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Titel der Diplomarbeit

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

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1 INTRODUCTION

Interactions between ants and plants range from very loose associations to obligate and highly specialised 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 (specialised 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.,* 2001) or may be indirectly acquired from hemipteran trophobionts tended by the ants (Gaume *et al.*, 1998; Stadler and Dixon, 2008). Flow of resources in such associations was for a long time thought to be directed mostly from the plant to its resident ants, but recent studies have shown that nutrient transfer from ants to plants may also be important. Ants accumulate organic matter in their nesting sites by storing discarded debris or faeces, which 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 significant (Janzen, 1974; Rico-Gray *et al.,* 1987; Gay, 1993; Treseder *et al.,* 1995). Several domatia-forming epiphytic myrmecophytes sacrifice a considerable amount of tissue potentially useful for water storage in order to provide nesting space for ants, which indicates that ant-provided nutrients

may be important for their survival. Nutrient transfer has been demonstrated in some well-known myrmecophytic epiphytes like *Dischidia* (Apocynaceae), which forms domatia from folded leaves avoiding loss of tissue (Treseder *et al.*, 1995), *Lecanopteris* (Polypodiaceae), developing hollow rhizomes (Gay, 1993), or *Myrmecodia* and *Hydnophytum* (Rubiaceae) exhibiting a prominent caudex with natural cavities (Huxley, 1978). By far less known are two genera of myrmecophytic Orchidaceae providing 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 two 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 the limiting factors for the growth and reproductive success of most epiphytes *in situ* (Benzing, 1990; Zotz and Hietz, 2001; Zotz and Richter, 2006). For *Caularthron*, the second genus of myrmecophytic Orchidaceae, so far no similar experiments had been performed at all.

This study is the first to focus 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 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). 32 different ant species were found to be inhabitants of hollow pseudobulbs of *C. bilamellatum* (Yanoviak *et al.,* in press). 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). *C. bilamellatum* pseudobulbs, on the other hand, lose up to 50 % of fresh weight through desiccation and formation of the hollow chamber (G. Zotz, pers. comm.) imposing the question whether this dramatic loss of parenchyma cells and water storage capacity could be outweighed by positive effects through the inhabiting ants. In an experiment performed by Fisher (1992), ant occupied young pseudobulbs produced significantly more flowers and fruits than those with ants and ant debris removed. The reason for this has not yet been investigated in detail but it may be hypothesized that nutrient gain from debris or faeces of the inhabiting ants poses a significant advantage for the hostplant, as the epiphytic habitat is known to be strongly nutrient limited (Benzing, 1990; Grime, 2001; Zotz and Richter, 2006).

The aim of this study therefore was to demonstrate that *C. bilamellatum* has the capability to acquire nitrogen through its hollow pseudobulbs and that transfer to reproductive structures occurs. To achieve this, I (1) determined the potential uptake rates and uptake kinetics for ammonium, urea and glutamine, each labelled with the stable isotope ^{15}N, (2) investigated, whether feeding the inhabiting ants with a ^{15}N labelled bait in the field led to ^{15}N uptake into the orchids tissue, (3) monitored a possible translocation of the ^{15}N tracer into reproductive structures of the plant, and (4) studied the pseudobulb morphology and inner surface properties to identify possible specialised uptake structures.

2   MATERIALS AND METHODS

2.1   Study site

The study was conducted from Nov. to Dec. 2007 on Barro Colorado Island (BCI), Republic of Panama (9°10'N, 79°51W). The island's surface is almost entirely covered by tropical moist forest, receiving an annual precipitation of 2600 mm. The rainy season lasts from April to December, a short but distinct dry season occurs from late December until March (Croat, 1978; Leigh, Rand and Windsor, 1982; Windsor, 1990). Primarily a canopy species, *C. bilamellatum* is also extremely abundant on *Annona glabra* (Annonaceae), a small evergreen tree growing along the southern shoreline of BCI and rarely exceeding 7 m (Zotz, 1999; Croat, 1978). This habitat is readily reachable by boat and specimens could easily be sampled and monitored in large numbers.

2.2   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 fixated in 70 % ethanol for further analyses at University of Vienna. For light microscopy, 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) and investigated with a Nikon Eclipse E 200 microscope. For scanning electron microscopy, samples were re-fixated 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 01960, USA).

2.3   Labelling experiments

In order 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 V and the hollow chamber V_c is expressed by the ratio between the total radius r and the radius of the chamber r_c.

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

To determine the plants potential for nutrient uptake through the pseudobulb chamber, mature uninhabited pseudobulbs of different sizes were sorted into two size classes. *Large* (L), ranging from 12–25 cm and *Small* (S) ranging from 3–6 cm. Label was carefully injected through the basal slit 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 using 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 across the tested concentrations ($P > 0.050$, two-way ANOVA, Holm-Sidak). Samples of the two size groups were therefore pooled.

In order 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 *C. 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 days. 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.

2.4   Stable isotope analysis

Samples were dried for 24 h at 60 °C and homogenised with a ball mill (RetschMM2, Haan, Germany). Aliquots of 1.5–2 mg were weighed into tin-capsules and submitted to isotope ratio mass spectrometry. For measuring stable nitrogen isotope ratios ($^{15}N/^{14}N$), an elemental analyzer (EA1110, CE Instruments, Milan, Italy) was connected to an IRMS (Delta^{PLUS}, Finnigan MAT, Bremen, Germany) by a ConFlo II interface (Finnigan MAT). 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.

