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Isotopic evidence of partial mycoheterotrophy in *Burmannia coelestis* (Burmanniaceae)

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Abstract

The Burmanniaceae contain several lineages of achlorophyllous mycoheterotrophic plants that associate with arbuscular mycorrhizal fungi (AMF). Here we investigate the isotopic profile of a green and potentially mycoheterotrophic plant in situ, *Burmannia coelestis* and associated reference plants. We generated $\delta^{13}C$ and $\delta^{15}N$ stable isotope profiles for five populations of *B. coelestis*. *Burmannia coelestis* was significantly enriched in ^{13}C relative to surrounding C_3 reference plants and significantly depleted in ^{13}C relative to C_4 reference plants. No significant differences were detected in ^{15}N enrichment between *B. coelestis* and reference plants. The isotopic profiles measured are suggestive of partial mycoheterotrophy in *B. coelestis*. Within the genus *Burmannia* transitions to full mycoheterotrophy have occurred numerous times suggesting that some green *Burmannia* species are likely partially mycoheterotrophic but may be undetectable using natural abundance stable isotopic methods under many conditions.

Keywords: ^{13}C; ^{15}N; mycorrhizae; stable isotopes

INTRODUCTION

It is widely recognized that plant symbioses with fungi to enhance nutrient uptake was a key innovation in the diversification of plants. At least 80 genera and approximately 10 % of all plant species use carbon from a mycorrhizal association at some stage of their life cycle (Leake and Cameron, 2010, Leake, 1994). This relationship is most dramatic and obvious in achlorophyllous mycoheterotrophic plants (fully mycoheterotrophic) that typically have a ghostly appearance. However, partial mycoheterotrophy (or mixotrophy) has emerged as a

generally accepted precursor to chlorophyll loss (e.g. Abadie et al., 2006, Motomura et al., 2010) though may also be a stable evolutionary condition.

Stable isotope data have been applied effectively to provide insights into carbon and nitrogen sources in obligate and partial mycoheterotrophic plants (reviewed in Hynson et al., 2013). Various mycoheterotrophic plant and fungal lineages intimately linked by mycorrhizal symbioses can generate consistent patterns of ^{13}C and ^{15}N enrichment in the mycoheterotrophic plant. Diagnostic ^{13}C and ^{15}N enrichment in mycoheterotrophic and partial mycoheterotrophic plants have been demonstrated in numerous studies of plants with ectomycorrhizal associates (ECM) (e.g. Gebauer and Meyer, 2003, Trudell et al., 2003, Liebel et al., 2010, Tedersoo et al., 2007, Hynson et al., 2009, Zimmer et al., 2007) and saprotrophic orchid-mycorrhizal associates (SAP) (e.g. Martos et al., 2009, Ogura-Tsujita et al., 2009). Arbuscular mycorrhizal (AMF) fungi in symbiotic relationships with Burmanniaceae and Gentianaceae (Cameron and Bolin, 2010, Courty et al., 2011, Merckx et al., 2010) have received the least study and the patterns of mycoheterotroph enrichment in ^{15}N apparently do not closely mimic those for ECM or SAP and some ^{13}C profiles of putative mycoheterotrophs have been inconclusive relative to reference plants.

The Burmanniaceae are a fascinating lineage of achlorophyllous mycoheterotrophic and autotrophic green species that occur primarily in tropical regions of the world and include approximately 130 species and 14 genera (Merckx et al., 2006). All Burmanniaceae form AMF associations and only the genus *Burmannia* includes green plants. Interestingly, molecular phylogenetic data suggests at least 8 independent losses of chlorophyll in Burmanniaceae

