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Japonolirion osense, a close relative of the mycoheterotrophic genus *Petrosavia*, exhibits complete autotrophic capabilities

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Abstract

The plant kingdom exhibits a diversity of nutritional strategies, extending beyond complete autotrophy. In addition to full mycoheterotrophs and holoparasites, it is now recognized that a greater number of green plants than previously assumed use partly of fungal carbon. These are termed partial mycoheterotrophs or mixotrophs. Notably, some species exhibit a dependency on fungi exclusively during early ontogenetic stages, referred to as initial mycoheterotrophy. *Japonolirion osense*, a rare plant thriving in serpentinite soils, emerges as a potential candidate for initial mycoheterotrophy or mixotrophy. Several factors support this hypothesis, including its diminutive sizes of shoot and and seeds, the establishment of *Paris*-type arbuscular mycorrhizal associations, its placement within the Petrosaviales—largely composed of fully mycoheterotrophic species—and its ability to face the challenging conditions of its environment. To explore these possibilities, our study adopts a multidisciplinary approach, encompassing stable isotope abundance analyses, in vitro experiments, anatomical analyses, and comparative plastome analyses. Our study aims to (1) determine whether *J. osense* relies on fungal carbon during germination, indicating initial mycoheterotrophy, (2) determine if it employs a dual carbon acquisition strategy as an adult, and (3) investigate potential genomic reductions in photosynthetic capabilities. Contrary to expectations, our comprehensive findings strongly indicate that *J. osense* maintains complete autotrophy throughout its life cycle. This underscores the contrasting nutritional strategies evolved by species within the Petrosaviales.

Keywords *Japonolirion osense*, Petrosaviales, Stable isotopes, Mixotrophy, In vitro, Plastome

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Introduction

In general, photosynthesis is essential for plants to obtain carbon. However, achlorophyllous plants exist in nature [1, 2]. Among achlorophyllous plants, parasitic plants are well-known for their direct connections to the phloem and xylem of a host plant, from which they extract water, nutrients, and assimilates [2–4]. More recently, however, mycoheterotrophic plants have received increased attention. These "second-hand" or indirect plant parasites are obligately dependent on fungal carbon. In symbioses with arbuscular or ectomycorrhizal fungi, the fungal carbon has been fixed primarily by plants and exchanged for soil nutrients in mutualistic mycorrhizal relationships, whereas, in symbioses with saprophytic fungi, the fungal carbon comes from dead plant materials [1, 5, 6].

Mycoheterotrophy can be restricted to the early developmental stages of a plant's life cycle. These so-called "initial mycoheterotrophs" develop functional photosynthesis as they mature. In addition to full (FMH) and initial mycoheterotrophs (IMH), which do not perform photosynthesis for at least part of their life, research in the last decade has shown that their relatives are sometimes mixotrophic (or partially mycoheterotrophic; hereafter as MX): combining photosynthesis with fungal carbon gain simultaneously [7]. Mycoheterotrophy has been confirmed in about 9% of plant species, mostly in orchids [8, 9]. However, recent studies have shown that a much larger number of plant species may be capable of mixotrophy, namely those establishing *Paris*-type arbuscular mycorrhiza [10–15].

FMH has evolved multiple times within angiosperms [7]. It has been hypothesized that MX or IMH may be an evolutionary predisposition to FMH, as mixotrophic and IMH species are frequently within the same taxonomic group as FMH [16, 17].

The majority of FMH species belong to monocots [7]. The earliest diverging order of monocots, Petrosaviales, comprises a single family, Petrosaviaceae, with two genera: *Petrosavia* (two species) and *Japonolirion* (single species) (APG IV, 2016; Cameron et al., 2003). *Japonolirion* and *Petrosavia* differ in their ecology (Funamoto 2023). Namely, both *Petrosavia* species are FMH [19, 20], growing in dark (sub)tropical humid forest understory, and are capable of autogamy. *Japonolirion* is generally considered to be autotrophic, relying on insect pollination and inhabiting open meadows at colder-northern latitudes and higher altitudes on serpentinite soils [19, 21]. Petrosaviales are distributed throughout eastern and southeastern Asia [18, 21], and *J. osense* is endemic to Japan, known from only three localities [22, 23].

Classification of *J. osense* as autotrophic is based solely on the presence of green leaves [20]. Additionally, a large endosperm [22] may support this. However, its diminutive size and relatively small seeds raise questions. It is unknown whether this species can be MX or IMH. It should also be noted that all members of Petrosaviaceae, including *J. osense*, are associated with arbuscular mycorrhizal (AM) fungi, especially those that form *Paris*-type AM associations [20]. Recently, stable isotope natural abundance data have suggested that green plants forming *Paris*-type AM can be frequently MX [10], so the classification of *J. osense* as autotrophic requires re-evaluation. In addition, the work of Murata-Kato et al. (2022) highlights the need for a more detailed investigation of MX in green plants associated with *Paris*-type AM, emphasizing that ^{13}C enrichment in such plants could be indicative of factors beyond the carbon nutritional mode. Consequently, the nutritional mode of *J. osense* remains an intriguing puzzle, largely unexplored, and remains to be explored.