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

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

Uptake rates (J) were calculated as follows:

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

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

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

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} could also be derived from linear regression 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.

2.5   *Statistical analysis*

Statistics were performed using SigmaPlot11 (Systat Software GmbH, Ekrath, Germany), and STATISTICA 8.0 (StatSoft, Inc. 2008, data analysis software system). Differences between size groups, types and concentrations of labelling substrates as well as between labelled and unlabelled control samples were analysed by one- or two-way analysis of variance (ANOVA) following a Holm-Sidak test. Log-transformation was applied to datasets failing to show normal distribution in order to fulfil the criteria for ANOVA testing. If not stated otherwise the standard error of mean (SE) was chosen to represent variability in all figures and tables.

3   RESULTS

3.1   *Pseudobulb anatomy and surface characteristics*

While in growth and immature, pseudobulbs of *C. bilamellatum* are bright green, fleshy and exhibit a very high water content (Fig. 1B). The centre is completely clear, gel-like and lacks cellular structures (Fig. 1C). Since material was collected at the end of rainy season, the preformed basal slits had just opened and the desiccation of the parenchyma tissue in the pseudobulbs was at a very early state. Ants rarely inhabited the small space within these immature pseudobulbs.

Mature pseudobulbs with fully desiccated parenchyma tissue which had formed the year before and were not, or only weakly inhabited by ants at the time of sampling exhibited a smooth and yellow brownish inner surface around the slit, turning darker toward the middle

region of the pseudobulb, becoming very rough towards the apex (Fig. 1D). Cross sections revealed a distinct outer layer of unusually large dead and partially torn open cells, forming a crater-like landscape at the apical regions (Fig. 1E). Pseudobulbs of all sizes and plant ages shared these features. The 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.

Very few of the mature pseudobulbs examined 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. Such pseudobulbs looked different from those exposed to the environment. Remains of desiccated parenchyma cells cover the entire surface giving it a white-yellowish colour (Fig. S1, see appendix).

In contrast, mature pseudobulbs inhabited by a large number of ants at the time of sampling differ in surface characteristics and colour. Apparently depending on species and number of ants living within, the surface is often covered with ant waste and ant-made carton and is of dark-brownish appearance, caused by fungal growth (Fig. 1F).

Ant waste is preferably stored in the outmost tip and may form a soil-like coat totally covering the cells of the surface. Remains of prey, dead nestmates, plant material, mites, nematodes and even coccids could be identified in the detritus, for the largest part it appears to consist of fungal hyphae (Fig. 1G,H). Ants of the genera *Azteca* and *Crematogaster* were the most common inhabitants of the pseudobulbs, *A.* cf *velox* and *C. crinosa* the most common species. *A.* cf *velox* is known to build carton to divide and extend the plant cavity (Fig. 1I).

The average ratio between mature pseudobulb volume to chamber volume was 2.36 ± 0.08 : 1 meaning that the hollow chamber took up about 42 % of the total pseudobulb volume.
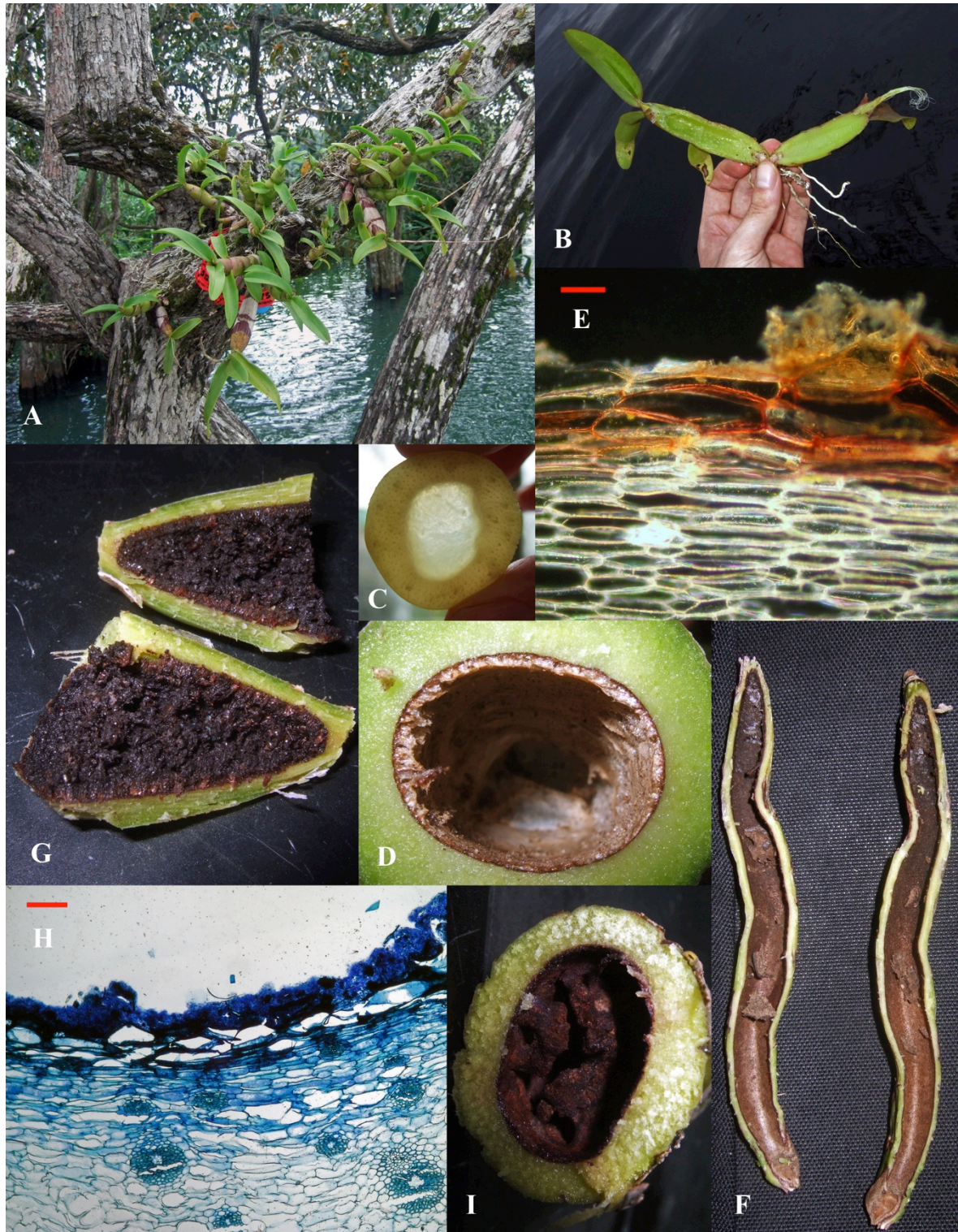
FIG**. 1.** Morphology of *Caularthron bilamellatum* pseudobulbs. Shown are anatomical characteristics of a myrmecophytic neotropical orchid collected at a tropical moist forest, BCI, Panama; (A) plant growing on *Annona glabra* (Annonaceae) in natural habitat; a small plastic bottle (blue) containing ^{15}N enriched honey was mounted beneath the orchid to label inhabiting ants and 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 of immature pseudobulb exhibiting clear gel-like parenchyma tissue at the centre; during pseudobulb maturation this tissue desiccates forming a hollow chamber; (D) cross section (near apex) of a mature hollow pseudobulb not inhabited by ants; (E) dark field microscopy image of a mature hollow pseudobulb not inhabited by ants (cross section near apex); The surface of the pseudobulb cavity (top) consists of a layer of unusually large

