Photosynthetic limitations in two Antarctic vascular plants: importance of leaf anatomical traits and Rubisco kinetic parameters

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Abstract

Particular physiological traits allow the vascular plants Deschampsia antarctica Desv. and Colobanthus quitensis (Kunth) Bartl. to inhabit Antarctica. The photosynthetic performance of these species was evaluated in situ, focusing on diffusive and biochemical constraints to CO₂ assimilation. Leaf gas exchange, Chl a fluorescence, leaf ultrastructure, and Rubisco catalytic properties were examined in plants growing on King George and Lagotellerie islands. In spite of the species- and population-specific effects of the measurement temperature on the main photosynthetic parameters, CO₂ assimilation was highly limited by CO₂ diffusion. In particular, the mesophyll conductance ($g_{m}$) — estimated from both gas exchange and leaf chlorophyll fluorescence and modeled from leaf anatomy — was remarkably low, restricting CO₂ diffusion and imposing the strongest constraint to CO₂ acquisition. Rubisco presented a high specificity for CO₂ as determined in vitro, suggesting a tight co-ordination between CO₂ diffusion and leaf biochemistry that may be critical ultimately to optimize carbon balance in these species. Interestingly, both anatomical and biochemical traits resembled those described in plants from arid environments, providing a new insight into plant functional acclimation to extreme conditions. Understanding what actually limits photosynthesis in these species is important to anticipate their responses to the ongoing and predicted rapid warming in the Antarctic Peninsula.

Key words: Antarctic plants, leaf traits, mesophyll conductance, photosynthesis, Rubisco, temperature.

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Introduction

Antarctica is the coldest, driest, and windiest continent on Earth, with mean summer air temperatures <0 °C in continental Antarctica and between 0 and 2 °C in Maritime Antarctica (Convey, 1996). The study of how organisms behave in this hostile habitat is of particular interest to unravel functional adaptations to extreme conditions (see Alberdi et al., 2002; Cavieres et al., 2016).

Deschampsia antarctica Desv. (Poaceae) and Colobanthus quitensis (Kunth) Bartl. (Caryophyllaceae) are the only two vascular species naturally colonizing these harsh conditions, being distributed along the west coast of the Antarctic Peninsula and its associated islands (Maritime Antarctica) down to Lazarev Bay on the north-west coast of Alexander Island (69°22.0'S, 71°50.7'W) (Convey et al., 2011). Although several differences have already been described between these two species in dealing with the Antarctic conditions (Pérez-Torres et al., 2007; Cavieres et al., 2016), the performance of both Antarctic vascular species relies on a robust CO2 assimilation machinery, including a high activation state of Rubisco and stromal fructose-1,6-biphosphatase (Pérez-Torres et al., 2006). Deschampsia antarctica is more abundant and widely distributed along the Antarctic Peninsula than C. quitensis (Smith, 2003), colonizing different habitats ranging from mineral to organic soils, and from dry to waterlogged areas. In contrast, C. quitensis seems to be less tolerant to extreme conditions, preferring sparsely vegetated and sheltered sites growing under different environmental conditions (Peguero-Pina et al., 2015a). Contrasting environmental conditions can induce changes in several leaf traits that affect $g_m$ (Evans et al., 1994; Kogami et al., 2001; Terashima et al., 2001). Differences in leaf anatomical traits and chloroplast ultrastructure have been reported for D. antarctica growing along the Maritime Antarctica (Jellings et al., 1983; Gielwanska and Szczuka, 2005). Regarding C. quitensis, plants growing at higher latitudes have smaller and thicker leaves than those grown at lower latitudes, evidenced by a smaller leaf cross-section, with higher mesophyll thickness, narrower adaxial surface, and reduced epidermis thickness (Cavieres et al., 2016). Although these changes have been related to plastic responses to the Antarctic climate (Romero et al., 1999), it is not clear to what extent they may affect the photosynthetic performance of these plants. More specifically, do ultrastructural mesophyll traits induce changes in $g_m$ and other photosynthetic parameters affecting the carbon assimilation capacity in these Antarctic plant species?

The main objective of the present study was to evaluate in situ the photosynthetic performance of two natural populations of D. antarctica and C. quitensis located at different latitudes within the Maritime Antarctica. In particular, we analyzed the response of photosynthesis to varying measurement temperatures and the diffusive and biochemical constraints to CO2 assimilation. To the best of our knowledge, this is the first field study thoroughly analyzing the responses and limitations of photosynthesis on these species.

Materials and methods

Study area and plant material

Two study sites were selected (Fig. 1): one located in King George Island (KGI), near to Henryk Arctowski Polish Antarctic Station (62°09'S, 58°28'W), and the other in Lagotellerie Island (LAG), in Marguerite Bay (67°53'20"S, 67°25'30"W). These sites were selected based on the visual similarity of the vegetation, exposure, and elevation, as well as their distance from the coast. The sites are characterized by wide open and relatively flat areas (KGI) or terraces (LAG) covered by vegetation. In both sites, individuals of D. antarctica and C. quitensis (see Supplementary Fig. S1 at JXB online) were randomly selected, within an area of ~300 m², for photosynthesis measurements and sampling of leaves during six sunny days in late January 2015 in LAG and during February 2015 in KGI.

Climate

Precipitation in both sites falls mainly as snow. Annual precipitation, which occurs mostly during summer, is higher in the northernmost
population (~1249 mm), while in Marguerite Bay it is ~360 mm (Turner et al., 2002). During summer, the day length in KGI is ~15 h, and the mean air temperature is 0.7 °C, with average maximum and minimum air temperature of 2.9 °C and –4.1 °C, respectively. In LAG, summer day length is ~17 h, with mean air temperature of –3.5 °C and average maximum and minimum of 1 °C and –10.9 °C, respectively (Utah State University Database; https://climate.usurf.usu.edu).

