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Uptake of ant-derived nitrogen in the myrmecophytic orchid *Caularthron bilamellatum*

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• *Background and Aims* Mutualistic ant–plant associations are common in a variety of plant families. Some myrmecophytic plants, such as the epiphytic orchid *Caularthron bilamellatum*, actively form hollow structures that provide nesting space for ants (myrmecodomatia), despite a substantial loss of water-storage tissue. This study aimed at assessing the ability of the orchid to take up nitrogen from ant-inhabited domatia as possible trade-off for the sacrifice of potential water storage capacity.
• *Methods* Nitrogen uptake capabilities and uptake kinetics of ^{15}N-labelled compounds (NH_4^+, urea and L-glutamine) were studied in field-grown *Caularthron bilamellatum* plants in a tropical moist forest in Panama. Plants were either labelled directly, by injecting substrates into the hollow pseudobulbs or indirectly, by labelling of the associated ants *in situ*.
• *Key Results Caularthron bilamellatum* plants were able to take up all tested inorganic and organic nitrogen forms through the inner surface of the pseudobulbs. Uptake of NH_4^+ and glutamine followed Michaelis–Menten kinetics, but urea uptake was not saturable up to 2 mM. ^{15}N-labelled compounds were rapidly translocated and incorporated into vegetative and reproductive structures. By labelling ants with ^{15}N *in situ*, we were able to prove that ants transfer N to the plants under field conditions.
• *Conclusions* Based on ^{15}N labelling experiments we were able to demonstrate, for the first time, that a myrmecophytic orchid is capable of actively acquiring different forms of nitrogen from its domatia and that nutrient flux from ants to plants does indeed occur under natural conditions. This suggests that beyond anti-herbivore protection host plants benefit from ants by taking up nitrogen derived from ant debris.

Key words: Ant–plant interactions, mutualism, nutrient uptake, Michaelis–Menten kinetics, ^{15}N labelling, myrmecophytes, epiphytes, *Caularthron bilamellatum*, Orchidaceae, BCNM.

INTRODUCTION

Interactions between ants and plants range from very loose associations to obligate and highly specialized mutualisms (Heil and McKey, 2003; Rico-Gray and Oliveira, 2007). Many studies have demonstrated plant protection by opportunistically attracted ants (Oliveira *et al.*, 1999; Sobrinho *et al.*, 2002), though variation in the abundance of ant species or species composition can lead to variation in protective effects (Rico-Gray and Thien, 1989; Di Giusto *et al.*, 2001). In specific and obligate mutualisms, plants offer food rewards and nesting space (specialized hollow structures called 'myrmecodomatia'), which ensure more constant and long-term associations with ants. In return, the resident ant colony often protects the host-plant against herbivores, fungal pathogens and competing vegetation. The food provided by plants is thought to play an essential role in plant-ant symbioses (Heil and McKey, 2003). It can be provided in liquid form by extrafloral nectaries or glandular trichomes, as energy rich solid food bodies (Fiala and Maschwitz, 1992; Alvarez *et al.*, 2001; Fischer *et al.*, 2002) or may be indirectly acquired from hemipteran trophobionts tended by the ants (Gaume *et al.*, 1998; Stadler and Dixon, 2008). For a long time, flow of resources in such associations was thought to be directed from the plant to its resident ants, but more recent studies have shown that nutrient transfer from ants to plants may also be important as ants accumulate organic matter (e.g. discarded debris or faeces) in their nesting sites that may constitute a nutrient source for their host plant (Treseder *et al.*, 1995; Fischer *et al.*, 2003; Solano and Dejean, 2004). Especially in epiphytes, which often face strong limitation in nutrient availability, the impact of nutrient input by ants on growth and successful reproduction may be substantial (Janzen, 1974; Rico-Gray, 1987; Gay, 1993; Treseder *et al.*, 1995). However, there is a consensus that water is generally even more limiting for growth and survival of epiphytes (Zotz and Hietz, 2001). Thus, the observation that several epiphytic myrmecophytes sacrifice a considerable amount of tissue potentially useful for water storage in order to provide nesting space for ants suggests that ant-provided nutrients are ecologically important. Nutrient transfer has been demonstrated in some well-known myrmecophytic epiphyte genera like *Dischidia* (Apocynaceae) which form domatia from folded leaves avoiding loss of tissue

(Treseder *et al.*, 1995), *Lecanopteris* (Polypodiaceae), which develop hollow rhizomes (Gay, 1993), or *Myrmecodia*and *Hydnophytum (*Rubiaceae) which exhibit a prominent caudex with natural cavities (Huxley, 1978). Much less studied are two genera of myrmecophytic Orchidaceae which provide hollow pseudobulbs as nesting space for ants: *Myrmecophila* and *Caularthron*. Labelling experiments to assess the nutrient uptake capabilities of *Myrmecophila* (syn. *Schomburgkia*) *tibicinis* pseudobulbs were performed by Rico-Gray *et al.* (1989). *Solenopteris* ants fed with ^{14}C-labelled glucose were killed and then placed within the pseudobulbs. After 2 weeks of exposure the label could be detected in leaves, roots and pseudobulbs demonstrating carbon uptake from the ant debris. This experiment did not, however, test the uptake of nitrogen or phosphorus, which are most likely severely limiting growth and reproductive success of many epiphytes *in situ* (Benzing, 1970; Zotz and Hietz, 2001; Zotz and Richter, 2006).