(Merckx et al., 2008) and it has been posited that the shift from partial to complete mycoheterotrophy may have occurred in response to low light conditions under the canopy and subcanopy of tropical forests (Bidartondo et al., 2004). Stable isotopic investigations of an achlorophyllous Burmanniaceae, *Dictyostega orobanchoides* (Hook.) Miers clearly indicate mycoheterotrophy via enriched ^{13}C values, while the same methods applied to a putative green mycoheterotroph *Burmannia capitata* Mart. were inconclusive (Merckx et al., 2010). Moreover, Merckx et al. (2010) grew four green *Burmannia* species (including the focal species of this paper *Burmannia coelestis* D.Don) from seed in pots to flowering with no access to heterotrophic carbon sources. Merckx et al. (2010) were very clear in stating that while their isotopic data did not show partial mycoheterotrophy in green *Burmannia* spp., they also did not rule it out. Courty et al. (2011) reported significantly enriched ^{13}C values for AMF spores and the shoots of two achlorophyllous mycoheterotrophs (*Voyria aphylla* Pers*., Voyria tenella* Guild. Ex Hook.) relative to understory reference plants but no difference relative to canopy reference plants. Due to conflicting or inconclusive $\delta^{13}C$ patterns at other populations studied, Courty et al. (2011) prudently encourages cautious interpretations of $\delta^{13}C$ data because mycoheterotrophic tissue $\delta^{13}C$ abundances seem indistinguishable from canopy leaves

The aim of this paper is to evaluate partial heterotrophy in Burmanniaceae using *Burmannia coelestis* in situ, as a model. A weedy Asian species of wet grassy areas, *B. coelestis* is distributed from eastern India, southern China, to Peninsular Malaysia, Borneo, and northern Australia (Zhang and Saunders, 2000). Authoritative works differ when addressing the putative mycoheterotrophic status of *B. coelestis*. The Flora of China and a monograph of the *B. coelestis* complex regard the species as “semi-mycotrophic” (Wu et al., 2010, Zhang and Saunders, 2000)

while in Flora Malasiana and the Flora of Singapore, *B. coelestis* is considered "autotrophic" (Keng et al., 1998, Jonker, 1948). Merckx et al. 2010 demonstrate that *B. coelestis* can grow autotrophically under ideal conditions thus the concept of green *Burmannia* as partial mycoheterotropic organisms remains an open question. Here we address a lack of empirical evidence for partial mycoheterotrophy in green Burmanniaceae (Courty et al., 2011, Merckx et al., 2010) by applying stable isotopic methods to the tropical herb *B. coelestis.*

MATERIALS AND METHODS

Study Area — All plant materials of *B. coelestis* were collected (1 Aug. 2010 to 3 Aug 2010) in Brunei Darussalam. The five sampling areas (Lambak Kanan Estate: 04˚ 58.530′ N, 114˚ 58.422′ E; Labi: 04˚ 28.3116′ N, 114˚ 28.133′ E; Qlap: 04˚ 54.259′ N, 114˚ 55.508′ E; Tungku Link: 04˚ 58.445′ N, 114˚ 52.779′ E; UBD Water Tank: 04˚ 58.291′ N, 114˚ 53.818′ E) were an average of 7.2 km ± 2.5 SE to the nearest next sampling site. All sampling areas were from similar ruderal plant communities, and were composed of irregularly mowed and grassy roadside areas within a few meters of native or naturalized forests. The dominant species in each sampling area was a mixture of C_4 grasses *Axonopus affinis* Chase*, Ischaemum ciliare* Retz.*,* and a *Paspalum sp.* (Table 2).

Isotopic methods—For stable isotopic analyses of *B. coelestis* and its surrounding reference plants, at each sampling site (n = 5), a single *B. coelestis* plant (shoot and root) was collected at the area center point. Newly expanded leaves or tissues of the first five vascular plant species (other than *B. coelestis*) within a 5 m radius of the sampling area center point were collected for comparison to the putative partially mycoheterotrophic plant. At least one

