Mycobiont identity and light conditions affect belowground morphology and physiology of a mixotrophic orchid Cremastra variabilis (Orchidaceae)

Takahiro Yagame, Tomáš Figura, Eiji Tanaka, Marc-André Selosse, Tomohisa Yukawa

DOI:
https://doi.org/10.1007/s00572-024-01138-8

Mycorrhiza
https://doi.org/10.1007/s00572-024-01138-8

RESEARCH

Mycobiont identity and light conditions affect belowground morphology and physiology of a mixotrophic orchid *Cremastra variabilis* (Orchidaceae)

Takahiro Yagame[1,2] · Tomáš Figura[3,4,5] · Eiji Tanaka[6] · Marc-André Selosse[5,7,8] · Tomohisa Yukawa[9]

Received: 3 May 2023 / Accepted: 12 February 2024

Abstract

We have investigated whether mycobiont identity and environmental conditions affect morphology and physiology of the chlorophyllous orchid: *Cremastra variabili*s. This species grows in a broad range of environmental conditions and associates with saprotrophic rhizoctonias including Tulasnellaceae and saprotrophic non-rhizoctonian fungi from the family Psathyrellaceae. We cultured the orchid from seeds under aseptic culture conditions and subsequently inoculated the individuals with either a Tulasnellaceae or a Psathyrellaceae isolate. We observed underground organ development of the inoculated *C. variabilis* plants and estimated their nutritional dependency on fungi using stable isotope abundance. Coralloid rhizome development was observed in all individuals inoculated with the Psathyrellaceae isolate, and 1–5 shoots per seedling grew from the tip of the coralloid rhizome. In contrast, individuals associated with the Tulasnellaceae isolate did not develop coralloid rhizomes, and only one shoot emerged per plantlet. In darkness, δ^{13}C enrichment was significantly higher with both fungal isolates, whereas δ^{15}N values were only significantly higher in plants associated with the Psathyrellaceae isolate. We conclude that *C. variabilis* changes its nutritional dependency on fungal symbionts depending on light availability and secondly that the identity of fungal symbiont influences the morphology of underground organs.

Keywords Coralloid rhizome · Orchidaceae · Symbiotic culture · *Cremastra variabilis* · Stable isotope

Introduction

All orchids are initial mycoheterotrophs, i.e., are fully dependent on fungi during early ontogenesis (Merckx et al. 2013; Dearnaley et al. 2016). Most become autotrophic at adulthood (AT), but some retain their nutrient dependence on mycorrhizal fungi, a trophic mode called mycoheterotrophy (MH). In temperate regions, some chlorophyllous orchids were found to obtain carbon not only from photosynthesis, but also from associated mycorrhizal fungi at adulthood. They are called mixotrophs (MX) or partial mycoheterotrophs.

We may generalize, albeit with exceptions, that MH orchids are rather specialized and autotrophic orchids are

✉ Takahiro Yagame
sebacina@gmail.com

1 The Mt. Fuji Institute for Nature and Biology, Showa University, 4562 Kamiyoshida, Fujiyoshida, Yamashi 403-0005, Japan

2 Division of Academic Resources and Specimens, Hokkaido University Museum, Kita 10-jo, Nishi 8-chome, Kita-ku, Sapporo, Hokkaido 060-0810, Japan

3 Department of Mycorrhizal Symbioses, Institute of Botany, Czech Academy of Sciences, Lesní 322, 25243 Průhonice, Czechia

4 Naturalis Biodiversity Center, Darwinweg 2, 2333 CR Leiden, The Netherlands

5 Institut de Systématique, Evolution, Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, Sorbonne Université, EPHE, CP, 39, 57 rue Cuvier, F-75005 Paris, France

6 Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan

7 University of Gdańsk, Faculty of Biology, Department of Vertebrate Ecology and Zoology; ul. Wita Stwosza 59, 80-308 Gdańsk, Poland

8 Institut Universitaire de France, Paris, France

9 Tsukuba Botanical Garden, National Museum of Nature and Science, 4-1-1, Amakubo, Tsukuba, Ibaraki 305-0005, Japan

more often generalists (Jacquemyn et al. 2010; Bougoure et al. 2009; Barrett et al. 2010), while MX orchids seem to use both strategies with respect to their choice of mycorrhizal fungal symbionts (e.g., Girlanda et al. 2006; Jacquemyn et al. 2021). Most orchids, namely those considered as autotrophic in adulthood, associate with a polyphyletic assemblage of saprotrophic fungi collectively called rhizoctonias (Dearnaley 2007; Bidartondo et al. 2004; Bougoure et al. 2005; Těšitelová et al. 2013; Rammitsu et al. 2019). Association with rhizoctonias is unique for orchids and Selosse et al. (2022) hypothesized that this specific association evolved from a common ancestor that formed arbuscular mycorrhiza. In addition, most MX and MH orchids form mycorrhizal association with "ectomycorrhizal" fungi (e.g., Jacquemyn et al. 2021; Selosse et al. 2002, 2022; McKendrick et al. 2002; Lallemand et al. 2018; Taylor et al. 2003), i.e., fungi which form ectomycorrhiza, mostly with forest trees. Recently, saprotrophic fungi in the tropics and subtropics were described as main mycobionts feeding MH orchids. For example, three *Gastrodia* species, *G. confusa, G pubilabiata*, and *G. nipponica*, associate with Mycenaceae and Marasmiaceae (Kinoshita et al. 2016), *Gastrodia similis* associates with *Resinicium* spp., *Wullschlaegelia aphylla* with *Gymnopus* and *Mycena* spp. (Martos et al. 2009), *Epipogium roseum* with Psathyrellaceae (Yamato et al. 2005), and *Eulophia zollingeri* with Psathyrellaceae (Ogura-Tsujita and Yukawa 2008). However, MX orchids associating with non-rhizoctonias saprobic mycobionts were rarely detected in previous studies. Suetsugu and Matsubayashi (2021) reported a tiny terrestrial orchid, *Calypso bulbosa,* associated with wood-decaying fungi belonging to the *Protomerulius* genus within the Auriculariales, and the MX status of this orchid was determined by increased stable isotope abundance.

Several orchids change fungal partner during their ontogenesis, e.g., MH species *Gastrodia elata* and *Cyrtosia septentrionalis* associate with *Armillaria* spp. at adulthood (Cha and Igarashi 1995; Terashita and Chuman 1987), whereas fungal partners of the juvenile stage belong to *Mycena* spp. (Xu and Guo 2000; Park and Lee 2013) or unidentified Polyporales spp. (Umata et al. 2013), respectively. Yamato and Iwase (2008) detected ectomycorrhizal fungi of the Thelephoraceae and Russulaceae families in MX *Cephalanthera falcata* but large individuals of this species mainly associated with fungi from an ectomycorrhiza-forming clade of Sebacinales (Yamato et al. 2021). The results of these studies indicate that MX and MH orchids can associate with several fungal species.

Stable isotope abundance analyses (Gebauer and Meyer 2003; Bidartondo et al. 2004) together with molecular identification of mycobionts has become a standard tool of research on trophic strategies in orchids (e.g., Hynson et al. 2013; Schiebold et al. 2017; Selosse et al. 2017). In recent decades, this research has revealed that most MH orchids are enriched in the stable isotopes ^{13}C and ^{15}N, while autotrophic orchids are relatively depleted. In general, MX plants show high variation in stable isotopic values (Julou et al. 2005; Suetsugu and Matsubayashi 2021; Yagame et al. 2021) ranging between those of AT and MH. When both AT and MH references are available at a given site, carbon gain of the target MX plant from the associated fungi can be calculated from its ^{13}C abundance (Hynson et al. 2013).

Light availability affects efficiency of photosynthesis but also the dependency of MX species on their fungal symbionts. The MX plant *Pyrola japonica* (Ericaceae) displays higher heterotrophy levels in shaded conditions, as estimated by carbon and nitrogen stable isotope values (Matsuda et al. 2012). High light availability allowed fully autotrophic survival of mixotrophic orchid *Epipactis helleborine* (May et al. 2020). Effect of light on orchids has primarily been studied in the germination stage, as demonstrated by research on species such as *Bletia purpurea* (Johnson et al. 2011) and *Laelia speciosa* (Díaz et al. 2009). In contrast, influence of light on morphological development of MX orchids had not been investigated, to our best knowledge. We expected that light availability would affect the morphological development of an MX orchid, because light affects the amount of carbohydrates produced as well as the dependency upon the mycobionts for nutrient uptake (May et al. 2020).

