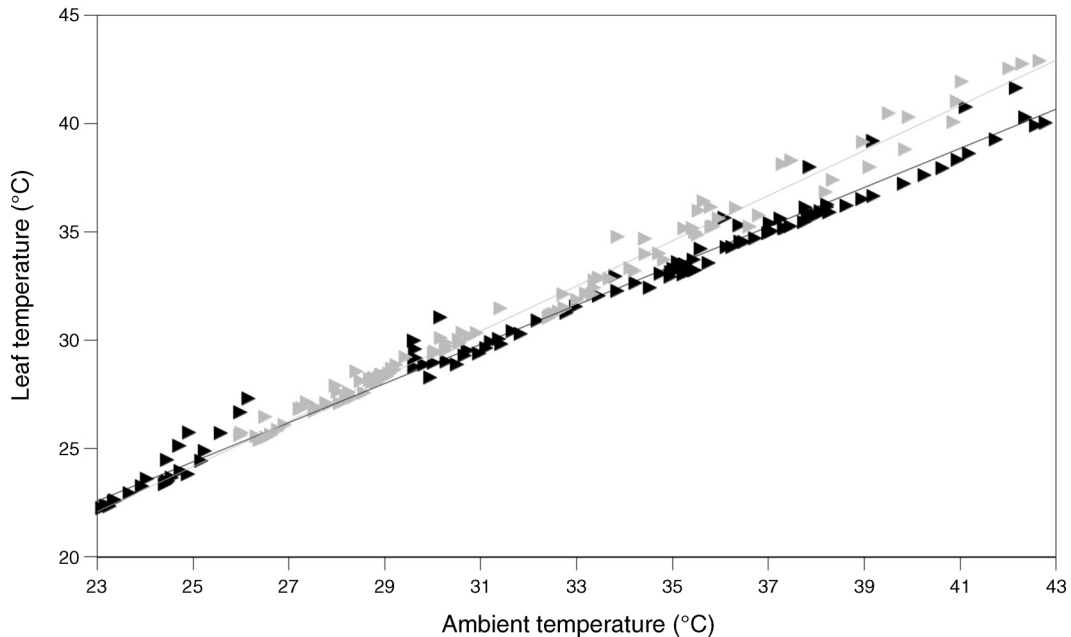


FIG. 4. Observed leaf temperature at different temperatures of surrounding air over two days (24 and 25 June 2007) at Mon, for albinos (gray triangles, gray regression line) and green individuals (black triangles, black regression line).

Seasonal variations in root mycorrhizal colonization

Mycorrhizal colonization, expressed as the area of root sections colonized by fungi (classes c0 to c3, see *Methods*), was highly variable from one root section to another within individuals (Appendix F), or even within single roots (not shown). Thus, high variance was observed at each sampling date in individual plants (not shown). The mean area of root sections colonized was higher for albinos at each date, except in July (Fig. 6), but this was significant in January only (Kruskal-Wallis test, $K=69.8$, $P < 10^{-10}$). Colonization decreased from January to July in both phenotypes (Kruskal-Wallis test, $K=182.4$, $P < 10^{-15}$; Fig. 6). In more detail, albinos in January had 100% of root sections at colonization level c3 (i.e., $>60\%$ of colonized cells; Appendix F), but were no more colonized in July (all sections non-colonized); green individuals, starting from a plateau ($\sim 30\%$ of root sections at c3 in January, March, and May; Appendix F), displayed 87% of non-colonized sections in July.

Susceptibility to herbivory and pathogens

At Mon in June 2008, herbivory damage was 3 \times more frequent on albino leaves (Table 3); similarly, at Boi, damage was more frequent on albinos in 2007 and 2008 (Appendix G). Accordingly, at Mon, herbivory was significantly more frequent on vegetative parts of albinos (Table 3), and albino fruits and flowers more frequently had herbivory damage and attracted more mites and aphids, respectively. More Thomisidae spiders (which eat pollinators; Appendix G) and more ants

(which tend the aphids) were recorded on albinos (Table 3). Mon and Boi leaves of both phenotypes sometimes presented black spots (Appendix B) caused by a *Pseudocercospora* sp. Since nondrying leaves were sometimes infected, while some drying leaves were uninfected (Appendix B), the drying did not correlate with infection. Infections were significantly more frequent on albinos at Mon in 2007 (Table 3) and Boi in 2007 and 2008 (in 2008 only albinos were infected; Appendix B). In all, albinos appeared to be more sensitive and attractive to herbivores and to pathogens than green individuals.

Survival and reproductive traits

We estimated several parameters related to fitness. Albinos were more frequently dormant in 2007 at Mon (by 22.4 \times ; Table 4), and at Boi (23% vs. 0%; Appendix H). The number of fruits or flowers in 2006 did not influence 2007 dormancy in Mon albinos (data not shown). Among individuals absent in 2008 compared with 2007 at Mon (i.e., dormant or dead), those that were albinos dried 4.3 \times more often in 2007 than those that were green; reciprocally, albinos that dried in 2007 were 1.3 \times more often absent in 2008 than green individuals (Table 4; similar trend applies to 2007, data not shown). Thus, drying was more linked to dormancy (and perhaps survival) in albinos.

The frequency of nonflowering individuals did not differ among phenotypes at Mon (Table 4) and Boi (Appendix H), but it was higher in Boi than in Mon (site effect; Table 4). The percentage of individuals flowering in two consecutive years (2006 + 2007 or 2007 + 2008)