In the present study, we focused on the life strategy of *J. osense*, in particular its nutritional mode and plastome composition. First, we examined the anatomical structure of seeds, post-germination ontogenetic development, and nutritional requirements to determine whether the initial stages of the *J. osense* life cycle rely on mycoheterotrophy. Secondly, using stable isotope abundance analyses we explored the possibility of MX in adult plants, to determine if *J. osense* exhibits this dual approach to nutrition in its mature phase. Finally, we examined the plastid genome of *J. osense* for any signs of pseudogenization or gene losses, particularly those genes associated with photosynthesis, which could provide insights into potential evolutionary shifts toward reduced photosynthetic dependence or efficiency [24, 25]. By addressing these questions, our study aims to unravel the trophic strategies of the *J. osense* and contribute to the broader understanding of plant-fungal interactions in an evolutionary context.

Materials and methods

Plant material

Mature seeds of *Japonolirion osense* were collected in Japan, Hokkaido Prefecture., Teshio County, Horonobe Town, Nakatoikan, 44.97572, 142.13208, 161 m a.s.l., on 17 September 2021 into paper bags. In addition, above- and below-ground parts of *Japonolirion osense* and surrounding plants were collected for stable isotope analysis from four different plots at the above site on 22 July 2009 (see Table S1 for details). Samples were oven-dried and stored on silica gel until further processing.

Identification of plant material

The identification of the plant material was carried out in the field by a plant taxonomist, Dr Kenji Suetsugu. A voucher specimen of *Japonolirion osense* has been prepared and is currently stored in the herbarium at Kobe University, while the herbarium does not have a formal

system for issuing unique specimen IDs. For the isotope reference plants, we did not create voucher specimens as these are common species.

In vitro cultivation

Based on observations that BM-1 medium is a suitable medium for the germination of pyroloids and many orchids, (and our preliminary experiment testing media ¼-2 and BM-1 showed equally good germination rates) we focused on this medium for our experiments [26, 27]. To test which carbon source is required for germination, a modified BM-1 medium was prepared. Since BM-1 medium contains sucrose, glutamine, glycine, and casein and this combination is suitable for *J. osense*, we prepared five variants of this medium with different carbon sources (no carbon source - "bm-", a variant with amino acids: glutamine, glycine, and casein - "aa", excess of amino acids: glutamine, glycine, and casein - "aa+", variant with sucrose - "bms" and a variant with both, sucrose and amino acids - "full"). The detailed composition of the media is shown in Table S2. The pH was adjusted to 5.8 using 1 M KOH and 0.2 M HCl, and the media were autoclaved at 144 kPa, 121 °C (Tuttnauer 2540 EK-N) for 20 min and poured into 9 cm plastic Petri dishes.

Seeds were disinfected in 5 mL syringes as previously described [27, 28]. Briefly, seeds were preincubated in 70% ethanl for 5 min, washed three times with deionized water (<0.2 µm cm−1), and treated in $Ca(OCl)_2$ solution for 5 min. In the end, the seeds were washed three times with sterile deionized water. The $Ca(OCl)_2$ solution was prepared by dissolving 20 g of chlorinated lime (Kittfort, Czech Republic) in 100 mL of deionized water, filtering through filter paper, and adding a drop of Tween 20. This solution was used within 30 min after filtering to prevent coagulation. Approximately 10 seeds seeds per Petri dish were sown using sterile tweezers. Six Petri dishes sealed with air-permeable foil (Parafilm M) were prepared for each experimental treatment. The cultures were incubated in the dark at 20 °C.

Cultures were observed monthly using a Zeiss Stemi 305 stereomicroscope. The total germination rate was counted when no further seeds were germinating, i.e., 45 days after sowing. To assess the germination rate, seeds lacking embryos or those underdeveloped seeds were excluded. Only well-developed seeds with ruptured testa were considered as germinated according to Figura et al. [27]. After these measurements, i.e. days after sowing, the whole experiment (all Petri dishes) was transferred to the 16/8 photoperiod maintaining the same temperature of 20 °C We also measured the total length of the seedlings (roots, hypocotyl, and shoot) after 22 days of cultivation in the dark, as plants on some media started to develop leaves after this time. Plants with developed leaves were transferred into Erlenmeyer flasks and kept at 20 °C and 16/8 photoperiod. Images of seedlings were taken using a Canon EOS 60D camera equipped with a Canon EF 100 mm f/2.8 L macro lens.

At the end of the experiment, which lasted 70 days (20 days on photoperiod), the plants were harvested and their fresh weight was measured. As most of the seedlings (and the largest ones) were observed on the "full" and "bms" media, the weight of some randomly selected plants was measured, while the rest were transplanted to fresh BM-1 or Orchid Maintenance Medium (Himedia, product code: PT072) to cultivate older plants that could be transferred to pots.

Statistical analyses were performed using RStudio 2023.09.1+494 "Desert Sunflower" release. The normality of the data was assessed using the Shapiro-Wilk test [29], and the homogeneity of variances by Bartlett test [30]. ANOVA with a nested factor (Petri dish) was used to analyze the seedling length data followed by the Tukey-Kramer test (Kramer, 1956) for pairwise comparisons between treatments.

