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Leaf wettability, anatomy and ultra-structure of *Nothofagus antarctica* and *N. betuloides* grown under a CO₂ enriched atmosphere

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ABSTRACT

Increasing CO₂ air concentration may affect wettability, anatomy and ultra-structure of leaves of Patagonian forest species, evergreen and deciduous plants potentially responding differently to such CO₂ increases. In this study, we analysed the wettability, anatomy and ultra-structure of leaves of *Nothofagus antarctica* (deciduous) and *N. betuloides* (evergreen) grown under high CO₂ concentrations. Leaf wettability was affected by increasing CO₂, in different directions depending on species and leaf side. In both species, soluble cuticular lipid concentrations per unit leaf area raised with higher CO₂ levels. Stomatal parameters (density, size of guard cells and pores) showed different responses to CO₂ increasing depending on the species examined. In both species, leaf tissues showed a general trend to diminish with higher CO₂ concentration. Cuticle thickness was modified with higher CO₂ concentration in *N. betuloides*, but not in *N. antarctica* leaves. In both species, chloroplasts were often damaged with the increase in CO₂ concentration. Our results show that several surface and internal leaf parameters can be modified in association with an increase in atmospheric CO₂ concentration which may vary among plant species.

1. Introduction

Nothofagus species forests are one of the main carbon (C) reservoirs of Southern Patagonia (Argentina and Chile) (Baswald et al., 2002; Peri et al., 2017), which gives them a relevant role as C sinks for mitigating climate change (Denman et al., 2007). These native forests also play a crucial role as regulators of nutrient and hydrological cycles, both as protection of associated water streams, as well as for the ecosystem services provided across different watersheds and landscapes (Jobbágy, 2011; Bahamonde et al., 2015; Rosas et al., 2019). The deciduous forests of *Nothofagus antarctica* (G. Forst.) Oerst. Have the widest natural distribution of all South American *Nothofagus* spp. However, in general these forests are restricted to the most severe environmental conditions (e.g., from rocky to poorly drained or waterlogged soils such as peat bogs), and are prone to occupy locations with low water availability and exposure to strong winds in the limit with the Patagonian steppe (Frangi

et al., 2004). On the other hand, the evergreen species *N. betuloides*, forms the southernmost evergreen forests in the world (Promis et al., 2008). In southern Patagonia, it is located in pure or mixed stands together with *Drimys winteri*, *Embothrium coccineum* or *N. pumilio* mainly in riparian areas or growing in lake shores (Donoso and Donoso, 2006; Peri et al., 2019), associated with higher levels of precipitation and less severe winters in terms of temperature than in *N. antarctica* forests (Peri et al., 2012).

Climate change modelling studies for Southern Patagonia (Tierra del Fuego and Santa Cruz Provinces; Kreps et al., 2012) predicted increases of 2 °C in mean annual temperature, and increases of 20 mm over the current mean annual precipitation for the next 70 years in native forest areas. On the other hand, the Intergovernmental Panel on Climate Change (IPCC) has reported increases of up to 47% in the concentration of atmospheric CO₂ since 1750 to the present day, predicting an accelerating trend in this rate (IPCC, 2021). To date, are no studies which evaluated the response of Patagonian native forest species to the effect of

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Abbreviations

SEM	scanning electron microscopy
SLA	specific leaf area
TEM	transmission electron microscopy

climate change and increased atmospheric CO₂ concentration.

The physico-chemical characteristics of plant surfaces affect their interaction with liquids, solid particles, insects or microorganisms deposited onto leaves (Fernández and Khayet, 2015). The combination of leaf roughness and chemical composition determine its degree of wettability, polarity and hydrophobicity, and therefore its potential capacity to absorb water from the atmosphere (Fernández et al., 2014a; Papierowska et al., 2019; Guzmán-Delgado et al., 2016). This may vary between species, varieties or environmental conditions (Muhammad et al., 2020; Boanares et al., 2021). Working with *Fagus sylvatica* and *Quercus petraea* Bahamonde et al. (2018) reported variations in the wettability of canopy leaves due to different levels of radiation. Furthermore, the physicochemical characteristics of leaf surfaces will influence their interactions with atmospheric water deposited onto the foliage due to e.g., rain or dew and involving drop repellence or adhesion (Fernández et al., 2017, 2021). This phenomenon is receiving increasing attention, since it is considered an important factor for the interception of rain by the leaves of trees and consequently in water balance of forest ecosystems under climate change scenarios (Smith and McLean, 1989; Rosado and Holder, 2013; Holder, 2020). In the north-west of Argentinean Patagonia, Brewer and Nunez (2007) evaluated the wettability and water droplet retention of leaves of 37 species growing in a moisture gradient that included plants from the steppe to the Andean Patagonian forests.