In the orchid subtribe Calypsoeae, *Cremastra variabilis* (= *Cremastra appendiculata*) is a common chlorophyllous species widely distributed from Japan and Sakhalin in Russia to Taiwan, and further to the foothills of the Himalayas (Maekawa 1971; Yukawa 1999). It occurs in dry roadside and wet sites along small streams in forests dominated by ectomycorrhizal and arbuscular mycorrhizal trees. This species associates both with saprobic rhizoctonias such as *Tulasnella* spp. (Tulasnellaceae) and saprotrophic non-rhizoctonias such as *Coprinellus* spp. (Psathyrellaceae) (Yagame et al. 2021). Individuals with branched rhizomes mainly were associated with specific Psathyrellaceae mycobionts, whereas individuals associated with rhizoctonias usually did not form a rhizome in their natural habitat (Yagame et al. 2021). However, several mycobionts were usually detected from a single individual of *C. variabilis*, the influence of each mycobiont on morphological development of this orchid being unclear. In addition, *C. variabilis* displayed relatively high $\delta^{15}N$ levels compared to neighboring C3 plants (Yagame et al. 2021), suggesting its mixotrophic status. Furthermore, *C. variabilis* with coralloid rhizomes collected from a natural habitat showed high fungal carbon dependency based on its enrichment with C and N stable isotopes (Yagame et al. 2021). Many MH orchid species often form large belowground coralloid rhizomes, while AT species develop roots. For example, *Corallorhiza*, which is a genus closely related to *Cremastra*,

comprises 11 MH species that do not form roots but develop branched coralloid rhizomes instead (Freudenstein 1997). This morphological feature was also found in many other MH species, such as leafless *Cremastra* species, *Cremastra aphylla*, *Chamaegastrodia sikokiana*, *Yoania japonica*, *Cymbidium macrorhizon*, and *Epipogium aphyllum* (Yagame et al. 2018; Maekawa 1971; Roy et al. 2009; Seidenfaden 1994; Yukawa 1999; Yamashita et al. 2020). The formation of coralloid rhizomes in orchids may thus be an adaptation to maintain mycoheterotrophy. In order to test whether mycobionts affect the level of plant dependency on the fungal symbionts, one of the most suitable methods is a symbiotic pot experiment with orchid and mycobiont.

In the present study, we investigated (i) whether morphological differences in underground structures in *C. variabilis* are caused by inoculation with different isolates (Tulasnellaceae versus Psathyrellaceae) in symbiotic pot culture, and whether degree of heterotrophy differs (ii) between individuals of *C. variabilis* associated by Tulasnellaceae and Psathyrellaceae, and (iii) under different light conditions.

Materials and methods

Due to the complex experimental design, a flowchart including timeline is shown in Supplementary Fig. 1.

Fungal isolates

Isolation and cultivation of *Cremastra variabilis* mycobionts

In this study we used two isolates SI1-1 and FU1-1. The first one, SI1-1, was isolated in a previous study (Yagame et al. 2013) while the second one, FU1-1, was isolated in the present study.

Yagame et al. (2013) obtained three isolates, SI1-1, SI1-2, and SI2-1 from a coralloid rhizome of a single *C. variabilis* individual, collected near Fujinomiya city (35 18′ 36″N, 138 36′ 00″ E), Shizuoka Prefecture, in forest dominated by *Quercus serrata* and *Cryptomeria japonica* in May 2005 and one of the isolates, called "SI1-1" stored in the Tottori University Fungal Culture Collection (TUFC) under the name TUFC 13516 showed symbiotic ability for this orchid. The isolation was performed according to Warcup and Talbot (1967), with slight modifications (see: Yagame et al. 2013). The isolate SI1-1 was identified as *Coprinellus domesticus* belonging to Psathyrellaceae, which is closely related to the mycobiont of a mycoheterotrophic orchid, *Epipogium roseum*, with high sequence similarity (98.8%) of the ITS region (Yagame et al. 2013). The sequence of the fungal isolate in the ITS region including the 5.8S region of rDNA was deposited in the DDBJ database under the number AB597771 (Yagame et al. 2013).

Isolate FU1-1 was obtained in this study as follows: One individual of *C. variabilis* was collected in a forest near Hatto town (35 24′ 44″N, 134 20′ 22″ E), Tottori Prefecture, dominated by *Carpinus japonica*, *Mallotus japonicus*, and *Quercus glauca.* The individual developed roots without coralloid rhizome. The collected plants were kept at 4 °C in individual plastic bags until processed, within 1 day after sampling. To determine the root-colonizing fungi, two yellowish roots (indicating fungal colonization) were sampled. Roots were washed with tap water and cut into 5 mm long pieces. The surface was sterilized in 70% ethanol for 30 s and in sodium hypochlorite solution with 1% available chlorine for 30 s. The root fragments were put into a mortar with 1 mL of sterilized distilled water and ground using a sterilized pestle to disperse the fungal coils. After rinsing the fungal coils in sterilized distilled water five times, they were transferred onto Petri dishes of a modified Czapek Dox agar (sucrose 0.5 g, $NaNO_3$, 0.33 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, KCl 0.1 g, yeast extract 0.1 g, agar 15 g, distilled water 1000 mL) and incubated at 25.0 ± 0.5 ℃ in the dark for 3 days. Fungal colonies growing from the pelotons were transferred using a sterilized scalpel and cultivated on potato dextrose agar (PDA, Difco, Franklin, New Jersey, USA) medium for 3 weeks.

The two fungal isolates were cultured on PDA medium and the medium cubes (1 cm × 1 cm) were also used for symbiotic culture as inoculum. In order to estimate stable isotope values of the isolates, pure hyphae were prepared. SI1-1 and FU1-1 isolates cultured on solid PDA medium cubes (1 cm × 1 cm) were put into sterilized liquid medium (malt extract 0.1 g, yeast extract 0.1 g, distilled water 1000 mL) and incubated at 25.0 ± 0.5 ℃ in the dark. Three weeks after the incubation, PDA cubes were removed from the fungal clumps to obtain pure hyphae of SI1-1 and FU1-1.

Molecular identification of mycobionts

The molecular identification method based on the intergenic transcribed spacer (ITS) region of ribosomal DNA was applied as in Yagame et al. (2013). DNA was extracted from FU1-1 and ITS was amplified from the extracted DNA by polymerase chain reaction (PCR) with the primers ITS1F/ITS4 (Gardes and Bruns 1993) After cloning procedure, the obtained FU1-1 sequence was subjected to BLAST searches (Altschul et al. 1997).

After the end of the symbiotic experiment, the sequence identities of two isolates and fungal DNA extracted from roots of the cultured plantlets were confirmed using the same procedure as described in Yagame et al. (2021). Roots displaying mycobiont colonization were washed in tap water and hand sectioned for examination by microscopy in order to confirm fungal colonization. Total DNA was extracted from the colonized root segments by the

cetyltrimethylammonium bromide method (Weising et al. 1995). After additional purification with the Mag Extractor Plant Genome Kit (Toyobo, Osaka, Japan), DNA was dissolved in 50 µL of Tris-EDTA buffer for PCR. PCR was performed as described above. Identical sequences of the isolates and the root-colonizing fungi were confirmed in MEGA5 (Tamura et al. 2011).

Seed collection and sowing

Pod collection and seed sowing

One pod of *C. variabilis* was collected from a *Quercus serrata* and *Cryptomeria japonica* forest site close to Sagamihara city (35 35′ 17″N, 139 17′ 28″ E), Kanagawa Prefecture, Japan in early October 2006. The pod surface was sterilized by soaking in 70% ethanol and subsequent combustion of the ethanol covering the surface (Yamato and Iwase 2008). The capsules were then cut open with a sterilized scalpel and the seeds were dispersed in sterilized 0.05% Tween 20 solution. They were sown on five Petri dishes (9 cm diam.) with BM1 medium (Van Waes and Debergh 1986). One mL of the suspension containing around 200 seeds was spread onto each Petri dish and incubated at 25.0 ± 0.5 ℃ in the dark. Sixty days after seed sowing, the germination rate was determined under a stereo microscope (SMZ-745N, Nikon, Japan). Seed germination was defined as the emergence of the swollen embryo from the seed coat.

Cultivation of seedlings

Three months after seed sowing, 10 seedlings were transferred into a culture bottle (6 cm in diameter and 30 cm in height: Supplementary Fig. 2) containing 200 mL of Hyponex agar medium (Kano 1968) slightly modified [2 g of Hyponex (N–P–K: 6.5–6–19), 25 g of sucrose, 1 g of peptone, 10 g of agar, 1000 mL of distilled water, pH 5.8], and incubated at 25.0 ± 0.5 ℃ under a photoperiod (165 µmol m^{-2} s^{-1} / 12 h per day). Photosynthetic photon flux density was measured using a Basic Quantum Meter BQM-1 (Apogee, Logan, USA). Transplantation of plantlets was repeated every 12 months, during which *C. variabilis* were propagated asexually under aseptic conditions. Five years after seed germination, we obtained seedlings (Supplement Fig. 3a) which were used for subsequent symbiotic culture. Since they were allopatric to the two sites from which the fungi were isolated, no bias due to local adaptation was expected.

