Responses to simulated nitrogen deposition and a stable isotopic assessment for the neotropical epiphytic orchid *Laelia speciosa*

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ABSTRACT

The accelerated increase of nitrogen deposition is the third cause of biodiversity loss, as a result of saturation of ecosystems worldwide. The effects of nitrogen deposition on the endemic and endangered neotropical epiphytic orchid, *Laelia speciosa,* were evaluated via a dose-response experiment and a stable isotopic field assessment for individuals from a city and from an oak forest, in order to evaluate the potential risk facing this orchid, and record the history of the nitrogen deposition of series of consecutive annually produced pseudobulbs. Lower doses of nitrogen of up to 20 kg N ha yr^{-1} the dose that led to optimal performance of plants, acted as fertilizer. For instance, chlorophyll content and chlorophyll fluorescence (Fv/Fm) peaked at 0.66 ± 0.03 g m^{-2} and 0.85 ± 0.01, respectively. In contrast, toxic effects were observed at the higher doses of 40 and 80 kg N ha yr^{-1}, leading a decrease of 38% of the chlorophyll content and 23% of the chlorophyll fluorescence. For the field assessment, a tissue nitrogen content of 1.2 ± 0.1% (dry mass basis) for the orchids suggested non-toxic deposition rates both at the city and the oak forest. However, their respective isotopic signatures revealed different sources of N at each site. Indeed, in the oak forest $\delta^{15}N$ amounted –3.1 ± 0.3‰, typical of places with low industrial activities, while in the city the $\delta^{15}N$ reached 5.6 ± 0.2‰, typical of sites with some degree of industrial and automobile activity. *Laelia speciosa* would be an adequate bioindicator of nitrogen deposition because its ability to take up nitrogen from the atmosphere while preserving its isotopic signature and showing a clear physiological response to increasing inputs of nitrogen. However, its limited geographical distribution precludes the orchid as an ideal candidate for biomonitoring. Thus other vascular epiphytes should be considered for this purpose.

Key words: bioindicators; CAM; conservation physiology; $\delta^{15}N$; nitrogen pollution

1. INTRODUCTION

Atmospheric nitrogen deposition is the third global cause of biodiversity loss (Sala et al., 2000). While nitrogen is an essential nutrient for all living organisms, an accelerated increase of atmospheric deposition of this element has caused saturation of various ecosystems around the world, causing significant biodiversity loss by direct toxicity, acidification, and nutrient imbalances between nitrogen and other major nutrients (Hatteenschwiller and Körner, 1998; Le Bauer and Treseder, 2008; Bobbink et al., 2010; Templer et al., 2012). Indeed, the release of reactive nitrogen species to the atmosphere has doubled over the last 50 year owing to an accelerated increase of industrial and agricultural activities, predominantly from burning fossil fuels, production and application of nitrogenous fertilizers, as well as, nitrogen-fixing plants cultivation for human consumption (Phoenix et al., 2006; Galloway et al., 2008).

Most studies regarding the effects of nitrogen deposition on biodiversity have been conducted in the USA and some European countries (Bobbink et al., 2010). In contrast, studies from megadiverse countries are scant. Considering that these countries tend to have developing economies and accelerated industrialization, it is urgent to determine the effects that current and future nitrogen deposition rates have on their local biodiversities (Austin et al., 2013). However, because instrumenting deposition monitoring networks is often cost prohibitive, the use of naturally occurring bioindicating organisms is a possible alternative for determining the rate of nitrogen deposition over large areas with a moderate cost (Whitton and Kelly, 1995). In addition, atmospheric nitrogen deposition can be traced to its source by means of its isotopic signature. For example, $\delta^{15}N$ values are positive for the combustion of coal in energy plants and vehicular emissions, whereas it becomes negative in less polluted areas (Moore, 1977; Ammann et al., 1999; Pearson et al., 2000). In this respect, bryophytes are effective bioindicating organisms, given that their nitrogen content can be related to the rate of deposition and their isotopic fractionation during nitrogen uptake is minimal (Bragazza et al., 2005; Solga et al., 2005; Wilson et al., 2009). Epiphytic vascular plants are another potentially apt group for bioindication of nitrogen deposition. Unlike bryophytes, many vascular epiphytes are physiologically active year round thus increasing their exposure to atmospheric pollution (Titus and Wagner, 1984; Benzing, 1990, Hietz et al., 2002; Silvera et al., 2009; Zotz and Bader 2009). Moreover, because some species of epiphytic orchids annually produce pseudobulbs, a water and carbon storage organ that persist over time, it is likely that a history of nitrogen deposition can be reconstructed from these longevous organs (Dressler, 1981; Ng and Hew, 2000).

Responses to simulated nitrogen deposition were determined for the neotropical and endangered epiphytic orchid *Laelia speciosa* (Kunth) Schltr (Orchidaceae) by means of a dose-response shadehouse experiment, in order to evaluate the risk that increasing nitrogen deposition poses for this species, as well as, a proof of concept for the potential use of vascular epiphytic plants as biomonitoring organisms. In addition, the natural abundance of stable isotopes of C and N was determined for *Laelia speciosa* growing at two sites with contrasting land use to gain insight of the sources at either site. Finally, it was determined whether this plant can record the nitrogen deposition history by determining the stable isotopic signature for series of consecutive annually produced pseudobulbs.

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2. MATERIALS AND METHODS

2.1. Dose-response experiment

2.1.1. Plant material

Two-year old plants of *Laelia speciosa* obtained by *in vitro* propagation were transferred into 2L pots containing Tezontle, a volcanic rock that is porous and inert, placing in a shade house for 2 years at the Universidad Nacional Autónoma de México, Campus Morelia (19° 38' 55.9" N; 101° 13' 45" W). A total of 120 plants were selected at random and assigned to one of six groups, each with 20 plants that received different doses of nitrogen.

2.1.2. Nitrogen deposition scenarios

Plants were watered weekly during two months with 12.5 ml of a modified Hoagland No. 2 solution from which the nitrogen was omitted (Hoaglan and Arnon, 1950; Nobel and de la Barrera, 2002). A the end of this period, six simulated atmospheric deposition scenarios were applied by adding NH_4NO_3 to the water solution, amounting to the equivalent of 2.5, 10, 20, 40, or 80 kg of N ha^{-1} yr^{-1}. The application of the different doses of nitrogen was carried out over 26 weeks, from December 2011 to June 2012. This period corresponds to the seasons of growth and reproductive development for *L. speciosa* (Halbinger and Soto-Arenas, 1997; Soto-Arenas, 1994; Soto-Arenas and Solano-Gómez, 2007).

2.1.3. Growth and development

The emergence of flowers, which remain for only few weeks, was recorded weekly, while the total production of new leaves and pseudobulbs, which are persistent, was recorded at the end of the experiment.

2.1.4. *Chlorophyll content*

Leaf discs were obtained with a cork borer (12-mm in diameter) from 5 plants per nitrogen deposition scenario to determine the concentration of chlorophyll a, chlorophyll b, and total chlorophyll in the plant tissue. The photosynthetic pigments were extracted by macerating leaf tissue with a chilled (3°C) mortar and pestle in an aqueous solution of acetone (80% v/v) and brought to a final volume of 20 ml. Absorbance was measured at 663 and 646 nm with an EZ 301 spectrophotometer (Perkin Elmer, Waltham, Massachusetts, USA). Chlorophyll concentration was calculated following Lichtenthaler (1987).

