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*RESEARCH ARTICLE*

Short Title: Suetsugu et al.—Mixotrophy in a leafless orchid

**Partial mycoheterotrophy in the leafless orchid *Cymbidium macrorhizon***

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**PREMISE OF THE STUDY:** The evolution of full mycoheterotrophy is one of the most interesting topics within plant evolution. The leafless orchid *Cymbidium macrorhizon* is often assumed to be fully mycoheterotrophic even though it has a green stem and fruit capsule. Here, we assessed the trophic status of this species by analyzing the chlorophyll content and the natural $^{13}C$ and $^{15}N$ abundance in the sprouting and the fruiting season.

**METHODS:** The chlorophyll content was measured in five sprouting and five fruiting individuals of *C. macrorhizon* that were co-occurring. In addition, their $^{13}C$ and $^{15}N$ isotopic signatures were compared with those of neighboring autotrophic and partially mycoheterotrophic reference plants.

**KEY RESULTS:** Fruiting individuals of *C. macrorhizon* were found to contain a remarkable amount of chlorophyll compared to their sprouting counterparts. In addition, the natural abundance of $^{13}C$ in the tissues of the fruiting plants was slightly depleted relative to the sprouting ones. Linear two-source mixing model analysis revealed that fruiting *C. macrorhizon* plants obtained approximately 73.7 ± 2.0% of their total carbon from their mycorrhizal fungi when the sprouting individuals were used as the 100% carbon gain standard.

**CONCLUSIONS:** Our results indicated that despite its leafless status, fruiting plants of *C. macrorhizon* were capable of fixing significant quantities of carbon. Considering the autotrophic carbon gain increases during the fruiting season, its photosynthetic ability may contribute to fruit and seed production. These results indicate that *C. macrorhizon* should, therefore, be considered a partially mycoheterotrophic species rather than fully mycoheterotrophic, at least during the fruiting stage.

**KEY WORDS:** carbon acquisition; chlorophyll content; mixotrophy; mycorrhizas; Orchidaceae; photosynthesis; stable isotopes

The evolution of mycoheterotrophs, which have lost their photosynthetic ability, is one of the most interesting and challenging topics in the study of plant evolution (Merckx, 2013). Full mycoheterotrophy has been observed in a wide range of plant taxa and is estimated to have evolved independently ca. 50 times (Merckx, 2013). Among these lineages, full mycoheterotrophy is surprisingly common among the Orchidaceae, with greater than 1% of all orchid species having completely lost the ability to photosynthesize (Bidartondo, 2005).

The dust-like seeds of orchids are the smallest seeds among angiosperms and are typically wind-dispersed (but also see Suetsugu et al., 2015; Suetsugu, 2018). Orchid seeds lack endosperm and contain only marginal C reserves within their embryos. (Dearnaley et al., 2016). Consequently, the early seedling (protocorm) stage of orchids is dependent on nutrient resources from fungal partners, a nutritional strategy termed initial mycoheterotrophy (Merckx, 2013; Dearnaley et al., 2016). This mycoheterotrophic stage has predisposed orchids to the evolution of life-long mycoheterotrophy, which is achieved by retaining the achlorophyllous status throughout the entire life cycle (Leake, 1994). In addition, some chlorophyllous orchids utilize C resources from their mycorrhizal fungi during their adult stage, a nutritional strategy known as mixotrophy or partial mycoheterotrophy (Gebauer and Meyer, 2003; Selosse et al., 2004; Selosse and Roy, 2009; Hynson et al., 2013; Suetsugu et al., 2017). This strategy is used by various ericaceous species that, interestingly, also produce dust seeds (reviewed by Selosse and Roy, 2009; Hynson et al., 2013).