Symbiotic culture of *C. variabilis*

Two fungal isolates, SI1-1 and FU1-1, were used for experimental symbiotic culture with *C. variabilis*. The medium for this culture consisted of sawdust of *Fagus crenata* and two types of volcanic soils—Kanuma (collected from Tochigi Prefecture, Japan), and Kiryuu (collected from Gunma Prefecture, Japan)—mixed at a ratio of 10:1:1 (v/v/v). The pH and moisture content of the medium after autoclaving were 5.5 and 80%, respectively. The composition of the medium was slightly modified compared to the one used in Yagame et al. (2007). This medium is useful for long-term symbiotic culture of orchids associating with Psathyrellaceae. Hyphal growth of Tulasnellaceae had also been confirmed on this medium in a preliminary experiment (Yagame unpublished data). A transparent polycarbonate container (cultivation container: 100 × 110 × 100 mm^3) used for the symbiotic experiment had high ventilation using Merck Milli-Seal (Merck Biopharma Japan Co., Ltd., Tokyo, Japan) on the cover. In order to avoid excess moisture in the medium, ca. 10 mL of Kanuma volcanic soil with large particle sizes (15–20 mm) was first placed at the bottom of the container. This Kanuma layer was overlaid with ca. 150 mL of the medium for symbiotic culture to make an approximately 1 cm deep layer. The containers containing the medium for symbiotic culture were autoclaved at 121 ℃ for 1 h with steam sterilization under pressure.

Since the plantlets used for the symbiotic cultivation had been cultured on agar medium containing sucrose, the nutrient-rich medium remained attached to the roots. In order to prevent excessive growth of mycobiont hyphae, roots were cut by sterilized scissors before the symbiotic culture (Supplement Fig. 3a). One plantlet and one PDA medium cube (1 cm × 1 cm) was placed on the symbiotic culture medium per container with a sterilized scalpel (Supplement Fig. 3b). In order to achieve proper colonization by the isolate, we buried the bottom of the plantlets ca. 5 mm deep into the medium using sterilized tweezers (Supplement Fig. 3b). Then, the fungal isolates were introduced, resulting in three inoculation treatments (inoculated with SI1-1, inoculated with FU1-1, non-symbiotic control). The plantlets of each inoculation treatment were grown either under light or in the dark, which resulted in 6 treatments in total (n = 12 replicates of each treatment). In order to compare $\delta^{13}C$ and $\delta^{15}N$ with an autotrophic reference plant under the symbiotic culture conditions, *Pinus densiflora* seedlings were added to each *C. variabilis* seedling under light. All cultures were incubated at 25 ± 0.5℃. The plantlets were cultured for 192 days.

Mycorrhiza staining

To check fungal colonization in the inoculated treatments, roots and rhizomes of *C. variabilis* were fixed in FAA (4% [v/v] formaldehyde, 5% [v/v] glacial acetic acid, and 50% [v/v] ethanol) for 1–3 days. The fixed samples were dehydrated in an ethanol series and tert-butyl alcohol, embedded in paraffin (Paraplast Plus; Monoject Scientific, St. Louis, Missouri), and sectioned (10 µm thick) using a microtome

(RM2125RT; Leica, Wetzlar, Germany). The sections were mounted on glass slides, dried, deparaffinized twice in Lemosol (Wako Pure Chemical, Osaka, Japan) for 15 min and hydrated in an ethanol series. The tissues were stained with periodic acid-Schiff (PAS) and Alcian blue (pH 2.5). Bright-field images were obtained using a differential interference contrast microscope (E-800; Nikon, Tokyo, Japan).

Stable isotope analysis

Pure hyphae cultured in liquid medium, dried symbiotic culture medium without any previous fungal inoculation and plant material (leaves of *C. variabilis* from the different treatments as well as *Pinus densiflora* seedlings) were analyzed for stable isotope abundance. The samples were oven-dried at 45 °C, ground to a homogenous fine powder using a tissue lyser (TissueLyser Quiagen II), and stored over silica gel. Samples were weighed in tin capsules (using Mettler Toledo MX5 micro scales) and analyzed for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ abundances using a Thermo Flash 2000 elemental analyzer coupled to a Thermo Finnigan Delta V Advantage continuous-flow isotope-ratio mass spectrometer. Relative natural abundances of the stable isotopes (δ values) were calculated as follows: $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1{,}000$ (‰), where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the sample, and $R_{standard}$ is the $^{13}C/^{12}C$ ratio of Vienna Pee Dee Belemnite or $^{15}N/^{14}N$ ratio of atmospheric N_2, respectively. Samples were analyzed in two separate runs. Alanine ($\delta^{13}C = -22.16 \pm 0.05$‰; $\delta^{15}N = 0.59 \pm 0.05$‰) was used as internal standard, and the average and standard deviation of the replicated measurements of this standard were -21.41 ± 0.01‰ and -21.53 ± 0.08‰ for ^{13}C, both runs separately, and 0.64 ± 0.13‰ and 0.69 ± 0.05‰ for ^{15}N, both runs separately. As primary standards, we used caffeine IAEA-600 ($\delta^{13}C = -27.77 \pm 0.04$‰) and ammonium sulfate IAEA-N-1 ($\delta^{15}N = 0.40 \pm 0.20$‰).

Statistical analyses

Statistical analyses were performed using statistical software R 3.2.3 (R Development Core Team 2012). The normality of data and homogeneity of variances was tested using Shapiro–Wilk test (Shapiro and Wilk 1965) and Bartlett test (Bartlett 1937), respectively. Data of ^{13}C isotope abundances did not meet the assumptions of ANOVA and was therefore tested by Kruskal–Wallis test followed by pairwise Wilcoxon rank sum test. The dataset of ^{15}N values in the symbiotic experiment met normality but not homogeneity. In this case, differences between the measurements were statistically tested with Welch's analysis of variance (ANOVA). The two remaining datasets were tested by ANOVA followed by Tukey's honest significant difference test (Tukey 1949). Differences between treatments in the light and in the dark with both *C. variabilis* cultivated with SI1-1 and FU1-1 were tested using Student's *t*-test. Two-way ANOVA (influence of isolate × light or dark) was applied to evaluate the increase in plant fresh weight, number of shoots, and total root length. Mean values were then compared using Scheffe's *F* test ($P < 0.05$).

Results

Identification of the fungal isolates

The isolate FU1-1, stored in the National Institute of Technology and Evaluation under the name NBRC 116064, was isolated from roots of a *C. variabilis* individual collected at Hatto town, Tottori Prefecture in June 2011. The isolate FU1-1 was identified as a Tulasnellaceae sp. and deposited in the DDBJ database under the code LC440252 in present study, which was same sequence as phylotype H in Yagame et al. (2021).

Shoot development and survival of *Cremastra variabilis* under symbiotic culture conditions

Sixty days after seeds were sown on BM1 (aseptic culture conditions), the seed germination rate was $62.1 \pm 6.7\%$ (mean ± SE). Five years after seed germination, we obtained 232 plantlets (Supplement Fig. 3a). We selected 72 out of 232 plantlets for a symbiotic culture experiment (12 per treatment). Fresh weight, height, and width of the

Table 1 Number of plantlets with shoot formation and total shoot number in symbiotic culture with Psathyrellaceae isolate (SI1-1), Tulasnellaceae isolate (FU1-1), and non-fungal control in *Cremastra variabilis*

Days[a]	Fungal treatments and light condition					
	SI1-1		FU1-1		Control	
	Light	Dark	Light	Dark	Light	Dark
60	4 (4)	3 (3)	0	0	0	0
90	12 (15)	9 (13)	3 (3)	2 (2)	0	0
100	12 (20)	11 (22)	12 (12)	7 (7)	3 (3)	1 (1)

The total shoot numbers in each section examined are shown in parentheses

[a]Propagated period of 5-year-old aseptic seedlings under symbiotic culture conditions

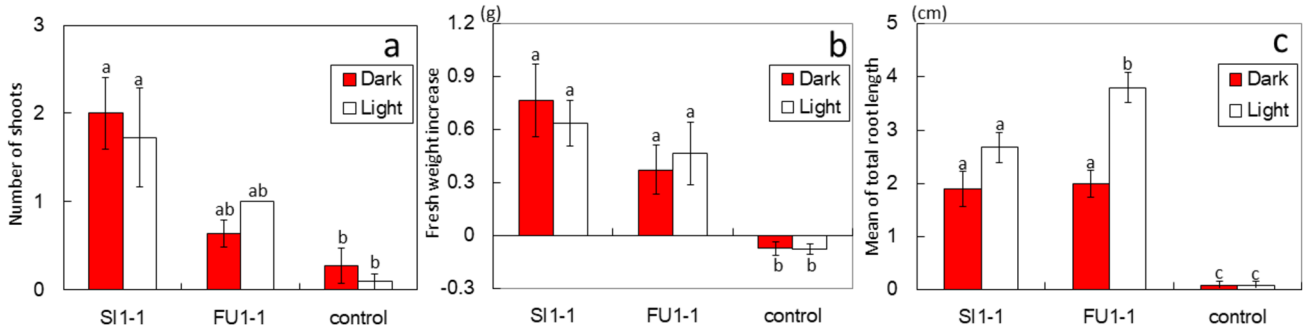

Fig. 1 **a** Average number of *C. variabilis* shoots formed in symbiotic in vitro culture with a Psathyrellaceae (SI1-1) or a Tulasnellaceae (FU1-1) species, and in non-symbiotic control treatment. **b** Fresh weight increase (in g) of *Cremastra variabilis* and **c** mean of total root length (in cm) under the same symbiotic conditions. Letters indicate significant differences according to two-way ANOVA followed by Scheffe's *F* test ($P < 0.05$). Error bars show standard error (SE)

selected plantlets were 1.86 ± 0.17 g, 1.54 ± 0.12 cm, and 2.14 ± 0.14 cm (mean ± SE), respectively. No significant differences were found in the fresh weight, height, and width of the selected plantlets among the treatments.