In addition, leaf morphology and anatomy are key aspects in the physiological potential of adaptation of plants to environmental changes (Smith and McLean, 1989; Hanba et al., 2002). In general, evergreen species have more robust leaf structures than deciduous species, such as thicker cell walls and epidermis, greater mesophyll volume and leaf density (Castro-Díez et al., 2000; Niinemets et al., 2005; Enrique et al., 2016). Besides, leaf morpho-anatomical characteristics show a high responsiveness to environmental factors such as temperature, lighting, soil water availability or atmospheric CO₂. In turn, this implies that in a scenario of increased CO₂ air concentration, evergreen species would be less affected than deciduous species in their leaf structure by reducing the photosynthetic efficiency through mesophyll conductance adjustment (Niinemets et al., 2011). Studies of leaf anatomy in *Nothofagus* forest species in southern Patagonia are very scarce (Ivancich et al., 2012) and the affectation level generated by variations in CO₂ levels is unknown. Structural leaf aspects related to physiological functions may vary according to the environment. For example, the specific leaf area and stoma characteristics (density, size) can reflect the photosynthetic capacity of plants or to environmental changes (light, temperature, water, nutrients) produced by wide geographic gradients (Hetherington and Woodward, 2003; Fernández et al., 2014b; Tian et al., 2016). The effects of increased CO₂ concentration on leaf anatomical parameters such as stomatal density (Beerling et al., 1998; Marchi et al., 2004) and size have also been documented (Ogaya et al., 2011; Driscoll et al., 2006). In addition, some studies (e.g., Pritchard et al., 1997; Velikova et al., 2009) focussed on the effect of high atmospheric CO₂ concentration on leaf internal structure (i.e., the mesophyll) and ultrastructure of chloroplasts (Pritchard et al., 1997). Working with *Pinus palustris* seedlings Pritchard et al. (1997) reported an increase in chloroplast size and number of starch grains when the CO₂ levels raised from 365 to 720 μmol mol⁻¹. On the other hand, an increase in chloroplast stromal volume and a reduction of the thylakoid system was observed in *Platanus orientalis* plants subjected to high levels of atmospheric CO₂ (Velikova

et al., 2009).

In light of the existing information on the effect of elevated atmospheric CO₂ in plant leaves and the importance of South-American *Nothofagus* forests, the aims of the present work were: i) to identify the potential mechanisms of water absorption through the leaves in both *Nothofagus* species; and ii) to determine potential leaf anatomical and ultrastructural modifications in response to an increase in the level of CO₂ of the growing atmosphere in evergreen (*N. betuloides*) and deciduous (*N. antarctica*) native tree species in Patagonia. The following hypotheses were tested: 1) despite *N. antarctica* and *N. betuloides* are distributed in environments with contrasting soil water availability, it is proposed that both species will present similar levels of leaf wettability and therefore a similar potential to absorb water through the foliage; and 2) owing to the softer nature of the deciduous leaves analysed, it is hypothesised that a high concentration of CO₂ in the growing atmosphere will generate leaf anatomical and ultra-structural modifications specially in *N. antarctica* compared to evergreen *N. betuloides*.

2. Material and methods

2.1. Plant culture

Seeds of *Nothofagus antarctica* (deciduous) and *N. betuloides* (evergreen) were obtained from 3 pure stands located in southern Patagonia (between 51° 13' and 51° 19' S latitude and between 72° 10' and 72° 15' W longitude for *N. antarctica*; between 54° 05' and 54° 50' S latitude and between 67° 15' and 68° 25' W longitude for *N. betuloides*). The selected seeds were first stratified and kept at 4 °C for 60 days (Prémoli, 1991). Afterwards, the stratified seeds were placed in a germination chamber for a period of 60 days (Bahamonde et al., 2011). The germinated seeds of each species were planted in 250 cm³ pots containing a 3:1 mixture substrate of peat (Floragard TKS2, Floragard Vertriebs gmbh, Oldenburg, Germany) and washed river sand. The soil substrate was supplemented with 2 kg m⁻³ of Osmocote Plus fertilizer (16-9-12 NPK + 2 micronutrients, Scotts, Heerlen, Netherlands).