Daily microclimatic records of air temperature at 10 cm above ground level, leaf temperature, and photosynthetically active radiation (PAR) were taken during 6 d in January 2015 (Fig. 2). According to these data, the daily integral of photosynthetic solar radiation in KGI was 30% greater than in LAG (Fig. 2A). Air temperature at KGI tended to be higher than at LAG during most of the daylight period (Fig. 2B). At both locations, leaf temperature of D. antarctica and C. quitensis was at least 2 °C above ambient temperature, reaching a maximum of ~10 °C (Fig. 2). Differences in leaf temperature between locations were evident during the night, where individuals of both species growing in LAG showed higher leaf temperature (~2 °C) compared with individuals from KGI (Fig. 2C, D). In contrast, during the day, there was a trend for higher leaf temperatures in KGI compared with LAG.

Fig. 1. The Antarctic continent (A), Antarctic Peninsula (B, inset from A), South Shetland Islands, and area of the Antarctic Peninsula (C, inset from B). Vascular plants from 62° to 67° South latitude, including the two study areas represented by an asterisk (*): King George Island (62°09’S, 58°28’W) and Lagotellerie Island (67°53’20”S, 67°25’30”W), Marguerite Bay (modified from Alberdi et al., 2002).

Fig. 2. Diurnal course of photosynthetically active radiation (A), air temperature (B), and leaf temperature in D. antarctica (C) and C. quitensis (D) in King George (KGI) and Lagotellerie Island (LAG) between 17 and 22 January 2015. Values are means ± SE.
Leaf gas exchange and chlorophyll fluorescence

Instantaneous gas exchange and Chl \(a\) fluorescence measurements (Li-6400XT, Li-6400-40 leaf chamber, LI-COR Inc., Lincoln, NE, USA) were performed on a group of leaves (from a branch of \(C.\) quitensis or a tiller of \(D.\) antarctica), trying to cover all the IRGA’s chamber area and avoiding leaf overlap. When the leaf area was lower than the chamber area, a correction by actual leaf area inside the chamber was performed.

The response of net photosynthesis \(\text{CO}_2\) uptake \((A_N)\) to varying substomatal \(\text{CO}_2\) concentration \((C)\) was studied with the so-called \(A_N-C_i\) curves. All measurements were made at 1000 \(\mu mol\) photons \(m^{-2}\) \(s^{-1}\), air relative humidity of 40–50\%, and at two leaf temperatures: 10 °C and 15 °C. These measurement temperatures corresponded to the maximal leaf temperatures recorded in the field (Fig. 2), and the average of optimal temperature for photosynthesis determined for these species in both populations, respectively (data not shown).

The \(A_N-C_i\) curves were initiated by allowing the leaf to reach steady state (typically 20–30 min after clamping the leaf). Thereafter, \(A_N-C_i\) curves were obtained at 11 different ambient \(\text{CO}_2\) concentrations \((C_i)\) from 0 to 2000 \(\mu mol\) \(\text{CO}_2\) mol\(^{-1}\). Leaves were left to equilibrate at least 5 min at each \(\text{CO}_2\) concentration. Dark mitochondrial leaf respiration \((R_{\text{dark}})\) was obtained at pre-dawn at a \(C_i\) of 400 \(\mu mol\) \(\text{CO}_2\) mol\(^{-1}\) and the two measuring temperatures (10 °C and 15 °C). Leaf temperature was measured with a fine-wire thermocouple touching the abaxial surface for the group of leaves. Corrections for the leakage of \(\text{CO}_2\) into and out of the leaf chamber of the Li-6400 IRGA’s chamber area and avoiding leaf overlap. When the leaf area was lower than the chamber area, a correction by actual leaf area inside the chamber was performed. The maximum velocity of carboxylation \((V_{\text{max}})\) was derived from \(A_N-C_i\) curves according to Farquhar et al. (1980) and using the kinetic constants for Rubisco determined for these species at the measurement temperatures (see below).

The approach of Tomáš et al. (2013) was used for anatomical modeling of \(g_m\). In the field, the central portions of leaves were fixed in formaldehyde, acetic acid, and ethanol, and 4% glutaraldehyde for optical and transmission electron microscopy (JEM1200 EXII, Japan), respectively. Six to 10 micrographs were randomly selected to measure the mesophyll thickness; the mesophyll area exposed to the intercellular air space \((S_a)\) to total leaf surface \((S)\) area ratio \((S_a/S);\) the chloroplast-exposed surface to total surface area ratio \((S_p/S);\) the chloroplast length \((L_p);\) the chloroplast thickness \((T_{\text{chl}});\) and the cell wall thickness \((T_{\text{cw}})\). All images were analyzed with image analysis software (ImageJ, Wayne Rasband/NIH, Bethesda, MD, USA). The one-dimensional gas diffusion model of Niinemets and Reichstein (2003) was employed to estimate the different leaf anatomical characteristics determining \(g_m\). The determinants of \(g_m\) were divided between gas-phase conductance and the different components of the cellular phase conductances: the cell wall \((\bar{g}_{\text{wall}})\), the plasmalemma \((\bar{g}_{\text{pl}})\), and inside the cells through the cytosolic path \((\bar{g}_{\text{cyt}})\).

**Rubisco kinetic characterization at varying temperature**

The Rubisco Michaelis–Menten constant for \(\text{CO}_2\) under 21% \(\text{O}_2\) \((K_{\text{cH}})\) was determined in crude extracts obtained as detailed in Galmés et al. (2014a). Replicate measurements \((n=3)\) were made using independent protein preparations from different individuals. To obtain the Rubisco carboxylase specific activity \((\bar{k}_{\text{cat}})\), the maximum rate of carboxylation was extrapolated from the Michaelis–Menten fitted curve and divided by the number of Rubisco active sites in solution, quantified by \([^{14}C]\text{CABP}\) (2-carboxyarabinitol-1, 5-bisphosphate) binding (Yokota and Canvin, 1985) as described in Galmés et al. (2013). The carboxylase catalytic efficiency was obtained as the ratio \(\bar{k}_{\text{cat}}/K_{\text{m}}\).