Twenty days after start of the symbiotic experiment, fungal hyphae had covered the surface of the medium in all the inoculated cultivation containers. Sixty days after the experiment was set up, we observed shoot formation of 3 plantlets in the dark and 4 plantlets in light with SI1-1 isolate (Table 1). Conversely, no shoots were formed with the isolate FU1-1 or in the non-symbiotic control at this time (Table 1). Ninety days after the experiment was set up, 2 plantlets in the dark and 3 in light formed shoots with FU1-1, whereas 9 plantlets in the dark and all 12 in light formed shoots with SI1-1 (Table 1). Seedlings in the non-symbiotic control still did not develop any shoots at this time. One hundred days after the experiment was set up, 3 seedlings of the non-symbiotic control under light developed shoots, whereas a single seedling formed one shoot in the dark (Table 1). At this time point, plantlets growing in light with the SI1-1 isolate formed 1 to 5 shoots per plant, and plantlets with FU1-1 one shoot per plant. Mean shoot number of plantlets cultivated with SI1-1 in the dark was 2.00 ± 0.40 (mean ± SE), under light 1.70 ± 0.19. In the case of plantlets cultivated with FU1-1, 1.00 ± 0.00 mean shoot was observed under light versus 0.63 ± 0.15 in the dark. In the non-symbiotic treatments, 0.27 ± 0.30 shoots were observed under light versus 0.08 ± 0.30 in the dark (Fig. 1a). Plantlets with SI1-1 formed multiple shoots; however, the shoot numbers did not significantly differ between light and dark treatments in any of the three inoculation treatments (Fig. 1a). The only significant differences in shoot number were found between plantlets cultivated with SI1-1 and the non-symbiotic controls independently of light conditions (Fig. 1a). At the end of the experiment (192 days after its start), all individuals of the non-symbiotic treatment in the dark had died (Table 2), while the maximum survival rate of *C. variabilis* was 92% (11/12) with SI1-1 under light. Survival was always better under light in spite of competition with the reference AT plant (the *Pinus* seedlings) (Table 2).

Morphological differences of *Cremastra variabilis* induced by isolates

Fungal coils were formed in the roots of *C. variabilis* with both inoculated isolates (Fig. 2), without any between-isolate morphological differences in the coils. Fungal ITS sequences amplified from the roots were identical to those of the SI1-1 and FU1-1 isolates, confirming successful inoculation. Concerning fresh weight increase in *C. variabilis*, no significant differences were found between the SI1-1 and FU1-1 treatments, but plants produced significantly higher fresh weights with either isolates than in the non-symbiotic control treatment (Fig. 1b; $P < 0.05$). The highest mean fresh weight increase, compared to the non-symbiotic control, was 0.76 g ± 0.2 in the SI1-1 treatment under light (Fig. 1b).

Conspicuous morphological differences were observed in the *C. variabilis* plants depending on the inoculated isolate (Fig. 3). Branched coralloid rhizomes with shoots

Table 2 Survival rates (%) of *Cremastra variabilis* in symbiotic culture conditions with Psatyrellaceae or Tulasnellaceae isolate (SI1-1 or FU1-1) and non-symbiotic control

Fungal treatments	SI1-1		FU1-1		Control	
Light condition	Light	Dark	Light	Dark	Light	Dark
Survival rates (%)	92	75	83	42	33	0

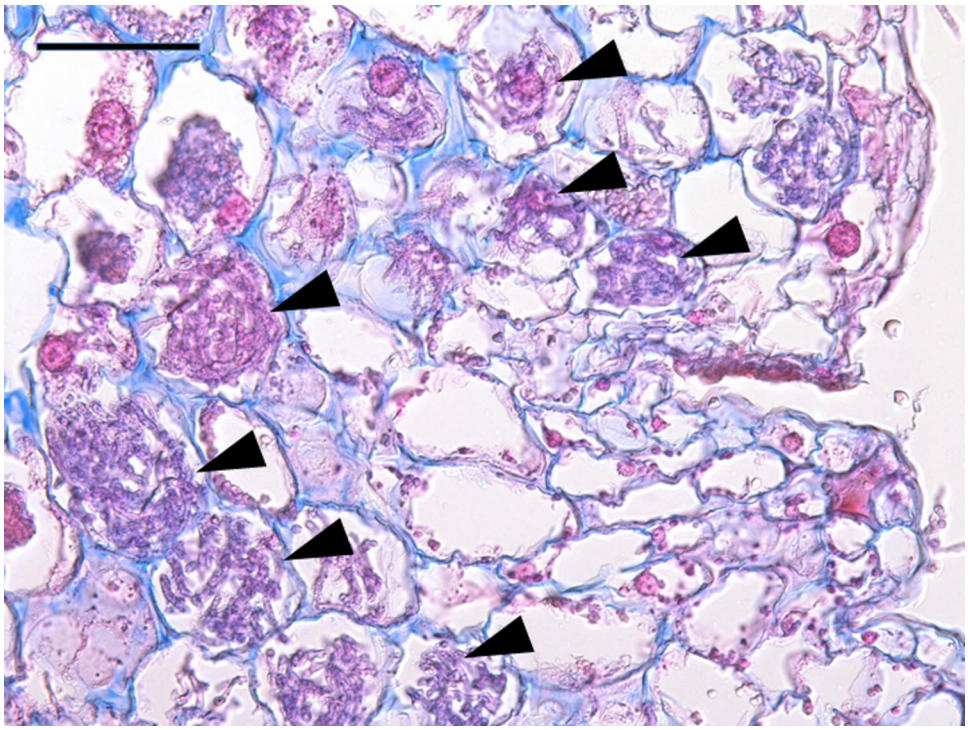

Fig. 2 Sections of a root of *Cremastra variabilis* colonized with the Tulasnellaceae isolate FU1-1 and stained with periodic acid-Schiff stain (PAS). Arrowheads point to formed fungal coils. Bar = 100 µm

developed only with the SI1-1 isolate (Fig. 3a, b), where browning of the original plantlets was observed in the dark (Fig. 3b). In contrast, plantlets with FU1-1 did not develop coralloid rhizomes, but shoots emerging directly from the bottom of the original plantlets (Fig. 3c, d). Total root length was generally longer in the newly formed plantlets exposed to light and longest with FU1-1 under light, whereas the shortest roots were observed in the non-symbiotically grown plantlets (Fig. 1c; $P < 0.05$). The highest mean total root length was 2.11 cm ± 0.23 in the FU1-1 treatment under light, whereas the lowest mean was 0.07 cm ± 0.08 in the non-symbiotic treatment.

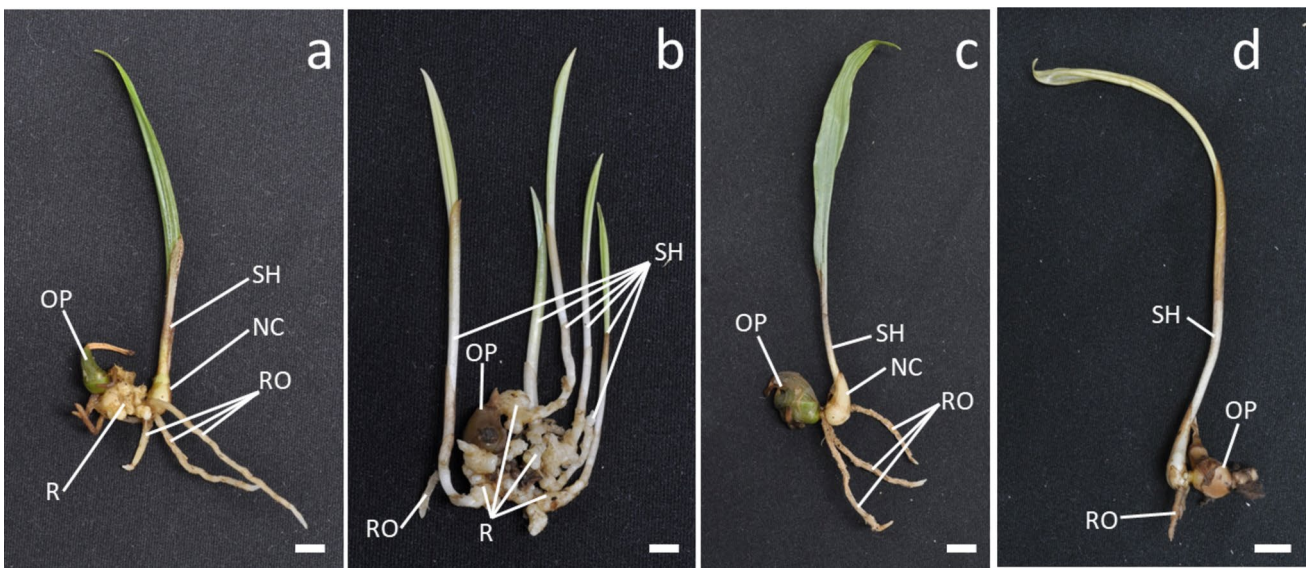

Fig. 3 Morphological differences in *Cremastra variabilis* plant bodies cultured for 192 days with the SI1-1 (Psathyrellaceae) isolate under light (**a**), SI1-1 in the dark (**b**), FU1-1 under light (**c**), and FU1-1 in the dark (**d**). Branched rhizome (R), root (RO), shoot (SH), original plantlet (OP), and newly formed corm (NC), respectively. Bars = 1 cm

Stable isotope analysis

Because all plants died in the non-symbiotic treatment in the dark, we determined the relative abundances of ^{13}C and ^{15}N stable isotopes in the leaves of *C. variabilis* plants from the five remaining treatments (Fig. 4). We first determined the relative abundances of ^{13}C and ^{15}N stable isotopes in the fungal isolates SI1-1 and FU1-1 cultivated on the same medium without plants. They both displayed a similar enrichment in ^{13}C and ^{15}N compared to the substrate (Fig. 4).