This study focuses on nutrient transfer from ants to plants in *Caularthron bilamellatum*, a pseudobulb-forming epiphyte distributed from southeast Mexico to Brazil (Govaerts *et al.*, 2010). According to Fisher *et al.* (1990) the parenchyma tissue inside young pseudobulbs desiccates upon maturation at the onset of the dry season, thus forming a hollow chamber. Ants can enter the hollow pseudobulbs through a vertical slit at the base, which forms during desiccation, and utilize them as nesting space (Dressler, 1981). Thirty-two different ant species were found to be inhabitants of hollow pseudobulbs of *Caularthron bilamellatum* (Yanoviak *et al.*, 2011). Apart from providing nesting space, the plant attracts ants through extrafloral nectaries on reproductive structures (pedicel, flowers and seedpods), on developing shoots and, as the only known orchid, on mature leaf bases thereby providing nectar throughout the year (Fisher and Zimmerman, 1988). Ants inhabiting the pseudobulbs clearly benefit from this association and, depending on ant species, colony size and alternative food sources, may gain up to half of their nutritional needs from the extrafloral nectaries of their host plants (Fisher *et al.*, 1990). Ant-occupied young pseudobulbs produce significantly more flowers and fruits than those with ants and ant debris removed (Fisher, 1992), but the reason for this has not yet been investigated in detail. *Caularthron bilamellatum* pseudobulbs lose up to 50 % of their fresh weight (G. Zotz, unpubl. res.) when the hollow chamber develops, which raises the question of a trade-off between water storage capacity and positive effects of the inhabiting ants. We hypothesized that nutrient gain from debris or faeces of the inhabiting ants may constitute such an advantage.

The aim of this study therefore was to demonstrate that *Caularthron bilamellatum* has the capability to acquire nitrogen through its hollow pseudobulbs and that transfer of this nitrogen to vegetative and reproductive structures occurs. To achieve this, we (*a*) studied the pseudobulb morphology and inner surface properties to identify possible specialized uptake structures, (*b*) determined the potential uptake rates and uptake kinetics for ammonium, urea and glutamine, each labelled with the stable isotope ^{15}N, (*c*) investigated, whether feeding of inhabiting ants with a ^{15}N-labelled bait in the field would lead to ^{15}N uptake into orchid tissue, and (*d*) monitored a possible translocation of the ^{15}N tracer into reproductive structures of the plant.

MATERIALS AND METHODS

Field study site

The *in situ* study was conducted from November to December of 2007 in the Barro Colorado Nature Monument (BCNM), Republic of Panama (9°10′N, 79°51′W). The reserve, which consists of various islands such as Barro Colorado Island (BCI) and a number of peninsulas, is almost entirely covered by tropical moist forest receiving an annual precipitation of 2600 mm. The rainy season lasts from April to December, a distinct dry season occurs from late December until March (Croat, 1978; Leigh *et al.*, 1982; Windsor, 1990). Primarily a canopy species, *Caularthron bilamellatum* is also very abundant on *Annona glabra* (Annonaceae), a small evergreen tree growing along the southern shoreline of BCI and rarely exceeding 7 m (Croat, 1978; Zotz *et al*, 1999). Here, this orchid is readily reachable by boat and can easily be sampled and monitored in large numbers.

Sample collection, light microscopy and SEM investigation

For studies of pseudobulb anatomy and surface characteristics, mature, hollow pseudobulbs of different size inhabited by or free of ants, as well as immature pseudobulbs that had not yet formed a hollow chamber, were harvested along the south coast of BCI and fixed in 70 % ethanol for further analyses at the University of Vienna. For light microscopy (LM), samples were embedded in Technovit 7100, a HEMA-based resin (Heraeus Kulzer GmbH, Wehrheim/Ts, Germany). Resin blocks were cut to slices of 5–10 μm using a Leitz 1515 microtome (Leica Microsystems AG, 35578 Wetzlarand, Germany). Samples were either stained with Toluidine Blue to enhance overall contrast, or with Sudan III to test for suberin and cutin in the cell walls. For scanning electron microscopy (SEM), samples were re-fixed in gluteraldehyde overnight, critical point dried in liquid CO_2, sputtered with gold and analysed in a JEOL JSM-6390 scanning electron microscope (JEOL USA Inc., Peabody MA, USA).

Labelling experiments

To estimate tissue loss during formation of the pseudobulb chamber, cross-sections of fresh mature pseudobulbs were taken, comparing the overall diameter to the diameter of the pseudobulb cavity. The ratio between total pseudobulb volume and the hollow chamber is expressed by the ratio between the total radius r and the radius of the chamber r_c.

$$r^2 : r_c^2 = V : V_c$$

To determine the plants potential for nutrient uptake through the pseudobulb chamber, label was carefully injected through the basal slit of mature uninhabited pseudobulbs using a syringe and bulbs were placed upside down for an incubation time of 1 h. We used ^{15}N-labelled NH_4Cl (99 at%), urea (98 at%) and L-glutamine (alpha-^{15}N, 98 at%) (Cambridge Isotope Laboratories, Andover, MA, USA) at concentrations of 50, 100, 250 and 500 μM, and 1·0 and 2·0 mM, with three replicates for each concentration and nitrogen form. After incubation, apoplastically bound ions

were removed by flushing the hollow pseudobulbs twice with a 10 mM $CaCl_2$ solution and washing the inner and outer surface of the pseudobulbs with distilled water. Small samples of each pseudobulb's apical region were cut out and dried at 50 °C for 48 h. Differences in nitrogen uptake rates between small and large pseudobulbs were not found to be significantly different (two-way ANOVA, $P > 0.050$). Samples of the two size groups were therefore pooled.