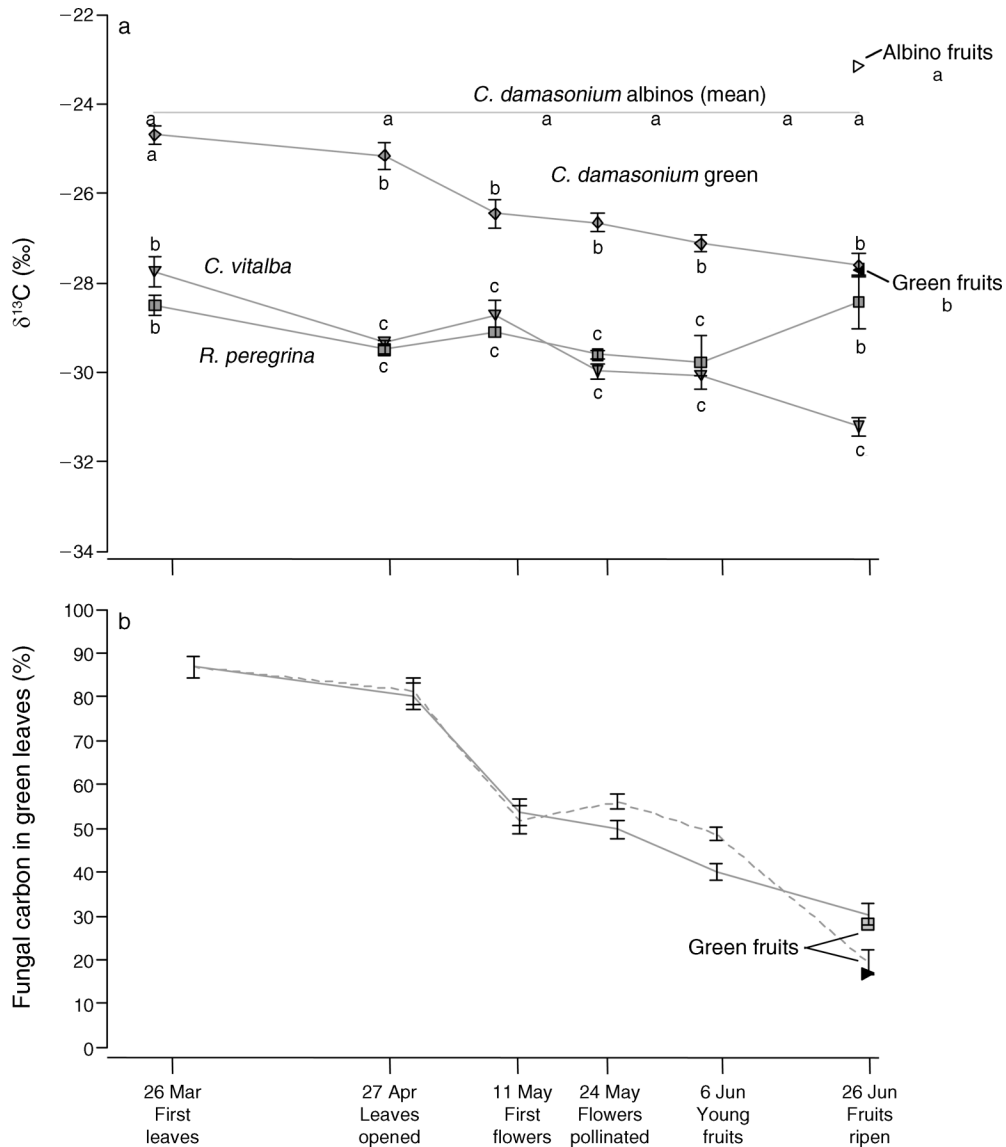


FIG. 5. Change in heterotrophy level in green *C. damasonium* between March and June 2007 at Mon, as estimated from $\delta^{13}\text{C}$. (a) Evolution of $\delta^{13}\text{C}$ in leaves: triangles show autotrophic *Clematis vitalba*; squares show autotrophic *Rubia peregrina*; diamonds show green *C. damasonium* leaves; the labeled black triangle shows green *C. damasonium* fruits in June; the labeled white triangle shows *C. damasonium* albino fruits in June; and the gray line is the mean value for *C. damasonium* albino leaves collected in April and mid-June. At each date, means followed by different letters significantly differ according to a Mann-Whitney test ($P < 0.001$). (b) Inferred change in the heterotrophy level of green leaves, as well as green fruits in late June, using a two-source mixing model with albino leaves as MH reference and, as autotrophic reference, either the mean value for autotrophic *C. vitalba* and *R. peregrina* at each date (dotted line, with a triangle for fruits) or the mean value for the two autotrophs over the whole sampling period (solid line, with a square for fruits).

did not significantly differ among phenotypes at Mon (data not shown), nor did numbers of flowers per shoot (Table 4). Similar numbers of flowers per shoot were also observed in Boi, except in 2007, when green individuals flowered significantly more (not shown), leading to a significant difference between sites (Table 4; Appendix H). Female fitness, assessed as the number of fruits per shoot or the percentage of remaining fruits on shoots surviving in late June (i.e., not taking into account shoot death), was higher for green individuals at

Mon (significant for the percentage of remaining fruits only; Table 4) and Boi (differences nonsignificant; Appendix H). Albino fruits were lighter and smaller in length and diameter; significantly at Mon (Table 4) and nonsignificantly at Boi (Appendix H). Seed number and total mass was about $3\times$ lower in albinos as compared with green individuals at Mon (Table 4). Female fitness was also influenced by site, since the percentages of nonflowering individuals and of remaining fruits, and length of fruits was significantly higher for both

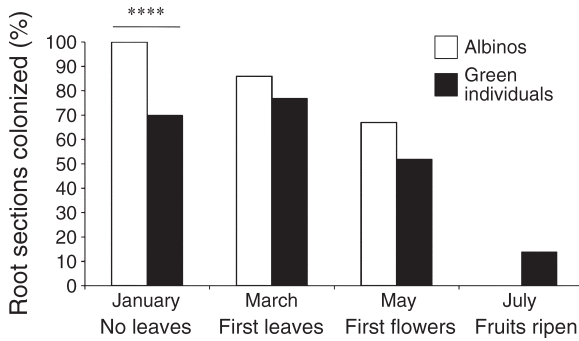


FIG. 6. Change in the mycorrhizal colonization at Mon in 2008 in roots of albino and green individuals (white and black columns, respectively). Values are mean percentage area of root section colonized by fungi based on inspection of multiple sections ($n = 25-110$) from two individuals per phenotype and sampling time (see Appendix F for detailed report).

****Significant difference between albino and green individuals according to a Kruskal-Wallis test, $P < 10^{-10}$.

phenotypes in Boi compared to Mon, while the number of flower per shoot and width of fruits were significantly lower in Boi (Table 4; Appendix H); as a result of these diverging trends, the net difference between sites was difficult to assess.

Green fruit volume roughly correlated with whole seed mass ($R^2 = 0.64$, $P < 0.001$; Appendix I), and to a lesser extent with seed number ($R^2 = 0.49$, $P = 0.02$; Fig. 7), but this trend was not significant for albino fruits, and the largest albino fruits had fewer seeds than their green equivalents (Fig. 7). Qualitatively, the percentage of seeds with embryos (viable seeds; Table 4) was significantly lower for albinos. Green seeds germinated better: After 18 months in soil, 3.5 \times more seeds from green individuals initiated germination (stage C), and 1.4 \times more developed into protocorms (stage D; the low protocorm number limited the significance; Table 4). To summarize, the flowering effort, to very limited extent, and especially the fruiting effort and seed production were lower for albinos than for green individuals.

Fruiting success in experimental crosses

In experimental crosses at Mon in 2008 (Fig. 8), the proportion of flowers producing fruits was lower for albinos in each cross, but not significantly ($P = 0.49$ for GLM on binomial proportions; Fig. 8). When summing all experiments, this proportion was higher in green individuals (66.6%) than for albinos (37.9%), but again without significance (GLM on binomial proportions; $P = 0.11$). Seed production was estimated using fruit volume, a good estimator of seed set for green individuals and a conservative one for albinos (as reported in the previous paragraph; Fig. 7; Appendix I). Geitono-, homo- or heterogamy crosses did not significantly differ from natural pollination for green individuals on the one hand, and for albinos on the other hand (pairwise Student tests, $P > 0.05$; Fig. 8), so that no cost of inbreeding and no pollen limitation were detected. In all, fruit volume was significantly lower in albinos than in green individuals (between 1.2 \times and 2 \times ; GLM $F_{29,28} = 24.53$, $P < 0.0001$); in pairwise Student's t tests, only fruits produced by heterogamy did not significantly differ between mother phenotypes ($P = 0.85$; Fig. 8). No significant interaction was detected between phenotypes and crosses over the whole experiment (GLM $F_{29,28} = 0.90$, $P = 0.46$). Moreover, albino fruits pollinated by green pollen were significantly bigger (Student's t test, $P = 0.009$) than other albino fruits, and green fruits pollinated by albino pollen were smaller than other green fruits, but not significantly (Student's t test, $P = 0.40$). Experimental crosses thus demonstrate a strong maternal and a limited paternal effect on seed set, which we may have underestimated in albinos by using fruit volume as a proxy.