Welch's ANOVA determined significant differences in the $\delta^{13}C$ of *C. variabilis* leaves between the five experimental treatments ($P < 0.001$), and the autotrophic co-cultivated *P. densiflora* seedlings were most depleted in ^{13}C (Fig. 4a). Under light, *C. variabilis* plants did not differ between the three treatments. In the dark, the symbiotic culture treatments with SI1-1 and FU1-1 did non-significantly differ between each other, but had significantly higher $\delta^{13}C$ than under light with FU1-1 ($P < 0.05$; Fig. 4a). Stronger enrichment in the dark whatever the mycorrhizal strain, may indicate that plant survival is dependent on carbon provided by the fungus.

For $\delta^{15}N$, Welch's test also determined significant differences between the five treatments ($P = 8.001e\text{-}16$), and the *C. variabilis* seedlings under non-symbiotic control were most depleted in ^{15}N (Fig. 4b). Under light, *C. variabilis* with SI1-1 and FU1-1 were enriched in ^{15}N compared to the non-symbiotic control, suggesting that N nutrition was modified by the fungal symbiont. In the dark, the treatment with SI1-1 was further enriched, while a non-significant decrease was observed in the treatment with FU1-1 ($P < 0.05$; Fig. 4b). To summarize, mycorrhizal colonization enriched *C. variabilis* in ^{15}N in the light indicating N supply by the fungus. This trend was enhanced in the dark only in association with the SI1-1 isolate (Fig. 4b).

Discussion

Role of isolate identity in plant belowground morphological development

We succeeded in cultivating *C. variabilis* from seed to mature plant and propagated multiple individuals from a single pod using aseptic culture conditions (5 years from seed until start of the experiment). We even maintained them in a long-term in vitro symbiotic culture for 192 days. A similar cultivation protocol for large-scale micropropagation of *C. variabilis* in aseptic culture has recently been developed also by Yang et al. (2022), so that this species is a suitable model for investigating the influence of fungal isolate identity on orchid physiology and morphology. In the present study, seedlings originating from a single pod were used for symbiotic culture. Since the seedlings had a more uniform genetic background compared to seedlings in natural habitat, the influences of the two isolates on the physiological and morphological properties of *C.variabilis* plantlets was truly estimated.

Cremastra variabilis formed a coralloid rhizome exclusively with isolate SI1-1 (Psathyrellaceae), but not with FU1-1 (Tulasnellaceae) or without fungi. The morphological characteristics of the non-symbiotic control plants under light were similar to those inoculated with the FU1-1 isolate, pointing to a specific role of SI1-1 in morphological development. Determining significant differences in numbers of shoots, fresh weights and stable isotope values between plants associated with the two isolates (Figs. 1a, b and 4) may have required even higher replicate numbers per treatment than n = 12. However, the dimorphism and differences in root length were clearly confirmed (Figs. 1c and 3). This corroborates a similar dimorphism that was observed, albeit

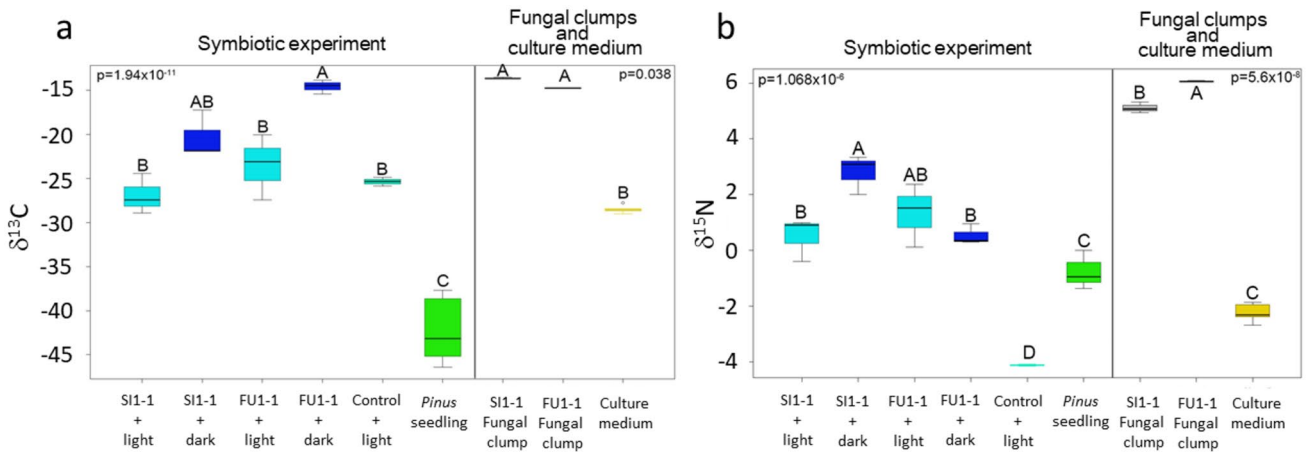

Fig. 4 Boxplots illustrating stable isotope abundances of $\delta^{13}C$ (**a**) and $\delta^{15}N$ (**b**) in *Cremastra variabilis* growing with the SI1-1 (Psathyrellaceae) isolate under light and in the dark, *C. variabilis* growing with the FU1-1 (Tulasnellaceae) isolate under light and in the dark, asymbiotically growing *C. variabilis*, autotrophic non-orchid control (*Pinus densiflora*), fungal clumps in SI1-1 and FU1-1, and culture medium. Letters indicate significant differences according to ANOVA followed by the Tukey HSD post-hoc test ($P < 0.05$)

on a correlative basis, in natural habitats for two species belonging (as *Cremastra*) to subtribe Calypsoeae: *Calypso bulbosa* and *Oreorchis indica* (Suetsugu and Matsubayashi 2021; Suetsugu et al. 2020). However, in these studies, the authors did not demonstrate whether morphological differences were caused by the root-colonizing fungal isolate or whether fungal identity and morphology co-varied as a response to another factor.

Mycorrhizal colonization is well known to induce morphological changes in the roots of most plants (Smith and Read 2008). In this study, we found fungal coil formation in roots (Fig. 2) and fresh weight gain in *C. variabilis* cultivated with FU1-1 (Fig. 1b). Moreover, we observed longer roots in the light than in the dark (Fig. 1c). The survival rate of *C. variabilis* with FU1-1 was higher than that of non-symbiotic individuals (Table 1), so we consider the FU1-1 isolate beneficial for *C. variabilis*. Longer root development with FU1-1 was induced under light condition, which is a first report of light effect on the morphological development of an orchid plantlet associating with a specific mycobiont. Interestingly, isolate FU1-1 did not induce seed germination of *C. variabilis* in our preliminary experiment, whereas SI1-1 stimulated seed germination and induced coralloid rhizome of plantlets (Yagame et al. 2013). Under aseptic culture conditions, *C. variabilis* forms a rhizome and propagates asexually (Yagame unpublished data). This orchid has an intrinsic ability to form rhizome under nutrient-rich conditions. SI1-1 may supply more nutrients to *C. variabilis* than FU1-1, and this ability of SI1-1 could lead to rhizome formation and seed germination in the orchid.

This is congruent with previous studies where Psathyrellaceae, namely *Coprinellus* species, are mostly found in protocorms (or coralloid rhizomes) and rhizoctonias in adult *C. variabilis* roots (Zahn et al. 2022; Suetsugu et al. 2022; Yagame et al. 2021). It is also possible that such selection of a fungal partner may be caused by an actual physiological need of the plant, e.g., for more (or different) nutrients, including carbon during early life and in adulthood.

Nutritional dependency on fungi

All plants asymbiotically grown in the dark died before the end of the experiment (192 days), whereas they survived with the fungal isolates, even in dark conditions (Table 2). These results suggest that the fungus (in the absence of any other carbon sources available to the plant) provides carbon to the orchid, adding to photosynthesis in the light or replacing it in the dark. In other words, this suggests MX nutrition in *C. variabilis*.