**Determination of the Rubisco specificity for \(\text{CO}_2/\text{O}_2\) \((S_{\text{CO}/\text{O}_2})\) at varying temperature**

The Rubisco \(\text{CO}_2/\text{O}_2\) specificity \((S_{\text{CO}/\text{O}_2})\) was measured on purified extracts as in Gago et al. (2013), except that values were not normalized to those of wheat Rubisco. Measurements were performed at 5, 15, and 25 °C, with 3–6 replicates per species and per assayed temperature. For comparative purposes, all Rubisco kinetic traits, including \(S_{\text{CO}/\text{O}_2}\), were also determined in wheat (\(Triticum aestivum\) ‘Cajermo’) at 25 °C.

For all Rubisco assays, the pH of the assay buffers was accurately adjusted at each measurement temperature. The concentration of \(\text{CO}_2\) in solution in equilibrium with \(\text{HCO}_3^-\) was calculated assuming a \(p\bar{K}_a\) for carbonic acid of 6.31, 6.19, and 6.11 at 5, 15, and 25 °C, respectively. The concentration of \(\text{O}_2\) in solution was assumed to be 400.5, 316.4, and 258.9 mmol ml\(^{-1}\) at 5, 15, and 25 °C, respectively (Truesdale and Downing, 1954).

**Temperature response of the Rubisco kinetic constants**

The temperature response of the Rubisco kinetic parameters was fitted for each individual temperature response data set by an Arrhenius-type temperature response function:

\[
\text{Parameter} = \exp(c - \Delta H / RT) \tag{1}
\]

where \(c\) is the scaling constant for the parameter, \(\Delta H\) (J mol\(^{-1}\)) is the activation energy, \(R\) is the universal gas constant \((8.314 \text{ J mol}^{-1} \text{K}^{-1})\), and \(T\) (K) is the temperature. Equation 1 was fitted to the data by iteratively minimizing the sum of squares between the measured and predicted values of each kinetic parameter using the Microsoft Excel Solver function.

**Estimation of \(g_m\) and \(C_i\)**

Mesophyll conductance for \(\text{CO}_2\) \((g_m)\) was calculated from both combined gas-exchange and Chl \(a\) fluorescence measurements, and anatomical modeling. From the combined gas-exchange and Chl \(a\) fluorescence measurements, \(g_m\) was calculated as in Harley et al. (1992):

\[
g_m = A_N/C_i - \left[ \left( \frac{1}{F_{\text{m}}} + \frac{1}{F_{\text{n}}} \right) \left( \frac{1}{4A_N + R_D} \right) \right] / \left( \frac{1}{4A_N + R_D} \right)
\]

where \(A_N\) and \(C_i\) were obtained from gas exchange measurements at saturating light. The non-photosynthetic \(\text{CO}_2\) evolution rate in the light \((R_D)\) was assumed to be half of \(R_{\text{dark}}\) and the chloroplast \(\text{CO}_2\) compensation point \((\Gamma^*)\) was calculated according to Brooks and Farquhar (1985) from the Rubisco specificity factor \((S_{\text{CO}/\text{O}_2})\) measured in vitro. Determination of \(g_m\) was used to calculate the chloroplast \(\text{CO}_2\) concentration \((C_i)\), converting \(A_N-C_i\) curves into \(A_N-C_i\) curves, as \(C_i = C_i - (A_N/g_m)\).


Statistical analyses

Fully factorial two-way ANOVAs were performed on each species to assess differences between populations and measurement temperatures. Differences between means were assessed by a posteriori Tukey test (P<0.05). These analyses were performed with the SPSS statistics 19.0 software package (IBM-software, New York, USA). A Pearson correlation analysis was performed to assess the relationship between the anatomical derived $g_m$ and studied anatomical traits. Goodness of fit to saturation curves was assessed in: $A_N/g_{tot}$, $A_N/C_i$, and $A_N/g_{stt}$. All these analyses were done in Statistica 7.0 (Stat Soft Inc. Tulsa, OK, USA).

Results

Leaf carbon exchange in Antarctic plants at different measurement temperatures

Plants from LAG exhibited higher net CO$_2$ assimilation rates ($A_N$) at ambient CO$_2$ concentration than those from KGI, with no differences between measurement temperatures in the studied populations, although in both species the interaction between these two factors was significant (Supplementary Table S1; Supplementary Fig. S2). In D. antarctica, $A_N$ was higher in LAG at 15 °C (15.11 $\pm$ 0.51 µmol CO$_2$ m$^{-2}$ s$^{-1}$) compared with KGI (5.93 $\pm$ 0.60 µmol CO$_2$ m$^{-2}$ s$^{-1}$; Fig. 3A), but $A_N$ was similar across the two sites at 10 °C (~12 µmol CO$_2$ m$^{-2}$ s$^{-1}$). In C. quitensis, the highest $A_N$ value was observed in LAG at 10 °C (9.88 $\pm$ 1.71 µmol CO$_2$ m$^{-2}$ s$^{-1}$) and the lowest in KGI at 10 °C (2.53 $\pm$ 0.37 µmol CO$_2$ m$^{-2}$ s$^{-1}$; Fig. 3A). There were no differences in the dark respiration rate ($R_{dark}$) between populations or measurement temperatures (Fig. 3C, D; Supplementary Table S1). This indicates that the observed differences in $A_N$ were not due to differences in $R_{dark}$, but to differences in the photosynthetic process and its determinants.