DISCUSSION

Comparisons of albinos and green individuals in *Cephalanthera* and *Epipactis* species have focused on mycorrhizae (Salmia 1989a, Selosse et al. 2004, Julou et al. 2005, Abadie et al. 2006), population dynamics (Abadie et al. 2006, Tranchida-Lombardo et al. 2010),

TABLE 3. Herbivory damage and parasitic load of albino ($n = 81$) and green ($n = 319$) *C. damasonium* individuals at Mon in June 2008, reported as the percentage of individuals displaying at least one indication of herbivory or one parasite over the survey (tested by a general linear model fitted on a binomial distribution).

Herbivory/parasite	Target organ	Green (%)	Albino (%)	Test
Herbivory damage	leaf†	15.5	43.0	****
Herbivory damage	flower or fruit	1.6	5.4	****
Presence of at least one herbivore	leaf + shoot	3.7	31.6	****
Ants‡	leaf + shoot	2.6	9.4	ns
Crickets	leaf + shoot	1.0	5.4	*
Caterpillars	leaf + shoot	0.0	4.0	***
Mites	flower	2.0	5.4	ns
Aphids	fruit	6.0	10.8	ns
Thomisidae spiders	all organs	2.4	9.5	***
Infection by <i>Pseudocercospora</i> sp.§	leaf†	35	55	*

† Identical trends and significance observed at Boi in the same year; see Appendix G.

‡ Although ants are not herbivorous, they tend aphids.

§ Putative causal agent identified molecularly; see GenBank accession number HQ395393.

* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant.

TABLE 4. Reproductive traits and survival of albino and green *C. damasonium* individuals at Mon from 2006 to 2008.

Organ/stage and parameter studied	Year	Green		Albino		Test
		Mean \pm SD	<i>n</i>	Mean \pm SD	<i>n</i>	
Reproduction						
Individuals not flowering (%)	2006–2008†,‡	14.5		17.17		GLM ns
Dormant individuals (%)§	2007¶	0.84	129	18.84	69	GLM****
Impact of drying						
Individuals absent in 2008 that dried in 2007 (%)#		11.2	187	48.6	74	GLM****
Individuals dried in 2007 absent in 2008 (%)#		66.6	30	86.1	36	GLM*
Flowers						
Flowers per shoot	2006–2008†,‡	5.54 \pm 1.85	1657	4.86 \pm 1.51	145	GLM ns
Fruits						
Number of fruits per shoot	2006–2008†,¶	3.94 \pm 3.26	1021	3.46 \pm 1.46	107	GLM ns
Remnant fruits (%)	2006–2008†,‡	61.3 \pm 19.3	1098	49.2 \pm 21.0	137	GLM****
Fruit width (cm)	2007–2008†,‡	0.62 \pm 0.11	95	0.39 \pm 0.13	67	GLM****
Fruit length (cm)	2007–2008†,‡	1.87 \pm 0.29	95	1.44 \pm 0.41	67	GLM***
Fruit dry mass (mg)	2008	73.2 \pm 12.3	33	43.6 \pm 34.2	29	GLM*
Seeds						
Number of seeds per fruit	2006 and 2008†	7339 \pm 2371	56	2554 \pm 1157	31	GLM****
Total seed mass (mg)	2008	17.46 \pm 2.09	16	6.01 \pm 2.05	14	GLM****
Seeds with embryo (%)	2006 and 2008†	53.8 \pm 10.3	56	31.3 \pm 8.7	31	GLM****
Germination						
Swollen seeds, stage C (%)††	2006	75.7 \pm 10.1	8437	21.1 \pm 13.1	830	GLM bin.****
Protocorms, stage D (%)††	2006	0.79 \pm 0.02	75	0.55 \pm 0.01	28	GLM bin.*

Notes: Abbreviations are: *n*, number of individuals; GLM, generalized linear model; and GLM bin., GLM fitted on a binomial distribution.

† The effect of year was not significant according to a GLM, and data were thus pooled for all investigated years.

‡ In a GLM including Boi data (see Appendix H), the effect of site was significant, although similar trends occurred in Boi.

§ Dormant individuals were recorded in 2006 and 2008, but they did not form shoots in 2007.

¶ In a GLM including Boi data (see Appendix H), the effect of site was not significant, and similar trends occurred in Boi.

Individuals absent in 2008 are either dead or dormant (see footnote §).

|| In late June, calculated on surviving shoots.

†† After 18 months in the soil; *n* is the number of seeds reaching stage C (swollen pro-embryo with cracked testa) or D (protocorm with trichomes); data were pooled from 37 seed packets.

* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant.

and morphology and photosynthetic abilities (Salmia 1986, Salmia et al. 1989b, Julou et al. 2005, Stöckel et al. 2011). Unfortunately, it is difficult to infer a general view from different species and populations, often with few albinos, and we are not aware of any comparative study of fitness. Here, the numerous Mon albinos revealed several differences in shoot life cycle and fitness as compared with green individuals. We discuss our main findings about (1) variations of shoot C resources over the growing season, which suggest some C limitation in albinos; (2) the drying phenomenon and its potential link to thermal and water stress in albinos; (3) the higher load of pathogens and herbivores on albinos; and (4), probably as a result of previous points, the reduced fitness of albinos. Whenever we provide values for these trends, the reader should keep in mind that they reflect the Mon population, with some support from Boi, in the years of study. Based on a general recapitulating model (Fig. 9) linking these various trends, we then discuss (5) the evolutionary potential of albinos in orchid populations and (6) important adaptations in the evolutionary transition to MH nutrition.

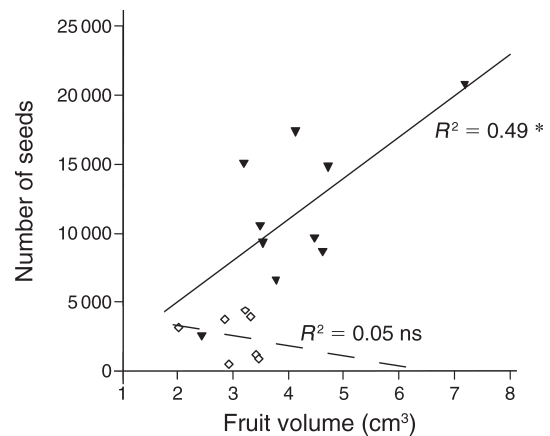


FIG. 7. Relationship between total seed number and fruit volume for albinos (white diamonds, from $n = 7$ fruits) and green individuals (black triangles, from $n = 10$ fruits) at Mon in 2009. The significance of the comparison between distribution observed and that predicted by the regression is indicated according to a *F* test.

* $P < 0.05$; ns, not significant.