Cremastra variabilis cultivated in the dark with FU1-1 displayed significantly higher ^{13}C enrichment compared to the individuals cultured in the light (Fig. 4a), which indicates survival on carbon provided by the fungus. The axenic cultivation of the fungi and their high ^{13}C enrichment on the medium suggests that carbon was gained from the fungi. Light availability often drives the dependency of orchids on fungal carbon: this dependency has been observed in temperate MX plants such as *Epipactis* and *Cephalanthera* (May et al. 2020; Preiss et al. 2010; Gonneau et al. 2014). *Epipactis helleborine*, which is normally associated with ectomycorrhizal fungi in shady forests, can occasionally grow in full light even in a field, far from forests and thus from ectomycorrhizal fungi (Rydlo 2008; May et al. 2020) In such case, this species was found to be able to switch to rhizoctonia (May et al. 2020).

Similarly, it seems that although *C. variabilis* is usually found in shady forests, this species may change its mycobionts leading to lower dependence on fungi at sufficient light availability (see Yagame et al. 2021). To summarize, enrichment of *C. variabilis* tisssues by ^{13}C was higher in the dark with both fungi, suggesting that this orchid can compensate the lack of self-fixed carbon with carbon provided by the fungus when light availability is low (Fig. 4a).

Cremastra variabilis associated with the Psathyrellaceae SI1-1 isolate formed a coralloid rhizome (Fig. 3), and the morphological characteristics of this underground organ strongly resembled those of the related achlorophyllous species, *C. aphylla*, also associated with Psathyrellaceae (Yagame et al. 2018). As expected for MH species, high $\delta^{13}C$ values were reported for *C. aphylla* compared to co-occurring C3 plants in a recent study by Suetsugu et al. (2022). The present study revealed no statistical differences in $\delta^{13}C$ between *C. variabilis* associated with a Psathyrellaceae isolate (coralloid rhizome development) and with a Tulasnellaceae isolate (no coralloid rhizome development). Because of limited coralloid rhizome growth in our experiment, the level of carbon heterotrophy in *C. variabilis* may not have differed between Psathyrellaceae (SI1-1) and Tulasnellaceae (FU1-1) under symbiotic culture conditions. In addition, the type and amount of substrate may also have affected the isotopic composition of the plant. Noteworthy, a relative from the same subtribe, *Calypso bulbosa* var. *bulbosa*, which also displays underground organ dimorphism, had relatively high carbon heterotrophy in coralloid rhizome (based on $\delta^{13}C$), but without statistically significant differences between individuals with and without coralloid rhizome (Suetsugu and Matsubayashi 2021).

While dependence on carbon from the mycobiont varied in *C. variabilis* significantly due to light conditions, fungal nitrogen level was rather stable (Fig. 4b). Little is known about nitrogen metabolism in orchid mycorrhizas, but orchid species that are more dependent on fungi often contain higher total N and higher $\delta^{15}N$ levels (Liebel et al. 2010; Hynson et al. 2009; Minasiewicz et al. 2023). However, ^{15}N enrichment is not universal determinant of all MX/MH species (see Merckx et al. 2013). Interestingly, $\delta^{15}N$ values of *C. variabilis* plants with SI1-1

were higher in the dark than in the light in our study. However, the difference was not significant between the individuals with FU1-1 in the dark, which suggests lower dependency on fungal nitrogen (Fig. 4b). The *C. variabilis* individuals associating with SI1-1 developed coralloid rhizomes, from which several shoots emerged (Fig. 3a, b). Furthermore, the shoot development observed in plantlets with SI1-1 in the dark (Fig. 3b) may reflect consumption of storage nutrients from the original plantlets in each individual. By contrast, several seedlings with FU1-1 formed a single shoot per seedling without forming coralloid rhizome and the young plantlets remained green (no browning occurred; Fig. 3d). This could be the main difference in nitrogen metabolism of *C. variabilis* between the isolates SI1-1 and FU1-1. In rhizoctonias, *Tulasnella* is unable to utilize nitrate, while it can readily grow on other nitrogen sources (Nurfadillah et al. 2013; Figura et al. 2021). Moreover, Fochi et al. (2017) found two functional ammonium transporters in *Tulasnella calospora* but no nitrate transporters. In further studies, it would be interesting to investigate differences in nitrogen metabolism between the two isolates, using RNA sequencing, which could clarify the mechanism of nitrogen utilization in *C. variabilis* with both isolates. It is worth mentioning that several orchid species associating with rhizoctonias are believed to be "cryptic mycoheterotrophs" (these orchids are green associating with rhizoctonia but based on ^{18}O and ^{2}H analyses receive some organic matter from mycobionts; Minasiewicz et al. 2023; Gebauer et al. 2016; Schiebold et al. 2018; Schweiger et al. 2018), but $\delta^{13}C$ and $\delta^{15}N$ analyses are insufficient to detect such possible cryptic mycoheterotrophy. This phenomenon may at least partly explain the incongruence of $\delta^{15}N$ versus $\delta^{13}C$ analyses. Some papers assume MX in rhizoctonia associated orchids based on enrichment by ^{2}H and ^{18}O in plant issues (Gebauer et al. 2016; Schweiger et al. 2018). These analyses show that plants indeed receive organic compounds from their fungal hosts but do not necessarily prove mycoheterotrophic carbon gain (Jacquemyn et al. 2021; Murata-Kato et al. 2022).

Adaptation ability of orchids with multiple mycobionts to different environmental conditions

The chlorophyllous orchid, *Oeceoclades maculata* (a tropical African species naturalized in America; Stern 1988) has similar mycobionts as *C. variabilis* and the main mycobiont of *O. maculata*, *Psathyrella candolleana* (Psathyrellaceae), stimulates germination of the orchid (Bayman et al. 2016). Typical mycobionts of chlorophyllous orchids, *Ceratobasidium* and *Tulasnella* spp., were also detected in the roots of this species (Bayman et al. 2016). Because both orchid species, *O. maculata* and *C.variabilis* have large distribution ranges, these orchids may adapt to a broad scale of environmental conditions. They can grow in various shaded wet and/or bright dry forests with ectomycorrhizal and/or arbuscular mycorrhizal trees.

Furthermore, we found that *C. variabilis* can develop a specific organ when associated with a Psathyrellaceae sp. and changes its root length as response to different mycobiont and light conditions. The closely related MH species, *C. aphylla,* uniformly develops a large branched rhizome and extremely short roots (Yagame et al. 2018). This species only occupies cool and wet valleys, with broadleaved forests dominated by *Ulmus laciniata* (Ulmaceae) and *Cercidiphyllum japonicum* (Cercidiphyllaceae), etc., *Cremastra aphylla* may have changed its niche toward shaded habitats by shortening its roots and developing narrower fungal specificity. However, *C. variabilis* not only grows sympatrically with *C. aphylla*, but also at much drier and warmer habitats. The morphological plasticity of the belowground organs and plurality of mycobionts in *C. variabilis* may increase its adaptability toward a broader range of environmental conditions. In general, mycoheterotrophic species (including orchids) have a rhizome and/or shortened roots while long branched roots, which are typical for autotrophic plants, are usually missing (McKendrick et al. 2000; Imhof 2010). In fact, heavy fungal colonization was found in the branched rhizome of *C. aphylla* (Yagame et al. 2018). Chlorophyllous orchids seem to depend on longer roots to absorb water and nutrients in case of autotrophy and/or relatively low fungal dependency. In the present study, we have shown that *C. variabilis* may combine autotrophic and mycoheterotrophic nutrition depending on light and mycobiont availability.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00572-024-01138-8.

Acknowledgements We would like to thank E. Funabiki for assistance with sampling and fungal isolation, and H. Abe and Y. Yamashita for support and technical assistance in obtaining molecular data. We also would like to thank K. Ishii and M. Matsumoto in Tokyo Metropolitan Mizuho Nogei High School for support in culture of isolates fungal clumps.

Author contribution T. Yagame conceived the idea of the study. T. Yagame and T. Figura developed the statistical analysis plan and conducted statistical analyses. T. Yagame, T. Figura, and E. Takana contributed to the interpretation of the results. T. Yagame and T. Figura drafted the original manuscript. T. Yagame, T. Figura, M. A. Selosse, and T. Yukawa supervised the conduct of this study. All authors reviewed the manuscript draft and revised it critically on intellectual content. All authors approved the final version of the manuscript to be published.

Funding This work was supported by a research grant from the National Museum of Nature and Science, The Global COE Program "Advanced Utilization of Fungus/Mushroom Resource for Sustainable Society in Harmony with Nature (15K14442)," and JSPS KAKENHI Grant Numbers (15H04417). T. Figura was supported by the Czech Science Foundation (project ThiefHunt GN23-05310O) and the Czech Academy of Sciences within the long-term research development program RVO 67985939.

Availability of data and materials Deposited DNA data in the DNA Data Bank of Japan were used in this study. Other data were generated or analyzed during this study are included in this article and its supplementary file.

Declarations

Ethical approval This article does not contain any studies with human or animal subjects performed by any of the authors.

Competing interests The authors declare no competing interests.