To detect possible translocation of label to reproductive structures, *Caularthron bilamellatum* plants were collected at Barro Colorado Island and cultivated at the Botanical Garden of Vienna (HBV). At the onset of flower buds, 2 mL of a 2·0 mM $^{15}NH_4Cl$ solution were injected into the pseudobulb cavity, and plants mounted upside down to keep the label in the pseudobulb apex. After 12 weeks the ripened seedpods were harvested and seeds dried at 50 °C for 48 h.

To investigate nutrient transfer from ants to plants isolated trees of *Annona glabra* along the south coast of BCI and north coast of neighbouring Gigante peninsula carrying *Caularthron bilamellatum* plants of different sizes and inhabited by different ant species were randomly selected for an ant feeding experiment. A small plastic bottle containing a solution of honey amended with $^{15}NH_4Cl$ was mounted to each host tree (Fig. 1A). Small holes drilled in the upper part of the bottle allowed ants to access the bait while preventing it from leaking or being washed out by heavy rain. The bottle was located beneath the orchids and active roots were removed to prevent contamination by patrolling ants carrying the label. The bait was usually taken up overnight and refilled every 2–3 d. After 2 weeks small plants were harvested in total, while only individual pseudobulbs were sampled from very large plants. Adult ants, larvae, detritus as well as ant carton made by some species were collected from each sampled plant and dried at 50 °C for 48 h. Pseudobulbs were washed, cut, and dried as described above.

Stable isotope analysis

Samples were dried for 24 h at 60 °C and homogenized with a ball mill (RetschMM2, Haan, Germany). Aliquots of 1·5–2 mg were weighed into tin-capsules and subjected to isotope ratio mass spectrometry. For measuring stable nitrogen isotope ratios ($^{15}N/^{14}N$), an elemental analyzer (EA1110, CE Instruments, Milan, Italy) connected to an isotope ratio mass spectrometer (Delta^{PLUS}, Finnigan MAT, Bremen, Germany) by a ConFlo II interface (Finnigan MAT) was used. Reference gas (high purity N_2, Air Liquide, Vienna, Austria) was calibrated to the atmospheric N_2 (at-air) standard using reference material obtained from the International Atomic Energy Agency (Vienna, Austria).

^{15}N incorporation was determined from N concentrations (C_N) in dry mass (M_d) and the corresponding atom % ^{15}N and at% ^{15}N excess (APE) values.

$$\text{at\% } ^{15}\text{N } [\%] = \text{mol } ^{15}\text{N}/(\text{mol } ^{15}\text{N} + \text{mol } ^{14}\text{N})$$

$$\text{APE } [\%] = \text{at\% } ^{15}\text{N}_{\text{labelled sample}} - \text{at\% } ^{15}\text{N}_{\text{unlabelled control}}$$

Uptake rates (J) were calculated as follows:

$$J[\mu\text{mol}^{15}\text{N g}^{-1}M_d\text{h}^{-1}] = C_N \times \text{APE}/100/M_r \times 1000/t$$

M_r is the molecular weight of ^{15}N and t the incubation time in hours.

Kinetic constants were determined using SigmaPlot11 (Systat Software GmbH, Ekrath, Germany), fitting the uptake values to the Michaelis–Menten equation (regression analysis by hyperbola, single rectangular, two parameters). The equation for the hyberbolic regression was used to determine the Michaelis–Menten constant according to the equation

$$v = V_{max} \times [S]/(K_m + [S])$$

in which v is the uptake rate at a given substrate concentration [S], V_{max} the maximum uptake rate at substrate saturation and K_m the Michaelis–Menten constant (Leskovac, 2003; Wanek and Pörtl, 2008). K_m and V_{max} can also be derived from regression models using Lineweaver–Burk, Eadie–Hofstee and Hanes–Wolf equations (Markus *et al.*, 1976), but as hyperbolic regression delivered the best fitting to the datapoints ($R^2 > 0.9$) as well as the most robust results, it was chosen for further analyses.

Statistical analysis

Statistics were performed using SigmaPlot 12 (Systat Software GmbH, Ekrath, Germany), and STATISTICA 8·0 (StatSoft, Inc. 2008, data analysis software system). Differences between types and concentrations of labelling compounds, as well as between labelled and unlabelled control samples were determined by *t*-test or analysis of variance (ANOVA), followed by a Holm–Sidak *post hoc* test where appropriate. Log-transformation was applied to datasets failing to show normal distribution in order to fulfil the criteria for statistical testing. Unless stated otherwise the standard error of the mean (s.e.) was chosen as a measure of variability in all figures and tables.

RESULTS

Pseudobulb anatomy and surface characteristics

Growing pseudobulbs of *Caularthron bilamellatum* were bright green, fleshy and exhibited a very high water content (Fig. 1B). The centre was completely clear, gel-like and lacked cellular structures (Supplementary Data Fig. S1A). As material was collected at the end of the rainy season, the preformed basal slits had just opened and the desiccation of the parenchyma tissue in the pseudobulbs was at a very early stage. Ants rarely inhabited the small space within these immature pseudobulbs.