C_4 grass in immediate proximity to the sampled *B. coelestis* was collected at each sampling location, since C_4 grasses accounted for approximately 50-80% of the cover at each sampling area. Whole plants of *B. coelestis* were separated into root and shoot tissues for isotopic analyses. Root tissues were thoroughly rinsed in deionized water to remove soil debris. Then all tissues were dried at 70 ˚C in a drying oven until no further mass reduction was observed. Segregated dried tissues were pulverized to powder in a mortar and pestle and 5 mg samples were weighed into tin capsules. The weighed samples were analyzed for $\delta\,^{13}C$ and $\delta\,^{15}N$ by a continuous-flow mass spectrometry (PDZ Europa 2020 Isotope Ratio Mass Spectrometer and a PDZ ANCA GSL preparation Unit). Data were collected as $\%\,^{13}C$ and $\%\,^{15}N$ and re-expressed as delta (δ), relative to the isotope ratios of the sample and the standards Pee Dee Belemnite and atmospheric air, respectively. See Cameron and Bolin (2010) for an expanded explanation of standard $\delta\,^{13}C$ calculation used. For ^{13}C measurements, commercial poplar leaf standards were used at 10 sample intervals and similarly commercially available reference gases were used to calibrate the detector for ^{15}N. Total tissue nitrogen (N) concentrations using the sample mass were also simultaneously collected from the same samples. All statistical tests for isotope and chlorophyll data were conducted in IBM SPSS Statistics 21.0 (IBM Corp., Armonk New York USA).

RESULTS

Whole plant morphology— The leaves and roots of *B. coelestis* are reduced relative to typical autotrophic plants. The *B. coelestis* green plant body comprises a basal rosette (< 1 cm),

a slender stem, and foliose scales. Moreover roots are relatively short and coralloid (Fig. 1), reduced photosynthetic surfaces combined with coralloid roots are often hallmarks of mycoheterotrophic plants (Peterson et al., 2004).

Stable isotopic profiles presented as $\delta^{13}C$ and $\delta^{15}N$ values for *B. coelestis* (root and shoot) and reference plants (C_3 and C_4) are presented in Table 1 and summarized in Fig. 2. The root portions of *B. coelestis* were significantly enriched in ^{13}C relative to *B. coelestis* shoot portions (enriched by 6.6‰ $\delta^{13}C$) and surrounding C_3 vegetation (enriched by 9.2 ‰ $\delta^{13}C$), and significantly depleted relative to surrounding C_4 vegetation (depleted by -9.5 ‰ $\delta^{13}C$) (ANOVA for $\delta^{13}C$, $F_{3,24} = 82.1$, $P < 0.001$; TUKEY *post hoc* test; $\alpha = 0.05$). Shoot portions of *B. coelestis* were significantly enriched in ^{13}C relative to reference C_3 vegetation (enriched by 2.6 ‰ $\delta^{13}C$), and significantly depleted relative to surrounding C_4 vegetation (depleted by -16.0 ‰ $\delta^{13}C$). Profiles of $\delta^{15}N$ did not vary significantly among groups (*B. coelestis* root and shoot, C_3 and C_4 reference plants) (ANOVA for $\delta^{15}N$, $F_{3,24}= 0.68$, $P = 0.57$). The pattern of ^{13}C enrichment in *B. coelestis* relative to reference plants was consistent across all five study areas presented in supplementary material Fig. S1

DISCUSSION

Our isotopic study of *B. coelestis* shows significant ^{13}C enrichment relative to reference C_3 plants and significant ^{13}C depletion relative to C_4 reference plants (Table 1; Fig. 2). The significant difference in $\delta^{13}C$ profiles in *B. coelestis* relative to reference plants suggest a heterotrophic path of carbon to *B. coelestis* via AMF symbionts. Our data is congruent with two studies that collected and evaluated isotopic evidence with an eye towards resolving patterns