Seedlings were first grown in the greenhouse during 6 months with a thermal regime of 25/18 °C day/night temperature and sufficient water available. Later, the plants were divided in 2 sets and were distributed in 2 cultivation chambers (MP-BIO-M, Spain) under controlled environmental conditions (14/10 h day/night photoperiod, of 25/20 °C day/night temperature, 65% air relative humidity and 800 μmol m⁻²s⁻¹ photosynthetic photon flux density (PPFD) of at the top of plants). In each chamber, 10 plants of each species were randomly placed just at the beginning of bud sprouting. Each chamber had a different CO₂ of the atmosphere. One of them maintained an air concentration of 400 ppm (control) and the other one was raised to 800 ppm CO₂ (high level CO₂ treatment). At that time the seedlings were between 60 and 80 cm high and had low leaf cover (less than 30 leaves per seedling) in both species. However, for all evaluations, fully-expanded, healthy leaves of plants grown under the conditions of the chambers described above were used. Plants were grown in these chambers for 5 months and at the end of this period leaf samples were taken and several leaf traits were estimated as detailed below. Plants were changed between chambers two times during the experiment, for maintaining the same CO₂ levels established at the beginning of the experiment for each CO₂ treated set of plants. At the end of the 5-months growing period of controlled atmospheric CO₂ concentrations, 30 fully expanded leaves per species and treatment were sampled.

2.2. Wax extraction

For soluble cuticular lipid (wax) extraction, the central vein was discarded (Geyer and Schönherr, 1990) and the remaining 2 leaf lamina sections were first scanned, and their area was measured by image analysis (ImageJ; Rasband, 2004). Lamina sections were then clamped with forceps and washed by carefully applying chloroform

(Sigma-Aldrich) flushes on both sides of each leaf piece with a Pasteur pipette (6 flushes of 1.7 ml each per leaf side) and storing the liquid in separated glass beakers which were previously weighed in a precision balance. These beakers with the chloroform extracts were placed in a laboratory hood at 25 °C until the chloroform evaporated. Finally, the beakers of each sample were weighed and the amount of soluble cuticular lipids of each sample was determined gravimetrically and expressed as weight per unit leaf area.

2.3. Leaf surface wettability and physico-chemical parameters

Measurements were carried out to determine the wettability of the leaf surfaces of 60 leaves per each species, selecting 6 fully-expanded leaves from each plant (2 at the top, 2 in the middle and 2 at the bottom). The gross physico-chemical properties (i.e., the combination of surface structure and chemical composition) of the adaxial (upper) and abaxial (lower) side of the leaves were characterised by the 3-liquids method (Fernández and Khayet, 2015). For this purpose, contact angles of drops of three liquids of different degrees of polarity and apolarity (i.e., distilled water, glycerol and diiodomethane) were measured on each leaf surface, using a drop shape analysis system (DSA 100, Krüss, Germany). For each liquid, approximately 2 µl drops were deposited onto the adaxial and abaxial leaf surfaces (N = 30) and lateral images were taken through a camera incorporated in the system. Contact angles were hence automatically calculated using the tangent method. For all *Nothofagus* leaf surfaces, the total surface free energy (γ_s), its components (i.e., the Lifshitz-van der Waals (γ_s^{LW}) and acid-base (γ_s^{AB} , γ^+ and γ^-), surface polarity ($\gamma_s^{LW} \gamma_s^{-1}$ expressed as %) and solubility parameter (δ) were calculated considering the surface tension components of water ($\gamma_1 = 72.80 \text{ mJ m}^{-2}$, $\gamma_1^{LW} = 21.80 \text{ mJ m}^{-2}$, $\gamma_1^+ = \gamma_1^- = 25.50 \text{ mJ m}^{-2}$), glycerol ($\gamma_1 = 63.70 \text{ mJ m}^{-2}$, $\gamma_1^{LW} = 33.63 \text{ mJ m}^{-2}$, $\gamma_1^+ = 8.41 \text{ mJ m}^{-2}$, $\gamma_1^- = 31.16 \text{ mJ m}^{-2}$) and diiodomethane ($\gamma_1 = \gamma_1^{LW} = 50.80 \text{ mJ m}^{-2}$, $\gamma_1^+ = 0.56 \text{ mJ m}^{-2}$, $\gamma_1^- = 0 \text{ mJ m}^{-2}$, Fernández and Khayet, 2015).