2.1.5. *Chlorophyll fluorescence (Fv/Fm)*

The maximum yield of photosystem II (the ratio of variable vs. maximum fluorescence; Fv/Fm) was measured with an Opti-Science 05-30p Fluoreometer (Hudson, New Hampshire, USA) before dawn for the leaves of five individuals per dose of nitrogen on 29 June 2012 and 2 July 2012. At this time, while the dark period is coming to an end, the the photosystems are already active (González-Salvatierra et al., 2013).

2.2. **Field assessment**

2.2.1. *Study sites*

Two contrasting sites in the state of Michoacán, Mexico, were selected for comparing their nitrogen deposition sources using *Laelia speciosa*. The first site was the oak forest located at Cerro el Olvido in the municipality of Tzintzuntzán (19º 37’ 59” N, 101º 29’ 09” W, 2361 m). The dominant species is *Quercus deserticola* Trel. (Fagaceae), this tree is the most frequent phorophyte for *Laelia speciosa* (Soto-Arenas, 1994). At this site, the mean annual precipitation is

758 mm. Dominant winds blow from the southwest and the northeast, and the mean annual temperature is 16.1 ºC (Servicio Meteorológico Nacional, 2011).

At 30 km east from the oak forest, the second site was located at the UNAM Campus shadehouse, located in the periphery of Morelia, a city with approximately 600,000 people and 250,000 cars. The dominant winds blow from the southwest and northwest, the mean annual precipitation is 773 mm and the mean annual temperature is 18.3 ºC (Servicio Meteorológico Nacional, 2011). The plants utilized at this location were rescued in 2004 from an urban development site that was cleared at ca. 7 km west from campus (19º 38' 21" N; 101º 15' 30" W, 1942 m a.s.l.) and were maintained on their original substrate, namely branches of *Quercus deserticola*.

2.2.2. Plant material

To determine the photosynthetic metabolism of *Laelia speciosa* and to determine the sources of nitrogen for each site, tissue samples were collected for measuring the content and isotopic abundances for carbon and nitrogen. In particular, the most recent leaf, pseudobulb, and roots were collected from each one of 4 plants from each site that were at least 10 years old. For these individuals, leaf length ranged from 8 to 12 cm. Plant samples from the oak forest were collected under the Centro de Investigaciones en Ecosistemas institutional plant collection permit (Secretaría de Medio Ambiente y Recursos Naturales, Oficio SGPA/DGGFS/712/2767/14) from individuals growing in the outer canopy, at a minimum height of 5 m and a minimum radial distance of 3 m from the main stem of *Quercus deserticola* individuals, where the canopy cover is sparse. The same number and type of samples were collected from plants from the city, where the rescue individuals, still attached to the original branches of *Quercus deserticola*, where kept on a shadehouse bench. In order to assess whether pseudobulbs of *Laelia speciosa* can record the history of nitrogen deposition over multiple years, 10 consecutive pseudobulbs from 4 oak forest plants and from 4 shade house plants were collected.

2.2.3. Characterization of nitrogen sources

To characterize the nitrogen concentration and the $\delta^{15}N$ that orchids can receive from the atmospheric nitrogen deposition at each site, samples of the moss *Braunia* sp. growing in the oak branch near to the orchids were collected in both sites. Likewise, in order to have samples with no contamination with organic material from the host, and ensure to have the actual nitrogen from the atmosphere, additional samples of the moss growing on granite rock in the oak forest and moss growing on concrete in the city were collected, such mosses were totally exposed to the atmosphere and not received any input of other sources.

2.3. Isotopic analyses

Plant material from both the dose-response experiment and from the field assessment was dried at 80°C in a gravity convection oven until reaching constant weight. The dried materials were ground to a fine powder in a ball mill (Retsch MM300; Retsch, Vienna, Austria), wrapped into tin capsules (Costech Analytical, Inc. Valencia, California, USA), and weighed on a microbalance (0.01 mg, Sartorius, Göttingen, Germany). For each plant sample, the carbon and nitrogen content, as well as their isotopic proportions were determined with an isotope ratio mass spectrometer Carlo Erba EA 1110 (Costech Analytical Inc., Valencia, CA, USA). Isotopic values of $\delta^{13}C$ and $\delta^{15}N$ were expressed relative to Vienna Pee Dee Belemnite (V-PDB) or atmospheric air standards, respectively. All analyses were performed at the Stable Isotope Facility at the University of Wyoming (Laramie, Wyoming, USA). The natural abundances of ^{13}C and ^{15}N were calculated as:

$$\delta^{13}C_{\text{(‰ versus V-PDB)}} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

$$\delta^{15}N_{\text{(‰ versus at-air)}} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

where, R is the ratio of $^{13}C/^{12}C$ for carbon and $^{15}N/^{14}N$ for nitrogen isotope abundance for a given sample (Ehleringer and Osmond, 1989; Evans et al., 1996).

2.4. Statistical analyses

Production of organs was tested by means of the Kruskal-Wallis non-parametric ANOVA, followed by post-hoc Tukey test. Differences in the response to simulated nitrogen deposition among watered plants in the shade house were tested by means of one-way ANOVA followed by the Holm-Sidak post-hoc test for multiple comparisons. Comparisons for nitrogen content, C:N, $\delta^{13}C$, and $\delta^{15}N$ for organs from two sites plants of the field assessment and mosses were analyzed by means of two-way ANOVA (factors were site and plant organ) followed by the Holm-Sidak post-hoc test for multiple comparisons. Finally, the series of pseudobulbs were analyzed by means of nested ANOVA followed by the Holm-Sidak post-hoc test. All analyses were conducted on SigmaPlot 12 (Systat Software Inc. USA).

RESULTS

2.3. Dose-response experiment

2.3.1. Organ production

After 26 weeks of watering the plants with different doses of nitrogen, the development of new organs was greater for those individuals that received 20 kg N ha^{-1} yr^{-1} than under any other dose ($p < 0.05$, from a Kruskal-Wallis non parametric ANOVA; Table 1; Fig 1). In particular, 1.0 ± 0.1 leaves were produced per plant over the course of the experiment under most doses, except for the plants that received 20 kg N ha^{-1} yr^{-1}, which produced 35% more leaves.

Similar was the case for 0.9 ± 0.1 pseudobulbs per plant that increased by 36% under the optimum dose of nitrogen. Finally, 0.3 ± 0.1 flowers per plant developed over the course of the experiment for plants growing under the lower nitrogen doses, increasing to 0.8 ± 0.1 flowers per plant under 20 kg N ha^{-1} yr^{-1}, and decreasing to 0.2 ± 0.1 flowers per plant under the higher doses.

2.3.2. Chlorophyll fluorescence

The quantum efficiency of photosystem II (Fv/Fm) was similar among the orchids that received up to 20 kg N ha^{-1} yr^{-1} amounting to 0.8 (Fig 2), while a significant decrease of 23% was observed for plants irrigated with higher concentrations of nitrogen.

3.1.3. Chlorophyll content

Total chlorophyll content for the leaf tissue of *Laelia speciosa* increased as the nitrogen dose increased, peaking at 0.7 ± 0.0 g m^{-2} for plants irrigated with 20 kg N ha^{-1} yr^{-1} ($p < 0.05$; Fig 3; Table 2), while the higher doses of nitrogen resulted in a 38% reduction of the pigment. Similarly, a chlorophyll-a concentration of 0.5 ± 0.4 g m^{-2} was maximum for plants growing under 20 kg N ha^{-1} yr^{-1}, and it was 30% lower under all other nitrogen doses. In turn, chlorophyll-b did not respond to nitrogen, averaging 0.1 ± 0.0 g m^{-2} regardless of the dose under which plants grew ($p > 0.05$; Fig 3).

