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- 2 Corresponding author: Erick de la Barrera
- 3 mailing address: Antigua carretera a Pátzcuaro 8701. Col. Ex-Hacienda de San José de la Huerta, Morelia,
- 4 Michoacán, México 58190
- 5 Phone number: +52 (443) 3223810
- 6 e-mail address: delabarrera@unam.mx
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17 **A  $\delta^{15}\text{N}$  assessment of nitrogen deposition for the endangered epiphytic orchid *Laelia speciosa***  
18 **from a city and an oak forest in Mexico**

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21 Edison A. Díaz-Álvarez<sup>a,b</sup>, Casandra Reyes-García<sup>c</sup>, and Erick de la Barrera<sup>b\*</sup>

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23 <sup>a</sup>Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, México,  
24 Distrito Federal 04510, México

25  
26 <sup>b</sup>Instituto de Investigaciones en Ecosistemas y Sustentabilidad, Universidad Nacional Autónoma de México,  
27 Morelia, Michoacán 58190, México.

28  
29 <sup>c</sup>Centro de Investigación Científica de Yucatán, Mérida, Yucatán 97200, México.

30  
31 \*Correspondence: Erick de la Barrera; e-mail: delabarrera@unam.mx.

42 **ABSTRACT**

43 Atmospheric nitrogen deposition poses a major threat to global biodiversity. Tropical epiphytic plants are especially  
44 at risk given their reliance on atmospheric sources of nutrients. The leaf, pseudobulb, and root carbon and nitrogen  
45 content, C:N ratio, as well as the nitrogen isotopic composition were studied for individuals of *Laelia speciosa* from  
46 a city and from an oak forest in Mexico. The nitrogen content of leaves was similar between the city and the oak  
47 forest, reaching  $1.3 \pm 0.2\%$  (dry mass). The  $\delta^{15}\text{N}$  of leaves, pseudobulbs, and roots reached  $5.6 \pm 0.2\text{‰}$  in the city,  
48 values found in sites exposed to industrial and vehicular activities. The  $\delta^{15}\text{N}$  for plant from the oak forest amounted  
49 to  $-3.1 \pm 0.3\text{‰}$ , which is similar to values measured from sites with low industrial activities. Some orchids such as  
50 *Laelia speciosa* produce a single pseudobulb per year, i.e., a water and nutrient storage organ, so the interannual  
51 nitrogen deposition was studied by considering the ten most recent pseudobulbs for plants from either site formed  
52 between 2003 and 2012. The C:N ratio of the of ten most recent pseudobulbs from the oak forest, as well as that of  
53 the pseudobulbs formed before 2010 for plants in the city were indistinguishable from each other, averaging  $132.4 \pm$   
54  $6.5$ , while it was lower for the two most recent pseudobulbs in the city. The  $\delta^{15}\text{N}$  values of pseudobulbs from the oak  
55 forest averaged  $-4.4 \pm 0.1\text{‰}$  for the entire series. The  $\delta^{15}\text{N}$  ranged from  $0.1 \pm 1.6\text{‰}$  for the oldest pseudobulb to  $4.7$   
56  $\pm 0.2\text{‰}$  for the pseudobulb formed in 2008 onwards. Isotopic analysis and the C:N ratio for *L. speciosa* revealed that  
57 rates of nitrogen deposition were higher in the city than in the forest. The  $\delta^{15}\text{N}$  values of series of pseudobulbs  
58 showed that it is possible to track nitrogen deposition over multiple years.

59

60 **Key words:** Atmospheric pollution; Conservation Physiology; global change; industrial activities; Neo-Volcanic  
61 Axis.

## 62 **Introduction**

63 Atmospheric nitrogen deposition has more than doubled since the mid-20th century as a result of an increased  
64 release of reactive nitrogen species from industrial and agricultural origin (Phoenix et al. 2006; Galloway et al.  
65 2008). This important component of global change has driven a loss of biodiversity, which has been widely  
66 documented in Europe, the USA, and China (Xue-Yan et al. 2008; Bobbink et al. 2010). However, studies are scant  
67 for megadiverse countries like Mexico, which tend to have developing economies and ongoing industrialization  
68 processes, making it urgent to determine the effects of nitrogen deposition on their biodiversities (Austin et al.  
69 2013). In this respect, an interest on tracing sources of deposition has increased and it can be studied by means of the  
70 isotopic composition of the vegetation.

71 Atmospheric nitrogen deposition confers a distinctive isotopic signature to vegetation. The isotopic  
72 composition of the reactive nitrogen species (NO<sub>x</sub> and NH<sub>x</sub>) that result from the atmospheric N<sub>2</sub> rupture can be  
73 either positive or negative (Heaton 1990). In particular, the NO<sub>x</sub> from electric plants, coal, gasoline, diesel, and trash  
74 burning has δ<sup>15</sup>N between +3.7 and +26‰ (Widory 2007). However, these isotopic values can also be negative,  
75 ranging from -13 to -2‰ (Redling et al. 2013). Whether the values are negative or positive will depend on the type  
76 of material burnt, the nitrogen in the fuel, the various isotopic fractionations associated with the thermal production  
77 of NO<sub>x</sub>, when the fuel goes through the engine and is mixed with air O<sub>2</sub> and N<sub>2</sub> during the combustion (Liu et al  
78 2012; Felix et al. 2013; Felix and Elliot 2014). The δ<sup>15</sup>N of biogenic emissions (NH<sub>x</sub>) of soils in rural areas are  
79 negative and can fluctuate between -50 and -20‰, including, organic wastes of animals and volatilization of  
80 nitrogen from agriculture (Hoering 1957; Li and Wang 2008; Felix et al. 2012). Once emitted the NO<sub>x</sub> or the NH<sub>x</sub>  
81 react with various compounds in the atmosphere, such reactions involve isotopic fractionations, that result in  
82 compounds such as NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>, with different δ<sup>15</sup>N values in rain ranging from -15 to +15‰, the NH<sub>4</sub><sup>+</sup> is more  
83 negative than the NO<sub>3</sub><sup>-</sup> (Heaton 1990; Xiao and Liu 2002; Heaton et al. 2004; Elliot et al. 2009; Xiao et al. 2012).  
84 These isotopic values directly affect the <sup>15</sup>N composition of plants of different sites. In particular, plants exposed to  
85 industrial and vehicular emissions tend to be <sup>15</sup>N-enriched. For example, the δ<sup>15</sup>N of mosses growing close to busy  
86 urban roads range between -1 and 6‰, while plants from less polluted areas tend to be <sup>15</sup>N depleted as is the case  
87 for mosses in rural areas, whose δ<sup>15</sup>N values range from -2 to -12‰ (Ammann et al. 1999; Pearson et al., 2000;  
88 Stewart et al., 2002; Liu et al. 2007; Power and Collins 2010). However, in cities where the industrial activities