Anatomical analysis of seeds

Mature seeds of *J. osense* were sectioned using a Shandon cryomicrotome after cryoprotection by incubation in 2% sucrose for 12 h at 4 °C. Section (20 µm) were collected on alum gelatin-coated slides [31] and stained with Ponceau 2R in 2% acetic acid (10 min) and Azur (10s) according to [32] to detect proteins or Lugol's solution (1 min) to detect starch. Lipids were detected with Sudan Red 7B according to Soukup [33]. Stained sections were mounted in 65% glycerol and photographed using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) equipped with a Nikon Digital Sight 10 camera (Nikon Europe B.V.) and NIS-Elements AR 5.42.02 software (Laboratory Imaging, Prague, Czech Republic).

Stable isotope abundance analyses and elemental analyses

Samples were ground in 2 mL Eppendorf safe-lock tubes in a ball mill (MM200, Retsch Gmbh, Haan, Germany) and analyzed for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios using a Thermo Flash 2000 elemental analyzer coupled to a ThermoFinnigan DeltaV Advantage continuous-flow isotope-ratio mass spectrometer. The relative 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 $^{13}C/^{12}$ 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. As an internal standard we used alanine ($\delta^{13}C = -22.16 \pm 0.68$‰; $\delta^{15}N = 0.59 \pm 0.07$‰), and the average and standard deviation of the replicated measurements of this standard were -26.65 ± 0.05‰ for ^{13}C and 0.81 ± 0.08‰ for ^{15}N. As primary standards, we used caffeine IAEA-600 ($\delta^{13}C = -27.77 \pm 0.04$‰) and ammonium sulfate

IAEA-N-1 ($\delta^{15}N = 0.40 \pm 0.20‰$). The C/N ratio was calculated using the following formula: C/Nratio = (total % of carbon/12)/(total % of N/14).

Plastid genome annotation

The available complete plastome sequences of Petrosaviales species, namely *J. osense* and *P. stellaris*, were retrieved from GenBank (accession numbers NC_036154.1 and NC_023356.1, respectively). Subsequently, these sequences were annotated using GeSeq [34] with default settings.

We employed an alternative third-party stand-alone annotator, Chloë v0.1.0 (unpublished), which is offered in GeSeq. The Chloë output provides a comprehensive list of gene content and identifies missing exon(s), absent start codon, and premature stop codon, offering insights into potential pseudogenes.

To infer the trophic mode for both species based on the plastid annotations, we applied the model of gene loss in heterotrophic plants (Barrett & Davis, 2012 modified in Graham et al. [35]). This model facilitated the inference of the trophic mode changes by considering potential gene losses and pseudogenization events within the plastome sequences. Specifically, we referred to a list of genes observed to be lost in heterotrophic taxa provided by Graham et al. [35] to identify any specific losses or pseudogenization events in the studied species.

Since Chloë outputs only present genes and not missing ones, we individually cross-referenced against Graham et al. [35] and the summary of plastid-encoded genes in land plants provided by Wicke et al. [36] to ascertain the presence or absence of specific genes. This comprehensive cross-referencing allowed us to examine the complete gene landscape. During this process, synonymous gene names were validated using UniProt [37], ensuring accurate identification and interpretation of gene presence or absence.

Results

In vitro experiments

We successfully cultivated plants from seed to adult plants (Fig. S1). In preliminary experiments, seeds germinated equally well on both media tested (¼-2 and BM-1). Adult plants exhibited the best growth on the Orchid Maintenance Medium (Himedia, product code: PT072), slightly better than on BM-1 media, although this was not evaluated.

In the experiment with different carbon sources in BM-1 medium, there were no significant differences in the total germination rate between the experimental variants ($F4,24=0.145$, $P=0.963$). However, the highest median germination was observed on the full medium containing both amino acids and sucrose (full, Fig. 1A), whereas the lowest germination was observed on the medium containing no carbon source (bm-, Fig. 1A). No further germination was observed 45 days after sowing.

Seedling lengths were measured 22 days after sowing, just before the emergence of the first shoots. Once again, no significant differences were observed between the experimental variants ($P=0.226$), with the highest median value observed on the medium without any carbon (BM-; Fig. 1B) while the lowest was observed on the medium with only amino acids (aa; Fig. 1B). At the end of the experiment (day 70), living plants with multiple green leaves were present on all variants, except for aa+, However, their number and fresh weight decreased in the following order: bms>full>bm- > aa>aa+ (see Tables S3,4, Fig. S2,S3), while the average number of leaves per plant decreased in the order full>bms>bm- > aa>aa+ (see Table S4).

Anatomical analysis

Mature seeds of *J. osense* were approximately 0.6 mm long and contained a massive multi-layered endosperm surrounding a relatively small embryo (Fig. 2A-C). The embryo appeared oval in shape, resembling a late