2.4. Specific leaf area and microscopic examination

Thirty fresh leaves from each treatment were collected for anatomical analyses. Leaves were scanned without petioles to calculate leaf area by image analysis (ImageJ software), and then dried at 65 °C to a constant weight and weighed to estimate their specific leaf area (SLA). Fifteen sub-samples were taken from each leaf treatment for surface examination with a variable pressure scanning electron microscope (SEM; Hitachi S-3400 N, Tokyo, Japan). The images obtained by SEM were analysed with ImageJ software to measure the following leaf anatomical parameters: stomatal density (number of stomas per mm² of leaf area), length and width of stomata guard cells, length and width of stomata opening (see annex 1, image 1). These measurements were used to calculate the stomatal guard cell area, stomatal opening area, the potential conductance index (Holland and Richardson, 2009) and the relative stomata per pore area.

Another sub-sample of 15 leaves of the different CO₂ treatments, was collected for transmission electron microscopy (TEM) observations. For this purpose, leaves were cut in 4 mm² pieces and fixed in 2.5% glutaraldehyde 4% formaldehyde during 6 h at 4 °C, then they were rinsed 4 times in a period of 6 h with a phosphate buffer cooled in ice at pH 7.2, in which they were left overnight. After fixation, the samples were placed in a 1:1 solution of 2% aqueous osmium tetroxide and 3% potassium ferrocyanide for 1.5 h. Then, samples were washed with distilled water, dehydrated in a graduated series of 30, 50, 70, 80, 90, 95 and 100% acetone. Afterwards, the samples were infiltrated with acetone-Spurr's resin at a ratio of 3:1 (2 h), 1:1 (2 h), 1:3 (3 h) and pure resin (samples were left for 14 h). Tissues totally infiltrated by the resin were placed in blocks filled with pure resin and incubated for 3 days at 70 °C for complete polymerization. From this, ultrafine sections were cut mounted on nickel grids and stained with Reynolds' lead citrate for 5 min. Finally, the tissues were observed with a TEMJeol 1010 (Tokyo,

Japan) operated at 80 kV and equipped with a megaview CCD camera. Cuticle (adaxial and abaxial) thicknesses and the areas of chloroplasts were measured by image analysis (ImageJ software).

On the other hand, semi-thin leaf cross sections were also cut with a microtome, mounted on microscope slides and stained with toluidine blue, before observation with an epifluorescence microscope (Axioplan-2, Zeiss, Germany). From these images, the following parameters were derived from image analysis (ImageJ software): thickness of lamina, thickness of upper and lower epidermis, and percentage (%) area of the different tissues (upper and lower epidermis, palisade parenchyma and spongy parenchyma) (see annex 1, image 2).

2.5. Data analysis

The variables related to the determination of wettability and adherence/repellency (surface free energy, solubility parameter) of leaf surfaces were analysed through ANOVA with 3 main factors: species, leaf side and CO₂ level. All other parameters related to the external and internal anatomy of the leaves were analysed through two-factor ANOVA: species and CO₂ level. To evaluate the differences between factors when the F-test values were significant (P < 0.05) the Tukey test was used. When significant interactions between factors were detected, multiple comparisons were made (Willems and Raffaele, 2001).

3. Results

3.1. Surface properties of *N. betuloides* and *N. antarctica* leaves

When the two species were compared at 400 ppm CO₂, which represents the current atmospheric air concentration, the adaxial side of *N. antarctica* leaves had higher contact angles than *N. betuloides* leaves (P < 0.05) with the three liquids evaluated. Regarding the abaxial side of the *N. antarctica* leaves, contact angles were only higher with water (Table 1). When plants were grown at 800 ppm CO₂, the adaxial side of *N. antarctica* leaves had higher contact angles than *N. betuloides* with water and glycerol, but lower values with apolar diiodomethane (P < 0.05) (Table 1). Variations in the contact angles between the sides of species leaves depended on liquid type and CO₂ level (Table 1). The increase in atmospheric CO₂ concentration induced changes in the contact angles between drops of the different liquids and the leaf surfaces evaluated. Contact angles with water decreased in both species

Table 1

Contact angles of water (θ_w), glycerol (θ_g) and diiodomethane (θ_d), with adaxial and abaxial lamina surfaces of *Nothofagus antarctica* and *N. betuloides* collected from plants grown at two levels of CO₂ (400 y 800 ppm). The values are mean ± standard deviation (SD). For a same species and leaf side, lower case letters indicate significant differences (P < 0.05) between CO₂ levels. For a same CO₂ level and leaf side, capital letters indicate significant differences (P < 0.05) between species.