The natural abundance of stable isotopes (e.g., $^{13}C$ and $^{15}N$) provides a useful measure for assessing the degree of mycoheterotrophy in orchidaceous and ericaceous species (Gebauer and Meyer, 2003). Since ectomycorrhizal fungal, which are the main symbionts of orchidaceous and ericaceous mycoheterotrophs, are enriched in the heavy stable isotopes of C and N, fully mycoheterotrophic species end up possessing isotopic signatures that are similar to those of their fungal partners (Gebauer and Meyer, 2003; Trudell et al., 2003, but also see Lee et al., 2015). Meanwhile, partially mycoheterotrophic species exhibit intermediate isotopic signatures between those of autotrophic and fully mycoheterotrophic species (reviewed by Hynson et al., 2013). The analysis of isotopic signatures was refined by Gebauer and Meyer (2003), who used a linear two-source mixing model that assumes a linear correlation between the nutrient gain from fungi and the enrichment of $^{13}C$ to define the heterotrophic levels of autotrophic (0% organic nutrient gain from fungi) and fully mycoheterotrophic (100% nutrient gain from fungi) plants.

There has been some debate in the botanical literature regarding the photosynthetic capacity of leafless orchids, such as *Corallorhiza trifida*, that retain chlorophyllous stems (Girlanda et al., 2006; Zimmer et al., 2008; Cameron et al., 2009). In a recent study utilizing $^{13}C$ labeling, Cameron et al. (2009) found that *C. trifida* assimilated almost no C and possessed C levels that were similar to those of the fully mycoheterotrophic species *Neottia nidus-avis*. These results deviated from those of an older study (Montfort and Küsters, 1940), which reported that the $CO_2$ assimilation of *C. trifida* inflorescences was 2.2 times higher than the level of respiration. In contrast, Zimmer et al. (2008) found that the natural abundance of $^{13}C$ in the tissues of *C. trifida* was slightly depleted when compared to that of the neighboring full mycoheterotrophs *N.*

*nidus-avis* and *Monotropa hypopitys*. The study reported that *C. trifida* obtained only ca. 77 ± 10% of its C from its fungal partners, according to the two-source mixing model, although this could be overestimated if the plant's respiration $CO_2$ is sufficient to perform photosynthesis, owing to low C fixation; and consequently, the $^{13}C$ utilized by *C. trifida* for photosynthesis could be primarily obtained from *C. trifida* itself, rather than from the atmosphere. In addition, exchange measurements only provide a snapshot of photosynthetic activity, whereas the abundance of stable isotopes provides the insight into the origin of C resources over the entire life history of a plant or plant organ (Liebel et al., 2010). These differences probably explain the conflicting results reported for *C. trifida*, for which the isotopic abundance data suggest that the species is partially mycoheterotrophic (Zimmer et al., 2008), whereas results from exchange measurements were inconclusive regarding whether the species fixes $CO_2$ (Montfort and Küsters, 1940; Cameron et al., 2009).

However, even though measuring the abundance of stable natural isotopes can be a powerful method for assessing the nutritional mode of orchid species (Gebauer and Meyer, 2003), the C and N isotopic spectra of mycoheterotrophic plants often indicate interspecific differences in their C and N stable isotope profiles (Hynson et al., 2016; Schiebold et al., 2017). For example, there is evidence that the strategies for the use of C substrates by fungal symbionts can greatly influence the $^{13}C$ profiles of fully mycoheterotrophic plants, and the $^{15}N$ enrichment of ectomycorrhizal fungi can also vary greatly (Taylor et al., 2003; Hobbie et al., 2005; Mayor et al., 2009), owing to differences in the soil nutrient mining strategies and catabolic activities (Gebauer and Taylor, 1999; Emmerton et al., 2001; Pritsch and Garbaye, 2011). In addition, physiological differences can also influence how different plant and fungal species

interact during the exchange of C and N, thereby resulting in interspecific variation in $^{13}C$ and $^{15}N$ enrichment (Hynson et al., 2016). Consequently, the photosynthesis-derived C of partially mycoheterotrophic species can be overestimated when the $^{13}C$ and $^{15}N$ signatures associated with their fungal symbionts are more depleted than those of fully mycoheterotrophic plants (and vice versa). Therefore, stable isotopic signature data should be interpreted cautiously, especially when the contribution of mycoheterotrophy is predicted to be very high or low since the relative contributions of photosynthesis and mycoheterotrophy can be masked by differences in fungal associations and plant physiology. Accordingly, it will be difficult to precisely assess the trophic status of leafless orchid species that retain green stems and capsules and, thus, some potential for photosynthesis.