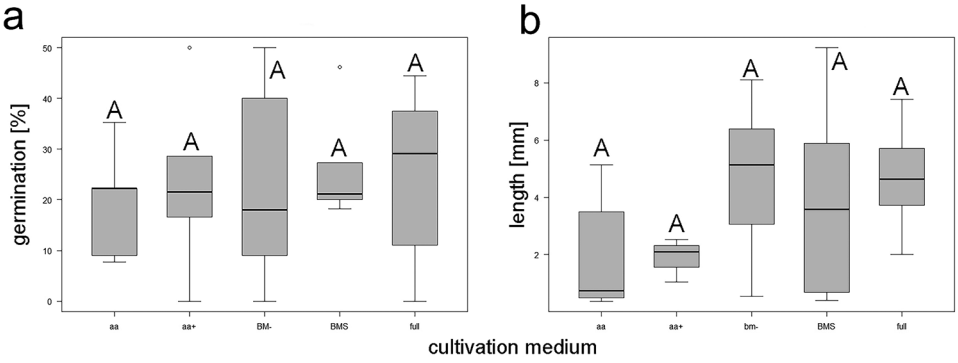

Fig. 1 Effect of different carbon sources on germination (**A**) and total length (**B**) of the *J. osense* seedlings. Final germination was recorded 46 days after sowing and the length of seedlings 22 days after sowing just before the emergence of leaves. The same letters indicate no significant difference according to the Tukey HSD post-hoc test for multiple comparisons. Cultivation media are named as follows: medium with no carbon as "bm-", with glutamine, glycine, and casein as "aa", with an excess of glutamine, glycine, and casein as "aa+", with only sucrose as "bms" and variant with all as "full"

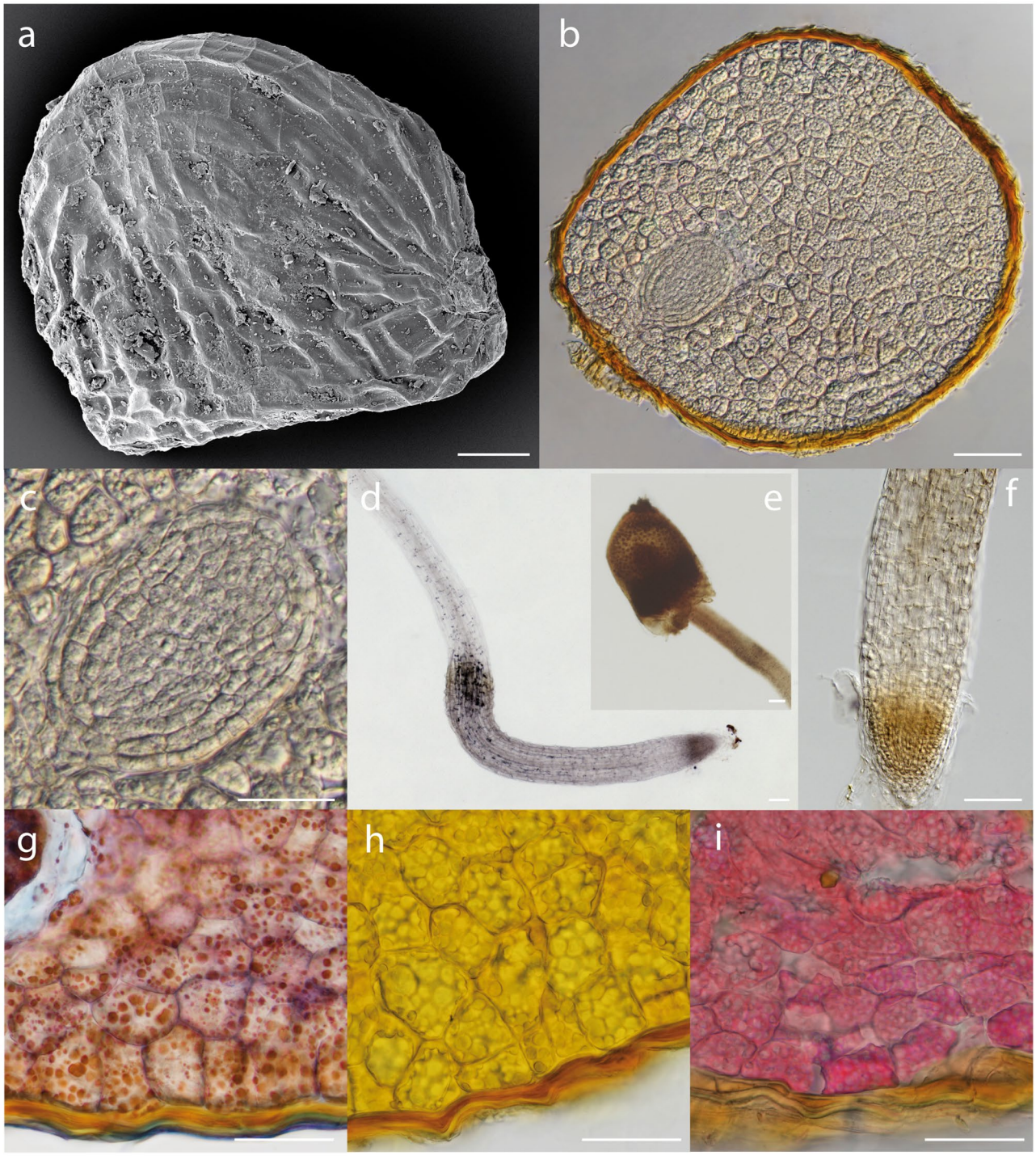

Fig. 2 Seed anatomy of *J. osense*: Seed surface appearance (**A**), Seed inner structure with thick endosperm and small oval embryo (**B**), detail of the embryo (**C**), details of the germinating seedling (**D**-**F**) including the root part (**D**), remnants of the seed (**E**) and detail of the root apical part (**F**), detail of the endosperm stained for proteins using Ponceau 2R and Azur (**G**), detail of endosperm stained for starch using Lugol's solution (H), detail of endosperm stained for lipids using Sudan Red 7B (**I**). TEM (**A**), DIC (**B**, **C**, **F**), bright field (**D**, **E**, **G**-**I**). Scale bars: 100 µm (**A**, **B**, **D**-**F**) or 50 µm (**C**, **G**-**I**)

globular state, and reached a length of up to 0,15 mm in length ($n=6$; Fig. 2B, C). Unfortunately, we could not fully elucidate the details of the embryo structure and observe the entire embryogenesis process in detail due to the limited number of seeds available for the study.