Diffusive limitations to photosynthesis

Stomatal ($g_s$) and leaf mesophyll ($g_m$) conductances for both species showed similar trends to those described for $A_N$ (Fig. 4; Supplementary Table S1). Values of $g_s$ for D. antarctica ranged between 0.08 $\pm$ 0.01 mol H$_2$O m$^{-2}$ s$^{-1}$ at KGI at 15 °C and 0.35 $\pm$ 0.03 mol H$_2$O m$^{-2}$ s$^{-1}$ in LAG at 15 °C. For C. quitensis, $g_s$ varied from 0.14 $\pm$ 0.02 mol H$_2$O m$^{-2}$ s$^{-1}$ in LAG at 15 °C to 0.23 $\pm$ 0.03 mol H$_2$O m$^{-2}$ s$^{-1}$ in KGI at 15 °C (Fig. 4A, B). Estimated $g_m$ values were lower than those of $g_s$, with the minimum value estimated in C. quitensis from KGI at 10 °C (0.01 $\pm$ 0.01 mol CO$_2$ m$^{-2}$ s$^{-1}$), and the highest in D. antarctica from LAG at 15 °C (0.13 $\pm$ 0.02 mol CO$_2$ m$^{-2}$ s$^{-1}$). The low $g_m$ determined low total leaf conductance to CO$_2$ ($g_{tot}$), which significantly correlated with $A_N$ (Fig. 5A). This indicates that the photosynthetic rates in the Antarctic vascular plants under field conditions were limited by diffusional components in general, and low $g_m$ in particular (Fig. 5B). The good correspondence between $A_N$ and the chloroplastic CO$_2$ concentration ($C_i$) further confirms that carbon fixation in these species was constrained by low availability of CO$_2$ at the carboxylation sites (Fig. 5C). In general, diffusion limitations to photosynthesis were more evident in C. quitensis (Figs 4, 5), which is consistent with the lowest CO$_2$ assimilation rates found in this species (Fig. 3B).

Mesophyll conductance modeled from leaf anatomy

The studied populations of D. antarctica showed similar leaf anatomy, with the exception of the mesophyll thickness ($T_{m}$), which was higher in plants from LAG, and the chloroplast surface area facing intercellular air spaces per leaf area ($S_{ch}$/$S$), which was larger in plants from KGI (Table 1). No differences between populations were found in ultrastructural leaf characteristics (Supplementary Fig. S3) such as cell wall thickness ($T_{cw}$), average distance between chloroplasts ($L_{cyl}$), and their size ($L_{chl}$ and $T_{chl}$). In addition, large numbers of organelles around chloroplasts were observed in both plant species (Supplementary Figs S3, S4). For C. quitensis, there were differences between populations...
in most of the anatomical parameters evaluated, except in $T_{mes}$ and the chloroplast length ($L_{chl}$) (Table 1). In particular, C. quitensis plants from KGI exhibited higher $T_{cw}$, $\Delta L_{cyt}$, and chloroplast thickness ($T_{chl}$) than plants from LAG (Table 1). The contrasting anatomical traits between both populations of C. quitensis determined differences in $g_m$ modeled from leaf anatomy ($0.013 \pm 0.001$ mol CO$_2$ m$^{-2}$ s$^{-1}$ in KGI versus $0.039 \pm 0.004$ mol CO$_2$ m$^{-2}$ s$^{-1}$ in LAG).

In general, anatomy-based $g_m$ values were lower than those estimated in vivo by the method of Harley et al. (1992) (Table 1). It should be noted that anatomical measurements do not consider the different leaf temperatures recorded between populations (Fig. 2); therefore, the comparison with the ‘Harley’ $g_m$ should be treated with caution. Despite this limitation, some of the described trends for $g_m$ estimated by the method of Harley et al. (1992) were also found with the anatomy-based $g_m$ (compare Fig. 4D with Table 1). For example, C. quitensis from LAG at 10 °C showed higher values compared with the KGI. In contrast, the higher ‘Harley’ $g_m$ found in D. antarctica from LAG compared with KGI (Fig. 4C) was not supported by the anatomical modeling of $g_m$ (Table 1).

Biochemical determinants of photosynthesis

The maximum rate of Rubisco carboxylation ($V_{\text{max}}$) increased with the measurement temperature in both plant species from KGI, but non-significant differences between measurement temperatures were found in plants from LAG (Fig. 4E, F). For all the range of $A_N$–$C_c$ curves, $A_N$ was linearly correlated to $C_c$ (Supplementary Fig. S5), which impeded the estimation of the maximum rate of electron transport ($J_{\text{max}}$).

The ratio between the electron transport rate and gross CO$_2$ assimilation rate (ETR/$A_G$) which is used as a proxy of the amount of reducing power per unit of fixed CO$_2$ (Flexas et al., 2008)
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2002), varied between measurement temperatures and populations in both species (Fig. 7). In plants from KGI, the increase in the measurement temperature induced an increase in ETR/A in *D. antarctica* but a decrease in *C. quitensis*. In plants from LAG, there were no temperature-driven changes in ETR/A in either of the two species. The relationship between ETR/A and C was highly significant and negative (Supplementary Fig. S6), indicative of enhanced photorespiration rates under low CO2 availability. Except for KGI at 15 ºC, *C. quitensis* exhibited higher values for ETR/A—consistent with lower values for Cc.

Rubisco in vitro kinetic parameters and their temperature dependence

The affinity of Rubisco for CO2 measured as Kc air at 25 ºC was 18.7 ± 0.4 µM and 23.4 ± 1.8 µM for *C. quitensis* and *D. antarctica*, respectively (Table 3). The maximum rate of the carboxylase catalytic turnover (kcat c) was 3.76 ± 0.29 s–1 in *C. quitensis* and 4.10 ± 0.75 s–1 in *D. antarctica*. The carboxylase catalytic efficiency assessed by the kcat c/Kc air ratio ranged between 0.18 s–1 µM–1 and 0.20 s–1 µM–1 in both Antarctic species. At 25 ºC, the values of the Rubisco specificity factor (Sc/o) were 99.5 ± 4.8 mol mol–1 in *D. antarctica* and 97.1 ± 2.4 mol mol–1 in *C. quitensis* (Table 3).

Differences were observed in the temperature response of Rubisco between *D. antarctica* and *C. quitensis* (Table 3). For instance, *D. antarctica* presented higher values for Kc air than *C. quitensis* at 5 ºC and 15 ºC. Differences in kcat c between *D. antarctica* and *C. quitensis* were found only at 5 ºC. In consequence, the Rubisco carboxylase catalytic efficiency did not differ between species at any of the assayed temperatures. Regarding Sc/o, differences between the two Antarctic plant species were observed only at 15 ºC, where *D. antarctica* presented higher values than *C. quitensis* (P<0.05).