The leafless orchid *Cymbidium macrorhizon*, which possesses chlorophyllous stems, could function as an ideal model to precisely determine the nutritional mode of leafless species that retain chlorophyllous stems. It is ideal because chlorophyll is strongly induced when it fruits (Fig. 1) and its flowering period is bimodal, with both sprouting and fruiting individuals co-occurring within the same habitat. The present study was, therefore, initiated to investigate the trophic status of both sprouting and fruiting *C. macrorhizon* specimens by analyzing their $^{13}C$ and $^{15}N$ isotopic signatures and chlorophyll content.

## <h1>MATERIALS AND METHODS

### *<h2>Study species and sampling locality*

*Cymbidium macrorhizon* is a leafless orchid that occurs in dense evergreen or deciduous broadleaf forests and mixed pine forests in Japan. The species produces 2–8 nectarless

flowers on a lax inflorescence that grows to ca. 10–30 cm tall (Fig. 1). Although *C. macrorhizon* is often assumed to be fully mycoheterotrophic (Motomura et al., 2010; Ogura-Tsujita et al., 2012), the species also appears to retain chlorophyll and photosynthetic ability in its stems and ovaries, especially at the fruiting stage (Suetsugu, 2015; K. Kobayashi, Osaka Prefecture University and K. Suetsugu, unpublished data).

A field study was conducted in Tsukuba City, Ibaraki Prefecture (36°00′N, 140°08′E) ,a warm, temperate area in Central Japan. The investigated *C. macrorhizon* population contained ca. 100 mature individuals. All samples were collected on 3 September 2016. At the study site, peak flowering occurs in June and October. Therefore, both sprouting and fruiting individuals could be sampled on 3 September 2016.

***<h2>Chlorophyll content***

The tips (ca. 3 cm long) of stems were removed from five individuals each of sprouting and fruiting *C. macrorhizon*, ground, mixed with *N*,*N*′-dimethylformamide, and then kept in the dark at −23°C for 8 d. After centrifugation, the absorbance of the supernatants was spectrophotometrically measured at 646.8, 663.8, and 750 nm (U-2010; Hitachi High Technologies, Tokyo, Japan), and the chlorophyll concentrations were calculated according to Porra et al. (1989): Chl$a$ = $12.00(A_{663.8} - A_{750}) - 3.11(A_{646.8} - A_{750})$

Chl$b$ = $20.78(A_{646.8} - A_{750}) - 4.88(A_{663.8} - A_{750})$

where $A$ is the absorbance at the specified wavelength.

The Chl$a$ + $b$ contents are given on a fresh mass basis (μg $g_{FW}^{-1}$). The chlorophyll concentrations of the sprouting and fruiting *C. macrorhizon* were compared using Student's *t*-test.

***<h2>Stable isotope analysis***

Leaves were collected from reference autotrophic and partially mycoheterotrophic plants in a 4-$m^2$ area around an individual *C. macrorhizon* specimen, and autotrophic reference plants were only sampled from understory saplings. This strategy was used to limit the influence of environmental factors, such as atmospheric $CO_2$ concentrations (which could affect C isotope values), and soil type (which could affect N isotope values) (Gebauer and Schulze, 1991). Each plot contained a sprouting plant and a fruiting plant of *C. macrorhizon,* plus an individual of two autotrophic reference species (i.e., *Paederia scandens* and *Viola verecunda*). We also collected a *Platanthera minor* specimen to serve as the partially mycoheterotrophic reference (Yagame et al., 2012) whenever the species was found in a plot. Furthermore, when collecting the sprouting and fruiting *C. macrorhizon* specimens*,* we ensured that the plants were at least 1 m apart to avoid the possibility that sprouting and fruiting specimens were connected by underground parts.