Despite the embryo's small size, its germination was macroscopically similar to many other monocots, such as *Acorus calamus* (for reference see Buell, 1935). It started with the growth of the primary root, enlargement of the hypocotyl, and elongation of the cotyledon, which turned green. The rest of the seed temporarily remained at the top of the cotyledon. The first adventitious root was formed at an early stage, as were the first leaves (Figs. 2D-F and 3). The endosperm contained numerous granular bodies that showed positive staining with Ponceau+Azur but not with Lugol's solution, indicating the presence of proteins but not starch (Fig. 2G, H). The positive histochemical staining with Sudan Red 7B, a dye that efficiently stains lipids in plant material [39], indicated the presence of lipid substances in the endosperm cells (Fig. 2I).

Stable isotope abundances and elemental analysis

Abundances of the stable isotope ^{13}C in *J. osense* tissues were comparable to those of control green plants and were not enriched (Fig. 4, Table S5, S6). Concerning ^{15}N abundances the tissues of *J. osense* were most depleted, with the leaves displaying the greatest depletion compared to the controls (Fig. 4, Table S5, S6). Additionally, the total percentage of nitrogen content was relatively low, with the roots of *J. osense* exhibiting the lowest values (Fig. S4, Table S5, S6). In the case of plot 3, the difference was even more significant (Fig. S4, Table S5, S6). The percentage of total C content of the *J. osense* samples tended to be higher than that of controls, with even a significant difference observed in the case of *J. osense* roots in plot 3. For the C/N ratio, the highest values were consistently observed in the roots of *J. osense* (Fig. S4, Table S5, S6). The majority of the control plants exhibited *Paris*-type AM, while two *Arum*-type AM plants – *Allium schoenoprasum* and *Drosera rotundifolia* – were rather enriched (Table S6, S7).

Plastid genome annotation

The complete plastid sequence lengths were 158,020 bp in *J. osense* and 103,835 bp in *P. stellaris*. The outputs from Chloë are shown in Table S8 and S9 for *J. osense* and *P. stellaris*, respectively. No indications of possible pseudogenes were identified in the *J. osense* plastid genome. However, in *P. stellaris*, certain genes were flagged as possible pseudogenes, including the NADH dehydrogenase-like complex (*ndh* genes), plastid-encoded polymerase PEP (*rpo* genes), and some of the principal photosynthesis genes (*pet* and *ccsA*). In *J. osense*, all ndh genes were present, whereas, in *P. stellaris*, there was a partial absence of these genes. Among the principal