The differences in the temperature response of Rubisco resulted in a trend for higher absolute values of the energy of activation (ΔH) of the different kinetic parameters in *C. quitensis*, although significant differences between both species were only observed in ΔH kcat c (78.8 ± 1.5 kJ mol–1 in *D. antarctica* versus 98.3 ± 6.6 kJ mol–1 in *C. quitensis*).

Discussion

The leaf anatomical and biochemical traits described here for the Antarctic vascular plants growing at different latitudes within Antarctica and their implications on the photosynthetic performance of these species constitute new insights into the plant functional responses to cold conditions. While ultrastructural traits of the leaf mesophyll restricted CO2 transfer and limited the photosynthetic capacity of the two Antarctic vascular plants, the kinetic traits of Rubisco, characterized by high affinity for CO2 and relative high values for kcat c, seem crucial to optimize carbon assimilation despite the restrictions of CO2 transport inside the leaf. The former suggests an important functional adaptation that, together with other traits (Cavieres et al., 2016), allows *D. antarctica* and *C. quitensis* to survive and grow in the harsh climate conditions of the Antarctica. As these two Antarctic species differ in their habitat requirements,
plant morphology, leaf anatomy, and photosynthetic optimum temperature (Cavieres et al., 2016), an interspecific comparison was not considered here, except for Rubisco kinetic parameters.

Leaf mesophyll conductance to CO₂ limits carbon assimilation in Antarctic plants

This is the first study assessing $g_m$ in Antarctic vascular plants, and how changes in the ultrastructure of the mesophyll affect $g_m$, and hence the carbon acquisition in these species. According to our results, the range of $g_m$ values and the $g_m/g_s$ ratio of these plant species are among the lowest reported so far for higher plant species (De Lucía et al., 2003; Flexas et al., 2009, 2014; Tomás et al., 2013; Peguero-Pina et al., 2015). Under certain conditions, $g_m$ can be the most significant photosynthetic limitation (Flexas et al., 2012; Tomás et al., 2013; Galmés et al., 2014; Niinemets and Keenan, 2014; Carriquí et al., 2014; Peguero-Pina et al., 2015a, b).

Table 1. The mesophyll thickness between the two epidermal layers ($T_{mes}$), the cell wall thickness ($T_{cw}$), the average distance between chloroplasts ($\Delta L_{cyt}$), the chloroplast thickness ($T_{chl}$), the chloroplast length ($L_{chl}$), the CO₂ transfer conductances across the intercellular air space ($g_{ias}$), the liquid phase ($g_{liq}$), and the mesophyll conductance for CO₂ ($g_m$) calculated from leaf anatomical measurements, the mesophyll ($S_m$) and chloroplast ($S_c$) surface area facing intercellular air spaces per leaf area, for D. antarctica and C. quitensis from King George (KGI) and Lagotellerie Island (LAG).

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<th>D. antarctica</th>
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<td></td>
<td>KGI</td>
<td>LAG</td>
<td>KGI</td>
<td>LAG</td>
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<tr>
<td>$T_{mes}$ (µm)</td>
<td>101.03 ± 5.43 a</td>
<td>136.27 ± 5.14 b</td>
<td>322.29 ± 13.42 a</td>
<td>290.77 ± 18.91 a</td>
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<tr>
<td>$T_{cw}$ (µm)</td>
<td>0.26 ± 0.01 a</td>
<td>0.22 ± 0.03 a</td>
<td>0.35 ± 0.06 b</td>
<td>0.21 ± 0.02 a</td>
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<tr>
<td>$\Delta L_{cyt}$ (µm)</td>
<td>0.68 ± 0.18 a</td>
<td>0.3 ± 0.16 a</td>
<td>0.53 ± 0.06 b</td>
<td>0.05 ± 0.03 a</td>
</tr>
<tr>
<td>$T_{chl}$ (µm)</td>
<td>2.96 ± 0.24 a</td>
<td>3.58 ± 0.52 a</td>
<td>5.12 ± 0.66 b</td>
<td>1.64 ± 0.12 a</td>
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<tr>
<td>$L_{chl}$ (µm)</td>
<td>4.40 ± 0.36 a</td>
<td>5.48 ± 0.56 a</td>
<td>5.32 ± 0.28 a</td>
<td>5.92 ± 0.36 a</td>
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<tr>
<td>$g_{ias}$ (m s⁻¹)</td>
<td>0.046 ± 0.006 a</td>
<td>0.031 ± 0.004 a</td>
<td>0.013 ± 0.001 a</td>
<td>0.019 ± 0.001 b</td>
</tr>
<tr>
<td>$g_{liq}$ (m s⁻¹)</td>
<td>0.0005 ± 0.0001 a</td>
<td>0.0005 ± 0.0001 a</td>
<td>0.0003 ± 0.0000 a</td>
<td>0.0001 ± 0.0001 b</td>
</tr>
<tr>
<td>$g_m$ (mol m⁻² s⁻¹)</td>
<td>0.022 ± 0.002 a</td>
<td>0.023 ± 0.004 a</td>
<td>0.013 ± 0.001 a</td>
<td>0.039 ± 0.004 b</td>
</tr>
<tr>
<td>$S_m$/$S$ (m² m⁻³)</td>
<td>6.40 ± 0.75 a</td>
<td>6.28 ± 0.51 a</td>
<td>4.33 ± 0.48 a</td>
<td>8.56 ± 0.53 b</td>
</tr>
<tr>
<td>$S_c$/$S$ (m² m⁻³)</td>
<td>2.24 ± 0.48 b</td>
<td>1.16 ± 0.08 a</td>
<td>2.06 ± 0.32 a</td>
<td>4.42 ± 0.63 b</td>
</tr>
</tbody>
</table>

Values are means ± SE ($n$=6–10). Different letters indicate statistically significant differences between populations for each species ($P<0.05$).
the photosynthetic rates of the Antarctic vascular plants were highly correlated with \( g_m \) (Fig. 5B), this factor seems to be the main constraint for the photosynthetic process in these plant species in the field. Positive correlations between \( A_N \) and \( g_m \) have been previously reported (e.g. Evans and Loreto, 2000; Warren, 2008), and recent meta-analyses suggest that \( g_m \) is also associated with the structure of leaves, determining the diffusion limitations of photosynthesis (Niinemets and Sack, 2006; Warren, 2008). Thus, our results highlight that morphoanatomical leaf characteristics regulating \( g_m \) are key determinants of the photosynthetic functioning in the two Antarctic vascular plant species.