89 release NH<sub>x</sub>, the δ<sup>15</sup>N of plants are more negative than in the field (Stewart et al. 2002; Liu et al. 2008; Xiao et al.  
90 2010).

91 A lifeform of particular interest for studying the effects of deposition is that of epiphytes, which account for  
92 up to 50% of vascular plant species in some tropical ecosystems and are particularly susceptible to atmospheric  
93 nitrogen deposition, given their reliance on atmospheric sources of nutrients (Mondragón et al. 2015). These plants,  
94 whose roots have no contact with the forest soil, where the nitrogen fractionation takes place, reflect the δ<sup>15</sup>N of the  
95 prevalent atmospheric nitrogen deposition (Delwiche and Steyn 1970; Hietz et al. 1999; Stewart et al. 2002; Zotz et  
96 al. 2010).

97 *Laelia speciosa* (Kunth) Schltr (Orchidaceae) is an endangered endemic epiphytic orchid from central  
98 Mexico with significant cultural importance (Halbinger and Soto-Arenas 1997). Vast numbers of individuals are  
99 illegally extracted and sold in streets and markets during May and June owing to their colorful flowers. This has  
100 caused a significant reduction of natural populations that has led to its special protection status by the environmental  
101 authority (Soto-Arenas and Solano-Gómez 2007; NOM-059-SEMARNAT-2010; Mondragón et al. 2015).  
102 Moreover, oak forests, the natural habitat for this orchid, are highly threatened by changes in land use and climate  
103 (Villers-Ruiz and Trejo-Vázquez 2000; Soto-Arenas and Solano-Gómez 2007; Rehfeldt et al. 2012; Gudiño et al.  
104 2015).

105 Under increasing rates of nitrogen deposition of up to 20 kg N ha<sup>-1</sup> y<sup>-1</sup>, the carbon and nitrogen content  
106 increase and the C:N ratio decreases in epiphytes responding to increased photosynthetic rates (Díaz-Álvarez et al.  
107 2015). But higher doses of nitrogen can produce noxious physiological effects on epiphytes, given their direct  
108 exposure to the atmosphere (Hietz et al. 1999; Zotz et al. 2010). For instance, higher nitrogen causes the reduction of  
109 chlorophyll content and chlorophyll fluorescence, as well as the reduction of leaf and pseudobulb production for  
110 *Laelia speciosa*, for which increased tissue nitrogen content is concurrent with nitrogen isotopic fractionation (Díaz-  
111 Álvarez et al. 2015). A plant's ability to accumulate foliar mass is limited by large quantities of nitrogen, because  
112 the availability of other nutrients is limited. For instance, excessive availability of nitrogen results in an imbalance of  
113 the nitrogen to magnesium ratio in the leaf because the cell releases protons (H<sup>+</sup>), which lowers the pH and inhibits  
114 chlorophyll production causing the loss of Mg<sup>2+</sup> (Mangosá and Berger, 1997; Sánchez et al. 2000; Nakaji et al.  
115 2001; Britto and Kronzucker, 2002; Wortman et al. 2012; Díaz-Álvarez et al. 2015). It is thus important to  
116 characterize the nitrogen deposition in the natural habitat of this species.

117 Environmental changes, such as variation in air temperature and available water, have been studied over  
118 multiple years by determining the natural abundance of some stable isotopes in the vegetation. For instance, H, C,  
119 and O isotopes of cactus thorns and tree rings indicate the occurrence of droughts in the Sonoran Desert and  
120 temperature and precipitation variations in Europe (West et al. 2006; English et al. 2007; 2010; Cufar et al. 2014).  
121 Also, changes in nitrogen deposition have been determined from  $\delta^{15}\text{N}$  of tree rings in the tropics (Hietz et al. 2011;  
122 Van Der Sleen et al. 2015). In this respect, some orchids, including *L. speciosa*, produce one pseudobulb per year,  
123 which remains attached to the plant for several years (Soto-Arenas and Solano-Gómez 2007). Thus, these storage  
124 organs of water, C, and other nutrients could be useful to reconstruct the interannual variation of nitrogen deposition  
125 over the plant's lifespan (Dressler 1981; Ng and Hew 2000; Soto-Arenas and Solano-Gómez 2007).

126 Leaf, pseudobulb, and root C and N content, C:N ratio, as well as the natural abundance of  $^{15}\text{N}$  were  
127 determined for individuals of *L. speciosa* growing in a city and growing in an oak forest in order to compare the  
128 signal that nitrogen deposition had on the tissue composition of this orchid at each site. In addition, series of  
129 annually produced pseudobulbs were analyzed to determine whether this orchid can record the historical changes on  
130 nitrogen deposition at either site.

131

## 132 **Materials and methods**

### 133 **Study sites**

134 The effect that atmospheric deposition had on tissue nitrogen composition was evaluated for the orchid *Laelia*  
135 *speciosa* from two contrasting sites in central Mexico. The first site was the Instituto de Investigaciones en  
136 Ecosistemas y Sustentabilidad, Universidad Nacional Autónoma de México (19° 38' 55.9" N; 101° 13' 45" W),  
137 located in the city of Morelia whose population is 800,000 inhabitants. Here, the mean annual temperature and annual  
138 precipitation are 18.3 °C and 773 mm, respectively, and dominant winds blow from the southwest and northwest  
139 (Servicio Meteorológico Nacional 2012; Instituto Nacional de Estadística y Geografía 2013). Orchids that had been  
140 rescued from a road construction site in 2004 were kept on the original oak branches of *Quercus deserticola* Trel.  
141 (Fagaceae) from which they were collected and placed inside a shadehouse. This shadehouse consists of a metallic  
142 structure surrounded with a shade-cloth (85% transmittance), including the top and sides, which allows the free  
143 movement of water and gases, while simulating a canopy leading to a temperature difference of up to 4 °C below  
144 ambient at midday.