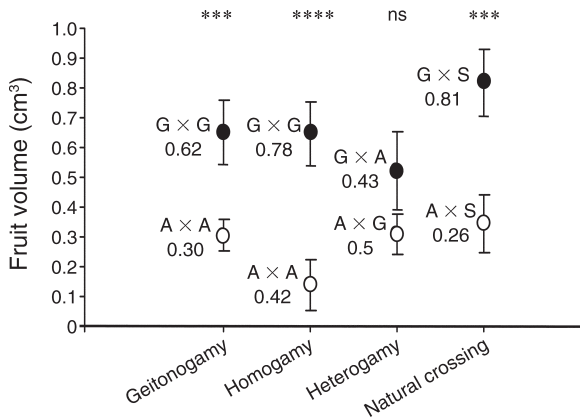


FIG. 8. Volume of fruits produced after experimental crosses (mean \pm SD; white circles show albino mother plant; black circles show green mother plant). The mother plant \times paternal plant combination is abbreviated as follows: A, albino; G, green individual; S, spontaneous albino or green pollen source. The number with each cross indicates the ratio of the number of fruits obtained to the number of flowers at the beginning of the experiment. Definitions are: geitonogamy, flowers manually pollinated with pollinia from the same inflorescence; homogamy, flowers manually pollinated with pollinia from an individual of similar phenotype; heterogamy, flowers manually pollinated with pollinia from an individual of different phenotype; and natural crossing, control flowers naturally pollinated and enclosed in a bag as in all other treatments. Significance of the comparison among phenotypes is given for each cross, according to Student's *t* tests.

*** $P < 0.001$; **** $P < 0.0001$; ns, not significant.

Green shoots increase autotrophy over the growing season, whereas albinos may be C limited

At their emergence, green and albino leaves have similar $\delta^{13}\text{C}$ values, and thus, a similar carbon source, i.e., C from fungi and/or from reserves. Over the next four months, green leaf $\delta^{13}\text{C}$ values decrease, supporting the incorporation of photosynthetic C after leaf expansion, and reach the values of the autotrophic *R. peregrina* at fruiting. During leaf ontogeny, the mobilization of C from reserves, usually enriched in ^{13}C (Cernusak et al. 2009), may contribute to the high $\delta^{13}\text{C}$ values at leaf emergence. For this reason, $\delta^{13}\text{C}$ usually decreases in herbaceous species over the growing season, but only by 2% at most (Smedley et al. 1991), as in autotrophic *C. vitalba* at Mon. We consider that this does not explain the $\delta^{13}\text{C}$ decrease in green individuals: First, the decrease reaches 4%; and second, since reserves in albinos only come from fungi, similar $\delta^{13}\text{C}$ values in green shoots at emergence suggest that they also mobilize fungal C (be it transiently stored in reserves, or directly obtained from fungi, in both phenotypes). Most importantly, water use efficiency, which may affect ^{13}C enrichment, did not change over the growing season (in 2007 [see Fig. 2], as well as in 2008 [data not shown]). Thus, the observed trend reflects a $\sim 4\times$ increase in autotrophy. One important caveat is that this part of the study was not replicated beyond Mon, so that we do not know whether the trend would be as pronounced in other populations, especially in lower light environments where MX plants assimilate less C through photosynthesis (Preiss et al. 2010).

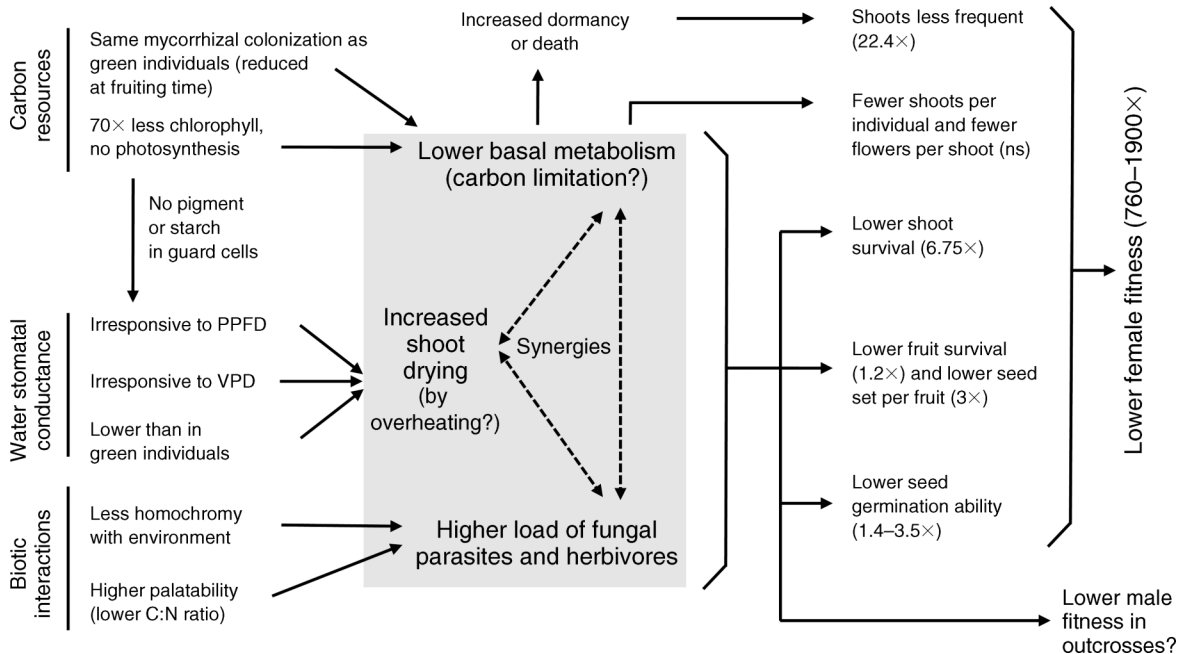


FIG. 9. A recapitulative model of factors reducing albino fitness, as compared with green individuals, with arrows suggesting possible causal links. Values are from the Mon population. Abbreviations are: ns, not significant; PPF, photosynthetic photon flux density; VPD, vapor pressure deficit.

For each phenotype, our isotopic measurements at Mon in late June show no significant differences between fruits and shoots (data not shown), and support that leaves are a good proxy for all aerial organs. Concerning catabolism, preliminary measurements performed in June 2007 show that $\delta^{13}\text{C}$ of the evolved respiratory CO_2 ($-24.9\% \pm 0.8\%$ for albinos and $-26.8\% \pm 1.3\%$ for green individuals; $n = 6$ each) did not significantly differ from the total leaf organic matter of each phenotype (Mann-Whitney test, $P > 0.05$; Fig. 5). Thus, catabolism does not bias assessment of C sources. MH nutrition usually entails low C:N and high $\delta^{15}\text{N}$ (Gebauer and Meyer 2003, Abadie et al. 2006): We observed an increase in C:N, but no decrease of $\delta^{15}\text{N}$ over four months, perhaps because too little N was acquired to modify isotopic abundances.