Large differences in \( g_m \) have been shown both between and within species with different leaf forms and habits (e.g. Flexas et al., 2008; Warren, 2008). The low \( g_m \) found in the Antarctic vascular plants in the field seems to be related to leaf anatomical traits that affect CO\(_2\) diffusion across the intercellular air space (Table 1), especially with the limitations associated with cell walls, cytoplasm, and stroma (Table 2). The leaf mesophyll diffusion limitations were especially evident in \( C. \) quitensis, which is consistent with the low photosynthetic rate of this species and with the high values of \( \text{ETR}/A_G \) (Fig. 7), indicative of enhanced photorespiration rates. Higher leaf mesophyll thickness is commonly associated with a greater number of leaf mesophyll cell layers (Niinemets et al., 2009). In \( C. \) quitensis, the highest leaf mesophyll thickness was accompanied by the largest area of leaf mesophyll exposed to intercellular air space (\( S_m \)), and thus the greatest \( S_m \) to total leaf surface area ratio (\( S_m/S \)) (Table 1). Provided that the numbers of chloroplasts are similar in mesophyll cells, a larger \( S_m/S \) also implies a greater ratio between the chloroplast-exposed surface area and the total surface area (\( S_m/S \)) (Terashima et al., 2005, 2006). As a larger \( S_m/S \) implies more parallel pathways for CO\(_2\) liquid-phase diffusion, \( g_m \) correlates positively with \( S_m/S \) (Fig. 6). Actually, higher leaf density has been associated with reduced gas-phase volume, and smaller and more densely packed leaf mesophyll cells with thicker cell walls (Niinemets, 1999; Niinemets and Sack, 2006). Such modifications could reduce the liquid-phase-diffusion conductance and \( g_m \) (Terashima et al., 2005; Evans et al., 2009). In addition, the observed differences in \( g_m \) between both populations of \( C. \) quitensis can be partially attributed to the variation in other leaf anatomical traits such as \( T_{cw}, \) \( \Delta L_{cyt} \), and \( T_{chl} \) (Table 1). Specifically, the cell wall and the resistances imposed by the cytoplasm and stroma exerted the highest limitations in the \( g_m \) (Table 2). Further, as was noted above, \( S_m/S \) and \( S_m/S \) are two of the most important anatomical traits influencing \( g_m \) (Tosens et al., 2012; Peguero-Pina et al., 2012; Carriquí Pina et al., 2014).

Anatomical characteristics could not be the only explanation for the observed differences in \( g_m \), as those were also found between measurement temperatures in plants growing in LAG (Fig. 4C, D). It seems likely that some (not yet fully understood) biochemical components of \( g_m \) could be involved in the regulation of \( g_m \). Among them, carbonic anhydrase (CAs) (Fabre et al., 2007) and aquaporins (AQPs) (Terashima and Ono, 2002) have been shown to modify \( g_m \) in vivo in response to varying measurement temperatures. The discrepancy between \( g_m \) estimates based on anatomical measurements and those based on conventional gas-exchange methods suggests the existence of these facilitating mechanisms for CO\(_2\) diffusion. However, to the best of our knowledge, there are no reports on the activity and abundance of these proteins in Antarctic vascular plants, and thus we cannot draw any general conclusion on this issue.

The anatomical features of the Antarctic species have been regarded as adaptive responses to the harsh climate conditions of Antarctica (Cavieres et al., 2016). Among them, the large number of organelles (mitochondria or peroxisomes) around the chloroplasts in both Antarctic species (Supplementary Table 2. Quantitative limitation analysis of the leaf mesophyll conductance to CO\(_2\) (\( g_m \)) for \( D. \) antarctica and \( C. \) quitensis from King George (KGI) and Lagotellerie Island (LAG) due to different anatomical components of the diffusion pathway: intercellular spaces (\( l_{icw} \)), cell wall (\( l_{cw} \)), plasmalemma (\( l_{pl} \)), and inside the cell (\( l_{cel, tot} \))

<table>
<thead>
<tr>
<th></th>
<th>( D. ) antarctica</th>
<th>( C. ) quitensis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KGI</td>
<td>LAG</td>
</tr>
<tr>
<td>( l_{icw} )</td>
<td>1.1 ± 0.1 a</td>
<td>1.7 ± 0.2 a</td>
</tr>
<tr>
<td>( l_{cw} )</td>
<td>34.4 ± 1.3 a</td>
<td>27.4 ± 2.1 b</td>
</tr>
<tr>
<td>( l_{pl} )</td>
<td>2.0 ± 0.1 a</td>
<td>2.3 ± 0.2 a</td>
</tr>
<tr>
<td>( l_{cel, tot} )</td>
<td>62.3 ± 1.5 a</td>
<td>68.4 ± 2.1 a</td>
</tr>
</tbody>
</table>

Values are means ± SE (\( n=4–10 \)).

Different letters indicate statistically significant differences between populations for each species (\( P<0.05 \)).