2.3.3. Carbon and nitrogen content

The carbon content of *Laelia speciosa* increased with the nitrogen dose peaking at 46.1 ± 0.3% (dry mass basis) at 20 and 40 kg N ha^{-1} yr^{-1} and decreased to 45.2 ± 0.3 % at 80 kg N ha^{-1} yr^{-1} (Table 1; Fig. 4A).

The nitrogen content for *Laelia speciosa* also increased with the nitrogen dose (Table 1; Fig. 4B). For the plants that received up to 10 kg N ha^{-1} yr^{-1} it averaged 1.2 ± 0.0% (dry mass basis), peaking at 2.4 ± 0.0% at 80 kg N ha^{-1} yr^{-1}.

2.3.4. Stable isotopes

The δ^{13}C for leaves of *Laelia speciosa* averaged –14.7 ± 0.2‰ and did not respond to the nitrogen dose (Table 1; data not shown). In contrast, the leaf δ^{15}N significantly decreased at higher nitrogen doses (Fig. 5). In particular, it averaged 0.9 ±0.1‰ for plants that received up to 10 kg N ha^{-1} yr^{-1}, a δ^{15}N similar to the isotopic signature of 1.1 ± 0.1‰ measured for the NH_4NO_3 that was utilized for the nutrient solution. The higher doses of N led to significant decreases of δ^{15}N, with a minimum of – 3.1 ± 0.2 ‰ for plants growing under 80 kg N ha^{-1} yr^{-1}.

3.2. Field assessment

3.2.1. Characterization of nitrogen isotopes

The carbon content of the mosses averaged 37.6 ± 0.9% (dry mass basis) and its nitrogen content averaged 1.7± 0.1% and neither differed between sites (Table 2). Similar was the case for their δ^{13}C, which averaged –26.0 ± 0.6‰. In contrast, δ^{15}N was 3.3 ± 0.1‰ for the mosses from the city and –4.6 ± 0.9‰ for the mosses from the oak forest (Table 2).

3.2.2. Nitrogen content for Laelia speciosa

The nitrogen content for the leaves of *Laelia speciosa* from the city was similar to that of plants from the oak forest and averaged 1.4 ± 0.2% (dry mass basis; Table 2; Fig 6). The nitrogen content of pseudobulbs and the roots was also similar for plants from the city and from the oak forest and respectively averaged 0.6 ± 0.1 and 1.0 ± 0.1%.

3.2.3. *Stable Isotopes*

The δ^{13}C of *Laelia speciosa* averaged –16.8 ± 0.5‰ and no significant differences were found in response to site nor organ (Table 2). In contrast, the δ^{15}N for *Laelia speciosa* responded significantly to the site of origin but not to organ (Table 2; Fig. 7). In particular, δ^{15}N averaged –3.9 ± 0.4‰ for plants from the oak forest and 4.9 ± 0.3‰ for the plants from the city.

3.2.4. *C:N*

The carbon to nitrogen ratio of plant tissue was generally higher for plants from the oak forest than for plants from the city (Table 2). In particular, the leaf ratio was 45.1 ± 2.5 for plants from the oak forest and 21.2% lower for plants from the city. In turn, the pseudobulb ratio was 127.6 ± 16.34 for plants from the oak forest and 62% for plants from the city. However, the root carbon to nitrogen ratio did not differed between sites, averaging 50.0 ± 2.1.

3.2.5. *Historical record of nitrogen deposition*

The δ^{15}N measured for the ten most recent consecutive pseudobulbs of plants from the oak forest remained constant over the most recent decade of growth and averaged –4.4 ± 0.1‰ (Table 3; Fig. 8). In contrast, the δ^{15}N for plants from the city appeared to respond to relocation, increasing from –0.1 ± 1.6‰ for the oldest pseudobulb to an average of 4.7 ± 0.2‰ for the six most recent pseudobulbs.

The C:N ratios of the entire series of pseudobulbs for plants from the oak forest, as well as those of the earlier 8 pseudobulbs for the series from the city were indistinguishable from each other and averaged 132.4 ± 6.5 (Table 3; Fig. 9). It was significantly lower for the two most recent pseudobulbs for the plants from the city.

4. DISCUSSION

4.1. Dose-response experiment

An intermediate nitrogen dose of 20 kg N ha^{-1} yr^{-1} was optimal for *Laelia speciosa*, while higher doses caused a significant reduction in the development of new organs. In this respect, large quantities of nitrogen limit other nutrients, restricting the plant's ability to produce foliar mass (Evans, 1989; Asner et al., 1997; Aber et al., 1998; Sánchez et al., 2000). For *Laelia speciosa* the reduction was also observed in the development of flowers and pseudobulbs.

The total chlorophyll content is proportional to the content of nitrogen in leaves, which typically ranges between 0.4 and 0.5 g m^{-2} (Evans, 1989; Nobel, 1999; Nobel and de la Barrera, 2002). For *Laelia speciosa* this chlorophyll content did increase with the dose of nitrogen, suggesting that this plant was able to assimilate and utilize the supplied nitrogen for the production of photosynthetic pigments. However, but higher doses also resulted in a drastic decrease of the chlorophyll content, as has been documented for other plant species (Baxter et al., 1992; Majerowicz et al., 2000; Lin et al., 2007; Arróniz-Crespo et al., 2008; Ying-Chun et al., 2010). Such a decrease in the chlorophyll content can be explained by the resulting imbalance of the nitrogen to magnesium ratio in the leaf (Nakaji et al., 2001; Wortman et al., 2012). Excessive nitrogen in the cell promotes release of protons (H^+) and accumulation of phenolic compounds and hydrogen peroxide, as a result, the pH can be altered impeding chlorophyll production and loss of Mg^{2+} (Mangosá and Berger, 1997; Sánchez et al., 2000; Britto and Konzucker, 2002). Changes in chlorophyll content for *Laelia speciosa* were accompanied by changes in the efficiency of photosystem II, which can be attributed to oxidative stress in the thylakoids that results in the blockage of electron transport to the oxidation site, as a consequence of low available energy for photosynthesis (Maxwell and Johnson, 2000; Poorter, 2000; Hogewoning and Harbinson, 2007; Lichtenthaler et al., 2007; Baker, 2008; Calatayud et al., 2008; Guidi and Degl'Innocenti, 2008; Massacci et al., 2008).

When nitrogen is added, plants tend to increase their rates of carbon fixation (Brown et al., 1996; Bauer et al., 2004; Le Bauer and Treseder, 2008). However, as the concentrations of nitrogen increase, the carbohydrates that are necessary substrate for carboxylation become limiting and this results in an eventual reduction of CO_2 fixation (Brown et al., 1996; Bauer et al., 2004). This also causes an increase and later reduction in carbon content for plants subjected to increasing doses of nitrogen, as was observed here for *Laelia speciosa.* However, $\delta^{13}C$ of *Laelia speciosa,* which was typical of CAM plants, did not change under the different N doses utilized, contrasting with $\delta^{13}C$ measured for C_3 plants subjected to supplementary nitrogen (Raven and Farquhar, 1990; Magalhaes et al., 1992).