145 The second site was one of the last natural refuges of *Laelia speciosa* in Mexico, an oak forest that is  
146 located at the Cerro el Olvido, Tzintzuntzán, Michoacán (19° 37' 59" N, 101° 29' 09" W, 2361 m; García-Cruz et al.  
147 2003). The dominant species at this site is *Quercus deserticola* which is the most common phorophyte for *L.*  
148 *speciosa* (Soto-Arenas 1994). Oak trees in this forest reach 10 m in height and grow in rocky, clay or clay-loam soils  
149 (unpublished observations). The mean annual temperature at Cerro el Olvido is 16.1 °C and the annual precipitation  
150 is 758 mm, with a rainy season from May to September, and the dominant winds blow from the southwest and the  
151 northwest (Servicio Meteorológico Nacional 2012).

152

### 153 **Characterization of the atmospheric nitrogen deposition**

154 Given that no infrastructure exists for measuring atmospheric pollution in the study region, the prevalent nitrogen  
155 deposition at each site was assessed by means of mosses. Indeed, mosses have been broadly utilized as bioindicators  
156 of atmospheric nitrogen deposition because their nitrogen content is correlated with the rates of deposition and  
157 because their isotopic fractionation during assimilation is null, reflecting the  $\delta^{15}\text{N}$  values of the prevalent  
158 atmospheric deposition (Bragazza et al. 2005; Zechmeister et al. 2008; Wilson et al. 2009; Power and Collins 2010).  
159 In particular, the genus *Braunia*, which is widely distributed in various Mexican ecosystems including urban  
160 environments, has shown to be an adequate bioindicator of atmospheric nitrogen deposition (Arciga-Pedraza 2009;  
161 Cardenas and Delgadillo 2009). In order to determine the  $\delta^{15}\text{N}$  values of the atmospheric nitrogen deposition as well  
162 as to determine the differences in rates of deposition by means of the nitrogen content of the mosses, samples of the  
163 moss *Braunia* sp. growing on the same oak branches that the orchids and less than 15 cm away of them in the oak  
164 forest and in the city were collected. In addition, to preclude any contamination from organic material from the  
165 phorophyte, i.e., the so called canopy soil, moss samples were also collected from exposed granite rock in the oak  
166 forest or exposed concrete in the city. The moss samples were analyzed as described below.

167

### 168 **Plant material**

169 *Laelia speciosa* (Kunth) Schltr is a sympodial epiphytic orchid with big and showy flowers, pink to lilac-purple  
170 petals, and a white lip. Flowers are produced during the spring. This orchid grows in sub-humid temperate climates,  
171 between 1250 and 2500 m (Soto-Arenas and Solano-Gómez 2007). The most recent leaf, pseudobulb, and root were  
172 collected from 4 individuals at each site, whose leaf and root length ranged from 8 to 12 cm and 5 to 10 cm

173 respectively. In the oak forest, orchids were collected from the outer portion of trees where canopy soil cannot be  
174 formed given a lack of plant cover. Indeed, these plants were not in contact with decomposing plant material and  
175 were at a minimum height of 5 m above the ground and a minimum radial length of 3 m from the stem of *Q.*  
176 *deserticola*.

177

### 178 **Interannual atmospheric nitrogen deposition**

179 In order to assess whether the nutrient-storing pseudobulbs of *L. speciosa* can record the changes of atmospheric  
180 nitrogen deposition over multiple years, series of consecutive pseudobulbs comprised by the ten most recent organs  
181 were collected for 4 individuals from each site (Fig. 1) for conducting the C and N analyses described below.

182

### 183 **Carbon and nitrogen content and isotopic $\delta^{15}\text{N}$**

184 Plant material was dried at 80 °C in a gravity convection oven until reaching constant weight (Drennan 2009; Díaz-  
185 Álvarez et al. 2015). The dried plant samples were ground to a fine powder in a ball mill (Retsch MM300; Retsch,  
186 Vienna, Austria), wrapped into tin capsules (Costech Analytical, Inc. Valencia, California, USA), and weighed with  
187 a microbalance (0.01 mg, Sartorius, Göttingen, Germany). For each sample, the carbon and nitrogen content, as well  
188 as their  $\delta^{15}\text{N}$  values were determined at the Stable Isotope Facility, University of Wyoming (Laramie, Wyoming,  
189 USA), with a Carlo Erba EA 1110 elemental analyzer (Costech Analytical Inc., Valencia, CA, USA) attached to a  
190 continuous flow isotope ratio mass spectrometer (Finnigan Delta Plus XP, Thermo Electron Corp, Waltham, MA).  
191 Nitrogen isotope ratios, reported in parts per thousand, were calculated relative to atmospheric air standards. The  
192 natural abundances of  $^{15}\text{N}$  were calculated as:

$$193 \quad \delta^{15}\text{N} (\text{‰ versus air}) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

194 where,  $R_{\text{sample}}$  and  $R_{\text{standard}}$  is the ratio of  $^{15}\text{N}/^{14}\text{N}$  for the sample and standard respectively. (Ehleringer and Osmond  
195 1989; Evans et al. 1996). The analytical precision for  $\delta^{15}\text{N}$  was  $\pm 0.06\text{‰}$  (SD).