In mature pseudobulbs which had formed during the previous growing season, the parenchyma tissue at the centre had fully desiccated forming a hollow chamber. Those pseudobulbs which were not (Fig. 1C), or only weakly inhabited by ants (Supplementary Data Fig. S1D), exhibited a smooth and yellow brownish inner surface around the slit, turning darker toward the middle region of the pseudobulb, and becoming very rough towards the apex (Supplementary Data Fig. S1D). SEM images revealed a distinct outer layer of unusually large dead and partially torn cells, forming a crater-like surface at the apical regions (Fig. 2A; Supplementary Data Fig. S2B). The cell walls of this outer layer are covered with

FIG. 1. Morphology of *Caularthron bilamellatum* pseudobulbs. All material is from plants naturally growing on *Annona glabra* (Annonaceae) in BCNM, Panama. (A) A small plastic bottle (blue, arrow) containing ^{15}N enriched honey solution was mounted beneath the orchids to determine a possible nutrient transfer from ants to plants. (B) Longitudinal section of an immature pseudobulb showing the transparent parenchyma tissue in the centre and the beginning desiccation at the base as light brown tissue. (C) Cross-section near the apex of a mature hollow pseudobulb not inhabited by ants. (D) Longitudinal section of the apical region of a mature pseudobulb inhabited by a large number of ants. The entire surface is covered with organic material containing remains of prey, dead ants, mites and coccids. (E) Longitudinal section of a mature pseudobulb inhabited by a large number of ants. The entrance is located at the base (right), the surface of the cavity is smooth in the lower third becoming increasingly rougher towards the apex (left) where waste is stored. Ant carton can be seen in the middle regions of the pseudobulb.

cutin and suberin accrustations, and the cell walls of the adjacent layers of living cells are densely pitted (Fig. 2C). The surface is highly hydrophilic and shows sponge-like properties; when wetted, moisture is quickly absorbed and distributed across the cavity. Pseudobulbs of all sizes and plant ages shared these features. The colonising ants seem to excavate the remains of the desiccated parenchyma and appear to be responsible for these surface characteristics indicating that at least at some point ants had visited these bulbs.

In contrast, the interior of mature pseudobulbs inhabited by large numbers of ants at the time of sampling differed in characteristics and colour. Apparently depending on ant species and colony size, the surface was often covered with ant waste and ant-made carton (Supplementary Data Fig. S1C).

Ant waste was preferably stored in the cavity's tip and developed into a dark brownish coat totally covering the cells of the surface (Fig. 1D, E). Remains of prey, dead nest mates, plant material, mites, nematodes and even coccids