The collected plants were dried at 60°C for 4 d and then ground using a Multi-Beads Shocker (MB601NIHS; Yasui Kikai Co., Osaka, Japan). The abundances of stable C and N isotopes were measured using a Delta V Advantage mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) that was coupled with a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific). The relative abundances of the stable isotopes were calculated as $\delta^{15}N$ or $\delta^{13}C = R_{sample} / R_{standard} - 1$, where $R$ is the molar ratio of the stable isotopes, $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Coplen, 2011). Differences in the $\delta^{13}C$ or $\delta^{15}N$ among the fruiting *C. macrorhizon*, sprouting *C. macrorhizon*, and autotrophic plant species were analyzed by ANOVA followed by Fisher's multiple comparison. The

relative contribution of fungal associations to C gain in the fruiting *C. macrorhizon* specimens (%Cx) was calculated as described by Gebauer and Meyer (2003), with slight modifications: $\%Cx = [(\delta C_{Cf} - \delta C_R) / (\delta C_{Cs} - \delta C_R)] \times 100$, where $\delta C_{Cf}$, $\delta C_{Cs}$, and $\delta C_R$ are the mean values of the fruiting *C. macrorhizon*, sprouting *C. macrorhizon*, and surrounding autotrophic plant species (reference plants), respectively. However, it should be noted that the idea that sprouting *C. macrorhizon* plants obtain 100% of their C from fungal associations may underestimate the contribution of fungal associations to C gain in fruiting *C. macrorhizon* and *P. minor*, when compared to usual two-source mixing model because (1) the photosynthates acquired during previous years can be stored in the underground structures of sprouting *C. macrorhizon* plants and (2) sprouting *C. macrorhizon* plants can already perform low levels of photosynthesis. For the values of $\delta^{13}C$ and $\delta^{15}N$ in *C. macrorhizon*, enrichment factors ($\varepsilon$) were also calculated according to the method of Preiss and Gebauer (2008) to allow the comparison of samples of fully mycoheterotrophic species collected from different sites.

The elemental concentrations and isotope ratios of C and N were calibrated against three laboratory standards: DL-alanine ($\delta^{13}C = -25.36‰$, $\delta^{15}N = -2.89‰$), L-alanine ($\delta^{13}C = -19.04‰$, $\delta^{15}N = 22.71‰$), and L-threonine ($\delta^{13}C = -9.45‰$), which are traceable back to international standards (Tayasu et al., 2011). The analytical standard deviations (SD) of these standards were 0.03‰ ($\delta^{13}C$, $n = 8$) and 0.02‰ ($\delta^{15}N$, $n = 10$) for DL-alanine, 0.04‰ ($\delta^{13}C$, $n = 8$) and 0.17‰ ($\delta^{15}N$, $n = 10$) for L-alanine ($n = 8$), and 0.06‰ ($\delta^{13}C$, $n = 8$) for L-threonine.

**<h1>RESULTS**

***<h2>Chlorophyll content***

The total chlorophyll concentrations (Chl*a* + Chl*b*) of the sprouting and fruiting *C. macrorhizon* differed significantly ($t$ = 16.969, df = 8, $P$ < 0.001). On average, the fruiting individuals contained 126.5 ± 15.7 µg total chlorophyll $g_{FW}^{-1}$, whereas the sprouting individuals contained only 6.1 ± 2.1 µg total chlorophyll $g_{FW}^{-1}$. However, there was no significant difference in the Chl*a*:Chl*b* ratios of the sprouting (1.8 ± 0.2) and fruiting (1.9 ± 0.2) specimens ($t$ = 0.708, df = 8, $P$ = 0.50).

***<h2>Stable isotope analysis***

The $\delta^{13}$C value of fruiting *C. macrorhizon* (−25.3 ± 0.2‰) was significantly lower than that of sprouting *C. macrorhizon* (−22.6 ± 0.3‰) and significantly higher than those of the partially mycoheterotrophic plant *P. minor* (−28.5 ± 0.8‰) and autotrophic references plants (−32.9 ± 1.7‰; $P$ < 0.005 in all combinations tested; Fig. 2). Similarly, the enrichment factors ($\varepsilon$) for the $^{13}$C relative abundance of the sprouting and fruiting *C. macrorhizon* were 10.3 ± 0.3‰ and 7.6 ± 0.2‰, respectively. If we consider that sprouting *C. macrorhizon* plants obtain 100% of their C from the fungal association, our model predicts that fruiting *C. macrorhizon* obtains 73.7 ± 2.0% of its C from the fungal association and that *P. minor* obtains 43.0 ± 7.6% of its C from the fungal association.