An increase in autotrophy parallels two other observations. First, mycorrhizal colonization sharply decreases between January and July. A minimum is reached in summer, as already reported in *Cephalanthera longifolia* (Látr et al. 2008), *C. falcata* and *C. erecta* (Matsuda et al. 2009), and other orchids (Rasmussen and Whigham 2002). One possible reason is that the summer dryness limits fungal vegetative activity. Thus, the fungal C flux likely diminishes over the growing season. Second, photosynthetic ability is higher in June than in May, with higher net CO_2 assimilation and a two times lower compensation point. In spite of the limited efficiency of the *C. damasonium* photosynthetic apparatus (Cameron et al. 2009), this may compensate for the reduction of fungal colonization and C flow. Such seasonal variations of photosynthetic parameters are not reported in autotrophic orchids (e.g., Zhang et al. 2010), but are relevant shade acclimations to forest canopy closure in late spring. The plausible hypothesis that photosynthesis in MX plants compensates for canopy closure and fungal absence in summer deserves further investigation.

Conversely, albinos are fully MH, with 60–70× lower chlorophyll content than green individuals and absence of CO_2 fixation (as we found; Julou et al. 2005). Their ability to compensate for the absence of photosynthesis by a more efficient mycorrhizal interaction has been postulated from reports of a denser colonization than in green individuals, for *Epipactis* species (Salmia 1989a, Selosse et al. 2004), *C. damasonium* (Renner 1938), and *C. longifolia* (Abadie et al. 2006). Here, the mycorrhizal colonization at Mon was higher only until flowering (this was significant only in January), but no longer at the time of fruiting. Accordingly, most previously cited investigations were carried out before or at flowering. Thus, albinos may receive less carbon than green individuals in late spring and summer.

MX nutrition is described as dynamic and flexible in *C. damasonium*, where the increased $\delta^{13}\text{C}$ values in MX plants that are less photosynthetically active, due to leaf variegation (Stöckel et al. 2011) or ambient light conditions (Preiss et al. 2010, Matsuda et al. 2012),

may result from a higher compensatory fungal C input. However, since we lack estimates of the total plant C budget, we do not know whether less photosynthetically active plants recover fungal C more efficiently, or not, in which case diluting equivalent amounts of ^{13}C -enriched fungal carbon in lower amounts of photosynthates also leads to increased $\delta^{13}\text{C}$ values. Most importantly, the 2–4× lower basal metabolism in albinos observed in this study and by Julou et al. (2005) indicates C limitation (Williams and Farrar 1990, Amthor 1994), so that fungal C may not fully compensate for loss of photosynthesis. Among other deficiencies, the shorter average life of albino shoots (earlier flowering and fruiting in spite of a similar mean date of appearance) may result from elimination of shoots with slower development, since long growth periods are not compatible with C shortage; similarly, the fact that flowering parameters are less impacted than fruiting parameters in albinos suggests that differences among phenotypes become more pronounced late in the growing season.

To summarize, even if some compensation occurs early in the season, albinos are likely more C limited than green individuals, especially in late spring and summer when photosynthesis becomes physiologically relevant due to decreasing fungal colonization. We note that MX *Cephalanthera* species strongly differ in this respect from MH orchids, which display denser and continuous colonization (Rasmussen and Whigham 2002, Tatarenko 2002), including in summer, as exemplified by the sympatric *Neottia nidus-avis* (Selosse et al. 2002; M.-A. Selosse, *personal observation*) and *Epipogium aphyllum* (Roy et al. 2009b); alternatively, MH orchids seasonally lacking fungal colonization accumulate reserves in tuberized roots or rhizomes (such as the tropical MH *Wulfschlaegelia aphylla* during the dry season; Martos et al. 2009). The latter patterns allow a constant C supply, which albino mycorrhizae cannot ensure.

*Albino shoots dry more frequently, perhaps
due to stomatal deficiencies*

The most dramatic trend in albinos is shoot drying, which occurred earlier and 6.75× more frequently than in green individuals at Mon. Starting from the leaves and then extending to the whole shoot, it is the main factor of shoot loss, and is associated with a 4.3× lower sprouting probability in the next year. Salmia (1986) reported that Finish *E. helleborine* albinos often “turn brown” in July. Given its timing in the season, the higher drying in albinos could result from C limitation as suggested in the previous section. We nevertheless investigated factors related to water and thermal physiology that may be proximal causes.

The measured 2.5–5× lower water conductance in albino leaves at Boi and Mon invalidated our earlier report, on a few Boi plants, of higher conductance in albinos (Julou et al. 2005). This lower conductance correlates with the lower stomatal density in albinos, in

spite of a thinner cuticle. Water stomatal conductance of green individuals showed limited response to PPFD and VPD, as expected for species adapted to low light (Björkman 1981), while albinos did not respond at all. Stomatal response to light has two components: a photosynthesis-independent response to blue light that saturates at low PPFD, and a photosynthesis-mediated response to red and blue light (active at higher PPFD; Shimazaki et al. 2007, Lawson 2009). The second pathway is obviously impaired in albino stomata that lack green plastids. Accordingly, albino *Vicia faba* individuals and albino leaf patches in *Chlorophytum comosum* do not respond to red light, although they retain sensitivity to blue light (Roelfsema et al. 2006), and the green orchid *Paphiopedilum harrisianum*, whose stomata are devoid of green plastids, is only responsive to blue light (Talbot et al. 2002). The absence of response to blue light in albinos (which we specifically tested at Mon, data not shown) is more unexpected. However, this pathway is not universal (Lawson 2009) and requires plastidial functions, such as degradation of starch to produce malate (Shimazaki et al. 2007), which may be altered in albinos: Actually, starch in the guard cells of *C. damasonium* albino is absent or not abundant (Renner 1938, Mairold and Weber 1950; M. Roy and M.-A. Selosse, unpublished data).

Regulation of leaf temperature by water evaporation largely depends on stomatal opening (Merlot et al. 2002), and lower transpiration plus absence of PPFD and VPD responsiveness may explain why albino leaves are warmer than green ones over 35°C, in spite of the higher reflectance of albinos. At high PPFD, albinos cannot dissipate the energy absorbed into photosynthetic metabolism and this may turn into overheating. On the one hand, we observed no differential heating at high PPFD in our short-term measurements (data not show), nor zeaxanthin from the xanthophyll cycle, a short-term response to light stress (Björkman and Demmin-Adams 1995). On the other hand, a role of high PPFD or environmental temperature could explain why drying is less pronounced at Boi, a dense forest that shows colder temperatures and lower PPFD than Mon (see *Methods*).

Low transpiration of course limits water loss, but insufficient water resources in albinos may also cause drying. If albinos use the same water resources but have a higher ratio of water loss to water influx as compared with green individuals, this predicts an increase in $\delta^{18}\text{O}$ of water in albinos (Cernusak et al. 2003, Keitel et al. 2003). Our preliminary measurements in May and June 2010 revealed similar water $\delta^{18}\text{O}$ for both phenotypes in shoots, leaves, and flowers (or fruit; $n = 4$ samples per organ, phenotype, and date; T. Bariac, P. Richard, and M.-A. Selosse, unpublished data), and do not support imbalanced water loss in albinos. To summarize, C limitation and overheating due to high PPFD or temperature, but not limited water availability, may contribute to the higher albino drying. Since we tested healthy albinos, we still do not know precisely what

happens at the point where physiological status or environment or both initiate drying.