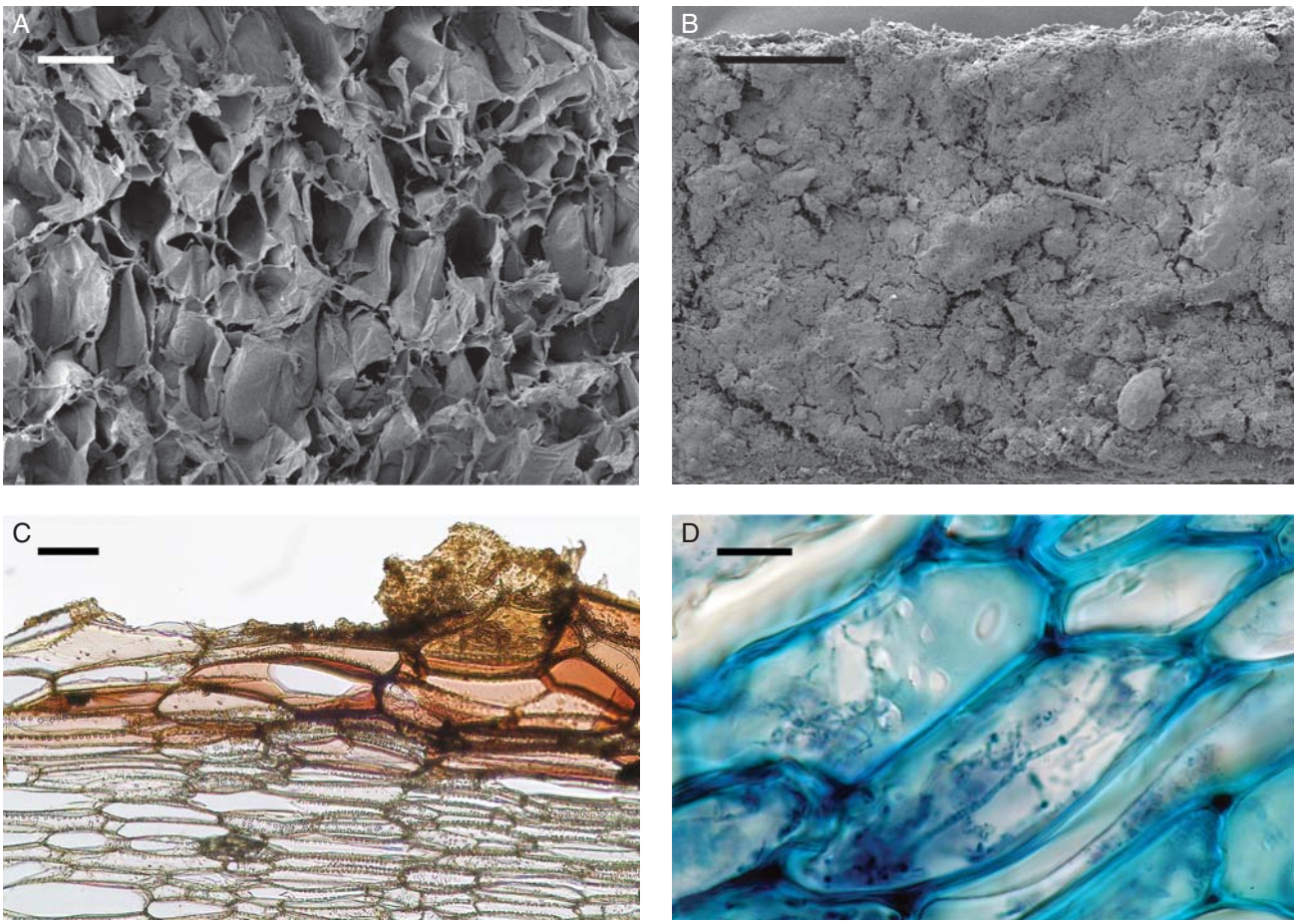

FIG. 2. Pseudobulb anatomy of *Caularthron bilamellatum*, collected in BCNM, Panama. (A) Scanning electron microscope image of a mature, uninhabited pseudobulb cavity near the apex. The rough surface consists of large dead and often torn cells. (A higher magnification of this image can be found in Supplementary Data Fig. S2B). (B) SEM image of a mature ant-inhabited pseudobulb cavity near the apex. The surface is completely covered with a layer of organic material. Note the mite down to the right. (A higher magnification of this image can be found in Supplementary Data Fig. S2D). (C) Light microscopic image of a mature pseudobulb inhabited with only few ants. The cross-section was made near the apex of fresh plant material. Sudan III staining reveals thick suberin/cutin accrustations on the cell walls of the cavity surface. Organic material and few fungal hyphae can be seen in the upper right corner. The cell wall in deeper tissue layers (colourless) are densely pitted. (D) LM image of a mature pseudobulb inhabited by a large number of ants (cross section near the apex). Toluidine Blue staining was used to enhance contrast. Intracellular fungal hyphae are present within the cells below the surface layer. Scale bars: (A) = 200 μm; (B) = 500 μm; (C) = 100 μm; (D) = 25 μm.

could be identified in the organic material, although for the largest part it appeared to consist of fungal hyphae (Fig. 2B and Supplementary Data Fig S2C, D). Hyphae were not restricted to the surface but could also be detected in the adjacent layer of living cells (Fig. 2D). Ants of the genera *Azteca* and *Crematogaster* were the most common inhabitants of the studied pseudobulbs, primarily *Azteca* cf *velox* and *Crematogaster crinosa*. *Azteca* cf *velox* built carton to divide the plant cavity into compartments (Supplementary Data Fig. S1C).

Very few of the examined mature pseudobulbs had failed to form a slit at the base and thereby remained closed. Though the centre had desiccated, the hollow chamber remained completely inaccessible to ants. The interior of such pseudobulbs differed from those exposed to the environment. Remains of desiccated parenchyma cells covered the entire surface of the pseudobulb cavity, giving it a white-yellowish colour (Supplementary Data Fig. S1B).

On average, the hollow chamber took up about 42 % of total pseudobulb volume (range 33 % –53 %, $n = 15$).

Potential N uptake and kinetics

Pseudobulbs of *C. bilamellatum* were able to take up all supplied forms of nitrogen and showed significant enrichment in ^{15}N compared with unlabelled controls at all concentrations. Plants preferably took up NH_4^+, which was taken up significantly faster than urea and glutamine (two-way ANOVA, $F_{8;69} = 2{\cdot}466$, Holm–Sidak, both $P < 0.001$). Uptake rates of urea and glutamine were not significantly different (Holm–Sidak, $P > 0.05$) from each other (Table 1).

Ammonium and glutamine exhibited Michaelis–Menten type uptake kinetics. In contrast, uptake of urea was linearly related to substrate concentrations within the tested range (up to 2 mM) (Supplementary Data Fig. S3). Calculated V_{max} values were $1{\cdot}01 \pm 0{\cdot}21$ μmol ^{15}N g^{-1} M_d h^{-1} for NH_4^+

and 0·66 ± 0·07 μmol ^{15}N g^{-1} M_d $h^{-1}$for glutamine. The affinity of the uptake system was slightly higher for ammonium, with a K_m value of 0·41 mM compared to 1 mM for glutamine. Catalytic uptake efficiency, calculated as V_{max}/K_m, was surprisingly low in both cases, but approx. 3-fold higher for NH_4^+ than for glutamine (Table 2).

Translocation of tracer to reproductive structures

Seeds harvested from plants labelled by injecting $^{15}NH_4^+$ solution into the pseudobulb cavity, allowing incubation until capsules matured, were significantly enriched in ^{15}N (*t*-test, $t = -17.311$, $P < 0.001$, $n_{control} = 6$, $n_{labelled} = 4$) exhibiting mean $\delta^{15}N$ values of 317.6 ± 23.1 ‰ compared with 1.6 ± 0.25 ‰ of the unlabelled control group (Fig. 3).