There were no significant differences between the $\delta^{15}$N values of the sprouting (5.7 ± 1.6‰) and fruiting *C. macrorhizon* (5.2 ± 0.7‰; $P$ = 0.75). Yet, the $\delta^{15}$N values of both were significantly higher than those of the reference autotrophic plants (−0.9 ± 2.6‰). In addition, there are at least marginally significant differences in the $\delta^{15}$N values between *C. macrorhizon* [5.7 ± 1.6‰ in sprouting plants ($P$ = 0.03) and 5.2 ± 0.7‰ in fruiting plants ($P$ = 0.06)] and *P. minor* (1.9 ± 4.0‰).

**<h1>DISCUSSION**

The present study demonstrates that both sprouting and fruiting *C. macrorhizon* specimens are significantly enriched in $^{13}C$ when compared to autotrophic plants growing in close proximity. Furthermore, the $^{13}C$ enrichment factors of both the sprouting and fruiting specimens ($\varepsilon$ = 10.3 ± 0.3‰ and 7.6 ± 0.2‰) were similar to the range reported for mycoheterotrophic orchids that form associations with ectomycorrhizal fungi ($\varepsilon$ = 8.2 ± 1.3‰; Hynson et al., 2013). As discussed by Motomura et al. (2010), CAM photosynthesis can be excluded as an explanation for the observed $\delta^{13}C$ values of *C. macrorhizon.* Consequently, the high $\delta^{13}C$ values should be due to mycoheterotrophic nutrition. In addition, although the $\delta^{15}N$ values of the sprouting and fruiting specimens were lower than those reported for other mycoheterotrophic orchids associated with ectomycorrhizal fungi ($\varepsilon$ = 11.6 ± 3.1‰; Hynson et al., 2013), both types ($\varepsilon$ = 6.6 ± 1.6‰ and 6.1 ± 0.7‰) had higher $\delta^{15}N$ levels than the surrounding autotrophic plants. It was also interesting to note that the $\delta^{13}C$ value of the fruiting plants was significantly higher than that of the partially mycoheterotrophic orchid *P. minor* (Yagame et al., 2012) and that, even during fruiting, *C. macrorhizon* is physiologically more similar to fully mycoheterotrophic plants than typical partially mycoheterotrophic orchids, possibly due to its leafless habit.

In addition, the present study found that the fruiting *C. macrorhizon* specimens contained much higher chlorophyll levels than their sprouting counterparts and that the Chl*a*:Chl*b* ratio of *C. macrorhizon* was similar to that found in $C_3$ plants (Larcher, 2003). When we compared the $\delta^{15}N$ and $\delta^{13}C$ of fruiting *C. macrorhizon* specimens using a linear two-source mixing model with sprouting plants as the standard for 100% C gain from fungi and autotrophic plants as the 0% baseline, we found that the fruiting *C.*

*macrorhizon* plants obtained approximately 73.7 ± 2.0% of their total C from their fungal partners. This value could have been underestimated, as described above (see materials and methods), or overestimated, if respiration $CO_2$ is the main source of C for *C. macrorhizon* photosynthesis and $^{13}C$ content utilized for photosynthesis is therefore more depleted from the atmosphere. However, because functional stomata can be observed on the *C. macrorhizon* pericarp surface (K. Kobayashi, Osaka Prefecture University and K. Suetsugu, unpublished data), some $CO_2$ was likely obtained from surrounding atmosphere. In either case (under- or overestimation), our results provided strong evidence that *C. macrorhizon* is able to fix C, despite being leafless. Therefore, since the species satisfies a significant proportion of its C demands through photosynthesis, *C. macrorhizon* should only be considered partially mycoheterotrophic, at least during its fruiting stage. Nonetheless, further studies will be needed to understand how the functionality of photosynthetic machinery is adapted to the nutritional mode in *C. macrorhizon.*