Isotopic discrimination against ^{15}N increases with nitrogen concentration (Evans, 2001), as was observed for *Laelia speciosa*. This is especially true when the N source is NH_4^+ (Ariz et al., 2011). In contrast, the sole addiction of NO_3^- leads to increased $\delta^{15}N$ values (Ariz et al., 2011). Because NO_3NH_4 was used here, the observed decrease in $\delta^{15}N$ suggests that these plants preferentially absorbed NH_4^+ at the higher doses. This has also been observed for various species, such as *Oryza sativa, Pinus sylvestris*, and *Trapa japonica,* species that discriminate between 0.9 and 13‰ when nitrogen is added (Yoneyama et al., 1991; Högberg et al., 1999; Maniruzzaman and Asaeda, 2012). Indeed, supplementary NH_4^+ causes failure in NO_3^--fixing enzymes and is generally toxic to the plant (Cruz et al., 1993; Soares and Pearson, 1997; Majerowicz et al., 2000; Nakaji et al., 2001; Persson and Näsholm, 2002; Liu et al., 2011).

4.2. Field assessment

Industrial activities and fossil fuel burning are sources of nitrogenous pollutants enriched in ^{15}N (Moore, 1977; Ammann et al., 1999; Power and Collins, 2010). As a result, plants that grow near air pollution emitters show enriched isotopic signatures (Ammann et al., 1999; Power and Collins, 2010). In this study the differences found for δ^{15}N of mosses growing in the city and mosses from an oak forest suggest that the atmospheric nitrogen deposition in the urban conditions resulted from industrial activities and vehicular emissions. However, the tissue nitrogen content and C:N ratio for mosses at both sites were not different, suggesting that the atmospheric nitrogen deposition in Morelia was not sufficiently high to affect the moss nitrogen content. This contrasts with very increasing nitrogen reported for mosses growing along a pollution gradient in London, where nitrogen deposition amounts to 36.6 kg ha^{-1} y^{-1}(Power and Collins, 2010).

The δ^{13}C measured for *Laelia speciosa* from the oak forest and the urban site were similar and consistent with Crassulacean acid metabolism (CAM; typical values exceed –22‰; Hietz et al., 1999). However, this type of photoshynthesis is more prevalent among orchids that grow below 1,500m, where air temperature is higher and precipitation is scarcer than at higher elevations. In the present case, *Laelia speciosa* may express CAM owing to the seasonal drought that spans for at least six consecutive months within its distribution range, including at the sites considered in the present work (Hietz et al., 1999; Silvera et al., 2009; Gudiño and de la Barrera 2014).

Nitrogen content in plant tissue usually amounts to 1.5% of the dry weight, approaching 2.5% in some monocots (Chapman, 1965; Mills and Jones, 1996 Epstein, 1972; 1999). Here, the leaf nitrogen for *Laelia speciosa* was 1.2% of dry weight, similar to the content found for leaves of several species of epiphytic orchids from environments with low nitrogen deposition, indicating that the two sites considered had low atmospheric nitrogen deposition rates (Hietz et al., 1999; Cardelús and Mack, 2010; Mardegan *et al.*, 2011).

The lowest tissue nitrogen content was found for the pseudobulbs of plants from the oak forest, because these organs preferentially store starch (Davidson, 1960; Hew and Ng, 1996; Ng and Hew, 2000). However, in places with some degree of nitrogen deposition as was the city of Morelia, the pseudobulbs were able to accumulate enough nitrogen to lower the C:N ratio. When compared with the mosses, the pseudobulbs of *L. speciosa* were less sensitive to small changes in atmospheric nitrogen deposition due to the accumulation of nitrogen via translocation from older to newly formed pseudobulbs.

The distinct δ^{15}N signatures found for *L. speciosa* were indicative of the nature of the nitrogen available at each site considered in the present study, as discussed above for the mosses. Plants from the city took up nitrogen from industrial and vehicular emissions, while the plants from the oak forest received nitrogen with δ^{15}N typical from less polluted environments. The very similar δ^{15}N signatures found at a given site for the different organs of orchids and for the mosses suggest that isotopic fractionation was very low or absent from the orchid. In addition, the tissue nitrogen content found for plants growing in Morelia suggests that the deposition rates in this city were not high enough to induce the fractionation observed under higher doses in the shadehouse experiment.

Differences between the rates of nitrogen deposition at the two sites considered were too small to be detected from elemental analysis of the bioindicating organisms considered in this study. However, the orchid pseudobulbs, which persist for several years, were a good model for detecting historical changes in the rates of nitrogen deposition or at least for characterizing the nature of this nutrient. Indeed, the nitrogen isotopic signature became increasingly enriched with ^{15}N for the most recently formed pseudobulbs of plants that had been transplanted to the city. However, the isotopic signatures of the earlier pseudobulbs that developed after transplanting the plants to the city had intermediate values, which indicates a buffering by translocation of "clean" nitrogen from the older pseudobulbs that had been formed in the field until its dilution for the most recent pseudobulbs (Bazzaz, 1997).

While the rates of nitrogen deposition in Morelia appeared to be similar to those from an oak forest located at 30 km, the persistence of the endemic and endangered orchid *Laelia speciosa*, which takes up nitrogen from atmospheric deposition, can be further threatened if the air pollution released from industrial and vehicular activities increases to toxic levels. The exact threshhold between 20 and 40 kg ha^{-1} year^{-1} needs to be determined. Isotopic analyses for this orchid indicated that it utilizes CAM and distinguished the nitrogen deposited in the oak forest from that of the city. Elemental analyses for series of pseudobulbs showed that the deposition rates in the city were low, but slightly higher than in the oak forest as the nitrogen content increased when the orchids were transplanted. In addition, isotopic analyses of series of pseudobulbsof *L. speciosa* enables tracking historical changes in nitrogen deposition. Future studies should consider the effect of the nitrogen deposition originated from industrial and from agricultural zones on wild populations of different species of orchids. Additionally, the use of other epiphytic plants that are more widely distributed should be considered for the biomonitoring of nitrogen deposition in neotropical ecosystems.

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Table 1. Kruskal-Wallis one-way ANOVA for the response of *Laelia speciosa* individuals growing in a shadehouse under various rates of simulated nitrogen deposition.

	Response to nitrogen dose		
	d.f.	*F*	*p*
Leaves	5	8.47	0.001
Pseudobulbs	5	7.04	0.001
Flowers	5	1.94	0.077
Total chlorophyll	5	15.68	0.001
Chla	5	6.67	0.001
Chlb	5	10.47	0.001
Fv/Fm	5	82.5	0.001
Carbon content	5	6.44	0.001
Nitrogen content	5	177.5	0.001
$\delta^{15}N$	5	15.68	0.001
$\delta^{13}C$	5	2.65	0.001

Table 2. Two-way ANOVA for individuals of *L. speciosa* growing in two contrasting environments.

		Nitrogen content		Carbon content		$\delta^{13}C$		$\delta^{15}N$	
	d.f.	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Organ	2	20.6	0.06	5.75	0.01	0.52	0.59	4.59	0.02
Site	1	4.07	0.001	6.40	0.02	3.13	0.09	828	0.001
Organ × Site	2	0.05	0.43	0.10	0.90	0.97	0.39	3.74	0.04

Table 3. Nested ANOVA for series of ten consecutive pseudobulbs of *L. speciosa* from two sites with contrasting land use in Michoacán, Mexico.