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cells with thick cell walls. Scale bar = 100 μm; (F) longitudinal section of a mature pseudobulb strongly inhabited by ants; The entrance is located at the base (bottom), the surface of the pseudobulb cavity is smooth in the lower third becoming increasingly rougher towards the apex (top) where waste is stored. Ant carton can be seen in the middle regions of the pseudobulb; (G) longitudilal section of the apical region of a mature pseudobulb inhabited by a large number of ants; The entire surface is covered by soil-like detritus containing prey, dead ants and coccids; (H) light microscopy image of a mature hollow pseudobulb strongly inhabited by ants (cross section near apex); The surface of the pseudobulb cavity (top) is covered by ant waste containing large amounts of fungal hyphae. Scale bar = 200 μm; (I) cross section of the middle region of a mature pseudobulb strongly inhabited by *Azteca* ants and filled with ant carton; this ant genus is known to use carton to divide nesting space into different compartments.

3.2   Potential nitrogen 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 to unlabelled controls at all applied concentrations (P < 0.050, two-way ANOVA, Holm-Sidak). Plants preferably took up NH_4^+, uptake rates of which were significantly higher than those of urea and glutamine (both P < 0.001, two-way ANOVA, Holm-Sidak). Uptake rates of urea and glutamine were not significantly different (P > 0.050).

Ammonium and glutamine exhibited Michaelis-Menten type uptake kinetics. In case of urea, linear uptake within the range of tested concentrations (up to 2 mM) was observed (Fig. S2). V_{max} values were 1.01 ± 0.21 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 411 μM than for glutamine with 999 μM, indicating greater dominance of low- than high affinity transport systems at the applied substrate concentrations. Catalytic uptake efficiency, calculated as V_{max} / K_m, was surprisingly low in both cases, but approximately 3-fold higher for NH_4^+, than for glutamine (Table1).

TABLE 1. Determination of kinetic constants by curvilinear regression showing net. nitrogen uptake of *Caularthron bilamellatum* pseudobulb cavities. Shown are uptake rates within the domatia of a neotropical myrmecophytic orchid collected at BCI, Panama. Three nutrient sources (NH_4^+, urea or glutamine) labelled with ^{15}N were injected into the hollow pseudobulbs. Michaelis-Menten constants (K_m) are given in μmol, maximum uptake rates (V_{max}) in μmol ^{15}N g^{-1} M_d h^{-1}. K_m and 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 (R²) show the quality of the regression fitting. Urea did not show Michaelis-Menten kinetics but linear uptake characteristics so K_m and V_{max} could not be calculated.