The fact that leaves dry first strongly suggests that they may somehow be causal in this phenomenon. Ironically, leaves and stomata, which, respectively, harvest light and circumvent CO_2 limitations for photosynthesis, are unnecessary features in albinos. Both features are absent or reduced in MH species (Leake 1994, Yukawa and Stern 2002, Zimmer et al. 2007, Roy et al. 2009a, b), such as the closely related *Cephalanthera austiniiae* and *C. exigua* (Pedersen et al. 2009). If they enhance drying, the vestigial leaves and stomata inherited from recent photosynthetic ancestors are not only useless C costs, but also maladaptations in albinos. Another problem is the duration of the aboveground phase, which is selected by the photosynthetic requirements of recent green ancestors. It may be longer than required for albino fruiting based on fungal C only, and unnecessarily enhance susceptibility to drying. As already mentioned, albino shoots have a shorter mean life span than green ones, perhaps because shoots with longer life span are progressively eliminated over the season. Congruently, MH species appear more sporadically (with very variable timing in the year, e.g., for *Epipogium aphyllum*; Roy et al. 2009b) and remain for a shorter time above ground, as compared with photosynthetic species (Leake 2004).

Albinos show higher sensitivity to pathogens and herbivores

Albinos display more fungal infections, attract more phytophagous arthropods, and display more herbivory damage. Several features may favor this. First, their thinner cuticle is less defensive, especially against fungi and mites. Second, albinos may induce weaker defenses than green individuals, due to their lower basal metabolism. Third, albinos have a $\sim 3\times$ lower C:N ratio than green individuals: High N content is attractive for herbivores that are often N limited (Cleland and Harpole 2010). N enrichment is a feature of MH plants (Selosse and Roy 2009) and albinos (Abadie et al. 2006, Stöckel et al. 2011), probably due to N re-concentration of the N-rich fungal biomass by plant respiration. The link between palatability and N content is complicated because N-rich plants often evolved higher defenses against herbivory, including N-containing toxins such as cyanogenic compounds or alkaloids (Gange and West 1994, Schoonhoven et al. 2005), whose production often correlates with N availability (Kempel et al. 2009). However, we speculate that such defensive traits have not been selected in recently emerged albinos, explaining their lack of herbivore and fungal avoidance.

Lastly, albino color (decreased blue and red absorption as found in our reflectance measurements; Julou et al. 2005) contrasts with nearby green plants. This may attract herbivores, such as aphids that are sensitive to blue/green ratio and brightness (Döring and Chittka 2007). Some local learning process may even reinforce

preference for albino color, as a clue for high-N food. Interestingly, most MH species are less whitish than albinos because of brown pigments and colored scales (Leake 1994), likely enhancing homochromy with the forest ground. A role in camouflage is demonstrated in the MH *Monotropis odorata*, whose brown shoot scales are highly homochromous with its substrate. Scale removal entails a 20–27% higher herbivory rate and a 7–20% lower fruit production as compared with control plants (Klooster et al. 2009). The albinos' color may also explain predation by large mammals and flower picking by humans, as often observed by field orchidologists (e.g., Scappaticci and Scappaticci 1998; during heavy deer grazing in June 2005 at Boi, all 12 albino shoots were fully eaten, vs. 6 out of 37 green shoots; M.-A. Selosse, *unpublished data*). If albino leaf color contributes to the herbivory load, this adds to the cost of these useless vestigial features. Although choice experiments are required to further support this, we hypothesize that albinos offer more attractive and palatable food. Abiotic factors and biotic aggressions might, in turn, act synergistically upon albino drying: Arthropods and pathogens are known to amplify, or be amplified by, C starvation and overheating (Breshears et al. 2005, McDowell et al. 2008).

Albinos' fitness is reduced as compared with green individuals

Albinos display more frequent dormancy (22.4× at Mon; similar trend at Boi), and thus reproduce in fewer years. In autotrophs, dormancy is an adaptation to stress, enhancing vegetative survival at the expense of immediate reproduction (Shefferson 2009). It is especially selected in perennial species whose fitness is sensitive to survival (Stearns 1992). An experimental defoliation on two long-lived orchids (including the MX *Cephalanthera longifolia* which can live 20+ years; Látr et al. 2008) did not affect survival, but enhanced dormancy relative to control plants (Shefferson et al. 2005). In terms of C budget, albinism is somewhat equivalent to defoliation, supporting a partial causal link between albinism and increased dormancy in Mon albinos. The observation that drying individuals are more often absent (dormant or dead) in the next year indicates that drying correlates with stress in albino and green individuals. In many natural populations of autotrophic orchids, dormancy correlates with lower survival (e.g., Shefferson et al. 2003, Shefferson and Tali 2007), so that albinos may be shorter lived. This remains speculative since we still do not know the exact survival rates and life span of the two phenotypes, although this is a major component of fitness in perennial species (Stearns 1992).

The word “dormancy” is a little misleading in the context of MX or MH plants (Shefferson 2009), which can receive C from fungi, even without shoots, while dormant individuals in autotrophic species survive on their reserves only (in any case, however, dormancy

suppresses seed production). In MH species, which do not require photosynthesis, dormancy restricts sprouting to years suitable for reproduction (Leake 1994). As a result, dormancy is frequent and all shoots form flowers in MH species. For example, in *Corallorhiza odontorhiza* populations, 84% of individuals are dormant and nearly all shoots flower (Shefferson et al. 2011). Although albinos are more often dormant than green individuals, such an optimization of sprouting for flower formation is lacking in albinos. For example, up to a half of albino shoots do not flower at Boi. Production of nonflowering shoots is again inherited from recent green ancestors, which sprout to achieve photosynthesis, not only for reproduction. Nonflowering shoots represent an unnecessary exposure to sprouting costs, as well as to biotic and abiotic stresses. To summarize, the dormancy of albinos indicates weakened plants and reduces fitness, while its suboptimal determinism entails costs and maladaptation.

Nondormant individuals of both phenotypes have similar reproductive effort (number of shoots, inflorescences, and flowers) early in the season, perhaps because of identical amount of C resources at that time. Later, albino fitness is reduced compared with green individuals because of (1) more frequent drying; (2) lower fruit survival per shoot, at least at Mon (1.2×); (3) smaller fruit size and mass, and thus lower seed set (3× at Mon); and (4) fewer viable seeds (germination was 1.4× to 3.5× less frequent at Mon, based on seeds reaching stage D or C, respectively). Our experimental crosses support a maternal effect on fruit size, without paternal (pollen) effect. All previously discussed factors (C limitation, overheating pathogens, and herbivores; Fig. 9) may reduce available resources in nondrying shoots. A shading experiment on MX *Cephalanthera longifolia* induced a reduction in flower number per shoot in the following years (Shefferson et al. 2006), further supporting the notion that limiting C resources interferes with fitness in MX orchids. Curiously, fruiting did not enhance detectable increase in dormancy of our albino and green individuals, contrasting with fruiting costs reported for diverse autotrophic orchids (Primack and Stacy 1998, Jacquemyn et al. 2010, Sletvold and Ågen 2011): Their ability to recover C from mycorrhizal fungi after fruiting may buffer the previous reproductive efforts.