		C:N ratio		δ^{15}N	
	d.f.	*F*	*p*	*F*	*p*
Year	1	7.30	0.001	4.13	0.045
Year × site	1	0.69	0.405	99.8	$1.49\text{x}10^{-5}$

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FIGURE LEGENDS

Figure 1. Number of new leaves (open bars), pseudobulbs (right hatched bars) and flowers (left hatched bars) that developed on plants of *Laelia speciosa* that were watered with different doses of nitrogen. Data are shown as mean ± S.E (n = 20 plants per dose of nitrogen). Different letters indicate significant differences ($p < 0.05$) for organs.

Figure 2. Predawn chlorophyll fluorescence (ratio of variable to maximum fluorescence) for *L. speciosa* individuals that were watered with different doses of nitrogen. Data are shown as mean ± S.E. (n = 5 plants per dose of nitrogen). Different letters indicate significant differences ($p < 0.05$).

Figure 3. Tissue content (area basis) for total chlorophyll (circles), chlorophyll-a (triangle), and chlorophyll-b (Square) for leaves of *Laelia speciosa* individuals that were watered with different doses of nitrogen. Data are shown as mean ± S.E. (n = 5 plants per dose of nitrogen). Different letters indicate significant differences ($p < 0.05$).

Figure 4. Carbon (A) and nitrogen (B) content (dry mass basis) for leaves of *Laelia speciosa* individuals that were watered with different doses of nitrogen. Data are shown as mean ± S.E. (n = 5 plants per dose of nitrogen). Different letters indicate significant differences ($p < 0.05$).

Figure 5. δ^{15}N (A) and fractionation (B) for leaves of *Laelia speciosa* individuals that were watered with different doses of nitrogen. Data are shown as mean ± S.E. (n = 5 plants per dose of nitrogen). The dashed line indicates δ^{15}N of the amonium nitrate solution utilized for watering

the plants. Data are shown as mean ± S.E. (n = 5 plants per dose). Different letters indicate significant differences ($p < 0.05$).

Figure 6. Nitrogen content for the organs of *Laelia speciosa* from two sites in Michoacán, Mexico. Data are shown as mean ± S.E. (n = 4 individuals per site). Different letters indicate significant differences ($p < 0.05$).

Figure 7. δ^{15}N for organs of *Laelia speciosa* individuals from two sites in Michoacán, Mexico. Data are shown as mean ± S.E. (n = 4 individuals per site). Different letters indicate significant differences ($p < 0.05$).

Figure 8. δ^{15}N for the 10 most recent consecutive pseudobulbs of *Laelia speciosa* individuals from two sites in Michoacán, Mexico. Data are shown as mean ± S.E. (n = 4 individuals per site). The vertical dashed line indicates the time when the city plants were transplanted.

Figure 9. Carbon to nitrogen ratio for the 10 most recent consecutive pseudobulbs of *Laelia speciosa* individuals from two sites in Michoacán, Mexico. Data are shown as mean ± S.E. (n = 4 individuals per site). The vertical dashed line indicates the city plants were transplanted.

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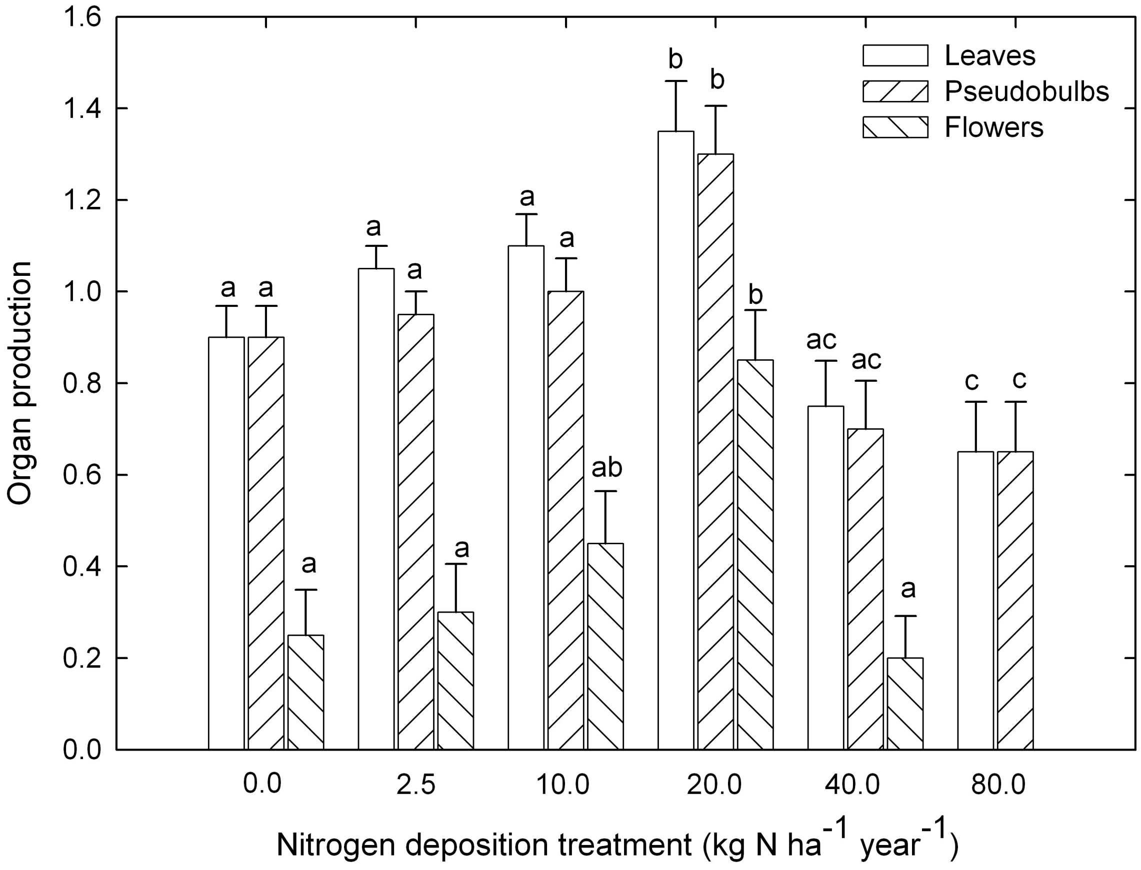
Figure 1



1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Organ production
Leaves
Pseudobulbs
Flowers
a
a
a
a
a
a
a
a
ab
b
b
b
ac
ac
a
c
c
0.0
2.5
10.0
20.0
40.0
80.0
Nitrogen deposition treatment (kg N ha^{-1} year^{-1})