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	0.094	0.0025	0.0190
glutamine	998.67 ± 226.14	0.66 ± 0.07	0.988	0.0007	0.0107
urea	---	---	0.999	---	0.0006

3.3   Translocation of tracer to reproductive structures

Seeds harvested from plants labelled by injecting ^{15}NH$_4^+$ solution into the pseudobulb cavity and incubating them until capsules had ripened were significantly enriched in ^{15}N (P < 0.0001, one way ANOVA, Holm-Sidak) exhibiting mean δ^{15}N values of 317.63 ± 23.08 ‰ compared to 1.61 ± 0.25 ‰ of the unlabelled control group (Fig. 2).
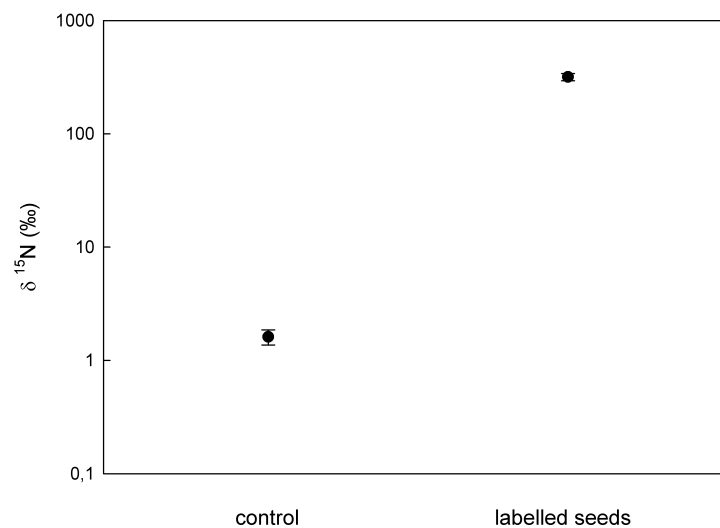

F$_{IG}$. 2. Translocation of nitrogen taken up by *Caularthron bilamellatum* pseudobulb inner surface to reproductive structures. Plants collected on BCI, Panama and cultivated at HBV Vienna, Austria were labelled by injecting 2.0 mM NH$_4^+$ into the hollow pseudobulbs during the onset of flowering. Seeds were harvested after 12 weeks and compared to an unlabelled control group. Groups were significantly different (P < 0.001). Note the logarithmic scale of the y-axis. Error bars represent standard error ($n_{control}$=6, $n_{labelled}$=4).

3.4   Transfer of label from ants to plants

Ants, which directly fed on a honey-solution highly labelled with NH_4^+, exhibited high $\delta^{15}N$ values ranging from 147.6 to 1457.1 ‰. Larval stages, only present in sufficient numbers for mass spectrometry in two samples showed very low $\delta^{15}N$ 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 considerably between the different sampling sites (Tab. S1, see appendix).

Samples taken from the apical pseudobulb regions of plants inhabited by labelled ants were also significantly enriched in ^{15}N (P < 0.001, one way ANOVA, Holm-Sidak) showing $\delta^{15}N$ values of 165.4 ± 34.5 ‰ compared to the control group with 0.61 ± 0.58 ‰ (Fig. 3).
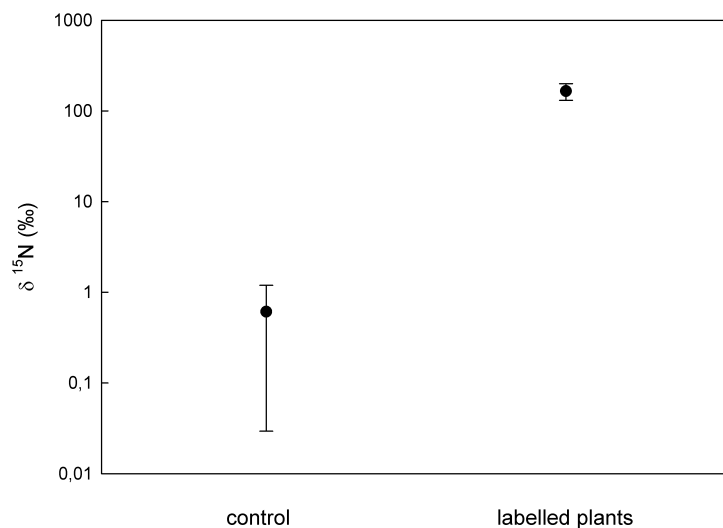

FIG**. 3.** Nitrogen transfer from ants inhabiting *Caularthron bilamellatum* pseudobulbs into plant tissue. Ants inhabiting specimens of the myrmecophytic orchid *C. bilamellatum* growing epiphytically on *Annona glabra* along the shoreline of BCI, Panama were labelled by feeding them a solution of honey containing $^{15}NH_4Cl$. Ants transported the label into the plants hollow pseudobulbs used as nesting space. Pseudobulbs were harvested after two weeks and compared to an unlabelled control group. Groups were significantly different (P < 0.001). Note the logarithmic scale of the y-axis. Error bars represent the standard error ($n_{control}$ = 8, $n_{labelled}$ = 90).