of $\delta^{13}C$ and $\delta^{15}N$ partitioning with AMF symbionts and achlorophyllous mycoheterotrophic plants compared to reference plants in tropical systems. Courty et al. (2011) evaluated a number of mycoheterotrophic plants including *Voyria aphylla* (Gentianaceae), *Apteria aphylla* Small, and a *Gymnosiphon* sp. (Burmanniaceae) in the shaded understory of tropical forests on La Guadalupe a Caribbean Island. Merckx et al. (2010) evaluated achlorophyllous *Dictyostega orobanchoides*, and *Voyria aphylla* from rainforest sites in French Guiana. Both previous studies demonstrated significant enrichment in ^{13}C for achlorophyllous mycoheterotrophs relative to understory plants (Merckx et al. 2010) and leaves of associated rainforest tree species (Courty et al. 2011), the same relationship we observed for the green *B. coelestis* and surrounding C_3 reference plants. Moreover, in both the Merckx et al. (2010) and Courty et al. (2011) studies no significant differences were observed between $\delta^{15}N$ values of achlorophyllous mycoheterotrophs and reference plants indicating that the mycoheterotrophic plants and reference plants may be using similar sources of N, congruent with the data presented here (Table 1) for *B. coelestis*. Two plant species in the reference plots showed much greater depletion in ^{15}N relative to *B. coelestis* fractions and other reference plants evaluated, *Calamus blumei* ($\delta^{15}N$ = -19.32) and *Melastoma malabathricum* ($\delta^{15}N$ = -13.96 ± 1.68). For, *C. blumei* the depleted ^{15}N values may be an artifact of a single sample among the 5 plots or may be suggestive of alternative N nitrogen sources as suggested for *M. malabathricum* an ammonia assimilating plant (Watanabe et al., 1998) though the interplay of mycorrhizae and internal fractionation of N may confound the use of ^{15}N a reliable indicator of N source (Emmerton et al., 2001). In summary, the $\delta^{13}C$ and $\delta^{15}N$ data for achlorophyllous mycoheterotrophic plants in tropical systems (Merckx et al. 2010, Courty et al. 2011) are similar to our findings for the green

and putative partial mycoheterotroph (Table 1; Fig. 2), *B. coelestis* providing a first line of evidence that *B. coelestis* is acting as a partial mycoheterotrophic plant.

Two factors contribute to complicate the conclusion of *B. coelestis* as a partial mycoheterotrophic plant using $\delta^{13}C$ data. First, Merckx et al. (2010) also evaluated the putative and green partial mycoheterotrophic plant *Burmannia capitata* from a grassland area of French Guiana and found that it was statistically indistinguishable from reference plants for both $\delta^{13}C$ and $\delta^{15}N$. Merckx et al. (2010) also established that *B. capitata* can grow autotrophically in pot culture; their isotopic and pot study results on *B. capitata* do not rule out partial mycoheterotrophy. However, we suggest that for many green *Burmannia* the $\delta^{13}C$ signal from AMF to the putative partial mycoheterotrophic plant may be difficult to detect, particularly under conditions where surrounding vegetation uses primarily C_3 photosynthesis, generating similar $\delta^{13}C$ values as autotrophic C_3 photosynthesis in *Burmannia*. Re-cycling of respired $C0_2$ from soil rich in ^{13}C might be considered a factor to obscure the signal of heterotrophic carbon in mycoheterotrophic plants, however pot studies suggest that the effect of respired soil $C0_2$ for hemiparasitic plants is negligible (Těšitel et al., 2010).

A second factor that may inform the framework of enrichment in ^{13}C for AMF and mycoheterotrophic plants relative to adjacent plants is the work by Nakano *et al.* (1999) on the AMF *Gigaspora margarita* (Gigasporaceae; Glomeromycota) in pot culture with C_3 and C_4 plants. Nakano *et al.* (1999) demonstrated that *G. margarita* spores are depleted (3.5‰) in ^{13}C relative to host roots and importantly that the $\delta^{13}C$ signature of the spores mirrors the photosynthetic pathway of the host (e.g. C_3 host: -31 to -37 $\delta^{13}C$ and C_4 host: -15 to -17 $\delta^{13}C$). Under a revised nomenclature of Glomeromycota (Krüger et al., 2012), *G. margarita* is

differentiated from *Glomeraceae*, and placed in a separate AMF family, and to our knowledge the only report of $\delta^{13}C$ values of AMF spores of *Glomeraceae* were collected by Courty et al. (2011) and they showed significant enrichment in AMF spores relative to understory tree leaves, and non-significant enrichment in AMF spores relative to mycorrhizal roots of canopy trees and canopy tree leaves, congruent with our results. Thus, it is evident that more species-specific measurements of the $\delta^{13}C$ profiles of Glomeraceae AMF spores and other AMF lineages are required to better understand these relationships. Diverse lineages of AMF have been identified as mycoheterotrophic symbionts outside of the conventional Glomeraceae (Glomus group A), even for Burmanniaceae (Merckx et al., 2012) and potential combinations of those symbionts within a single mycoheterotrophic plant should be considered.