Figure 2

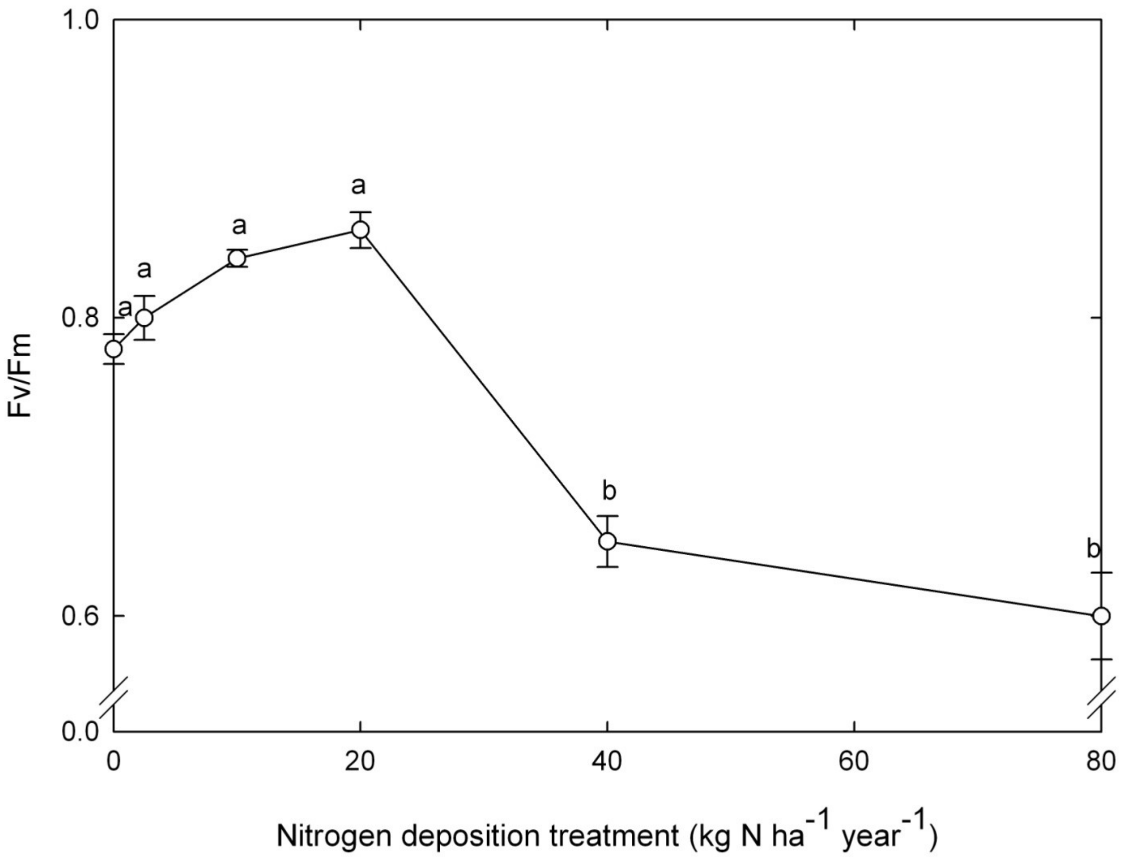
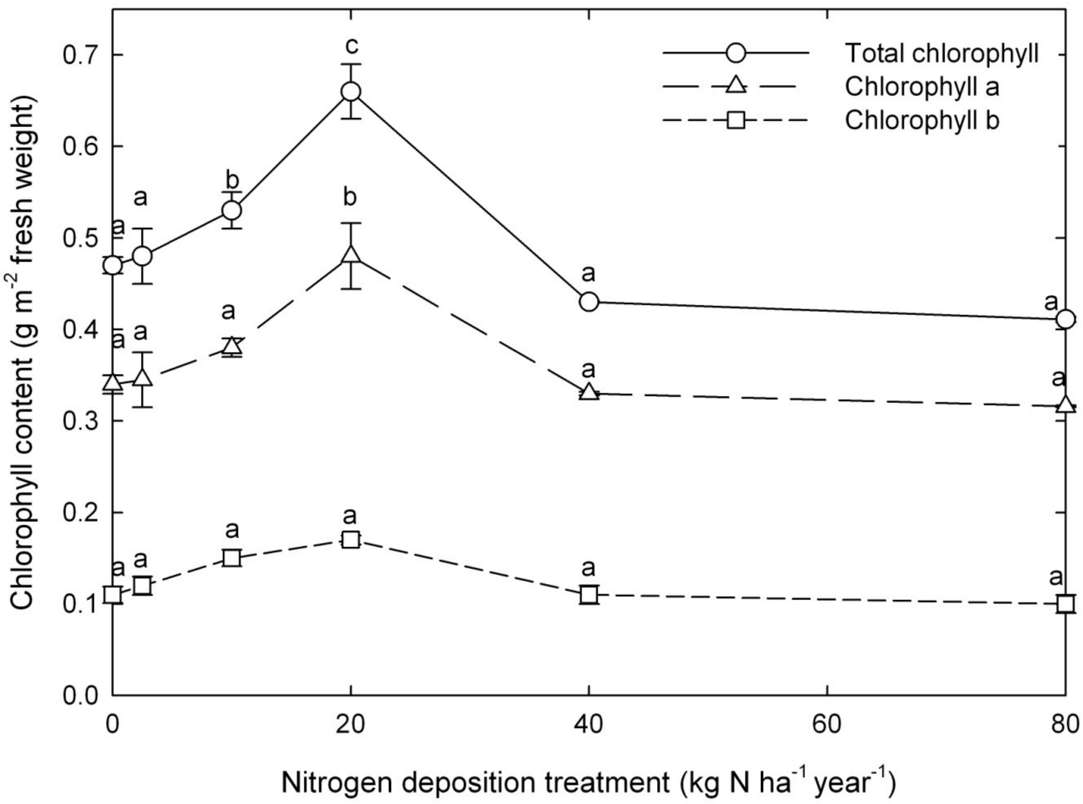
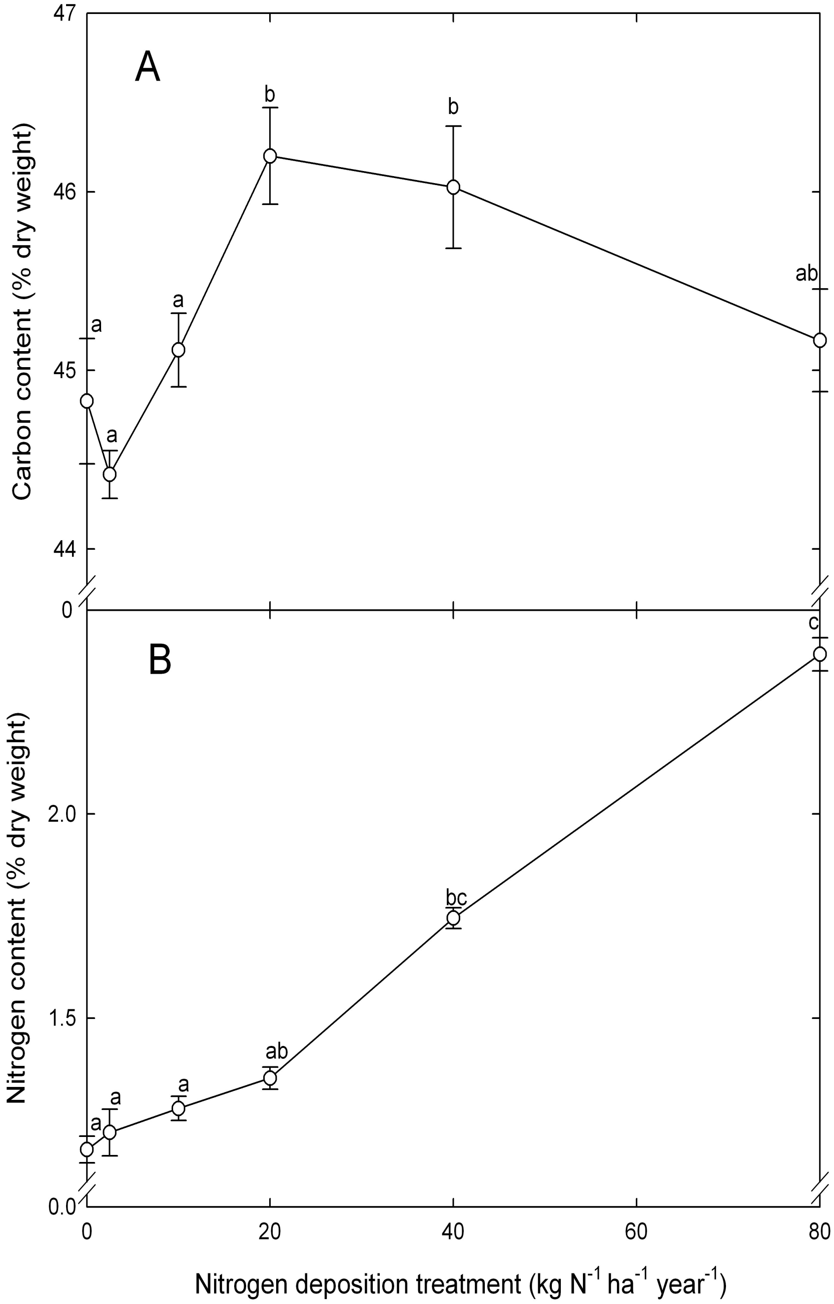