196

### 197 **Statistical analyses**

198 Data normality and homoscedasticity were confirmed before conducting the following statistical analyses.

199 Atmospheric nitrogen deposition, characterized by means of the moss *Braunia* sp. from the two sites, was analyzed  
200 with a one-way ANOVA followed by pairwise Holm-Sidak post-hoc tests ( $P < 0.05$ ). The effects of the site of



201 origin (two levels) on the nitrogen content, C:N ratio, and  $\delta^{15}\text{N}$  in *L. speciosa* organs (three levels) were analyzed by  
202 means of a two-way ANOVA followed by pairwise Holm-Sidak post-hoc tests ( $P < 0.05$ ). In turn, differences  
203 between the series of 10 consecutive pseudobulbs from two different sites were analyzed by means of a nested  
204 ANOVA followed by Holm-Sidak post-hoc tests ( $P < 0.05$ ). All analyses were conducted on R (version 2.12.2, R  
205 Core Team R Foundation for Statistical Computing, Vienna, Austria).

206

## 207 **Results**

### 208 **Characterization of the atmospheric nitrogen deposition**

209 The nitrogen content of the mosses averaged  $1.7 \pm 0.1\%$  (dry mass basis) regardless of the site (Tables 1, 2). Their  
210 C:N ratio averaged  $21.5 \pm 0.8$  and no significant difference was found between sites. The  $\delta^{15}\text{N}$  reached  $3.3 \pm 0.1\%$   
211 in the city, contrasting with the  $-4.6 \pm 0.9\%$  measured in the oak forest ( $P < 0.05$ ; Fig. 2).

212

### 213 **Carbon and nitrogen content and $\delta^{15}\text{N}$ for *Laelia speciosa***

214 The carbon content of leaves, pseudobulbs, and roots of *Laelia speciosa* in the city reached  $44.4 \pm 0.3\%$  (dry mass  
215 basis), it was 8.5% lower than for orchids in the oak forest ( $P < 0.05$ ; Tables 1,2). In the city, the carbon content of  
216 pseudobulbs was  $43.4 \pm 0.2\%$  and it was 10% lower than for the roots ( $P < 0.05$ ). No significant differences were  
217 found between leaves and pseudobulbs ( $P = 0.12$ ) nor between leaves and roots ( $P = 0.31$ ). In the oak forest, the  
218 carbon content was similar among organs averaging  $45.4 \pm 0.2\%$  ( $P \geq 0.05$ ).

219 The nitrogen content of pseudobulbs and roots in the city averaged  $0.9 \pm 0.1\%$  (dry mass basis), and it was  
220 lower than for the leaves that reached  $1.4 \pm 0.1\%$  ( $P < 0.05$ ; Fig 3). The nitrogen content of leaves, pseudobulbs, and  
221 roots was different among each other in the oak forest ( $P < 0.05$ ). The nitrogen content of leaves in the oak forest  
222 was similar to the nitrogen content of the leaves in the city and averaged  $1.3 \pm 0.2\%$  (Fig 3; Table 1). Similar was  
223 the case for pseudobulbs and roots whose nitrogen content averaged  $0.6 \pm 0.1$  and  $0.9 \pm 0.1\%$  respectively for both  
224 sites (Table 2).

225 The C:N ratio was similar among the organs of orchids from the city, where it averaged  $42.1 \pm 1.5$ . In turn,  
226 the C:N ratio for the organs of orchids from the oak forest was different between pseudobulbs and leaves ( $P < 0.01$ )  
227 and between pseudobulbs and roots ( $P = 0.02$ ). No significant differences were found between leaves and roots,  
228 whose C:N ratio averaged  $49.7 \pm 0.5$  ( $P \geq 0.05$ ). The C:N ratio of orchids growing at the oak forest was generally

229 the same as that of orchids growing in the city. Indeed, a C:N ratio of  $39.1 \pm 2.5$  was found for leaves of orchids  
230 from both sites, while it averaged  $50.0 \pm 2.0$  for the roots. An exception was observed for the C:N ratio of  $127.6 \pm$   
231  $16.34$  for pseudobulbs at the oak forest that was 62% higher than for the pseudobulbs in the city ( $P < 0.001$ ; Fig. 2;  
232 Table 1).

233 The  $\delta^{15}\text{N}$  values were positive for plants from the city and negative for plants from the oak forest ( $P <$   
234  $0.001$ ). For the plants in the city, differences were found between leaves and roots ( $P = 0.01$ ) and between  
235 pseudobulbs and roots ( $P = 0.02$ ). However, no differences were found between leaves and pseudobulbs, averaging  
236  $5.6 \pm 0.01\%$ . For the orchids from the oak forest,  $\delta^{15}\text{N}$  averaged  $-3.9 \pm 0.4\%$  and no significant differences were  
237 found among organs (Table 1; Fig. 2)

238

### 239 **Interannual atmospheric nitrogen deposition**

240 The C:N ratios for the entire series of pseudobulbs of the plants from the oak forest, as well as the C:N ratios for the  
241 pseudobulbs formed in 2010 and before in the city were similar and averaged  $132.4 \pm 6.5$  (Fig. 4, Table 3). In  
242 contrast, for the two most recent pseudobulbs that grew in the city the C:N ratios halved ( $P < 0.05$ ).

243 The  $\delta^{15}\text{N}$  values of pseudobulbs from the oak forest were constant over one decade and averaged  $-4.4 \pm$   
244  $0.1\%$  for the entire series ( $P \geq 0.05$ ; Fig 5). In contrast, the  $\delta^{15}\text{N}$  values of pseudobulbs from the city showed a  
245 gradual increase from  $0.1 \pm 1.6\%$ , for the oldest pseudobulb formed at the rescue site in 2003, until a plateau was  
246 reached at  $4.8 \pm 0.3\%$  for pseudobulbs formed 2007 onwards.