Fig. 7. The ratio of the electron transport rate and gross photosynthesis (ETR/A\(_G\)) for \( D. \) antarctica (A) and \( C. \) quitensis (B) in King George (KGI) and Lagotellerie Island (LAG), measured at 10 °C (white bars) or 15 °C (gray bars). Values are means ± SE (\( n=5–7 \)). Different letters indicate statistically significant differences for each species between populations and measurement temperatures according to Tukey (\( P<0.05 \)).
The Rubisco Michaelis–Menten constant affinity for CO₂ under 21% O₂ ($K_{\text{cat}}^c$), the maximum carboxylase catalytic turnover rate ($k_{\text{cat}}^c$), the carboxylase catalytic efficiency ($k_{\text{cat}}^c/K_{\text{cat}}^M$), and the specificity factor ($S_{\text{cat}}$). For each parameter, the scaling constant ($c$) and the activation energy ($\Delta H$) are shown.

<table>
<thead>
<tr>
<th>Assay temperature</th>
<th>$D. \text{antarctica}$</th>
<th>$C. \text{quitensis}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{\text{cat}}^c$ (μM)</td>
<td>$k_{\text{cat}}^c$ (s⁻¹)</td>
</tr>
<tr>
<td>5°C</td>
<td>10.9 ± 1.2 a*</td>
<td>0.4 ± 0.1 a*</td>
</tr>
<tr>
<td>15°C</td>
<td>14.7 ± 0.8 a*</td>
<td>1.4 ± 0.2 a</td>
</tr>
<tr>
<td>25°C</td>
<td>23.4 ± 1.8 b</td>
<td>4.1 ± 0.8 b</td>
</tr>
<tr>
<td>c</td>
<td>14.5 ± 1.8</td>
<td>33.2 ± 0.8</td>
</tr>
<tr>
<td>$\Delta H_c$ (kJ mol⁻¹)</td>
<td>28.2 ± 4.4</td>
<td>78.8 ± 1.5</td>
</tr>
</tbody>
</table>

Different letters indicate statistically significant differences among assay temperatures in each species, and an asterisk denotes statistically significant differences between $D. \text{antarctica}$ and $C. \text{quitensis}$ for each assay temperature ($P<0.05$). Values are means ± SE ($n=3-6$).

For comparative purposes, we also measured the kinetic parameters at 25°C in Rubisco from wheat (Triticum aestivum ‘Cajeme’). The values were as follows: $K_{\text{cat}}^c=16.0$ μM, $k_{\text{cat}}^c=2.2$ s⁻¹; $k_{\text{cat}}^c/K_{\text{cat}}^\text{air}=0.14$ s⁻¹ μM⁻¹; $S_{\text{cat}}=101.1$ mol mol⁻¹.

Figs S3, S4) have been suggested as facilitators for the CO₂ exchange between respiration and photorespiration processes (Gielwanańska and Szczuka, 2005). In addition, both species have xeromorphic leaf characteristics (Nobel, 1980; Vieira and Mantovani, 1995; Romero et al., 1999), which are related to the water limitations due to the low temperature and strong winds that characterize the Antarctic climate (Smith, 1993). Some features, such as the presence of two bundle sheaths in leaf vascular bundles (mestome), of $D. \text{antarctica}$ have been associated with an adaptation to high radiation in order to optimize photosynthesis and water use efficiency (WUE) (Pytkó, 1966). Evert et al. (1985) suggested that the mestome functions as a leaf endodermis, limiting apoplastic movement of water across the mesophyll. This trait, along with a high stomatal density and leaf mass area, confer a high capacity to control water loss on $D. \text{antarctica}$ (Vieira and Mantovani, 1995; Xiong et al., 2000; Alberdi et al., 2002). Decreased water movement across the leaf mesophyll could be advantageous to avoid heat loss in cold environments, being an important adaptation for these habitats (Sage, 2001). These characteristics are consistent with the low $g_{\text{sm}}$ values determined in the present study using both gas-exchange/fluorescence and anatomical methods.

The low $g_{\text{sm}}$ values resulted in low $g_{\text{cat}}$ values, and therefore low $C_c$. According to our results, $A_h$ was highly correlated with $g_{\text{sm}}$, $g_{\text{cat}}$, and $C_c$ (Fig. 5), confirming the predominant role of leaf CO₂ diffusion in the photosynthetic performance of Antarctic plant species. In this sense, reduced photosynthesis, due to low $g_{\text{sm}}$, seems to be the penalty of structurally robust leaves that the Antarctic plant species have to pay to survive in extremely stressful conditions.

Rubisco performance alleviates the low mesophyll conductance

The Rubisco kinetic parameters, and their temperature response, have been related to species differences in the photosynthetic performance under varying conditions (Galmés et al., 2014a, 2015, 2016; Sharwood et al., 2016). The temperature dependence of Rubisco kinetics revealed some differences between the two Antarctic angiosperm species. In particular, the energy of activation ($\Delta H_c$) for $k_{\text{cat}}^c$ was higher in $C. \text{quitensis}$ compared with $D. \text{antarctica}$, indicative of higher thermal sensitivity (Table 3). The values for $\Delta H_a$ of $k_{\text{cat}}^c$ in the two Antarctic species are among the highest in Streptophyta, and do not support the reported trend (see compilation by Galmés et al., 2015) that the Rubisco enzymes of C₃ species adapted to cool habitats have a lower plastic response to temperature changes compared with Rubisco of C₃ species from warm environments. The molecular and biochemical causes of this apparent discrepancy, already observed in other species from cool habitats such as Atriplex glabriflora (Badger and Collatz, 1977), are unknown and should be explored in depth. In contrast, the high values for both $S_{\text{cat}}$ and $\Delta H_a$ of $k_{\text{cat}}^c$ observed in the Antarctic species are in accordance with the transition state theory of Tcherkez et al. (2006).