References

Altschul SF, Madden TL, Schafer AA et al (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. https://doi.org/10.1093/nar/25.17.3389

Barrett CF, Freudenstein JV, Taylor DL et al (2010) Range wide analysis of fungal associations in the fully mycoheterotrophic *Corallorhiza striata* complex (Orchidaceae) reveals extreme specificity on ectomycorrhizal *Tomentella* (Thelephoraceae) across North America. Am J Bot 97:628–643. https://doi.org/10.3732/ajb.0900230

Bartlett NS (1937) The statistical conception of mental factors. Br J Psychol 28:97–104. https://doi.org/10.1111/j.2044-8295.1937.tb00863.x

Bayman P, Espinosa ATM, Aponte CMS et al (2016) Age-dependent mycorrhizal specificity in an invasive orchid, *Oeceoclades maculata*. Am J Bot 103:1880–1889. https://doi.org/10.3732/ajb.1600127

Bidartondo MI, Burghardt B, Gebauer G et al (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc Royal Soc B 271:1799–1806. https://doi.org/10.1098/rspb.2004.2807

Bougoure J, Ludwig M, Brundrett M et al (2009) Identity and specificity of the fungi forming mycorrhizas with the rare mycoheterotrophic orchid *Rhizanthella gardneri*. Mycol Res 113:1097–1106. https://doi.org/10.1016/j.mycres.2009.07.007

Bougoure J, Bougoure DS, Cairney JWG et al (2005) ITS-RFLP and sequence analysis of endophytes from *Acianthus*, *Caladenia* and *Pterostylis* (Orchidaceae) in southeastern Queensland. Mycol Res 109:452–460. https://doi.org/10.1017/s095375620500225x

Cha JY, Igarashi T (1995) *Armillaria* species associated with *Gastrodia elata* in Japan. Eur J Plant Pathol 25:319–326. https://doi.org/10.1111/j.1439-0329.1995.tb01347.xCit

Dearnaley J, Perotto S, Selosse MA (2016) Structure and development of orchid mycorrhizas. In: Martin F (ed) Molecular mycorrhizal symbiosis. Wiley-Blackwell, Hoboken, New Jersey, pp 63–86

Dearnaley JDW (2007) Further advances in orchid mycorrhizal research. Mycorrhiza 17:475–486. https://doi.org/10.1007/s00572-007-0138-1

Díaz IÁ, Oyama K, Alonso GC, Garciglia RS (2009) In vitro propagation of the endangered orchid *Laelia speciosa*. PCTOC 99:335–343. https://doi.org/10.1007/s11240-009-9609-8

Figura T, Tylová E, Jersáková J et al (2021) Fungal symbionts may modulate nitrate inhibitory effect on orchid seed germination. Mycorrhiza 31:231–241. https://doi.org/10.1007/s00572-021-01021-w

Fochi V, Chitarra W, Kohler A et al (2017) Fungal and plant gene expression in the *Tulasnella calospora–Serapias vomeracea* symbiosis provides clues about nitrogen pathways in orchid mycorrhizas. New phytol 213:365–379. https://doi.org/10.1111/nph.14279

Freudenstein JV (1997) A monograph of *Corallorhiza* (Orchidaceae). Harv Pap Bot 1:5–51

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2:133–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Gebauer G, Meyer M (2003) ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. New Phytol 160:209–223. https://doi.org/10.1046/j.1469-8137.2003.00872.x

Gebauer G, Preiss K, Gebauer AC (2016) Partial mycoheterotrophy is more widespread among orchids than previously assumed. New Phytol 211:11–15. https://doi.org/10.1111/nph.13865

Girlanda M, Selosse MA, Cafasso D et al (2006) Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. Mol Ecol 15:491–504. https://doi.org/10.1111/j.1365-294X.2005.02770.x

Gonneau C, Jersáková J, Tredern ED et al (2014) Photosynthesis in perennial mixotrophic *Epipactis* spp. (Orchidaceae) contributes more to shoot and fruit biomass than to hypogeous survival. J Ecol 102:1183–1194. https://doi.org/10.1111/1365-2745.12274

Hynson NA, Madsen TP, Selosse MA et al (2013) The physiological ecology of mycoheterotrophy. In: Merckx VSFT (ed) Mycoheterotrophy: The biology of plats living on fungi. Springer Science + Business Media, New York, pp 297–340

Hynson NA, Preiss K, Gebauer G (2009) Is it better to give than to receive? A stable isotope perspective on orchid–fungal carbon transport in the green orchid species *Goodyera repens* and *Goodyera oblongifolia*. New Phytol 182:8–11. https://doi.org/10.1111/j.1469-8137.2009.02778.x

Imhof S (2010) Are monocots particularly suited to develop mycoheterotrophy? In: Seberg O, Petersen G, Barfod AS (eds) Diversity, phylogeny, and evolution in the monocotyledons: Proceedings of the Fourth International Conference on the Comparative Biology of the Monocotyledons and the Fifth International Symposium on Grass Systematics and Evolution. Aarhus Universitetsforlag, Aarhus, pp 11–23

Jacquemyn H, Honnay O, Cammue PAB et al (2010) Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus Orchis. Mol Ecol 19:4086–4095. https://doi.org/10.1111/j.1365-294X.2010.04785.x

Jacquemyn H, Brys R, Waud M et al (2021) Mycorrhizal communities and isotope signatures in two partially mycoheterotrophic orchids. Front Plant Sci 12:618140. https://doi.org/10.3389/fpls.2021.618140

Johnson TR, Kane ME, Pérez HE (2011) Examining the interaction of light, nutrients and carbohydrates on seed germination and early seedling development of *Bletia purpurea* (Orchidaceae). Plant Growth Regul 63:89–99. https://doi.org/10.1007/s10725-010-9516-3

Julou T, Burghardt B, Gebauer G, Berveiller D, Damesin C, Selosse MA (2005) Mixotrophy in orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalanthera damasonium*. New Phytol 166:639–653. https://doi.org/10.1111/j.1469-8137.2005.01364.x

Kano K (1968) Acceleration of the germination of so-called hard-togermination orchid seeds. Amer Orchid Soc Bull 37:690–698

Kinoshita A, Ogura-Tsujita Y, Umata H et al (2016) How do fungal partners affect the evolution and habitat preferences of mycoheterotrophic plants? A case study in *Gastrodia*. Am J Bot 103:207–220. https://doi.org/10.3732/ajb.1500082

Lallemand F, Robionek A, Courty PE et al (2018) The ^{13}C content of the orchid *Epipactis palustris* (L.) Crantz responds to light as in autotrophic plants. Bot Lett 165:265–273. https://doi.org/10.1080/23818107.2017.1418430

Liebel HT, Bidartondo MI, Preiss K et al (2010) C and N stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean and Macaronesia. Am J Bot 97:903–912. https://doi.org/10.3732/ajb.0900354

Maekawa F (1971) The wild orchids of Japan in color [in Japanese]. Seibundo-shinkosha, Tokyo, Japan

Martos F, Dulormne M, Pailler T et al (2009) Independent recruitment of saprotrophic fungi as mycorrhizal partners by tropical