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 exceeding 1500 ‰ in several samples, presumably due to differences in ant visits. Generally the amount of incorporated ^{15}N decreased from the apex (106.5 ± 17.6 ‰) over middle (79.6 ± 16.0 ‰) to basal regions of the pseudobulbs (50.8 ± 8.3 ‰) where the slit is located. The difference between apex and base was significant (P = 0.036, one way ANOVA, Holm-Sidak) but not between apex and middle or middle and base (P > 0.050) (Fig. 4).
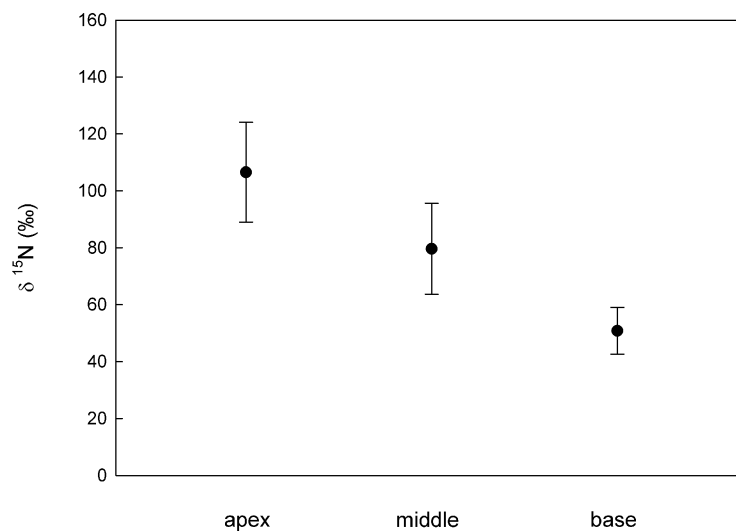

F$_{IG}$. 4. Spatial variations of ^{15}N uptake between basal, middle and apical regions of mature *Caularthron bilamellatum* pseudobulbs. Ants inhabiting specimens of the myrmecophytic orchid *C. bilamellatum* growing epiphytically on *Annona glabra* along the shoreline of BCI, Panama were labelled by feeding them a solution of honey containing $^{15}NH_4Cl$. Ants transported the label into the plants' hollow pseudobulbs used as nesting space. Pseudobulbs were harvested after two weeks. Spatial distribution of label within pseudobulbs was significantly different between apical and basal regions (P = 0.036, one way ANOVA, Holm-Sidak) but not between apical and middle or middle and basal regions (P > 0.050). Error bars represent the standard error (n = 9).

Immature pseudobulbs which were still mostly filled with parenchyma tissue, exhibited a highly significant (P < 0.001, one way ANOVA, Holm-Sidak) opposite trend: a higher amount of label was present at the already 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 inhabitants. (Fig. S3, see appendix).

4   DISCUSSION

Vascular epiphytes often have to deal with limited and/or irregular supply of nutrients, demanding highly specialised adaptions (Benzing, 1990). Myrmecophily may actually be one of these strategies. 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), especially in the case of myrmecophytic epiphytes, nutrient acquisition has to be recognised as another direct benefit for the host-plant. 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 *et al.,* 1987; Gay, 1993; Treseder *et al.,* 1995), however for many species and nutrients no or only little data exists and detailed work on uptake capabilities of different substrates has been lacking so far.

This study was the first to determine the nutrient transfer from ants to plants in a myrmecophytic epiphytic orchid using a ^{15}N stable isotope tracing approach. By labelling associated ants it could be unequivocally demonstrated that *C. bilamellatum* has the potential to take up nitrogen from ant waste through its hollow pseudobulbs under field conditions. The spatial distribution of labelled substrates within pseudobulbs, i.e. that apical parts of mature pseudobulbs were more strongly labelled than basal parts, may have two reasons. First, inhabiting 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 apex. Second, the roughness of pseudobulb surface strongly increases towards the apex due to unusually large cells in this area. Although not comparable to highly specialised surface structures like the warts in myrmecophytic Rubiaceae (Huxley, 1978; Rickson, 1979) these structures

increase the total surface considerably and therefore provide a higher waste storage capability and nutrient permeability, increasing uptake potential. In additional experiments nitrogen transfer into seeds could also be demonstrated, indicating that nutrients derived from pseudobulbs may be important for the plants' survival and reproductive success. Since growth of epiphytes is in many cases limited by nutrients such as nitrogen or phosphorus (Laube and Zotz, 2003; Winkler and Zotz, 2008) such additional inputs are likely to be beneficial for reproduction.

Nitrogen uptake kinetics for different organic and inorganic nitrogen sources showed significant and active uptake of all offered substrates. 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 increasingly expressed under nutrient starvation (von Wiren et al., 2000). Above 1 mM low affinity transport systems (LATS) with low substrate affinity but high capacity take over, facilitating the long-term uptake of larger substrate quantities. For *C. bilamellatum* a K_m value of 410 μM was calculated for NH_4^+ at a relatively low V_{max} of about 1 μmol ^{15}N g^{-1} M_d h^{-1}, leading to a small catalytic uptake efficiency. In studies with soft-bodied organisms (lacking a distinct cuticula) such as macroalgae and bryophytes K_m values in the range of 0.5–500 μM were found for ammonium and amino acid transport systems, 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 in this study for the inner surface of the pseudobulbs have been reported for amino acid and ammonium uptake by leaf tissue of an epiphytic tank bromeliad (Inselbacher et al., 2007). Interestingly, in both studies 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, having to deal with a broad variety of scarce or only temporarily available forms of nitrogen (Lambers *et al*., 1998) which demands a high flexibility to acquire potential nutrient sources.

In summary, this study was able to demonstrate that *C. bilamellatum* plants are capable of taking up nutrients (1) from organic matter deposited by ants at the inner surface of the hollow pseudobulbs and (2) 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 substrates and concentration ranges. As nitrogen was also translocated into reproductive structures it seems likely that nutrient input by ants may compensate for the loss of water storing tissue by the formation of the myrmecodomatia and increase plant fitness and ultimately reproductive success. All these findings 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.