In fact, the values of $S_{\text{cat}}$ measured at 25°C in both Antarctic plants (Table 3) are among the highest values reported so far for higher plant species (e.g. Galmés et al., 2005; Orr et al., 2016). We note that for comparative purposes between data sets, we also measured Rubisco kinetic parameters in wheat at 25°C (shown in the footnotes of Table 3) and the obtained values were similar to previous data (e.g. Galmés et al., 2005; Prins et al., 2016; Orr et al., 2016). Regarding $K_{\text{cat}}^\text{air}$ at 25°C, both Antarctic plants, but in particular $D. \text{antarctica}$, presented values comparable with those reported for species adapted to xeric environments (Galmés et al., 2014a). Notably, despite their high affinity for CO₂, Rubisco from the Antarctic plant species retained high $k_{\text{cat}}^c$, similar to species closely related to $D. \text{antarctica}$ such as Poa arctica and Poa pratensis (Sage, 2002). The high $k_{\text{cat}}^c$ leads to values for the carboxylase catalytic efficiency ($k_{\text{cat}}^c/K_{\text{cat}}^\text{air}$) higher than those reported for species from xeric habitats (Galmés et al., 2014a). Further, at 15°C (a temperature similar to the maximal diurnal leaf temperature recorded in the field; Fig. 2), Rubisco from both Antarctic plants increased the specificity for CO₂ to levels similar to that reported for xeromorphic species (Galmés et al., 2005).
Although it has been suggested that the selective pressures for greater CO₂ affinity for Rubisco have been high in species adapted to high temperature and low soil water availability (Galmés et al., 2005, 2014a), there is also evidence that in cold environments the selective pressures may favor Rubisco with higher \( k_{\text{cat}}^{\text{c}} \) (Sage, 2002; Yamori et al., 2009). Interestingly, the Antarctic species have evolved under both dry and cold conditions, and here we found that their Rubiscos show a high affinity for CO₂ and retain high \( k_{\text{cat}}^{\text{c}} \). The high \( S_{\text{clo}} \) in the Antarctic plants is additional evidence in favor of the hypothesis that CO₂ limitations shaped the evolution of their Rubisco kinetics (e.g. Raven, 2000; Young et al., 2012; Galmés et al., 2014b). In the case of the Antarctic plants, the low availability of CO₂ at the sites of carboxylation is driven by adaptive anatomical traits to resist the extreme climatic conditions of low temperature and strong winds. It has been demonstrated that under conditions that promote drought, higher \( S_{\text{clo}} \) reduces ribulose bisphosphate (RuBP) oxygenation and favors the carboxylase reaction (Galmés et al., 2005). Thus, it seems likely that the anatomical features that determine a low CO₂ diffusion in these species are partially counterbalanced by a highly efficient Rubisco. The concentration of active Rubisco, calculated from \( V_{\text{max}}=k_{\text{cat}}^{\text{c}} \times [\text{active Rubisco sites}] \), was notably high in both Antarctic species at 15 °C: ~44.57 ± 1.9 µmol m⁻² and 42.31 ± 2.84 µmol m⁻² in \( D. \) antarctica and \( C. \) quitensis, respectively. Although these data should be confirmed by direct measurements in leaf extracts, they suggest that the decrease in \( k_{\text{cat}}^{\text{c}} \) at low temperatures is compensated by increased amount of [active Rubisco sites], following trends already reported in other species (e.g. Yamori et al., 2005, 2006).

The high \( S_{\text{clo}} \) and low \( K_{\text{c}}^{\text{air}} \), as well as the constitutive low \( g_{\text{m}} \) of Antarctic plants, are not their unique traits, resembling those documented for drought-adapted species. The presence of bundle sheaths found in \( D. \) antarctica and also in \( C. \) quitensis (Vieira and Mantovani, 1995) is a characteristic of plants from xeric climates (Sage, 2001). In addition, Montiel et al. (1999) reported remarkably high values of instantaneous WUE in \( D. \) antarctica, more typical of \( C_{4} \) and Crassulacean acid metabolism (CAM) species than of \( C_{3} \) species. The high WUE of \( D. \) antarctica is likely to be related to the diffusive and biochemical determinants identified in the present study.

Concluding remarks

The present study provides new field data on the photosynthetic performance and diffusive and biochemical limitations of the two Antarctic plants. This work incorporates leaf anatomical traits related to CO₂ assimilation and increases the range of knowledge of the diversity of Rubisco kinetics parameters in two relevant species.

The ultrastructural traits of the leaf mesophyll in field-grown Antarctic plants ultimately restricted the CO₂ leaf transfer capacity and limited the photosynthetic capacity of these species. Under the cold and dry climate conditions of the Antarctica, the high Rubisco affinity for CO₂ and relatively high values for \( k_{\text{cat}}^{\text{c}} \) seem crucial to optimize carbon assimilation. Overall, these results constitute new insights regarding the functional adaptations to highly stressful conditions in plants and the properties that enable the distribution and abundance of vascular plants in Antarctica. Considering the strong climate changes experienced in the Antarctic Peninsula that include rapid warming during the last decades, and a pause of that warming during recent years (Turner et al., 2016), the next challenge should be to assess the effect of different durations of long-term exposure to warmer temperatures on the photosynthetic performance of \( D. \) antarctica and \( C. \) quitensis.

Supplementary data

Supplementary data are available at JXB online.

Table S1. ANOVA of the effects of populations, measurement temperature, and their interaction.

Fig. S1. \( D. \)eschampsia antarctica (left) and \( C. \)olobanthus quitensis (right).

Fig. S2. The response of the net photosynthetic CO₂ assimilation rate (\( A_{\text{N}} \)) to varying internal CO₂ concentration (\( C_{\text{i}} \)).

Fig. S3. Transverse section of the mesophyll and mesophyll cells of \( D. \) antarctica.

Fig. S4. Transverse section of the mesophyll and mesophyll cells of \( C. \) quitensis.

Fig. S5. The response of the net photosynthetic CO₂ assimilation rate (\( A_{\text{N}} \)) to varying chloroplast CO₂ concentration (\( C_{\text{c}} \)).

Fig. S6. The relationship between electron transport rate and gross photosynthesis ratio (ETR/\( A_{\text{G}} \)) and CO₂ concentration at the site of carboxylation (\( C_{\text{i}} \)).

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Dacrydium cupressinum


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