Transfer of label from ants to plants

Ants, which were fed a honey-solution labelled with NH_4^+, exhibited $\delta^{15}N$ values ranging from 148 ‰ to 1457 ‰. In contrast, larval stages, which were only present in sufficient numbers for mass spectrometry in two samples, showed a low enrichment with $\delta^{15}N$ values (10·2 and 12·4 ‰). Ant carton yielded intermediate $\delta^{15}N$ values (69·8 ± 36·0 ‰), the relative amount of label present in ant carton and plants varied greatly between the different sampling sites (Supplementary Data Table S1).

Samples taken from the apical pseudobulb regions of plants inhabited by labelled ants were also significantly enriched in ^{15}N (*t*-test, $t = 5.600$, $P < 0.001$, $n_{control} = 8$, $n_{labelled} = 90$) exhibiting mean $\delta^{15}N$ values of 165.4 ± 34·5 ‰ compared with the control group with 0·61 ± 0·58 ‰ (Fig. 3).

TABLE 1. *Two-way ANOVA and Holm–Sidak post-hoc test results for the net nitrogen uptake into pseudobulbs of* Caularthron bilamellatum *collected in BCNM, Panama*

Effect	SS	d.f.	MS	*F*	*P*
Intercept	3.925153	1	3.925153	74.61640	<0.000001
Label	1.446752	2	0.723376	13.75124	0.000009
Conc	2.377757	4	0.594439	11.30018	<0.000001
Label × conc	1.037714	8	0.129714	2.46584	0.020633
Error	3.629705	69	0.052604		

Three nutrient sources (NH_4^+, urea or glutamine) labelled with ^{15}N were injected into the plants' hollow pseudobulbs. SS, Single squares; d.f., degrees of freedom; MS, mean squares.

However, the amount of nitrogen taken up varied substantially between each plant and also in neighbouring pseudobulbs of the same plant, with $\delta^{15}N$ values ranging between 5·1 ‰ and 1684 ‰, presumably due to differences in ant visits. Generally the amount of incorporated ^{15}N decreased from apex (106.5 ± 17·6 ‰) over centre (79.6 ± 16.0 ‰) to base of the pseudobulbs (50.8 ± 8.3 ‰) where the slit is located (one way ANOVA, $F_{2;24} = 3.690$, $P < 0.05$). The difference between apex and base was significant (Holm–Sidak, $P < 0.05$) but not between apex and centre or centre and base (Holm–Sidak, $P > 0.05$), (Fig. 4; Supplementary Date Table S2).

Immature pseudobulbs that were still mostly filled with parenchyma tissue exhibited a highly significant (*t*-test, $t = -16.251$, $P < 0.001$, $n_{base} = 3$, $n_{middle} = 3$) opposite trend: a higher amount of label was present at the desiccated base (84.61 ± 6.35 ‰), which was accessible for ants, but declined towards the middle sections (20.51 ± 0.95 ‰) still filled with parenchyma tissue and therefore inaccessible to potential inhabitants. (Supplementary Data Fig. S4).

DISCUSSION

Most vascular epiphytes face limited and/or irregular supply of nutrients, demanding highly specialized adaptations, such as myrmecophily (Benzing, 1970). Even though research of ant–plant interactions in myrmecophytes has long focused on nutrient transfer from the plant to inhabiting ants, which in return defend their host against herbivores, encroaching vegetation and fungal pathogens (Rico-Gray and Oliveira, 2007), there is increasing evidence that nutrient acquisition has to be recognized as another direct benefit to the host-plant, especially in the case of myrmecophytic epiphytes. It has already been demonstrated that a number of myrmecophytic epiphytes in different plant families are capable of utilizing nutrients provided by inhabiting ants in form of waste and faeces (Janzen, 1974; Rico-Gray, 1987; Gay, 1993; Treseder *et al.*, 1995), but detailed information is still scarce.

Based on ^{15}N labelling experiments, our results for the first time provide direct evidence that transfer of nitrogen from ants to plants indeed occurs in a myrmecophytic epiphytic orchid. By labelling associated ants it was demonstrated unequivocally that *Caularthron bilamellatum* has the potential to take up nitrogen from ant waste through its hollow pseudobulbs under field conditions. The spatial distribution of labelled compounds within pseudobulbs, i.e. a stronger label in apical parts of mature bulbs, may have two reasons. First, inhabiting

TABLE 2. *Determination of kinetic constants by non-linear regression analysis of net nitrogen uptake in* Caularthron bilamellatum *pseudobulb cavities*

Label	K_m (μmol)	V_{max} (μmol ^{15}N g^{-1} M_d h^{-1})	R^2	V_{max}/K_m	*P*
Ammonium	410.94 ± 228.87	1.1 ± 0.21	0.940	0.0025	0.0190
Glutamine	998.67 ± 226.14	0.66 ± 0.07	0.988	0.0007	0.0107
Urea	–	–	0.999	–	0.0006

Shown are net nitrogen uptake rates within the domatia of plant material collected in BCNM, Panama. Michaelis–Menten constants (K_m) and maximum uptake rates (V_{max}) were derived from hyperbolic Michaelis–Menten fit ($n = 6$). The ratios K_m/V_{max}represent the catalytic uptake efficiencies and regression coefficients (R^2) show the quality of the regression fitting. Urea did not show Michaelis–Menten kinetics (see Supplementary Data Fig. S2).