The presence of at least one C_4 plant within each independent *B. coelestis* sampling location may have been an underlying driver for the observed enrichment in ^{13}C in *B. coelestis.* The C_4 graminoid genera *Axonopus, Ischaemum,* and *Papsalum* that were dominant in each sampling area are known to be colonized by AM fungi (Wang and Qiu, 2006, Grigera and Oesterheld, 2004, Karti et al., 2011, Nakano et al., 1999). Just as Nakano et al. (1999) demonstrated AMF spores mirroring the $\delta^{13}C$ signature of host C_3 and C_4 plants, the significantly enriched $\delta^{13}C$ signal of *B. coelestis* relative to C_3 vegetation may be attributed to a significant fraction of the carbon transferred from the AMF symbiont to *B. coelestis* coming from photosynthesis of C_4 grasses. In our view this proposed process does not weaken the argument for partial mycoheterotropy in *B. coelestis,* but rather strengthens it via hypothesized indirect C_4 labeled photosynthate passing through the AMF intermediate to *B. coelestis*.

The isotopic evidence suggestive of partial mycoheterotrophy in *B. coelestis* is also supported by observations of coralloid or star-like and stubby roots of *B. coelestis* (Fig. 1) that are hallmarks of many plants using mycorrhizal symbionts (Imhof, 2010). Additionally, total % N data, derived from continuous-flow mass spectrometry was significantly greater in both *B. coelestis* shoot (1.7 ± 0.1) and root (1.5 ± 0.1) relative to C_3 (1.2 ± 0.1) and C_4 (1.1 ± 0.1 % N) reference plants (data not shown in results; ANOVA, $F_{3,19}$ 7.3, $P < 0.001$) and was consistent with patterns of greater N concentrations in partial mycoheterotrophic plants relative to reference plants (e.g. Cameron and Bolin, 2010).

A phylogeny of Burmanniaceae indicates numerous losses of chlorophyll (Merckx et al., 2008). Transitions to achlorophyllous mycoheterotrophy may be common in monocots such as *Burmannia* due to low prostrate form, absence of secondary growth, and a fibrous root system characteristics outlined by Imhof (2010) that may predispose or support a mycoheterotrophic habit. The isotopic data collected *in situ* in this study supports the hypothesis that *B. coelestis* in Brunei Darussalam operates as a partial mycoheterotroph. However the broad geographic range of the *B. coelestis*, its taxonomic complexity (Zhang and Saunders, 2000) and potential corresponding AMF diversity may make *B. coelestis* an intriguing model for further study. We plan future experimental work to quantify the portion of carbon contributions from AMF to the partial mycoheterotrophic plant and any return on investment the AMF may receive.

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Table 2. Mean of $\delta^{13}C$ and $\delta^{15}N$ (± SE) isotope profiles of *Burmannia coelestis* (shoot and root) and surrounding (reference) plants. Reference plants parsed into C_3 and C_4 groups for comparison against *B. coelestis* root and shoot portions. Summary data analyzed with ANOVA followed by a *post hoc* TUKEY test (α = 0.05) to compare each group (*B. coelestis* root and shoot, C_3 and C_4 reference plants) (*n = 4*). Values in each column sharing the same letter are not significantly different.