5   SUMMARY

5.1   English summary

- *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 significant 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 substrates (NH$_4^+$, urea and L-glutamine) were studied in field-grown *C. 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* *C. 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 substrates were rapidly translocated and incorporated into reproductive structures. By labelling ants with ^{15}N *in situ*, this study was able to show that ants indeed transfer nitrogen to the plants under field conditions.

- *Conclusions* It is demonstrated here 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. The results suggest that apart from anti-herbivore protection, nitrogen derived from ant debris might be beneficial for survival and reproduction of the host plant, counteracting a possible trade-off for the loss of water storage capacity during domatia formation.

5.2 Deutsche Zusammenfassung

- *Hintergrund und Zielsetzung* Mutualistische Beziehungen zwischen Ameisen und Pflanzen kommen in einer Vielzahl unterschiedlicher Pflanzenfamilien vor. Traditionell wurde davon ausgegangen dass dabei die Pflanze den Ameisen Nistmöglichkeiten und Nahrung zur Verfügung stellt und dafür gegen herbivore Insekten und konkurrierende Pflanzen verteidigt wird. Einige myrmecophytische („ameisenbewohnte") Pflanzen wie die epiphytische Orchidee *Caularthron bilamellatum* bilden aktiv Hohlräume welche von den Ameisen als Nistplätze („Myrmecodomatien") genutzt werden, obwohl die Pflanzen dadurch beträchtliche Mengen von Wasserspeichergewebe verlieren. Diese Studie geht der Frage nach, ob Nährstoffe wie Stickstoff, welche von den Ameisen in Form von Ausscheidungen und Abfall in den zu Domatien umgebildetet Pseudobulben von *C. bilamellatum* eingelagert werden, für die Wirtspflanze einen zusätzlichen Vorteil darstellen um den Verlust an Wasserspeicherfähigkeit auszugleichen.

- *Methoden* Die Fähigkeit von *C. bilamellatum* unterschiedliche Stickstoffquellen in ihren zu Domatien umgebildeten Pseudobulben zu nutzen sowie die Aufnahmekinetik verschiedener organischer und anorganischer ^{15}N-markierter Substrate (NH$_4^+$, Harnstoff und L-Glutamin) wurden unter natürlichen

Bedingungen in einem tropischen Feuchtwald in Panama untersucht. Die Pflanzen wurden entweder direkt markiert indem die Substrate in flüssiger Form in die hohlen Pseudobulben injiziert wurden, oder indirekt *in situ* durch Markierung der die Pflanzen bewohnenden Ameisen welche die Substrate in die Pseudobulben der Pflanze eintrugen.

- *Ergebnisse C. bilamellatum* konnte alle getesteten Substrate über die Oberfläche des Hohlraumes im Inneren der Pseudobulben aufnehmen. Die Aufnahme von NH_4^+ und Glutamin folgte einer Michaelis-Menten Kinetik während die Aufnahme von Harnstoff bis zu einer Konzentration von 2 mM linear verlief, die entsprechenden Aufnahmesysteme demnach bei diesen Substratkonzentrationen nicht sättigbar waren. Die ^{15}N-markierten Substrate wurden rasch aufgenommen sowie in der Pflanze verteilt und in reproduktive Organe eingebaut. Durch die Markierungsexperimente mit Ameisen *in situ* konnte nachgewiesen werden dass diese unter natürlichen Bedingungen Stickstoff in die Pflanze transferieren.

- *Conclusio* Diese Studie demonstriert zum ersten mal dass eine myrmecophile Orchidee die Fähigkeit besitzt, aus ihren Domatien aktiv verschiedene Stickstoff-Formen aufzunehmen und liefert zudem den Nachweis dass ein Nährstofffluss von Ameisen zu Pflanzen unter natürlichen Bedingungen stattfindet. Diese Ergebnisse lassen darauf schließen, dass neben dem Schutz vor Herbivoren, auch der aus den Abfällen der Ameisen aufgenommene Stickstoff einen positiven Effekt auf die Fitness dieser Myrmecophyten hat. Diese zusätzliche Nährstoffquelle könnte den Pflanzen helfen, den Verlust von Wasserspeichergewebe, welcher durch die Bildung der Domatien entsteht, zu kompensieren.