These results provide some support for a previously proposed model that posits that photosynthates are not used for rhizome survival, but rather for fruit production, in partially mycoheterotrophic orchids (Roy et al., 2013; Gonneau et al., 2014). Actually, both Roy et al. (2013) and Gonneau et al. (2014) showed that photosynthates contribute little or nothing to the belowground reserves and emerging shoots are almost entirely composed of fungal resources, even in these partially mycoheterotrophic orchids with fully developed leaves. In contrast, photosynthesis is used for late building of the stem and for fruits production (Roy et al., 2013; Gonneau et al., 2014). This observation led Gonneau et al. (2014) to conclude that the dependence of partially mycoheterotrophic species on photosynthates for reproduction could impede their transition to full

mycoheterotrophy. In addition, Roy et al. (2013) reported that the photosynthetic ability of partially mycoheterotrophic *Cephalanthera damasonium* increased over the growth season, as in fruiting *C. macrorhizon*. It is interesting that we found a similar trend in *C. macrorhizon*, which belongs to a tribe (Cymbidieae) that has evolved partial mycoheterotrophy independently from tribe Neottieae.

Most partially mycoheterotrophic orchids produce fully expanded leaves for long periods of their life cycle, even during nonreproductive stages (e.g., Selosse et al., 2004; Suetsugu et al., 2017). However, fully mycoheterotrophic species only produce aboveground structures during reproduction, surviving for comparatively long periods in subterranean vegetative states that allow them to escape temporal effects, such as severe aboveground herbivory pressures (Shefferson et al., 2011, 2018). Because herbivores are often N-limited and, therefore, attracted to plants with high N contents, such as mycoheterotrophs (Roy et al., 2013), mycoheterotrophic species likely undergo selection to limit their sprouting period to the minimum duration required for flowering and seed dispersal. However, although *C. macrorhizon* produces shoots to coincide with reproduction, *C. macrorhizon* is present aboveground for a relatively long period (more than 4 mo). Considering that autotrophic C gain increases in *C. macrorhizon* during the fruiting season, the species' photosynthetic ability and prolonged aboveground shoot presence are likely to contribute fruit and seed production in *C. macrorhizon.* Chlorophyllous stems can serve as primary photosynthetic organs, even in autotrophic plants, although the efficiency of photosynthesis is lower than that of leaves (Aschan and Pfanz, 2003). In addition, photosynthesis by reproductive organs, such as fruits, can also offset the costs of reproduction (Aschan and Pfanz, 2003; Pélabon et al., 2015).

Even though *C. macrorhizon* is often assumed to be fully mycoheterotrophic, the

present study demonstrates that *C. macrorhizon* is not fully mycoheterotrophic, at least at the fruiting stage. The present study also demonstrates even at the fruiting stage, the $^{13}C$ enrichment of the species (7.6 ± 0.2‰) is similar to that of other mycoheterotrophic orchids associated with ectomycorrhizal fungi (8.2 ± 1.3‰; Hynson et al., 2013). Therefore, as reported by Motomura et al., (2010), the species would be concluded as fully mycoheterotrophic if the $^{13}C$ signatures of different stages had not been measured. In contrast, our results clearly showed that the absence of normal leaves, often used as a proxy to full mycoheterotrophy, is not necessarily a good predictor. As is the case with the present study, to accurately assess the trophic status of leafless orchid species that possess green stems and capsules, both chlorophyll concentrations and stable isotope abundance should be measured throughout the species' entire life cycle.

**<h1>ACKNOWLEDGEMENTS**

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**<H1>DATA ACCESSIBILITY**

Values of $\delta^{13}C$ and $\delta^{15}N$ in each sample of *Cymbidium macrorhizon* and surrounding plants are available in Appendix S1.

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**FIGURE 1.** (A) Sprouting and (B) fruiting plants of *Cymbidium macrorhizon* from the investigated population in Tsukuba City, Japan.