1.0
0.8
0.6
0.0
Fv/Fm
a
a
a
a
b
b
0
20
40
60
80
Nitrogen deposition treatment (kg N ha^{-1} year^{-1})

Figure 3


Total chlorophyll
Chlorophyll a
Chlorophyll b
Chlorophyll content (g m^{-2} fresh weight)
Nitrogen deposition treatment (kg N ha^{-1} year^{-1})
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
20
40
60
80
a
a
b
c
a
a
a
a
a
b
a
a
a
a
a
a
a
a

697

698 Figure 4



700

Figure 5

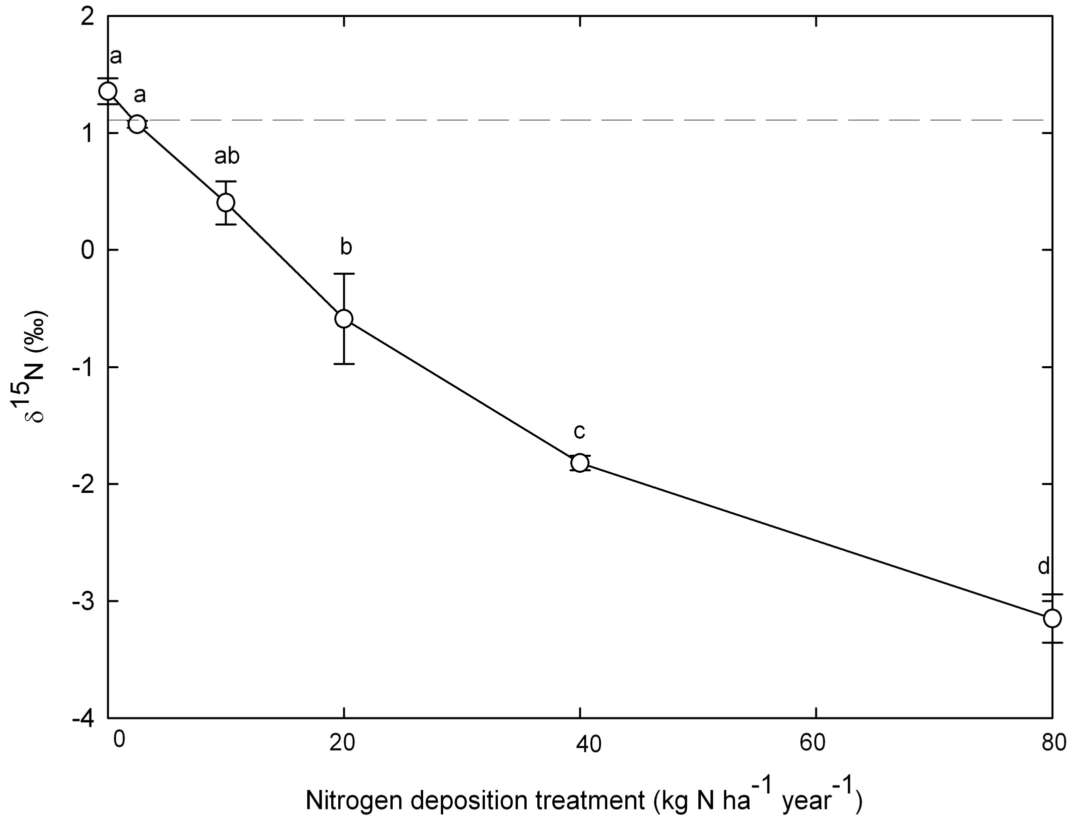
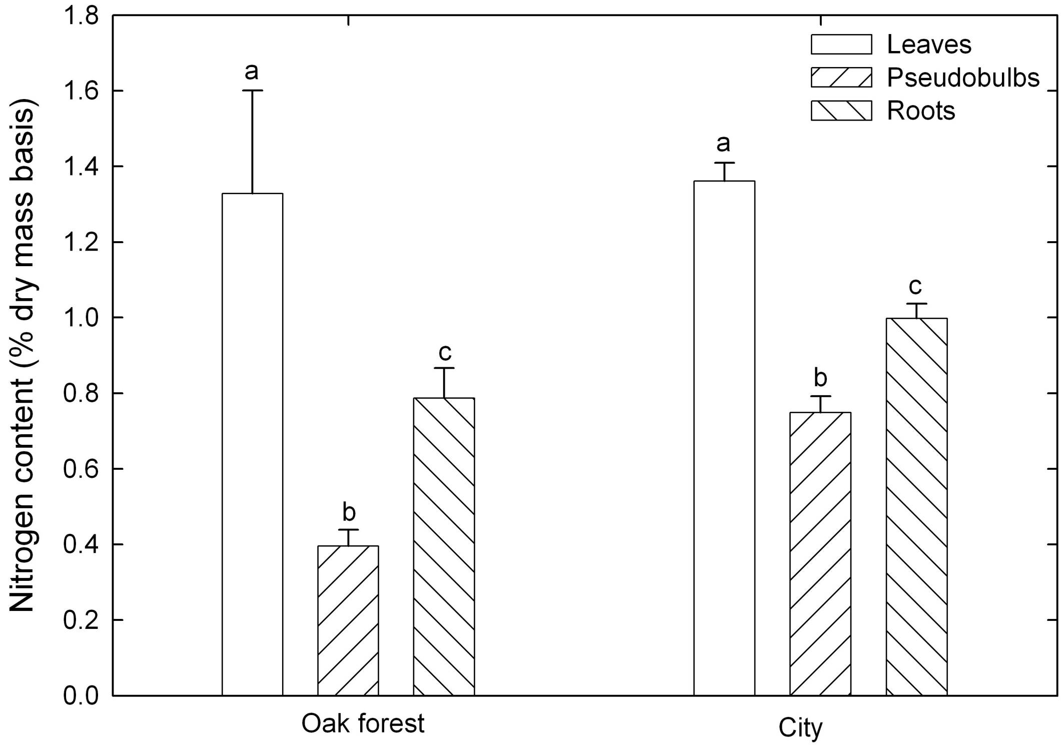