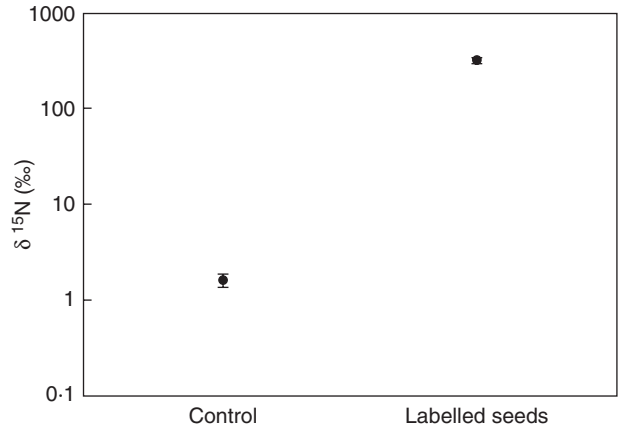

FIG. 3. Translocation of nitrogen taken up by *Caularthron bilamellatum* pseudobulbs to reproductive structures. Plants collected at BCNM, Panama and cultivated at HBV Vienna, Austria were labelled by injecting 2·0 mM NH_4^+into the hollow pseudobulbs at the onset of flowering. Seeds were harvested after 12 weeks and compared to an unlabelled control group. Groups were significantly different (*t*-test, $t = -17.311$, $P < 0.001$, $n_{control} = 6$, $n_{labelled} = 4$). Note the logarithmic scale of the y-axis. Error bars represent s.e.

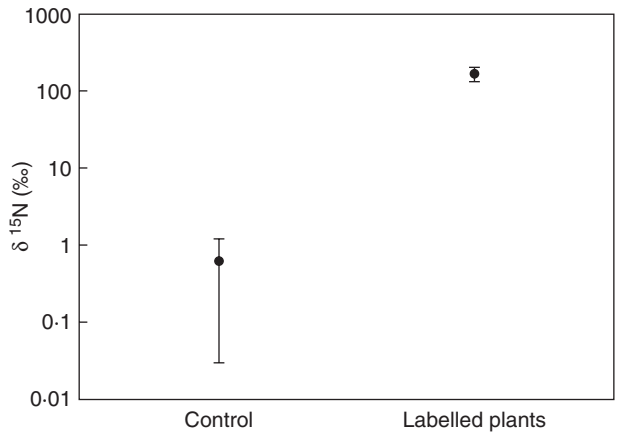

FIG. 4. Nitrogen transfer from ants inhabiting pseudobulbs into plant tissue. Ants inhabiting specimens of *Caularthron bilamellatum*growing naturally on *Annona glabra* at BCNM, Panama, were labelled by feeding them a solution of honey containing $^{15}NH_4Cl$. Ants transported the label into the hollow pseudobulbs. These were harvested after 2 weeks and compared with an unlabelled control group. Groups were significantly different (*t*-test, $t = 5.600$, $P < 0.001$, $n_{control} = 8$, $n_{labelled} = 90$). Note the logarithmic scale of the *y*-axis. Error bars represent the s.e.

ants generally tend to store their waste in the apical part of the pseudobulb and keep the entrance at the base clean, which leads to a concentration of detritus in the apical part. Second, the roughness of pseudobulb surfaces strongly increases towards the apex due to large and partly torn cells present in this area. Although not comparable to highly specialized surface structures like warts in myrmecophytic Rubiaceae (Huxley, 1978; Rickson, 1979) these structures increase the total surface considerably and therefore provide a higher waste storage capability. The strongly pitted cell walls in both, the outer layer of dead cells and in the adjacent layers of living cells below, may help to maintain nutrient permeability despite wall accrustation with suberin and cutin in the outermost surface cells. Though the surface has some sponge-like properties, a similarity to the *velamen radicum* of roots is not suggested. The cavity surface consists only of a single to very few layers of large dead cells with thick walls and is lacking typical helical cell wall thickenings of a *velamen radicum*. (Benzing *et al.*, 1982).

The growth of many epiphytes is thought to be limited by nutrients such as nitrogen or phosphorus (Laube and Zotz, 2003; Winkler and Zotz, 2008). It is therefore tempting to speculate that the input of nitrogen from ants established in this study may be beneficial for the growth of *Caularthron bilamellatum*. In additional experiments we were also able to demonstrate nitrogen transfer into seeds, indicating that nutrient input into pseudobulbs may also be beneficial for the plants' reproduction.