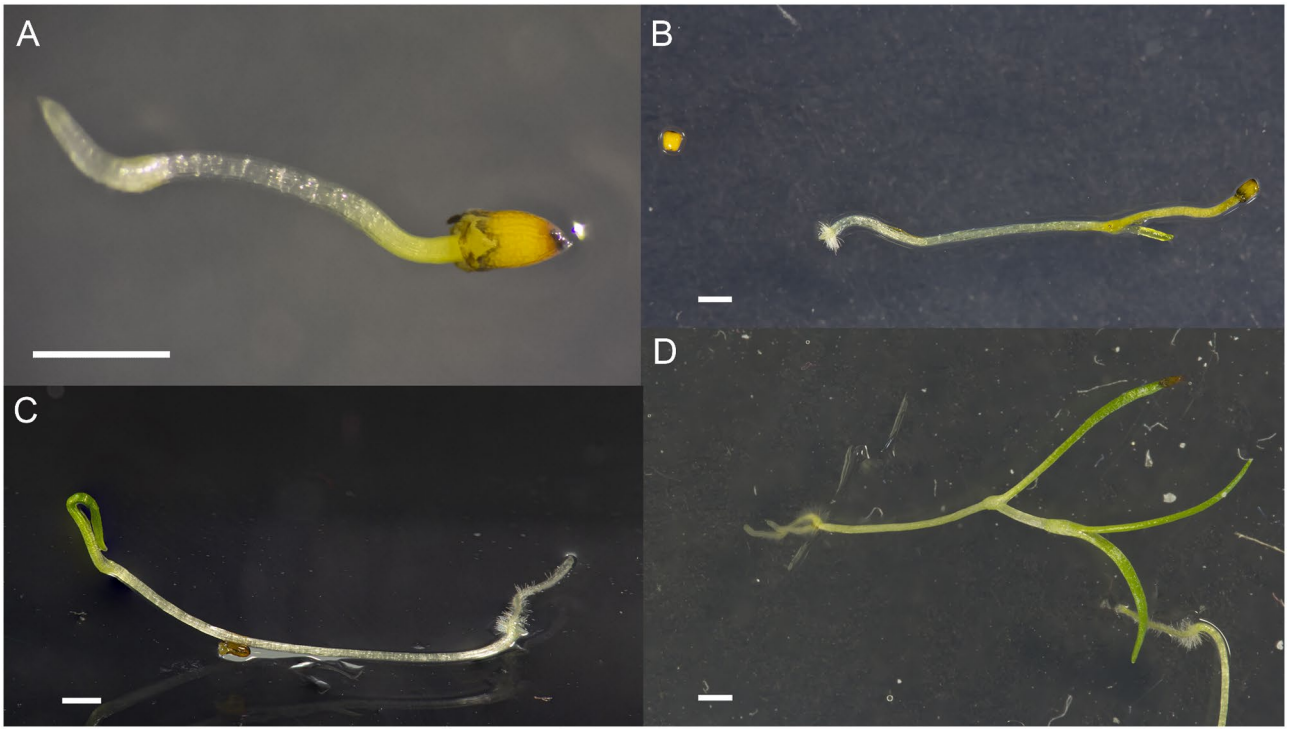

Fig. 3 Different stages of *J. osense* seedling development. (**A**) 27 days after germination on the medium BM-1 "full", (**B**) 70 days after germination on the medium "BM-", (**C**) 41 days after germination on the medium "full" (**D**), and after 70 days on the medium "full"

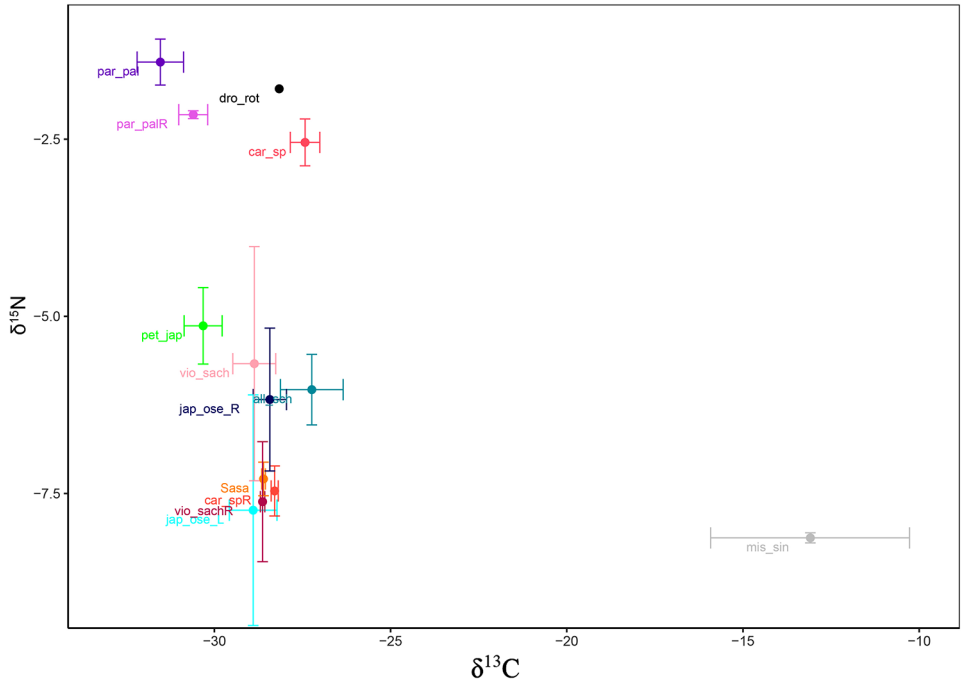

Fig. 4 Isotopic abundance of $\delta^{13}C$ and $\delta^{15}N$ in *J. osense* roots (dark blue, jap_ose_R), *J. osense* leaves (light blue, jap_ose_L), and surrounding autotrophic plants: *Allium schoenoprasum* var. *yezomonticola* leaves (azure; all_sch), *Carex* sp. leaves (pale orange, car_sp), *Carex sp.* roots (dark orange, car_spR), *Drosera rotundifolia* leaves (black, dro_rot), *Miscanthus sinensis* leaves (grey, mis_sin), *Parnassia palustris* leaves (dark_violet, par_pal) *Parnassia palustris* roots (light violet, par_palR), *Petasites japonicus* (green, pet_jap), *Viola sachalinensis* var. *alpina* leaves (light pink, vio_sach), *Viola sachalinensis* var. *alpina* roots (dark pink, vio_sachR), *Sasa* sp. leaves (yellow, Sasa). Bars represent the 95% standard deviation of the mean for each species

photosynthesis genes (*psa*, *psb*, *pet*, *ccsA*, *cemA*, *lhbA*, and *paf*), *J. osense* lacked *psbN* and *lhbA*, although synonymous genes (*pbf1* and *psbZ*, respectively) were present. In *P. stellaris*, these genes were partially lost, along with their synonymous counterparts. Other genes listed by Graham et al. [35] were all present in *J. osense* and partially lost in *P. stellaris* (Table S8, and S9). In summary, *J. osense* did not display any indications of pseudogenes or gene losses, whereas, in *P. stellaris*, only some genes were retained.

Discussion

Our results demonstrate that *J. osense*, despite its close relationship with *Petrosavia*, is fully autotrophic.

Mature seeds have a multi-layered endosperm

The seeds and embryogenesis of *J. osense* have been examined by Tobe (2008), who has documented the presence of endosperm of this species and its small embryo. In agreement with Tobe (2008), our anatomical analysis showed the presence of a multi-layered endosperm and small oval embryo in mature seeds of *J. osense*. In comparison, the seeds of *Petrosavia sakuraii* are of a similar size (0.4–0.8 mm long) according to Tobe (2009) but seem to have a slightly thinner endosperm than in *J. osense* [40], However, an accurate comparison of endosperm thickness is not possible at this time because Tobe (2009) did not provide detailed endosperm size data for *P. sakuraii* and we did not have enough seeds to accurately measure endosperm thickness in a reliable number of them.