Species	Physiology	Rep.	$\delta^{13}C$	$\delta^{15}N$
Blechnum orientale	C_3	1	-33.21	-1.88
Buchanania arborescens	C_3	1	-33.52	1.73
Calamus blumei	C_3	1	-31.42	-19.32
Desmodium heterphyllum	C_3	1	-30.87	2.27
Dillenia suffruticosa	C_3	2	-32.15 ±0.83	-0.40 ± 1.62
Lycopodiella cernua	C_3	3	-31.35 ± 1.27	0.45 ± 1.88
Macaranga gigantea	C_3	2	-31.01 ± 0.07	-0.85 ± 4.35
Melastoma malabathricum	C_3	2	-31.28 ± 0.42	-13.96 ± 1.68
Timonius eskerianus	C_3	1	-32.36	5.32
Torenia polygonoides	C_3	2	-33.06 ± 0.08	1.00 ± 1.37
Scleria ciliaris	C_3	2	-31.65 ± 1.64	-0.15
Axonopus affinis	C_4	2	-12.60 ± 0.17	2.08 ± 2.08
Eleocharis retroflexa	C_4	1	-13.39	-2.24
Ischaemum ciliare	C_4	3	-12.60 ± 0.54	0.78 ± 0.78
Papsalum sp.	C_4	3	-14.40 ± 0.16	0.85 ± 0.85
Summary Data	**Physiology**	**Rep.**	**$\delta^{13}C$**	**$\delta^{15}N$**
Mean of Reference Plants	C_3	5	-31.26 ± 0.38 a	-0.35 ± 1.80 a
Mean of Reference Plants	C_4	5	-12.64 ± 0.22 d	-1.09 ± 0.81 a
Burmannia coelestis (shoot)	C_3	5	-28.65 ± 0.64 b	1.47 ± 0.67 a
Burmannia coelestis (root)	C_3	5	-22.10 ± 1.99 c	-1.20 ± 1.18 a

Notes: ANOVA for $\delta^{13}C$, $F_{3,19} = 60.4$, $P < 0.001$; ANOVA for $\delta^{15}N$, $F_{3,19}$ 1.06, $P = 0.39$.

Fig. 1. Photographs of *Burmannia coelestis* (A) flowers and stem, (B) basal rosette, (C) coralloid roots (Scale Bars A = 2 cm; B = 1 cm; C = 0.5 mm).

Fig. 2. Mean $\delta^{13}C$ (Relative to PDB) and $\delta^{15}N$ (relative to air) for *Burmannia coelestis* root and shoot portions (circle symbol) relative to C_3 (diamond symbol) and C_4 (triangle symbol) reference plants. Error bars represent ± 1 SE (*N*=5 for *B. coelestis; N*=1-5 for reference plants).

Supplemental Fig. S1. Mean $\delta^{13}C$ (Relative to PDB) and $\delta^{15}N$ (relative to air) for *Burmannia coelestis* root (open circle symbol) and shoot portions (filled circle symbol) relative to C_3 (diamond symbol) and C_4 (triangle symbol) reference vegetation for each study area sampled (A – E). These plots of the raw data show the same pattern of ^{13}C enrichment from C_3 plants to *B. coelestis* to C_4 plants in each study area.