6 LITERATURE CITED

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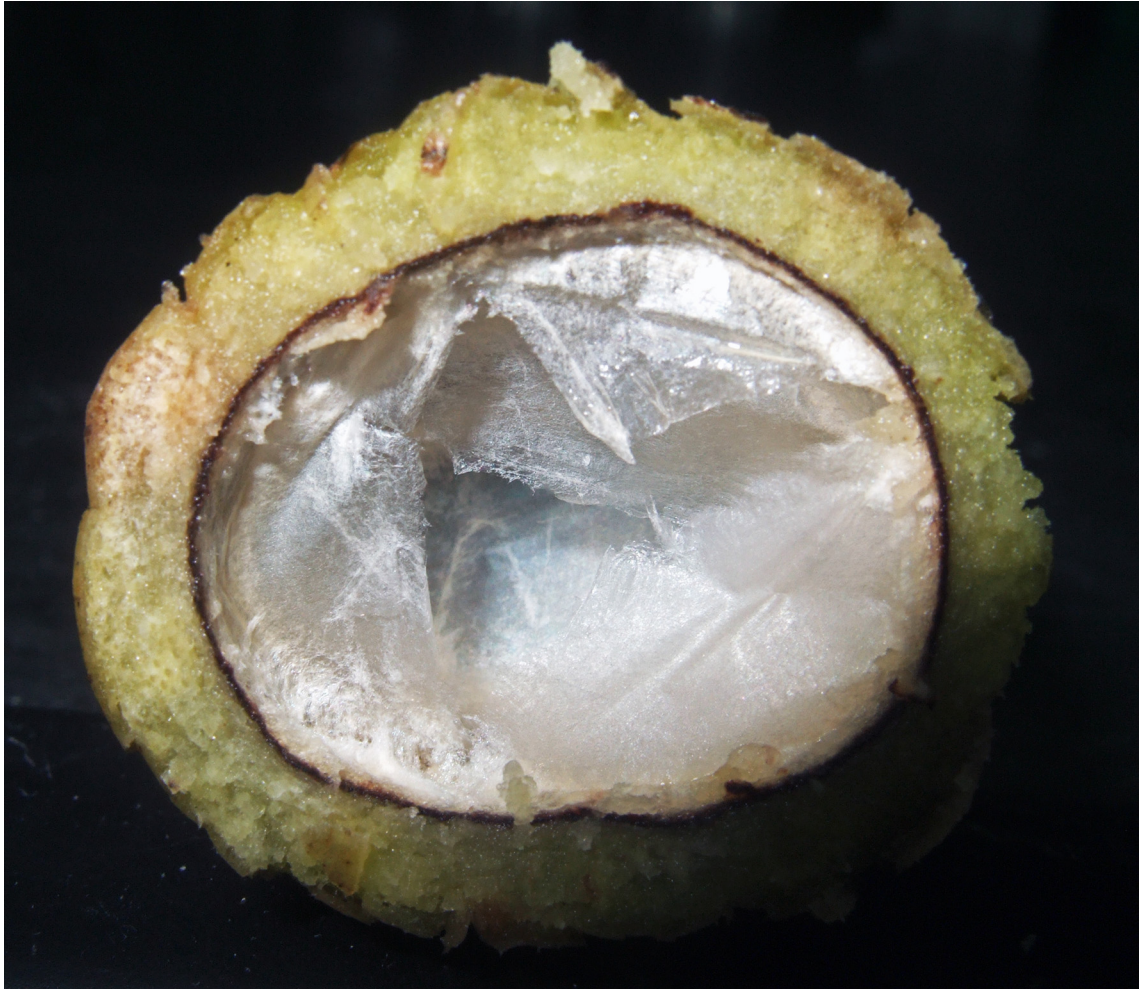
7 APPENDIX