247

### 248 **Discussion**

249 Plants tend to increase their photosynthetic rates as nitrogen becomes more available and, in consequence,  
250 they accumulate more carbon (Brown et al. 1996; Shangguan et al. 2000; Bauer et al. 2004; Le Bauer and Treseder  
251 2008; Díaz-Álvarez et al. 2015). For example, when *L. speciosa* is exposed to simulated nitrogen deposition, its leaf  
252 carbon content peaks at 46% of the dry mass under  $20 \text{ kg of N ha}^{-1} \text{ year}^{-1}$ , nearly 5% higher than for plants exposed  
253 to  $2.5 \text{ kg of N ha}^{-1} \text{ year}^{-1}$  (Díaz-Álvarez et al. 2015). In the present study, the carbon content of the bioindicator  
254 *Braunia* sp. and of the orchids, which was the same at both sites, suggest that nitrogen deposition is not high enough  
255 to induce significant changes in  $\text{CO}_2$  fixation nor changes in the tissue carbon content

256 In the present study, the nitrogen content for the moss from both sites was consistent with values measured  
257 under low deposition rates, which usually amounts to 1.5% and up to 2.4% in some monocots (Chapman 1965;  
258 Mills and Jones 1996; Epstein 1972; 1999). The leaves of *L. speciosa* from both sites also had similar nitrogen  
259 content with their values being similar to those of succulent leaves of several species of epiphytic orchids from low  
260 nitrogen deposition environments (Hietz et al. 1999; Wania et al. 2002; Cardelús and Mack 2010; Mardegan et al.  
261 2011). The nitrogen content for the plants in this study was substantially lower than for plants from cities with high  
262 rates of nitrogen deposition such as London or Mexico City (Power and Collins 2010; Arciga-Pedraza 2010).

263 While carbon and nitrogen content increase at different rate in response to nitrogen availability, the C:N  
264 ratio for the moss and for the leaves and roots of *L. speciosa* was similar at both sites. For the case of the orchid, this  
265 parameter was close to the ratio of 56 measured for orchids from the Central Brazilian Amazon, a clean environment  
266 (Mardegan et al. 2011). Contrasting with the lack of response from leaves and roots, the C:N ratio of pseudobulbs  
267 was different between the sites, which could be the result of a slightly higher accumulation of nitrogen in the city.  
268 The lowest nitrogen content of 0.6% was measured for pseudobulbs in the oak forest because these organs  
269 predominantly store carbohydrates, rather than minerals (Davidson 1960; Hew and Ng 1996; Ng and Hew 2000).

270 Plants that grow close to sources of pollution reflect the isotopic signals of the pollutants in their tissues  
271 (Stewart et al. 1995; Ammann et al. 1999; Stewart et al. 2002; Liu et al. 2008; Power and Collins 2010; Redling et  
272 al. 2013). For instance, negative isotopic values found in compounds derived from NH<sub>x</sub> (agriculture, animal  
273 manure, and sometimes vehicle exhaust) result from secondary compounds formed during reactions after emission  
274 (Redling et al. 2013). In contrast, compounds derived from the NO<sub>x</sub> that are released from industrial activities and  
275 fossil fuel burning have positive  $\delta^{15}\text{N}$  (Xiao and Liu 2002; Xiao et al. 2012). For example,  $\delta^{15}\text{N}$  of  $-11\text{‰}$  are found  
276 for epiphytic plants growing close to a city in Brazil where the main nitrogenous pollutants are NH<sub>x</sub> ( $-41\text{‰}$ ),  
277 meanwhile  $\delta^{15}\text{N}$  of plants from the field are less negative ( $-3.1\text{‰}$ ; Stewart et al. 2002). For epiphytes from a Costa  
278 Rican cloud forest (low nitrogen deposition) the  $\delta^{15}\text{N}$  reaches  $-4\text{‰}$  (Hietz et al. 2002; Wania et al. 2002). In  
279 contrast, the  $\delta^{15}\text{N}$  for spruce needles from Norway decreases from  $2\text{‰}$  in the proximity of a highway where nitrogen  
280 deposition is dominated by NO<sub>x</sub> to  $-3\text{‰}$  at 1 km into the forest (Ammann et al. 1999). The negative  $\delta^{15}\text{N}$  measured  
281 in the present study for the mosses and the orchids from the oak forest suggest that these plants assimilated nitrogen  
282 from a clean environment (low nitrogen deposition). Indeed, the total emissions in the municipality where the oak  
283 forest is located amounted to a 477 ton NO<sub>x</sub> and 73 ton NH<sub>3</sub> in 2008 (Sub-Sistema del Inventario Nacional de

284 Emisiones a la Atmósfera de México 2016). In contrast, positive  $\delta^{15}\text{N}$  were observed for both the moss and the  
285 orchid growing in the city, where the emissions were substantially higher than in the forest in 2008, reaching 57,081  
286 ton NO<sub>x</sub> and 2,732 ton NH<sub>3</sub> (Sub-Sistema del Inventario Nacional de Emisiones a la Atmósfera de México 2016).

287 The fact that the  $\delta^{15}\text{N}$  was similar among the different organs indicates that isotopic fractionation during the  
288 translocation process was very low or absent in plants exposed to the current rates of atmospheric nitrogen  
289 deposition at either the forest or the city. These observations contrast with an isotopic fractionation of 3‰  
290 determined for *L. speciosa* growing under a nitrogen deposition of 80 kg N ha<sup>-1</sup> year<sup>-1</sup> (Díaz-Álvarez et al. 2015).

291 Physiological responses to climate changes over the lifespan of a plant have been studied with tree rings  
292 and cactus spines (English et al. 2007; Hietz et al. 2011; Van Der Sleen et al. 2015). Here, pseudobulb  
293 chronosequences developed over a decade effectively recorded the interannual nitrogen deposition at the two sites  
294 considered in this study. The C:N ratio observed for the series of pseudobulbs was similar between sites until 2010,  
295 when a significant decrease became apparent for the plants in the city. Presumably, this is the result of 8 years of  
296 exposure to urban nitrogen deposition. However, it was not enough to decrease the C:N ratio of pseudobulbs before  
297 2011, suggesting that the rates of nitrogen deposition in the city were only slightly higher than in the oak forest. The  
298  $\delta^{15}\text{N}$  values confirmed that the pseudobulbs formed in 2004 and before grew at the rescue site, whereas the more  
299 recent pseudobulbs were formed in the city. It was interesting to observe that the first few pseudobulbs formed in  
300 city did not show a typical urban  $\delta^{15}\text{N}$ , but apparently the stored nitrogen that had been assimilated in the rescue site  
301 was translocated from the older pseudobulb developed in the city. Over time, the nitrogen from the rescue site was  
302 depleted, leaving the urban deposition as the only source of nitrogen for the ensuing pseudobulbs, as evidenced by  
303 the positive  $\delta^{15}\text{N}$  values of the six most recent pseudobulbs.