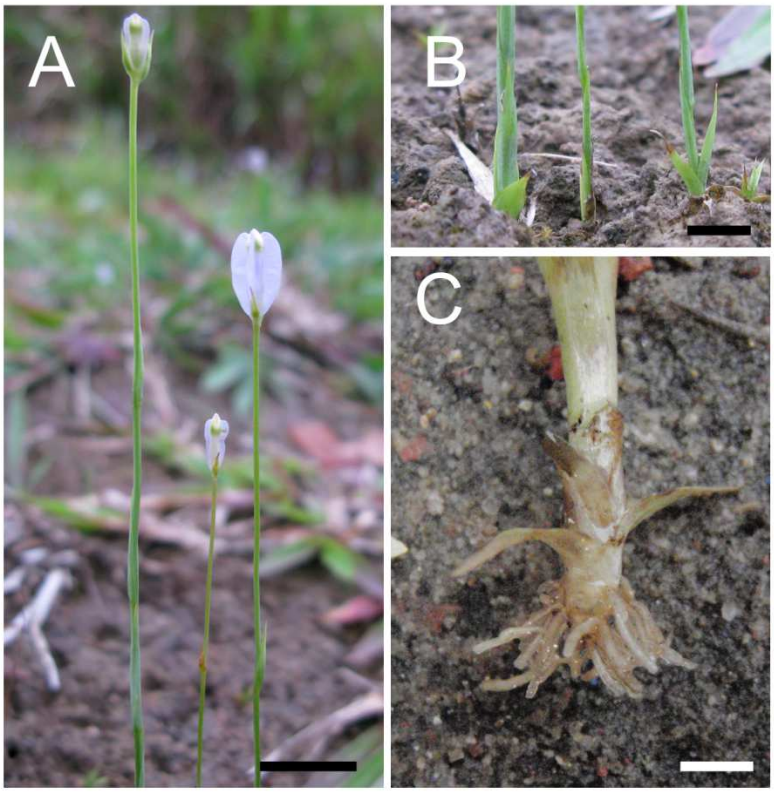
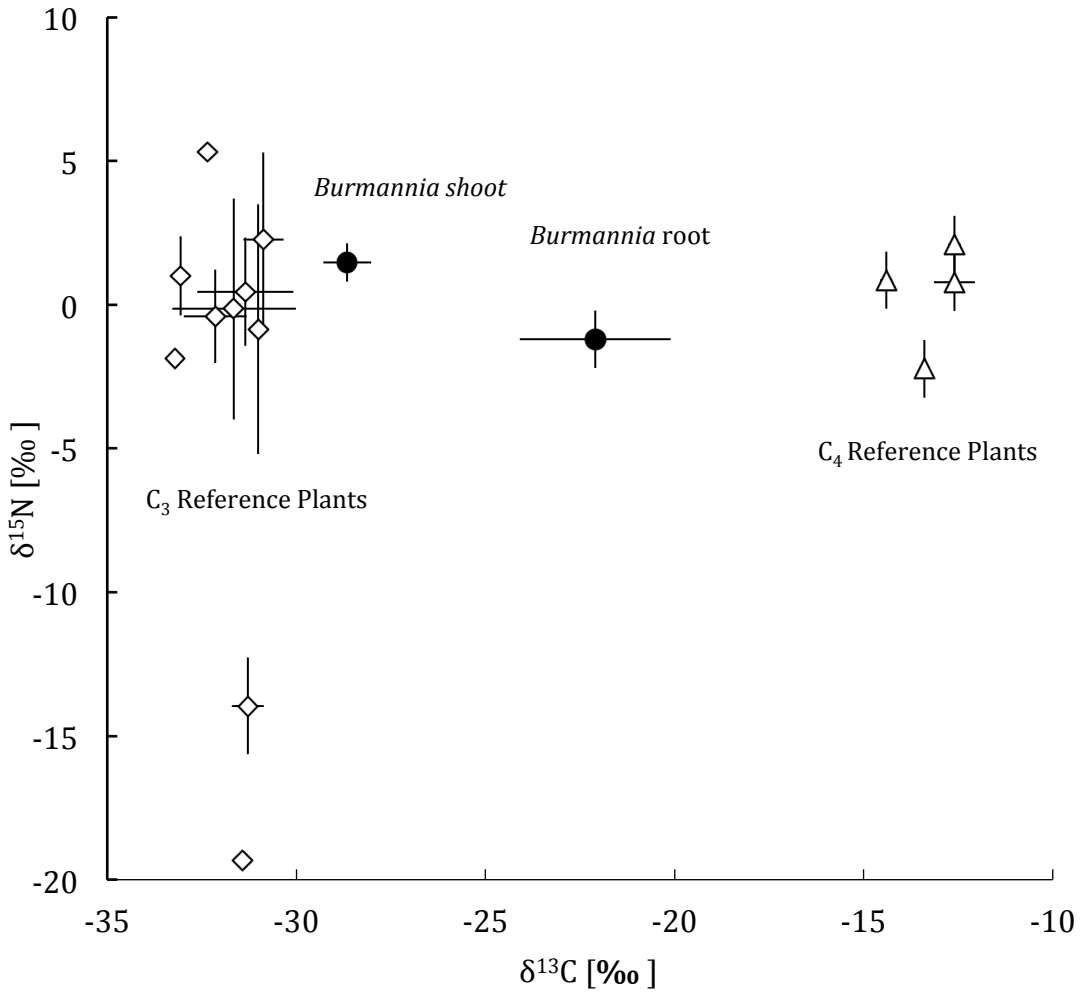


Fig. 1. Photographs of *Burmannia coelestis* (A) flowers and stem, (B) basal rosette, (C) coralloid roots (Scale Bars A = 2 cm; B = 1 cm; C = 0.5 mm).



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