No pollen limitation occurred for any phenotype, which, together with the absence of inbreeding effect, is expected because *C. damasonium* is autogamous (Scacchi et al. 1991, Claessens and Kleynen 2005, Micheneau et al. 2010, Tranchida-Lombardo et al. 2010). Because of autogamy, we did not assess male fitness. We admit that some pollen dispersal may entail a few outcrossings, although the low production of nectar (Claessens and Kleynen 2005) makes this unlikely. Estimation of pollen dispersal is notoriously difficult in *C. damasonium*, whose pollinia are granular and lack viscidia that allow full removal (Claessens and Kleynen 2005): Insects can

thus undetectably remove isolated pollen grains. If any outcrossing occurs, albino male fitness may be decreased by (1) lower shoot production (equal to higher dormancy), (2) higher shoot drying, which reduces floral display; (3) higher abundance of ants that may disturb pollinators; and (4) a 4× higher abundance of Thomisidae spiders, which eat pollinators (Thomisidae may prefer albinos as a substrate, making them less visible to their prey and to their own predators; Oxford and Gillespie 1998). Moreover, albino pollen tended to perform less well than green pollen in experimental outcrosses. The existence of some outcrossing and of a male component of fitness in *C. damasonium* populations deserves additional investigation.

To summarize, considering the species as autogamous, fitness can be approximated by seed production. At Mon, albino fitness is 760–1900× lower than that of green individuals (Fig. 9). Similar trends occur at Boi, where no precise calculation was possible. This model is conservative, since it does not take into account the lower percentage of flowering individuals and lower number of flowers per shoots, which are nonsignificant at Mon, and thus potentially underestimates the previous estimation by a factor $1.03 \times 1.14 = 1.44$. In addition, longevity, a major component of fitness in perennial species, is not considered either.

Are albinos evolutionary steps toward MH species?

The choice of populations with frequent albinos (up to 15% and 50% at Mon and Boi, respectively) may overlook some maladaptations that would make albinism even less fit in other populations and ecological conditions. We do not know whether stable microscale environmental conditions (microbial or abiotic) or genetic causes determine the stable albino phenotype in *Cephalanthera* spp. (Julou et al. 2005, Abadie et al. 2006). Moreover, genetic background and determinism likely vary among populations, as suggested by the presence of variegated individuals in some *C. damasonium* populations (Stöckel et al. 2011; variegations were never observed at Boi and Mon). Together with environmental differences, genetic differences may also explain the observed differences in performances between Boi and Mon albinos.

If albinism results from a mutation, it is unlikely to be fixed, because of its lower fitness. If albinism is purely phenotypic and reversible, then any mutation making albinism irreversible will not be fixed. Any genetic background making individuals prone to albinism may itself be counter-selected. Assuming genetic determinism, the mutation(s) involved would most probably be (1) nuclear, since more than 95% of plastid proteins are nuclear-encoded (Krause 2008, Keeling 2010), and (2) recessive, since albinism is likely a loss of function. On the one hand, albinism could be cryptopolymorphic (i.e., resulting from mutations only). On the other hand, our data suggest that albinos can reproduce, giving rise by autogamy to other albinos in *C. damasonium*. Indeed,

whereas cryptopolymorphism predicts a constant frequency equaling that of mutations abolishing photosynthesis, albino frequencies vary among populations (Tranchida-Lombardo et al. 2010). Although different dormancy rates may explain this, local genetic background and environmental conditions likely modify albino survival and fitness. A recessive nuclear mutation creates green heterozygotes that are not counter-selected, and repeatedly produce albinos. If some allogamy occurs, more heterozygotes are produced, slowing down the purge of the deleterious allele (in progenies of the orchid *Encyclia cochleata* germinated in vitro, albino seedlings were 35–60× more frequent in an outcrossing population than in a selfing one; Ortiz-Barney and Ackerman 1999). Although unfit, albinism would thus be difficult to erase by counter-selection (Lopez et al. 2008), and, under the assumption of a genetic determinism, the respective contributions of mutation and reproduction to albino frequency depend on the mating strategy of the population.

Our data do not support the hypothesis that albinos can produce directly a MH species, although additional mutations, or other genetic backgrounds, or even migration into other environments, may allow albino survival and fixation. Notably, the related MH *Cephalanthera exigua* and *C. austinae* are morphologically similar to *C. damasonium* albinos, although with smaller leaves and few or no stomata, and occupy very restricted, moist habitats (Correll 1950, Taylor et al. 1997, Pedersen et al. 2009). This suggests that fitness of such MH plants depends on environmental conditions, and indeed, some traits related to albino female fitness differ between Mon and Boi. Light, herbivory pressure, summer fungal activity, and so on, may modulate the maladaptations found in the present study. Indeed, wet years enhance shoot survival in *E. helleborine* albino (Salmia 1986), perhaps by counteracting their drying. Moreover, the duration of the vegetative period reduces temporal overlap between resource allocation to reproduction and to other functions (Sletvold and Ågren 2011). This may explain why albinos are more frequent at Boi than at Mon, where the dry Mediterranean summer selects for shorter shoot life span, although having only two populations limits any general conclusion about what could favor albino fitness.

Comparing albino and green individuals (Fig. 9) suggests several deleterious traits in albinos. These traits are vestiges from a long evolution as partly autotrophic plants that become unnecessary, if not maladaptive, in albinos (Table 5). Additional adaptations occur in fully MH species, as compared with albinos (Table 5; Leake 1994, 2004). The proposed albino syndrome (Fig. 9) offers some testable predictions: First, defoliation experiments may reduce the cost of leaves and stomata in albinos; second, shading may limit overheating that is not buffered by water conductance and stomatal response to environmental factors; third, cutting the stems when fungal colonization decreases may reduce

TABLE 5. A summary of the main vestigial traits inherited by albinos from their recent green mixotrophic (MX) ancestors that result in maladaptations, and a comparison with mycoheterotrophic (MH) plants.

Vestigial traits (and role in MX plants)	Resulting maladaptations in albinos	Corresponding adaptations in MH plants
Leaves (light capture for photosynthesis)	contribute to drying and susceptibility to fungi and herbivores	leaves absent or reduced to scales (Leake 1994)
Stomata (CO ₂ diffusion for photosynthesis)	may contribute to overheating and drying, partly because of impaired responsiveness to environmental cues	stomata absent or reduced in number (Leake 1994)
Temporal pattern of mycorrhizal colonization (minimal when photosynthesis is at highest)	C limitation in summer, at fruiting time	permanent mycorrhizal colonization (e.g., Selosse et al. 2002, Roy et al. 2009b); root or rhizome reserves when fungus is absent (Martos et al. 2009)
Long aboveground shoot presence, even without fruiting (C acquisition by photosynthesis)	susceptibility to drying or herbivores longer (or in more years); C investment higher than strictly required from fruits	short aboveground shoot presence, sprouting only when flowering (higher dormancy; e.g., Shefferson et al. 2011)
No pigment for homochromy (color from photosynthetic pigments is sufficient)	white plants more attractive (or recognizable) for herbivores, perhaps in combination with higher N content	brownish homochromous pigments and appendages (Leake 1994, Klooster et al. 2009); perhaps anti-herbivorous toxins (?)

the frequency of dormancy in the following years by suppressing the C costs unbalanced by photosynthesis. To summarize, albinos as we observed are not direct evolutionary links to MH species, unless they evolve additional traits (Table 5). Accordingly, MH species evolved in many other MX lineages that do not display albinos, such as Ericaceae (Tedersoo et al. 2007, Zimmer et al. 2007).