FMH and IMH species generally have small endosperms (e.g., one-layered endosperm in Pyroloideae [27]), or even no endosperm (e.g., Orchidaceae [41]). Therefore, the multi-layered endosperm of *J. osense* is indicative of autotrophic rather than mycoheterotrophic germination. In addition, we found abundant protein and lipid substances in the endosperm cells of *J. osense*. This is similar to the endosperm of IMH Pyroloideae [27], so the presence of storage compounds itself may not necessarily mean that the plant is autotrophic. However, our data show that the significantly larger endosperm of *J. osense* contains enough reserves to be able to sustain germination. Mycoheterotrophic plants commonly have dust seeds (Merckx, 2013), which are often smaller than those of *J. osense*, and usually, their embryogenesis is not completed [42]. We have observed small oval embryos in mature seeds of *J. osense*, which could indicate that embryogenesis ceases in the late globular stage in dry seeds, consistent with what Tobe (2008) observed. However, soon after germination, the seedlings form a primary root, elongated hypocotyl, and a greenish elongated cotyledon. This is followed by the emergence of true leaves and adventitious roots. The process macroscopically

resembles the germination of other monocotyledonous species, e.g., *Acorus calamus* [38] or *Allium cepa* [43]. It can therefore be assumed that further embryo differentiation occurs at the beginning of seed germination, including the formation of the cotyledon. However, this stage has not been analyzed in detail. The differentiation of cotyledons is common in autotrophic plants, but the development of cotyledons is extremely rare in mycoheterotrophic plants [44], present only as tiny structures in a single genus *Epirixanthes* [45, 46]. Therefore, the post-germination development of the plant also fits well with autotrophic plants. It is also noteworthy that the germination of *J. osense* started in less than 14 days after sowing, which is considerably faster than what is observed for IMH orchids (2–13 months; Figura et al. [27], Ponert et al. 2013) or pyroloids and *Monotropa* (2–12 months; Figura et al. [27]).

No need for external carbon and no reduction in photosynthetic capabilities

To test the possible dependence of *J. osense* on fungal carbon, we tested whether it could axenically germinate and grow without a source of organic carbon. The seeds germinated in all experimental variants, and the seedling size was also similar in all variants, including the medium without any carbon source, also supporting the view that *J. osense* is autotrophic. In addition, plants on the medium without any carbon source were also able to produce leaves and numerous roots.

The autotrophic mode of life for *J. osense* is further supported by its plastid genome, which did not experience pseudogenization and gene deletions, a clear contrast to *Petrosavia stellaris*, where some genes appear to be pseudogenes or partially lost. The model proposed by Graham et al. [35], which primarily associates *ndh* gene loss with MX and FMH, also suggests that *J. osense* does not fall into this category. Moreover, this indicates that the degradation of plastid genes in Petrosaviales started after the divergence of *Petrosavia* from its common ancestor with *Japonolirion*. It is important to note, however, that Graham et al. [35] included one species classified as partially mycoheterotrophic, *Neottia ovata* (L.) Hartm., which exhibited no signs of plastome degradation, suggesting that the existence of MX plants with no reduction in photosynthesis and plastid genes cannot be ruled out. This highlights the need to examine a broader range of MX species to gain a more comprehensive understanding of plastome evolution in mycoheterotrophic plants. The current model, based on just eight species—one from Ericaceae and seven from Orchidaceae, including one *Neottia* Guett species and six from *Corallorhiza* Gagnebin—provides a limited and potentially biased perspective on plastome degradation in MX.

The lack of stable isotope enrichment suggests autotrophy

The above-mentioned results do not exclude the possibility that this species could be MX under natural conditions. FMH and MX can be detected by the abundance of ^{13}C but also by that of ^{15}N [47, 48]. This method is commonly used, for example, in orchid research. Despite potential limitations [49, 50], it has been successfully applied in studies of arbuscular and ectomycorrhizal systems [13, 51, 52]. In our study, we found no enrichment by ^{13}C and even depletion by ^{15}N isotopes supporting autotrophy of the sampled *J. osense* plants in their natural habitat. Furthermore, elevated total endogenous nitrogen concentrations might be used as a sign of mycoheterotrophy as well [53, 54]. Our samples of *J. osense* from the natural habitat had lower (or similar) total nitrogen concentrations compared to the control plants, which also suggests autotrophy.

Conclusions

We have successfully cultivated *J. osense* from seed to mature plant, and our results demonstrate that *J. osense* can germinate and grow without heterotrophic carbon gain. Based on stable isotope natural abundances, no indication of fungal carbon gain in their natural habitat was found. Therefore, our results indicate that, despite its close relationship to FMH *Petrosavia*, *J. osense* sustains a fully autotrophic existence throughout its life cycle. This discovery places *J. osense* on the opposite - autotrophic side of the autotrophy-mycoheterotrophy continuum in contrast to all other species within the order Petrosaviales. The divergence between *Japonolirion* and *Petrosavia* has been estimated to have occurred at 40.9 [55] or 72.2 mya [56], and the evolution towards a FMH mode of life has therefore started after this divergence. Consequently, any IMH or MX taxa that were potentially part of this evolutionary path have become extinct. The cultivation protocol presented here can now be used for the *ex-situ* conservation or reinforcement of existing populations of the endangered *J. osense*.