Nitrogen uptake kinetics for different organic and inorganic nitrogen sources showed significant and active uptake of all offered nitrogen forms. It is well known, for example for NH_4^+, that plant roots often exhibit uptake kinetics dominated by high affinity transport systems (HATS) at substrate concentrations up to 1 mM, consisting of highly sensitive but quickly saturable transport proteins usually expressed under nutrient starvation (von Wiren *et al.*, 2000). Above about 1 mM low affinity transport systems (LATS) with low substrate affinity, but high uptake capacity take over, facilitating uptake at larger substrate concentrations. For *Caularthron bilamellatum* we calculated a K_m value of about 0·4 mM for NH_4^+ at a relatively low V_{max} of about 1 μmol $^{15}N\ g^{-1}\ M_d\ h^{-1}$, representing small catalytic uptake efficiency. In studies with soft-bodied plants such as macroalgae and bryophytes (lacking a distinct cuticula) K_m values in the range of 0·5–500 μM were found for ammonium and amino acid transport systems in leaves, but at a higher V_{max} causing distinctively higher catalytic uptake efficiencies than in this study (Tyler *et al.*, 2005; Wanek and Pörtl, 2008). However, V_{max} and K_m values comparable to those found for the inner surface of the pseudobulbs in our experiments have been reported for amino acid and ammonium uptake by leaf tissue of an epiphytic tank bromeliad (Inselbacher *et al.*, 2007). Interestingly, in both cases a linear uptake of urea up to a concentration of several mM was found, indicating low-affinity uptake systems, as a possible adaption to exploit the infrequent but intense nitrogen input by animal excretions. Such versatile uptake capacities seem especially important for epiphytes adapted to nutrient-poor ecosystems, which have to deal with a broad variety of scarce or only temporarily available forms of nitrogen (Lambers *et al.*, 1998) demanding a high flexibility to acquire potential nutrient sources.

Myrmecophytic epiphytes like *Caularthron bilamellatum* share some similarities with carnivorous plants, which have also developed sophisticated strategies to survive in extremely nutrient-poor environments (Juniper *et al.*, 1989, Krol *et al.*, 2012). The traps of some carnivorous plants, similar to ant domatia, often resemble microenvironments containing a large number of different organisms which may help to degrade detritus thereby increasing nutrient uptake of the host plant (Blatrix *et al.*, 2009, Paracer and Ahmadjian, 2000). In comparison with true carnivorous plants in a strict sense (Givnish *et al.*, 1984), active prey caption or glands secreting digestive enzymes are missing in *Caularthron*

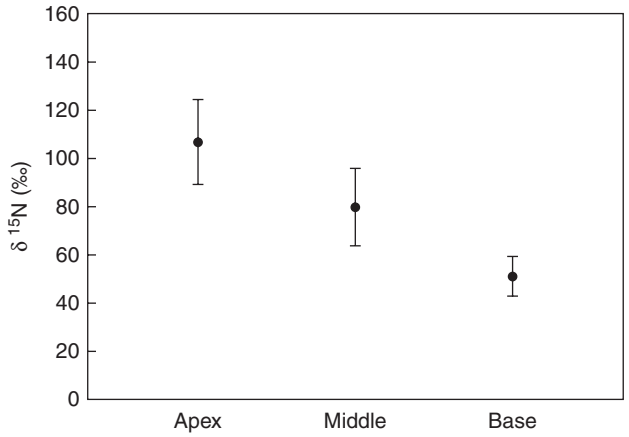

FIG. 5. Spatial variations of ^{15}N uptake between basal, middle and apical regions of mature pseudobulbs of *Caularthron bilamellatum*. For details compare legend of Fig. 3. Spatial distribution of label within pseudobulbs was significantly different between apical and basal regions (one way ANOVA, $F2;24 = 3.690$, Holm–Sidak, $P < 0.05$) but not between apical and middle or middle and basal regions (Holm–Sidak, $P > 0.05$) (see Supplementary Data Table S2). Error bars represent s.e. ($n = 9$).

bilamellatum. Some protocarnivorous plants, such as *Roridula gorgonias*, however, are also missing glands and digestive enzymes and do not digest trapped insects directly. They rather use the faeces of a mutualistic bug from the genus *Pameridea* Reut. (Heteroptera, Miridae) which feeds on trapped insects (Juniper *et al.*, 1989), thereby acquiring significant amounts of nitrogen (Ellis and Midgley, 1996; Anderson, 2005). Even though the amount and importance of nutrient transfer from symbiont to plant has yet to be quantified in many systems, these results suggest that plants have many and often subtle ways of exploiting animals as food sources.

In summary, we were able to demonstrate that *Caularthron bilamellatum* plants are capable of taking up nutrients (*a*) from organic matter deposited by ants at the inner surface of the hollow pseudobulbs and (*b*) from different organic and inorganic nitrogen forms injected into the pseudobulb cavity in liquid form. Uptake kinetics of the inner surface of the hollow pseudobulbs were comparable to results obtained from leaves of epiphytic bromeliads suggesting the presence of active transport systems capable of dealing with a broad variety of compounds and concentration ranges. As nitrogen was also translocated into reproductive structures we speculate that nutrient input by ants may generally increase plant fitness (vegetative growth and reproduction). All these features are especially useful for an epiphytic myrmecophyte having to cope with a harsh, unpredictable and nutrient-poor habitat where associations with ants acting both as a potential protection and a constant supply of nutrients may be the key to survival.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: morphological characteristics of *Caularthron bilamellatum* pseudobulbs in different stages of development and ant colonisation. Figure S2: SEM and LM images of *C. bilamellatum* pseudobulb cavity surface. Figure S3: ^{15}N uptake kinetics of *C. bilamellatum* pseudobulb inner surface. Figure S4: spatial variations of ^{15}N uptake between basal and middle regions of immature pseudobulbs, partially filled with parenchymous tissue. Table. S1: distribution of ^{15}N label within ants, ant larvae and carton in ant feeding experiment. Table. S2: one-way ANOVA and Holm-Sidak *post-hoc* test results for the distribution of ^{15}N label in basal, middle and apical regions of *C. bilamellatum* pseudobulbs.

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