304 Isotopic analyses for *L. speciosa* enabled the distinction between nitrogen deposition in the city and the oak  
305 forest. Also, the C:N ratio of the series of pseudobulbs showed that deposition was already slightly higher in the city  
306 than in the oak forest, but not enough to effect physiological damage. In turn, the  $\delta^{15}\text{N}$  values of the series of  
307 pseudobulbs showed that it is indeed possible to track nitrogen deposition and detect possible changes over various  
308 years. In addition to land use and climate changes, the risk for *L. speciosa* will increase as the atmospheric nitrogen  
309 deposition increases above 20 kg N ha year<sup>-1</sup> (Díaz-Álvarez et al. 2015). This is already happening in some regions  
310 of Mexico City, where nitrogen deposition rates reach 48 kg N ha<sup>-1</sup> year<sup>-1</sup> (Secretaría del Medio Ambiente del  
311 Distrito Federal 2012). Granted that these alarmingly high rates of nitrogen deposition do not overlap with the

312 distribution range of *L. speciosa*, but deposition rates will most likely exceed 25 kg N ha year<sup>-1</sup> by mid-century in  
313 the regions of central Mexico where *Laelia speciosa* is endemic (Galloway et al. 2004; Phoenix et al. 2006;  
314 Galloway et al. 2008). Thus, *L. speciosa* could disappear in the following decades if necessary actions are not taken  
315 to reduce the increasing rates of nitrogen deposition in the regions where this epiphytic orchid still grows.

316

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530 **Table 1.** Two-way ANOVA for nitrogen content, carbon content, C:N ratio and  $\delta^{15}\text{N}$  of *Laelia speciosa* growing in  
 531 the city and the oak forest.

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	<i>d.f.</i>	Nitrogen content		Carbon content		C:N Ratio		$\delta^{15}\text{N}$	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Organ	2	4.07	0.001	5.75	0.01	21.1	0.001	828	0.02
Site	1	20.6	0.06	6.40	0.02	27.0	0.001	4.59	0.001
Organ $\times$ Site	2	0.88	0.43	0.10	0.90	12.9	0.001	3.74	0.04

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544 **Table 2.** Carbon and nitrogen content (dry mass basis) for plant tissue from an oak forest and from a city in  
 545 Michoacán, Mexico. Data are shown as mean  $\pm$  1 s.e. (n = 4 individuals). For each element, different letters indicate  
 546 statistically significant differences from pairwise Holm-Sidak tests ( $P < 0.05$ ).

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	Oak forest		City	
	Carbon	Nitrogen	Carbon	Nitrogen
	(% dry mass)	(% dry mass)	(% dry mass)	(% dry mass)
<i>Braunia</i> sp.				
Inert substrate	39.1 $\pm$ 0.24 a	1.57 $\pm$ 0.03 c	37.6 $\pm$ 0.32 a	1.62 $\pm$ 0.09 c
Epiphytic	35.4 $\pm$ 0.24 a	1.66 $\pm$ 0.34 c	38.6 $\pm$ 0.64 a	2.00 $\pm$ 0.05 c
<i>Laelia speciosa</i>				
Leaves	45.6 $\pm$ 0.20 b	1.3 $\pm$ 0.30 d	44.5 $\pm$ 0.4 b	1.3 $\pm$ 0.10 d
Pseudobulbs	44.6 $\pm$ 0.30 b	0.4 $\pm$ 0.10 e	43.4 $\pm$ 0.2 b	0.9 $\pm$ 0.10 f
Roots	46.0 $\pm$ 0.10 b	0.8 $\pm$ 0.10 f	45.2 $\pm$ 0.2 b	0.9 $\pm$ 0.10 f

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552 **Table 3.** Nested ANOVA for the elemental and isotopic composition for series of ten consecutive pseudobulbs of *L.*  
553 *speciosa* from the city and the oak forest.

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		C:N ratio		$\delta^{15}\text{N}$	
	<i>df.</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Year	1	7.30	0.001	4.13	0.045
Year $\times$ site	1	0.69	0.405	99.8	$1.49 \times 10^{-5}$

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574 **Figure 1.** Morphology of *Laelia speciosa* showing the series of ten consecutive annually produced pseudobulbs. The  
575 most recent pseudobulb has been formed during the past year's growing season. The number inside each pseudobulb  
576 indicates the year it was formed.

577

578 **Figure 2.** C:N ratio and  $\delta^{15}\text{N}$  for *Laelia speciosa* organs and mosses growing near the orchid on the oak branches  
579 and mosses from granite or concrete from two sites in Michoacán, Mexico. Data are shown as mean  $\pm$  S.E. (n = 4  
580 individuals per site). The arrows indicate the mosses growing on exposed bare rock at the oak forest or exposed on  
581 concrete at the city.

582

583 **Figure 3.** Nitrogen content (dry mass basis) for the organs of *L. speciosa* from two sites in Michoacán, Mexico.  
584 Data are shown as mean  $\pm$  S.E. (n = 4 individuals per site). Different letters indicate significant differences ( $p <$   
585 0.05).