Needless to note, we cannot fully exclude the possibility of facultative MX, i.e., under some (environmental) conditions, *J. osense* could be MX, but in this work, we have found any signs favoring such a claim. More broadly, our findings lend support to the perspective proposed by Murata-Kato et al. (2022), asserting that the presence of *Paris*-type arbuscular mycorrhiza in green plants does not invariably denote MX. Moreover, in our study the *Arum*-type AM references were rather enriched, however, limited number of *Arum*-type AM species among our control species does not allow for further comparisons.

Therefore, a thorough study of MX in other green plants associated with *Paris*-type arbuscular mycorrhiza deserves detailed exploration.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05721-1.

Supplementary Material 1: Adult plants of J. osense still on cultivation medium before transferring to pot

Supplementary Material 2: Seedlings of J.osense, 70 days after sowing, growing on different variants of BM-1 medium containing different carbon source. Variant with glutamine, glycine, casein and sucrose (a; "full"), with sucrose only (b;"bms"), with no carbon (c,f; "bm – "), with glutamine, glycine, and casein (d; "aa"), with an excess of glutamine, glycine, and casein as (e; "aa +)

Supplementary Material 3: Average fresh weight in miligrams of J.osense seedlings recorded 70 days after sowing on media amended with different carbon source. Cultivation media are named as follows: medium with no carbon as "bm – ", with glutamine, glycine, and casein as "aa", with an excess of glutamine, glycine, and casein as "aa + , with only sucrose as "bms" and variant with all as "full"

Supplementary Material 4: Total concentrations of nitrogen (a, d, g, j), carbon (b, e, h, k) and C/N ratio (c, f, i, l) on 4 plots: plot 1 (a, b, c), plot 2 (d, e, f) and plot 3 (g, h, i), plot 4 (j, k, l). J. osense leaves are marked by light blue, roots dark blue, plants with C4 metabolism are marked dark green while C3 plants by light blue. Species shortcuts: J. osense roots (R), J. osense leaves (L), Allium schoenoprassum var. yezom leaves (all_sch), Carex sp. leaves (car_sp), Carex sp. roots (car_spR), Drosera rotundifolia leaves (dro_rot), Miscanthus sinensis leaves (mis_sin), Parnassia palustris leaves (par_pal) Parnassia palustris roots (par_palR), Petasites japonicus (pet_jap), Viola sachalinensis var. alpina leaves (vio_sach), Viola sachalinensis var. alpina roots (vio_sachR), Sasa sp., (Sasa). Different letters indicate a significant difference. Results of the Tukey HSD post-hoc test for multiple comparisons are indicated by upper case letters, and results of the pairwise Wilcoxon rank sum test are indicated by lower case letters. Plot 4 was statistically not evaluated due to limited number of samples

Supplementary Material 5: Supplementary Table 1: List of full names of analysed species with shortcuts used in figures and number of samples per variant (n). **Supplementary Table 2**: Cultivation media composition. **Supplementary Table 3**: Average fresh weight of plant per variant and percentual weight loss compared to medium on which biggest plants were growing (BMS). Counted at the end of the experiment (70 days after sowing). **Supplementary Table 4**: Total numbers of green and dead plants per each variant of medium, percentage of living plants per variant of medium and average number of leaves including cotyledons per each variant. Counted at the end of the experiment (70 days after sowing). **Supplementary Table 5**: Statistical test used, p-values of statistical test for each plot separately. δ13C, δ15N total percentage of carbon and nitrogen and C/N ration in tissues. KW stands for Kruskal-Wallis test, ANOVA for analysis of variance. Highlighted are significant values ($p < 0.05$). **Supplementary Table 6**: Raw data of all samples analysed by stable isotope and elemental analyses. **Supplementary Table 7**: List of analysed species, their carbon fixation mode (C3 and C4) and morphological status of arbuscullar mycorrhiza (Arum vs. Paris). Question mark denote some level of uncertainity in determination of PT/AT raised by authors

Supplementary Material 6: **Supplementary Table 8**: Chloë output of J. osense (NC_036154.1)

Supplementary Material 7: **Supplementary Table 9**: Chloë output of P. stellaris (NC_023356.1)

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Author contributions
TF, KS, JP and MAS conceived the idea for the study. TF, JP and MAS developed the research design. MK and KS were involved in the sample collection and species identification. Data analyses awere done by TF, ET and AG, stable isotope analyses by MAS and TF, in vitro analyses by AG and TF, anatomical analyses and visualisation by ET and plastome comparisions by ISBK. Interpretation and writing were done by VSFTM, TF and JP. Funding acquisition by TF and KS. All authors commented and approved the final version of the manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
The authors declared that experimental research works on the plants described in this paper comply with institutional, national, and international guidelines. Field studies were conducted under local legislation and permission was granted for sampling from relevant authorities. The collection of isotope samples and seeds was conducted with full permission from Hokkaido University. The sampling was performed in the Teshio Experimental Forest, owned and managed by Hokkaido University, with all necessary authorizations in place.

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

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