F$_{\mathrm{IG}}$. S1. Cross section of a closed *Caularthron bilamellatum* pseudobulb having failed to form a basal opening. Shown are morphological characteristics of the hollow pseudobulbs of a neotropical myrmecophytic orchid collected at a tropical moist forest, BCI, Panama. Very few pseudobulbs fail to form an opening after maturation. The centre desiccates nonetheless but the resulting cavity remains inaccessible to ants, which normally inhabit the plants. In these rare cases remaining parenchymous tissue partially covers the cavity inside the pseudobulb whereas it is usually removed by inhabiting ants (compare to Fig. 1).
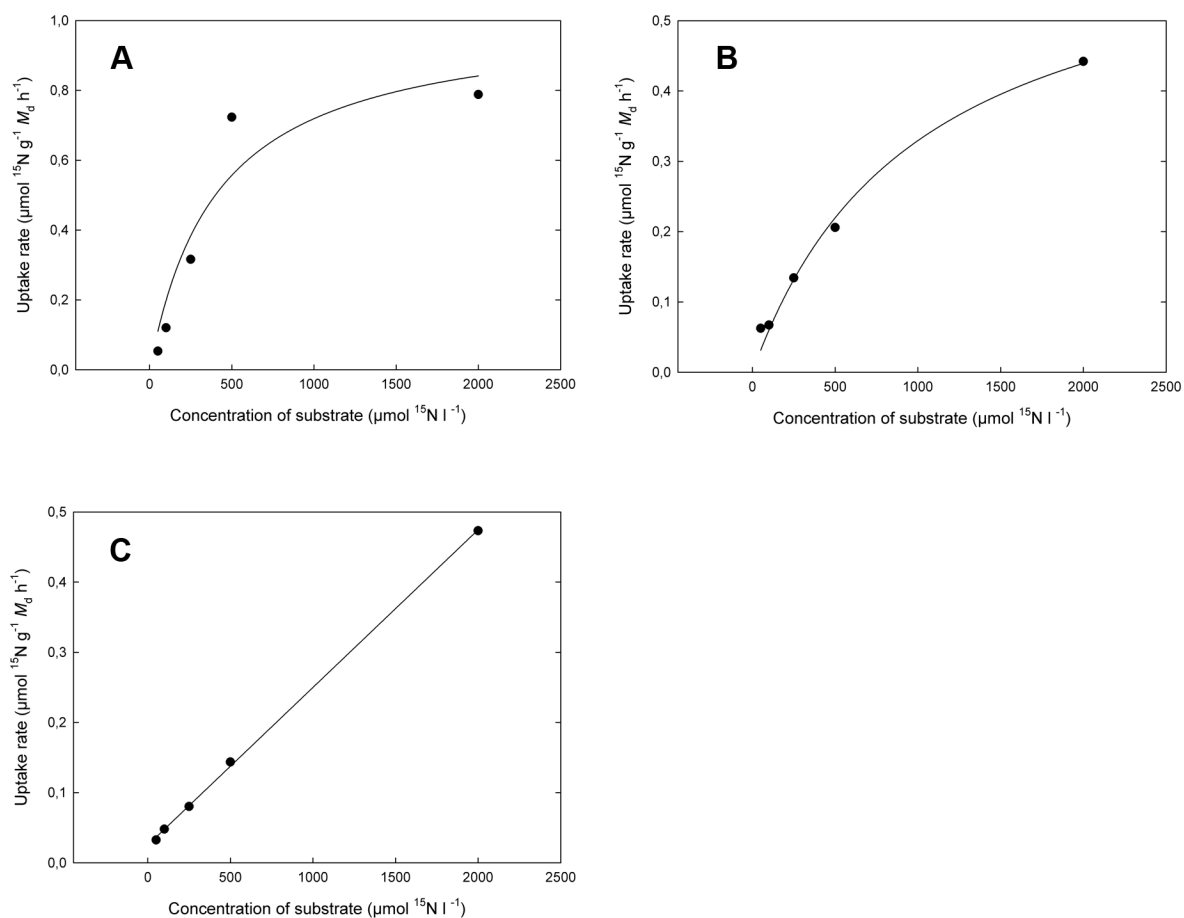
FIG. S2. [15]N uptake kinetics of *Caularthron bilamellatum* pseudobulb inner surface. Shown are uptake rates within the domatia of a neotropical myrmecophytic orchid collected at a tropical moist forest, BCI, Panama. Data is given for (A) NH_4^+, (B) glutamine, and (C) urea. Points indicate the mean of 6 replicates. Nitrogen concentrations of the labelling substrate in μM are given as on the x-axes, uptake rates in μmol [15]N g^{-1} M_d h^{-1} on the y-axes. Note the different scales. Regression lines are representing the fitting of Michaelis-Menten equation to data by hyperbolic regression for (A) and (C), and by linear regression for (B).
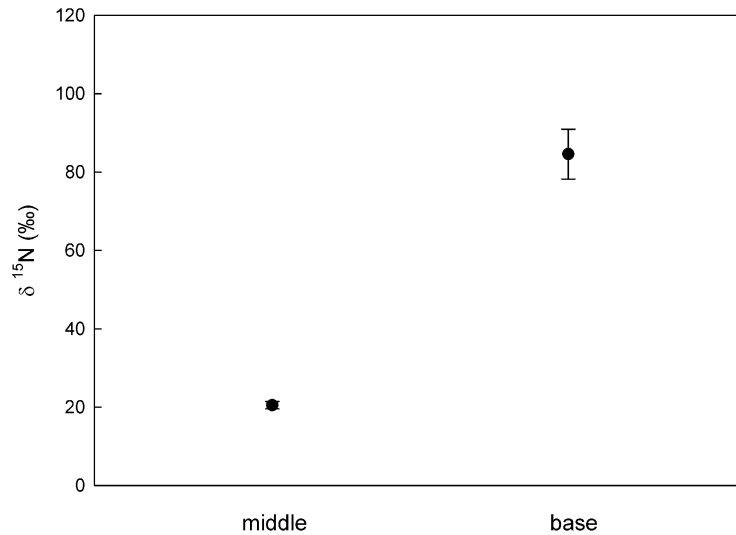
F_{IG}. S3. Spatial variations of ^{15}N uptake between basal and middle regions of immature pseudobulbs, partially filled with parenchyma tissue. Ants inhabiting specimens of the myrmecophytic orchid *C. bilamellatum* growing epiphytically on *Annona glabra* along the shoreline of BCI, Panama were labelled by feeding them a solution of honey containing ^{15}NH$_4$Cl. Ants transported the label into the plants' hollow pseudobulbs used as nesting space. Pseudobulbs were harvested after two weeks. Spatial distribution of label within immature pseudobulbs was significantly different ($P < 0.001$) between basal regions accessible by ants and inaccessible middle regions still filled with parenchymous tissue slowly desiccating from basal to apical regions. δ^{15}N values in middle regions were again significantly different from the unabelled control group ($P < 0.001$) indicating distribution of label within the plant. Error bars represent the standard error ($n = 3$).

TABLE S1. Comparison of nitrogen enrichment in *Caularthron bilamellatum* plants, inhabiting ants, ant larvae and ant carton across different sampling sites. Ants inhabiting specimens of the myrmecophytic orchid *C. bilamellatum* growing epiphytically on *Annona glabra* along the shoreline of BCI, Panama were labelled by feeding them a solution of honey containing $^{15}NH_4Cl$. Ants transported the label into the plants' hollow pseudobulbs used as nesting space. Pseudobulbs were harvested after two weeks. Ants and larvae could not be sampled in sufficient quantities for analysis in all plots.

Plot Nr.	pseudobulbs $\delta^{15}N$ (‰)	ants $\delta^{15}N$ (‰)	larvae $\delta^{15}N$ (‰)	ant carton $\delta^{15}N$ (‰)
1	78,99	---	12,41	33,86
2	163,35	290,11	10,23	27,15
3	69,70	1457,10	---	90,24
4	80,71	471,96	---	214,99
5	49,41	147,57	---	32,30

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