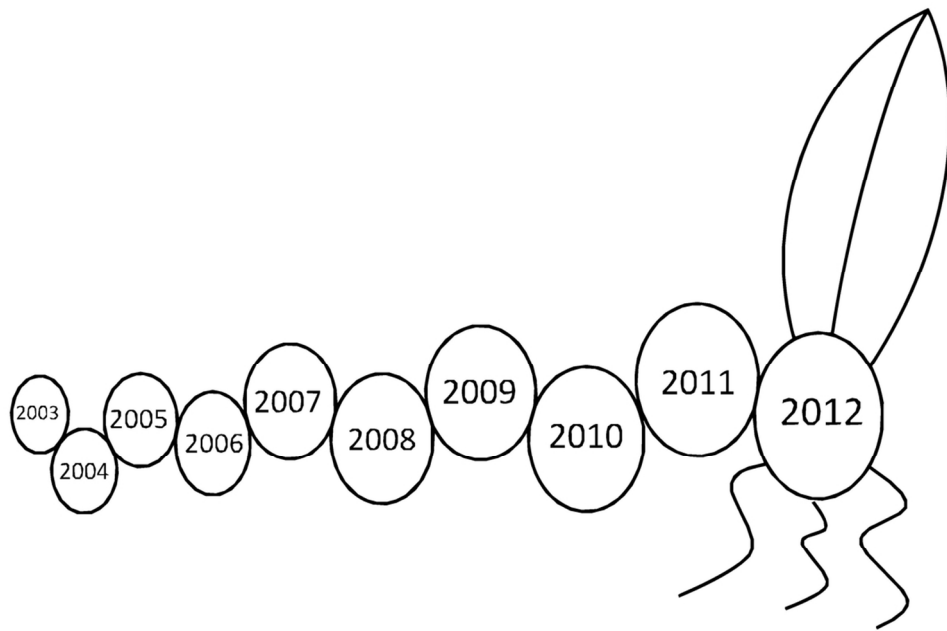
586

587 **Figure 4.** Carbon to nitrogen ratio for the 10 most recent consecutive pseudobulbs of *L. speciosa* individuals from  
588 two sites in Michoacán, Mexico. Data are shown as mean  $\pm$  S.E. (n = 4 individuals per site). The vertical dashed line  
589 indicates when the rescue plants were transplanted to the city.

590

591 **Figure 5.**  $\delta^{15}\text{N}$  for the 10 most recent consecutive pseudobulbs of *L. speciosa* individuals from two sites in  
592 Michoacán, Mexico (see Fig. 1). Data are shown as mean  $\pm$  S.E. (n = 4 individuals per site). The vertical dashed line  
593 indicates the time when the rescue plants were transplanted to the city.

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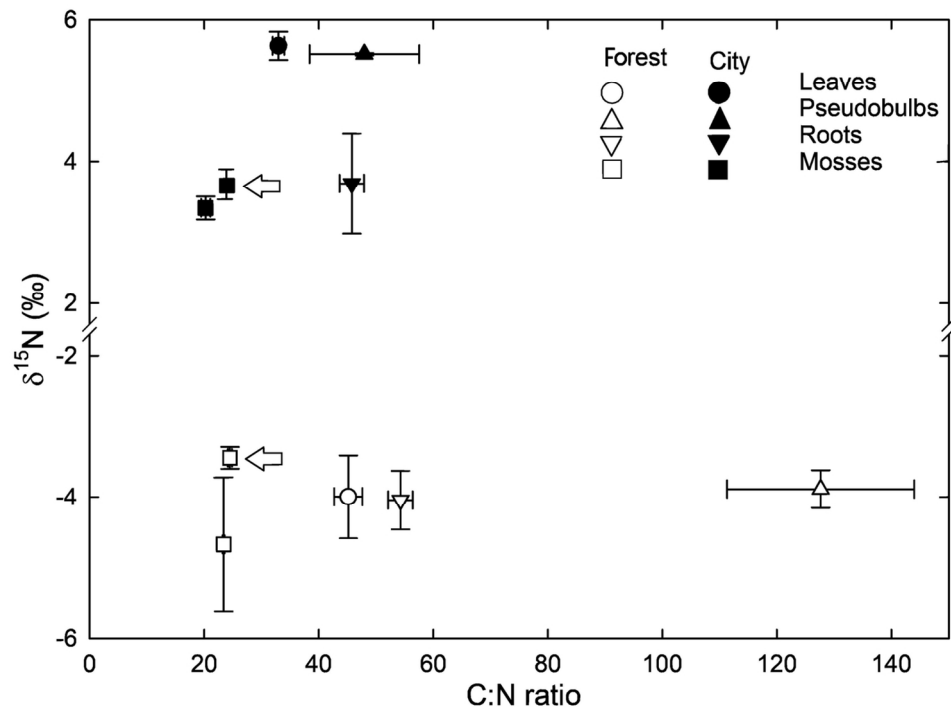
Morphology of *Laelia speciosa* showing the series of ten consecutive annually produced pseudobulbs. The most recent pseudobulb has been formed during the past year's growing season. The number inside each pseudobulb indicates the year it was formed.

106x68mm (300 x 300 DPI)