2
1
0
-1
-2
-3
-4
δ15N (‰)
a
a
ab
b
c
d
0
20
40
60
80
Nitrogen deposition treatment (kg N ha-1 year-1)

Figure 6



1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Nitrogen content (% dry mass basis)
Leaves
Pseudobulbs
Roots
a
b
c
a
b
c
Oak forest
City

Figure 7

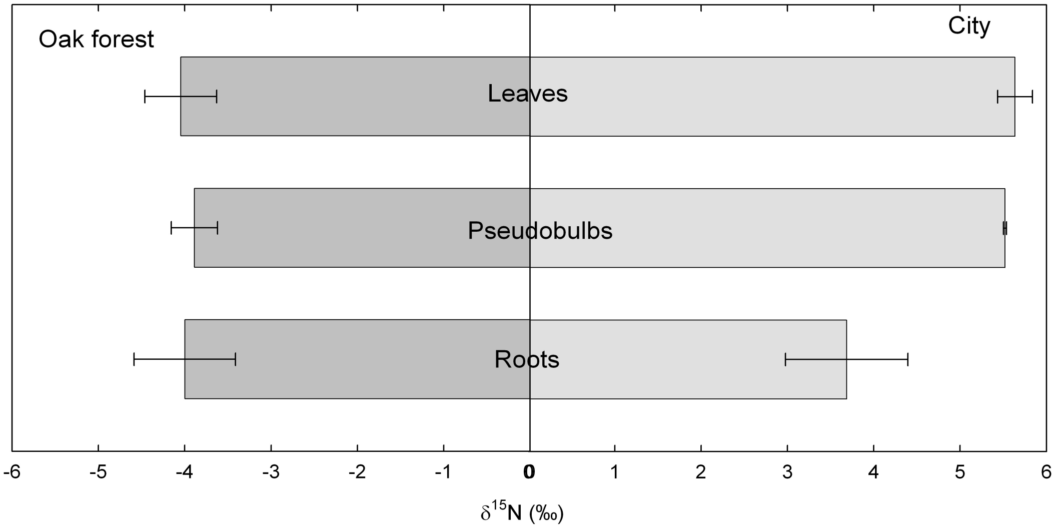

Oak forest
City
Leaves
Pseudobulbs
Roots
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
δ^{15}N (‰)

Figure 8

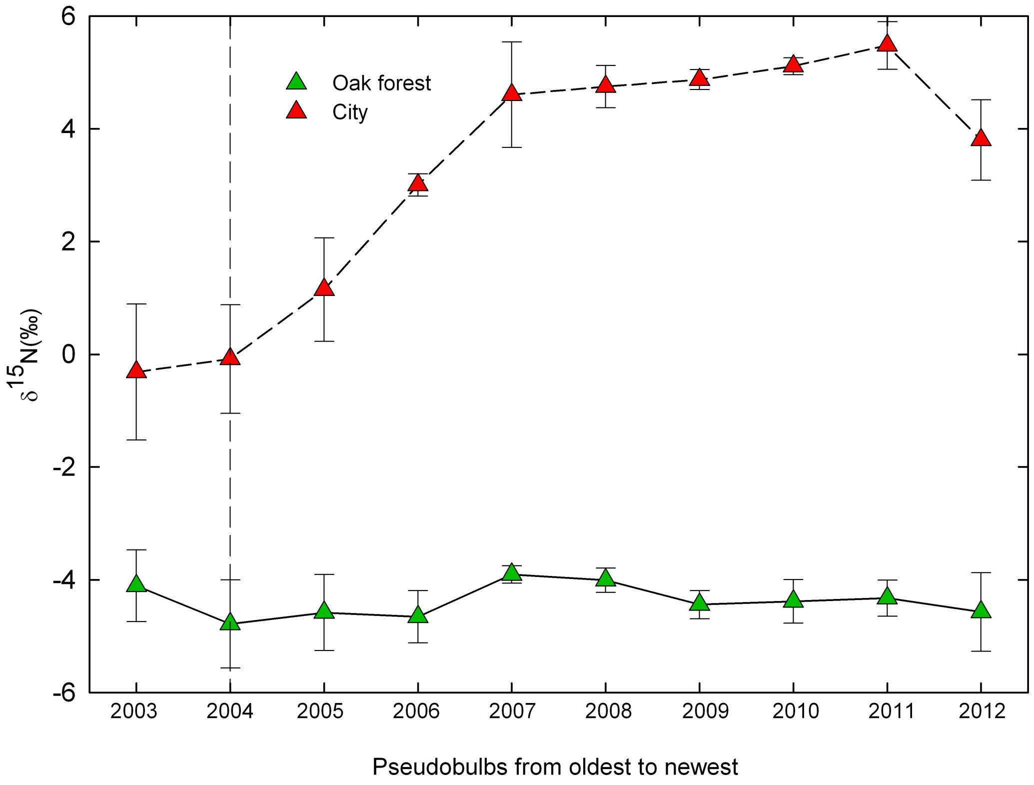
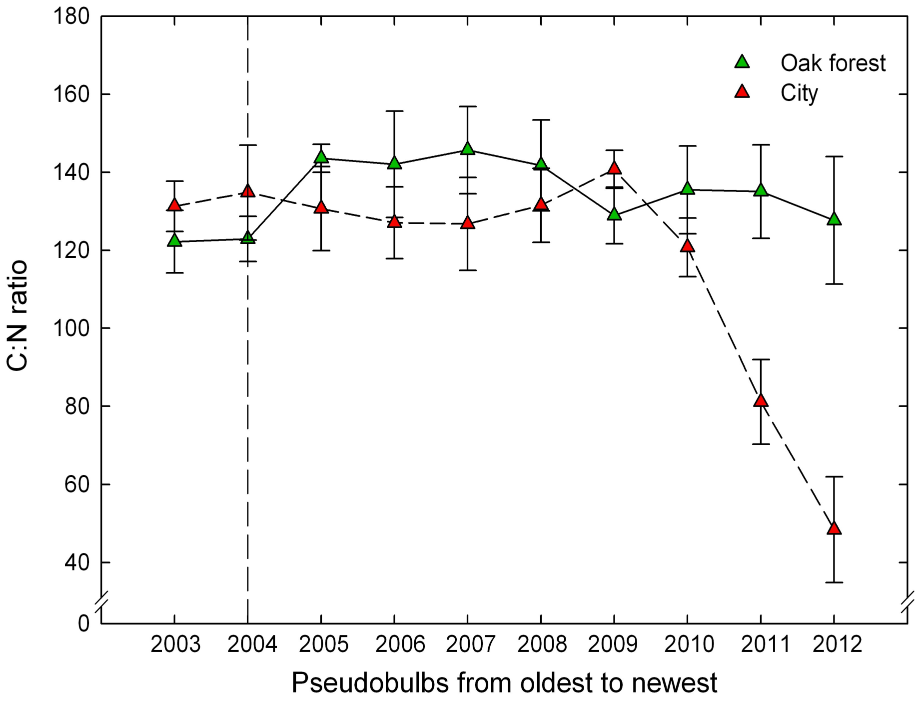

6
4
2
0
-2
-4
-6
$\delta^{15}N$(‰)
Oak forest
City
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
Pseudobulbs from oldest to newest

Figure 9


180
160
140
120
100
80
60
40
0
C:N ratio
Oak forest
City
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
Pseudobulbs from oldest to newest