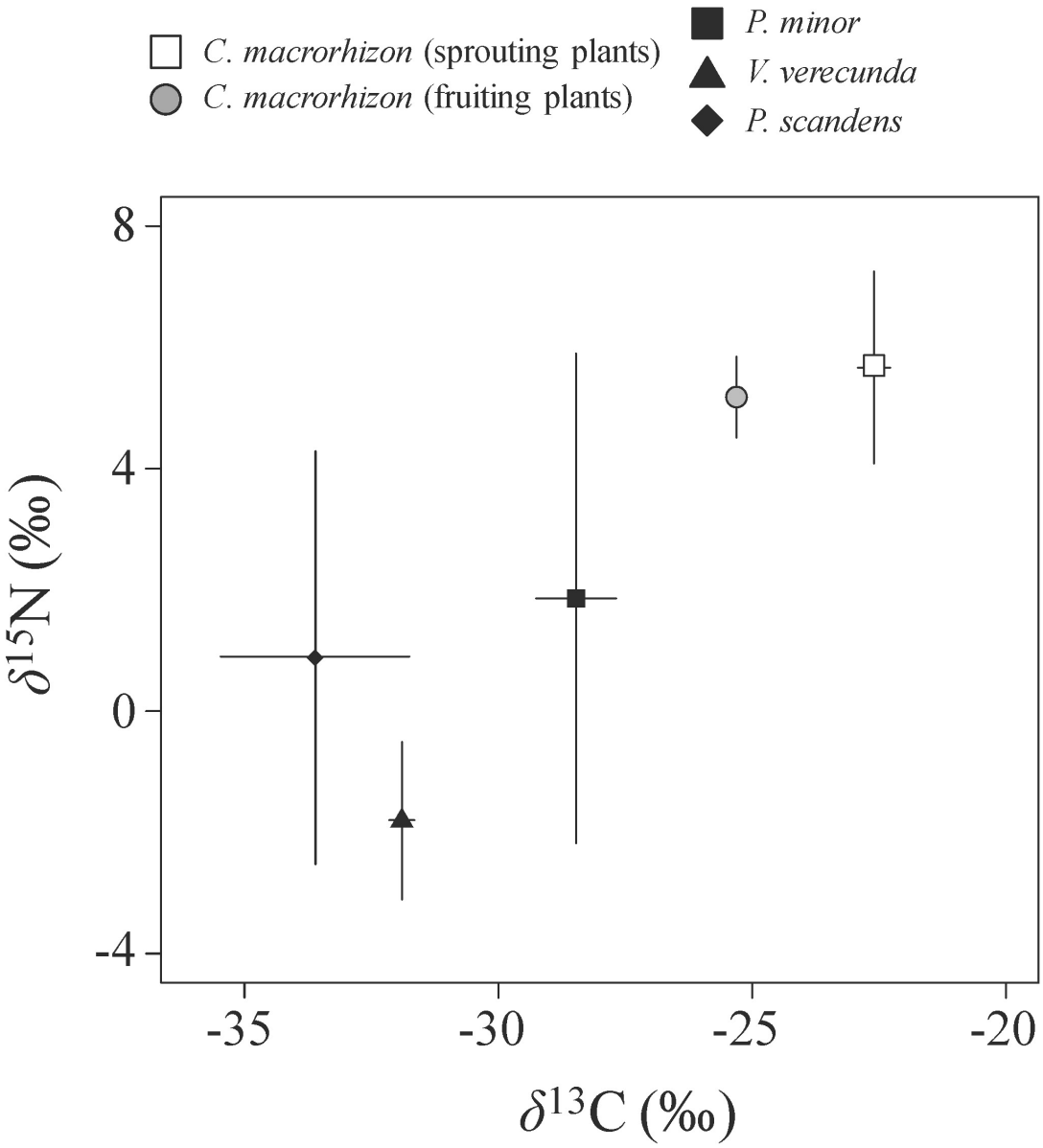


**FIGURE 2.** Mean relative abundances (±SD) of stable isotopes ($\delta^{15}$N and $\delta^{13}$C) in aboveground biomass of sprouting and fruiting *Cymbidium macrorhizon*, leaves of the partially mycoheterotrophic orchid *Platanthera minor*, and autotrophic *Viola verecunda* and *Paederia scandens*. ANOVA results: $\delta^{15}$N, $F_{3,20} = 10.91$, $P < 0.001$; $\delta^{13}$C, $F_{3,20} = 101.23$, $P < 0.001$.