Review

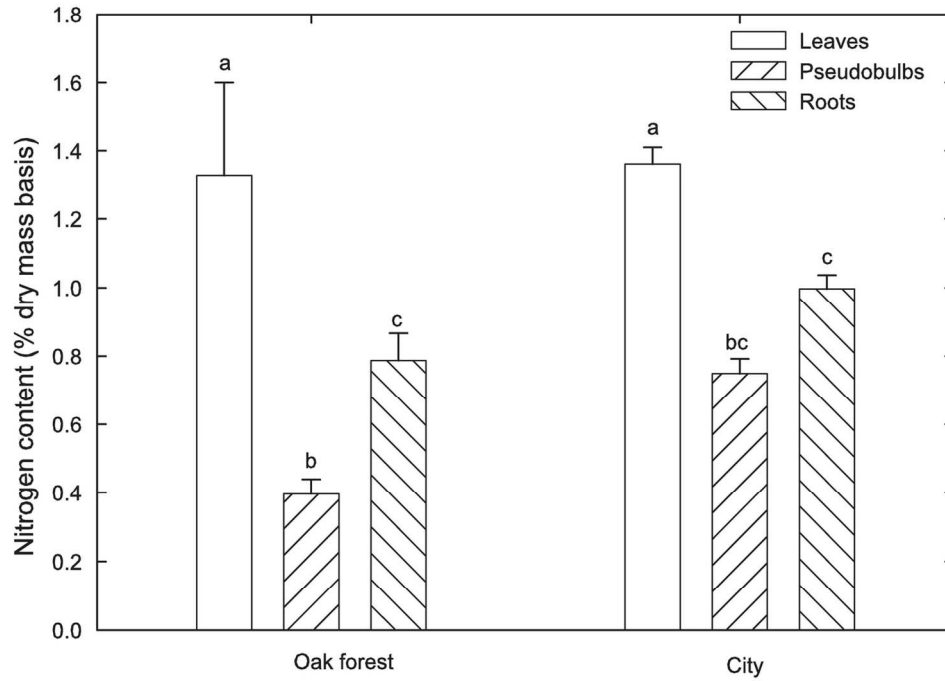
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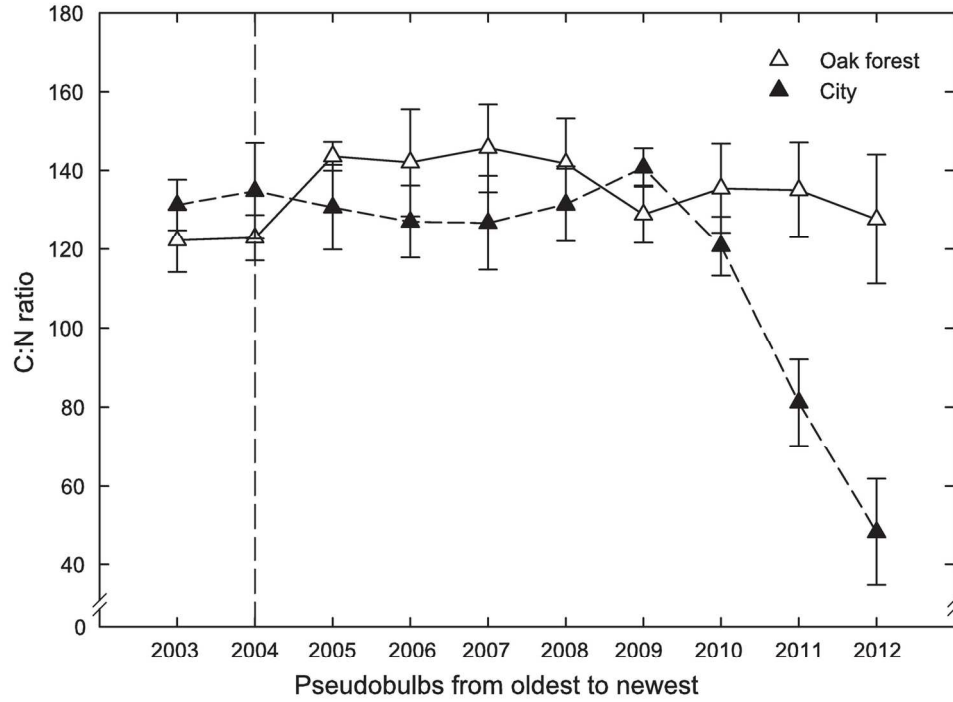
C:N ratio and  $\delta^{15}\text{N}$  for *Laelia speciosa* organs and mosses growing near the orchid on the oak branches and mosses from granite or concrete from two sites in Michoacán, Mexico. Data are shown as mean  $\pm$  S.E. ( $n = 4$  individuals per site). The arrows indicate the mosses growing on exposed bare rock at the oak forest or exposed on concrete at the city.

122x90mm (300 x 300 DPI)



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

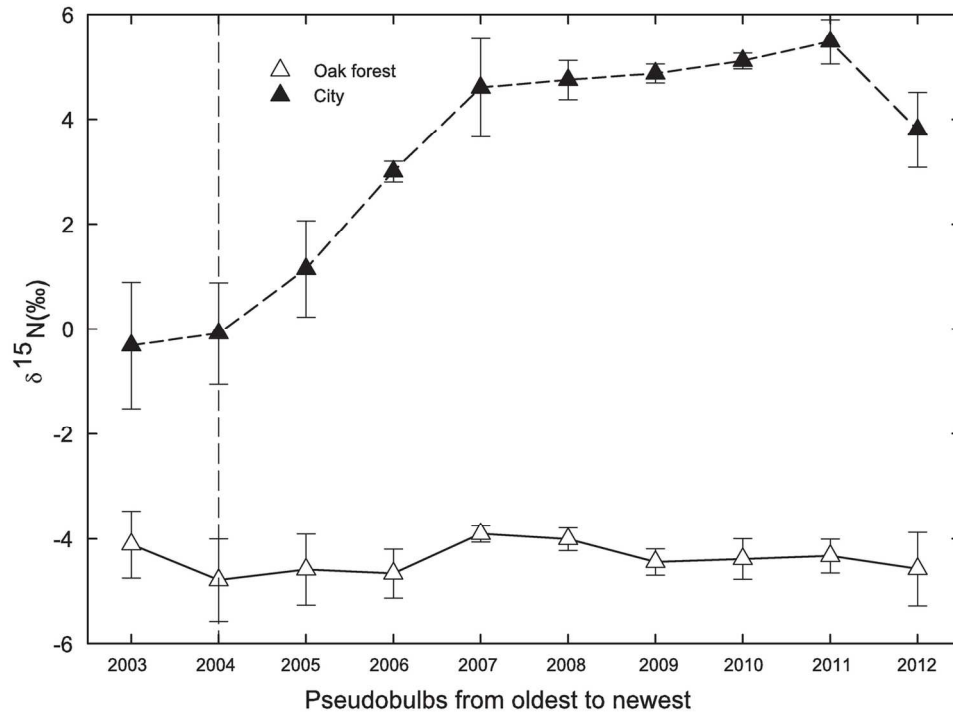
133x101mm (300 x 300 DPI)



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

147x123mm (300 x 300 DPI)





$\delta^{15}\text{N}$  for the 10 most recent consecutive pseudobulbs of *L. speciosa* individuals from two sites in Michoacán, Mexico (see Fig. 1). Data are shown as mean  $\pm$  S.E. ( $n = 4$  individuals per site). The vertical dashed line indicates the time when the rescue plants were transplanted to the city.

136x107mm (300 x 300 DPI)