Important adaptations and limitations for the evolution of MH nutrition

Albinos a contrario emphasize important adaptations to MH nutrition (Table 5), none of which alone may solve all albinos' deficiencies (Fig. 9). Rather, we speculate the need for a joint and progressive evolution of, at least: (1) more continuous mycorrhizal colonization (or large C reserves) for permanent C supply, (2) smaller leaves, with fewer or no stomata, (3) reduction of photosynthesis efficiency and pigment content, (4) optimal sprouting and dormancy, selected for fruiting more than for photosynthesizing, and (5) some homochromy and new defenses against parasites and herbivores. Evolution of some additional traits may be required, such as ecological preference for old growth, dark and moist forests (Correll 1950, Leake 1994), or belowground vegetative multiplication structures (Klimesnova 2007, Roy et al. 2009b). Since multiple loci are likely involved, evolution of MH plants from MX ancestors (or full fitness remediation in albinos) is unlikely to occur in a single mutational step, and probably involves a progressive, joint evolution among above-mentioned traits.

MX orchids in the Neottieae tribe display a continuum of phenotypes along this putative multi-trait trajectory to mycoheterotrophy. Some leafy MX orchids sprout over long periods, even without fruiting, to fulfill their photosynthetic needs, such as *C. damasonium* or *Epipactis helleborine*. Some smaller leaved MX orchids display shorter sprouting durations and have darker

colors due to a mix of violet anthocyanins and green chlorophylls (Rasmussen 1995), such as *Epipactis microphylla* (Selosse et al. 2004) or *Limodorum abortivum* (which lives below the compensation point and always flowers when sprouting; Girlanda et al. 2006); yet albinos remain rare, and likely unfit, in these species (Selosse et al. 2004). Fully MH species are often dormant, sprout over short periods, always display flowers, and have homochromous, brownish colors, such as *Neottia nidus-avis* (Selosse et al. 2002), *C. erecta* (Pedersen et al. 2009), or *Aphyllorchis* spp. (Roy et al. 2009a). These evolutionary trends to MH nutrition occurred convergently in Neottieae (Roy et al. 2009a) and are not unidirectional, since reversion to autotrophy is suspected in the *Epipactis* and *Neottia* = *Listera* lineages (Bidartondo et al. 2004, Abadie et al. 2006). Similar leaf reduction, brownish colors and stomata degeneration accompany the emergence of MH species within the genus *Cymbidium* (Yukawa and Stern 2002, Motomura et al. 2010). Despite previous work on other species (Rasmussen and Whigham 2002, Tatarenko 2002), the intensity and phenology of root colonization by fungi along these continua from MX to MH nutrition deserve further studies.

The difficulty of evolving a purely MH nutrition has several important consequences. First, it explains why MX species are evolutionarily stable, although the complexity of the photosynthetic apparatus allows many mutations to abolish photosynthesis (Feldmann 1991), e.g., in orchids (Ortiz-Barney and Ackerman 1999), and potentially entails a mutational drift to heterotrophy. Evolutionary transition to MH nutrition may superficially look like a loss of function, which can occur easily and frequently in MX species due to use of fungal C, but it turns out to be a complex process, antagonistic with traits selected for photosynthetic efficiency of green ancestors. This also applies to plants displaying microspermy, i.e., the 10% of land plant species propagating by small, reserveless seeds or spores and using fungal C for MH

germination (Leake and Cameron 2010). Microspermy may predispose these plants to evolve MH nutrition in adulthood, but ancestral adaptations to photosynthesis may complicate the transition. Second, the difficulty of evolving a purely MH nutrition is relevant for the stability of mycorrhizal symbiosis, which, like any mutualism, is susceptible to invasion by cheaters (i.e., non-rewarding plants or fungi). This is especially crucial since mycorrhizal symbiosis is a network, where fungi link different plants from the same or different species (Simard and Durall 2004, Selosse et al. 2006), allowing some of these to behave as C sinks. Nevertheless, mycorrhizal associations lasted >400 million years (e.g., Selosse and Le Tacon 1998). As an explanation for this, green plants and fungi were recently demonstrated to symmetrically reward more the most cooperative partners, i.e., plants furnishing the most C and fungi furnishing the most mineral nutrients (Kiers et al. 2011), and thus to counter-select cheaters from both sides (Selosse and Rousset 2011). However, MH and MX plants, which obviously avoid the fungal partners' choice based on C flow, somehow avoid this sanction. The difficult emergence of MH lineages, as we suggest here, may nevertheless contribute to limiting the strength of MX species as C sinks in mycorrhizal networks.

Last, the need for multiple, complex adaptations in transition to heterotrophy explains why heterotrophy arose in a limited number of cases in lineages with MX nutrition or microspermy. This may also apply far beyond mycoheterotrophy, in the numerous other mixotrophic lineages. Among the so-called "hemiparasitic" plants, i.e., green parasitic plants that tap into the sap of other plants, mixotrophy turns out to be the rule (Těšitel et al. 2010, Bell et al. 2011). Yet, the evolution into pure heterotrophy ("holoparasitism") occurred in a limited number of lineages only (Westwood et al. 2010). Although the adaptations required for holoparasitism differ somewhat from those in MH plants, they are complex, too (Westwood et al. 2010, Bell et al. 2011). Mixotrophic nutrition may be evolutionarily stable in hemiparasitic plants because, in this framework, simple loss of function cannot achieve successful transition to heterotrophy. Indeed, albino variants exist in hemiparasitic species such as *Striga* spp., but remain rare (Press et al. 1991). In unicellular planktonic algae, MX nutrition by phagocytosis or uptake of dissolved organic matter is widespread and evolved repeatedly (Crane and Grover 2010). Despite a few notable exceptions mentioned in the *Introduction* (Gockel and Hachtel 2000, Hannaert et al. 2003, Reyes-Prieto et al. 2008, Lim and McFadden 2010), there is less loss of photosynthesis than expected if a simple loss of function was sufficient. Beyond the selective advantages of photosynthesis, some metabolic or cellular constraints may also limit MH evolution in unicellular algae. Assessing the pressures that slow down evolution to heterotrophy in MX organisms needs more data and experimental approaches in plants (e.g., deriving predictions from Fig. 9 and Table 5). It

also needs replicate studies of fitness and physiology in phylogenetically independent lineages that display albino variants, e.g., in parasitic plants, whose metabolism is currently attracting considerable interest (Těšitel et al. 2010), and in easily tractable unicellular lineages, such as euglenids (Rosati et al. 1996).

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