achlorophyllous orchids. New Phytol 184:668–681. https://doi.org/10.1111/j.1469-8137.2009.02987.x
Matsuda Y, Shimizu S, Mori M et al (2012) Seasonal and environmental changes of mycorrhizal associations and heterotrophy levels in mixotrophic *Pyrola japonica* (Ericaceae) growing under different light environments. Am J Bot 99:1177–1188. https://doi.org/10.3732/ajb.1100546
May M, Jąkalski M, Novotná A et al (2020) Three-year pot culture of *Epipactis helleborine* reveals autotrophic survival, without mycorrhizal networks, in a mixotrophic species. Mycorrhiza 30:51–61. https://doi.org/10.1007/s00572-020-00932-4
McKendrick SL, Leake JR, Read DJ (2000) Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal *Salix repens* and *Betula pendula* to the orchid *Corallorhiza trifida* through shared hyphal connections. New Phytol 145:539–548. https://doi.org/10.1046/j.1469-8137.2000.00592.x
McKendrick SL, Leake JR, Taylor DL et al (2002) Symbiotic germination and development of the mycoheterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. New Phytol 154:233–247. https://doi.org/10.1046/j.1469-8137.2002.00372.x
Merckx VSFT, Mennes CB, Peay KG et al (2013) Evolution and diversification. In: Merckx V (ed) Mycoheterotrophy. Springer, New York, NY. https://doi.org/10.1007/978-1-4614-5209-6_5
Minasiewicz J, Zwolicki A, Figura T et al (2023) Stoichiometry of carbon, nitrogen and phosphorus is closely linked to trophic modes in orchids. BMC Plant Biol. https://doi.org/10.1186/s12870-023-04436-z. https://bmcplantbiol.biomedcentral.com/articles/
Murata-Kato S, Sato R, Abe S et al (2022) Partial mycoheterotrophy in green plants forming *Paris*-type arbuscular mycorrhiza requires a thorough investigation. New Phytol 234:1112–1118. https://doi.org/10.1111/nph.18049
Nurfadillah S, Swarts ND, Dixon KW et al (2013) Variation in nutrient-acquisition patterns by mycorrhizal fungi of rare and common orchids explains diversification in a global biodiversity hotspot. Ann Bot 111:1233–1241. https://doi.org/10.3390/jof9010088
Ogura-Tsujita Y, Yukawa T (2008) High mycorrhizal specificity in a widespread mycoheterotrophic plant, *Eulophia zollingeri* (Orchidaceae). Am J Bot 95:93–97. https://doi.org/10.3732/ajb.95.1.93
Park EJ, Lee WY (2013) In vitro symbiotic germination of mycoheterotrophic *Gastrodia elata* by *Mycena* species. Plant Biotechnol Rep 7:185–191. https://doi.org/10.1007/s11816-012-0248-x
Preiss K, Adam IKU, Gebauer G (2010) Irradiance governs exploitation of fungi: fine-tuning of carbon gain by two partially mycoheterotrophic orchids. Proc Royal Soc B 277:1333–1336. https://doi.org/10.1098/rspb.2009.1966
R Development Core Team (2012) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
Rammitsu K, Yagame T, Yamashita Y et al (2019) A leafless epiphytic orchid, *Taeniophyllum glandulosum* Blume (Orchidaceae), is specifically associated with the Ceratobasidiaceae family of basidiomycetous fungi. Mycorrhiza 29:159–166. https://doi.org/10.1007/s00572-019-00881-7
Roy M, Yagame T, Yamato M et al (2009) Ectomycorrhizal Inocybe species associate with the mycoheterotrophic orchid *Epipogium aphyllum* but not its asexual propagules. Ann Bot 104:595–610. https://doi.org/10.1093/aob/mcn269
Rydlo J (2008) *Epipactis helleborine* jako polni plevel. Muz a Současnost, Roztoky, ser natur 23:211–218
Schiebold JMI, Bidartondo MI, Karasch P et al (2017) You are what you get from your fungi: nitrogen stable isotope patterns in *Epipactis* species. Ann Bot 119:1085–1095. https://doi.org/10.1093/aob/mcw265
Schiebold JMI, Bidartondo MI, Lenhard F et al (2018) Exploiting mycorrhizas in broad daylight: partial mycoheterotrophy in a common nutritional strategy in meadow orchids. J Ecol 106:168–178. https://doi.org/10.1111/1365-2745.12831
Schweiger JMI, Bidartondo MI, Gebauer G (2018) Stable isotope signatures of underground seedlings reveal the organic matter gained by adult orchids from mycorrhizal fungi. Funct Ecol 32:870–881. https://doi.org/10.1111/1365-2435.13042
Seidenfaden G (1994) The genus *Chamaegastrodia* (Orchidaceae). Nord J Bot 14:293–301. https://doi.org/10.1111/j.1756-1051.1994.tb00603.x
Selosse MA, Weiss M, Jany JL et al (2002) Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) LCM Rich. and neighbouring tree ectomycorrhizae. Mol Ecol 11:1831–1844. https://doi.org/10.1046/j.1365-294x.2002.01553.x
Selosse MA, Charpin M, Not F (2017) Mixotrophy everywhere on land and water: the grand écart hypothesis. Ecol Lett 20:246–263. https://doi.org/10.1111/ele.12714
Selosse MA, Petrolli R, Mujica MI et al (2022) The waiting room hypothesis revisited by orchids: were orchid mycorrhizal fungi recruited among root endophytes? Ann Bot 129:259–270. https://doi.org/10.1093/aob/mcab134
Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591–611. https://doi.org/10.2307/2333709
Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic Press, San Diego, CA, USA
Stern WL (1988) The long-distance dispersal of *Oeceoclades maculata*. Am Orchid Soc Bull 57:960–971
Suetsugu K, Matsubayashi J (2021) Subterranean morphology modulates the degree of mycoheterotrophy in a green orchid *Calypso bulbosa* exploiting wood-decaying fungi. Funct Ecol 35:2305–2315. https://doi.org/10.1111/1365-2435.13864
Suetsugu K, Haraguchi T, Tanabe AS et al (2020) Specialized mycorrhizal association between a partially mycoheterotrophic orchid *Oreorchis indica* and a *Tomentella* taxon. Mycorrhiza 31:1–8. https://doi.org/10.1007/s00572-020-00999-z
Suetsugu K, Haraguchi TF, Tayasu I (2021) Novel mycorrhizal cheating in a green orchid: *Cremastra appendiculata* depends on carbon from deadwood through fungal associations. New Phytol 235:333–343. https://doi.org/10.1111/nph.17313
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumer S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739. https://doi.org/10.1093/molbev/msr121
Taylor DL, Bruns TD, Szaro TM et al (2003) Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. Am J Bot 90:1168–1179. https://doi.org/10.3732/ajb.90.8.1168
Terashita T, Chuman S (1987) Fungi inhabiting wild orchids in Japan IV: *Armillariella tabescens,* a new symbiont of *Galeora septentrionalis*. Trans Mycol Soc Japan 28:145–154
Těšitelová T, Jersáková J, Roy M et al (2013) Ploidy-specific symbiotic interactions: divergence of mycorrhizal fungi between cytotypes of the *Gymnadenia conopsea* group (Orchidaceae). New Phytol 199:1022–1033. https://doi.org/10.1111/nph.12348
Tukey JW (1949) Comparing individual means in the analysis of variance. Biometrics 5:99–114. https://doi.org/10.2307/3001913
Umata H, Ota Y, Yamada M et al (2013) Germination of the fully myco-heterotrophic orchid *Cyrtosia septentrionalis* is characterized by low fungal specificity and does not require direct seed-mycobiont contact. Mycoscience 54:343–352. https://doi.org/10.1016/j.myc.2012.12.003

Van Waes JM, Debergh PC (1986) Adaptation of the tetrazolium method for testing the seed viability, and scanning electron microscopy study of some Western European orchids. Physiol Plant 66:435–442. https://doi.org/10.1111/j.1399-3054.1986.tb05947.x

Warcup JH, Talbot PHB (1967) Perfect states of *Rhizoctonias* associated with orchids. New Phytol 66:631–641. https://doi.org/10.1111/j.1469-8137.1967.tb05434.x

Weising K, Nybom H, Wolf K, Meyer W (1995) DNA fingerprinting in plants in plants and fungi. CBC, Boca Raton

Xu J, Guo S (2000) Retrospect on the research of the cultivation of *Gastrodia elata* Bl a rare Chinese traditional medicine. Chin Med J 113:686–692

Yagame T, Yamato M, Mii M, Suzuki A, Iwase K (2007) Developmental processes of achlorophyllous orchid, *Epipogium roseum*: from seed germination to flowering under symbiotic cultivation with mycorrhizal fungus. J Plant Res 120:229–236. https://doi.org/10.1007/s10265-006-0044-1

Yagame T, Funabiki E, Yukawa T et al (2018) Identification of mycobionts in an achlorophyllous orchid, *Cremastra aphylla* (Orchidaceae), based on molecular analysis and basidioma morphology. Mycoscience 59:18–23. https://doi.org/10.1016/j.myc.2017.08.001

Yagame T, Funabiki E, Nagasawa E et al (2013) Identification and symbiotic ability of Psathyrellaceae fungi isolated from a photosynthetic orchid, *Cremastra appendiculata* (Orchidaceae). Am J Bot 100:1823–1830. https://doi.org/10.3732/ajb.1300099

Yagame T, Lallemand F, Selosse MA et al (2021) Mycobiont diversity and first evidence of mixotrophy associated with Psathyrellaceae fungi in the chlorophyllous orchid *Cremastra variabilis*. J Plant Res 134:1213–1224. https://doi.org/10.1007/s10265-021-01337-w

Yamashita Y, Kinoshita A, Yagame T et al (2020) Physisporinus is an important mycorrhizal partner for mycoheterotrophic plants: identification of mycorrhizal fungi of three *Yoania* species. Mycoscience 61:219–225. https://doi.org/10.1016/j.myc.2020.05.003

Yamato M, Iwase K (2008) Introduction of asymbiotically propagated seedlings of *Cephalanthera falcata* (Orchidaceae) into natural habitat and investigation of colonized mycorrhizal fungi. Ecol Res 23:329–337

Yamato M, Kosaka R, Masui Y, Goda Y, Shirasaka S, Maruyama A, Yukawa T (2021) Mycorrhizal associates of *Cephalanthera falcata* (Orchidaceae) in a habitat with giant individuals. Ecol Res 36:177–188. https://doi.org/10.1111/1440-1703.12187

Yamato M, Iwase K, Yagame T et al (2005) Isolation and identification of mycorrhizal fungi associating with an achlorophyllous plant, *Epipogium roseum* (Orchidaceae). Mycoscience 46:73–77. https://www.jstor.org/stable/23596213

Yang N, Wang D, Gao Y et al (2022) An efficient micropropagation protocol, chemical components, and hypoglycemic activity for *Cremastra appendiculata* (D. Don) Makino pseudobulbs. In Vitro Cell Dev Biol Plant 58:213–224. https://doi.org/10.1007/s11627-021-10222-z

Yukawa T (1999) *Cremastra aphylla* (Orchidaceae), a new mycoparasitic species from Japan. Annals of the Tsukuba Botanical Garden 18:59–60

Zahn FE, Lee YI, Gebauer G (2022) Fungal association and root morphology shift stepwise during ontogenesis of orchid *Cremastra appendiculata* towards autotrophic nutrition. AoBP Plants 14:1–10. https://doi.org/10.1093/aobpla/plac021