Species	Leaf side	[CO ₂] (ppm)	θ_w (°)	θ_g (°)	θ_d (°)
<i>N. betuloides</i>	Adaxial	400	78.0 ± 8.3 aB	60.9 ± 6.8bB	56.9 ± 7.2bB
		800	65.9 ± 10.0bB	73.7 ± 9.0 aB	61.4 ± 9.9 aA
	Abaxial	400	82.6 ± 9.8 aB	85.2 ± 6.2bA	69.2 ± 5.3bA
		800	80.5 ± 6.5 aB	93.4 ± 6.6 aA	73.3 ± 4.8 aA
<i>N. antarctica</i>	Adaxial	400	88.7 ± 8.7 aA	78.8 ± 6.2bA	63.8 ± 4.8 aA
		800	75.1 ± 7.7bA	84.8 ± 5.7 aA	55.9 ± 7.1bB
	Abaxial	400	96.5 ± 6.3 aA	87.7 ± 5.2 aA	65.3 ± 4.8 aA
		800	93.7 ± 7.7 aA	86.6 ± 6.2 aB	67.9 ± 6.4 aB

with the increase of CO₂ in the adaxial leaf side (beam), but without significant differences ($P > 0.05$) for the abaxial surface (Table 1). Contact angles of leaf surfaces with glycerol were higher at the higher CO₂ level on both sides of the leaves for both species, except for the abaxial side of *N. antarctica* where no differences ($P > 0.05$) between CO₂ levels were observed. This increased contact angle with higher CO₂ concentration occurred with diodomethane on both sides of the *N. betuloides* leaves, but in *N. antarctica* there was an inverse trend with higher adaxial surface contact angles with the lower CO₂ concentration, while on the lower side no difference was found ($P > 0.05$) (Table 1).

When comparing the two species at control CO₂ levels (400 ppm), the total surface free energy and its components (dispersive and non-dispersive) were higher for *N. betuloides* (Table 2). By contrast, on the leaf lower side no differences were found between species for the surface free energy (Table 2). This pattern changed with the increase in atmospheric CO₂, because differences were determined between species and leaf side (Table 2). The total surface free energy increased with high level of CO₂ on the lower side of *N. betuloides* and *N. antarctica* upper side, while it did not vary on the other leaf sides in both species. The dispersive component (γ^{LW}) significantly decreased in *N. betuloides*, both on the upper and lower side of the leaf, while there was no variation of this parameter in *N. antarctica* leaves with different CO₂ levels. The non-dispersive component (or acid base, γ^{AB}) increased with increasing CO₂ in both species and leaf side combinations, except on the lower side of *N. antarctica*. Regarding the polarity of the surfaces (i.e., $\gamma_s^{AB} \gamma_s^{-1}$ expressed as %), a higher air CO₂ led to approximately 2 times higher values both for the upper and lower surfaces of *N. betuloides*, and induced a major increase only in the upper side of *N. antarctica* leaves (approximately x 6 higher values compared to 400 ppm grown plants). For a similar atmospheric CO₂ level, the solubility parameter (δ) between species showed the same pattern observed for the surface free energy (Table 2). Increased solubility parameter (δ) values were only estimated with high CO₂ for the lower side and upper side of *N. betuloides* and *N. antarctica*, respectively.

The concentration of soluble cuticular lipids (waxes) was higher ($P < 0.05$) in *N. betuloides* leaves at the two CO₂ levels evaluated compared to *N. antarctica* (Table 3). Regardless of the ambient CO₂ concentration, specific leaf area (SFA) values were always higher in *N. antarctica* ($P < 0.05$). The high CO₂ level generated a significant increase ($P < 0.05$) in leaf soluble cuticular lipids in the two species (Table 3), while SLA did not change with CO₂ concentration in any of the species analysed.

3.2. Stomatal density, structure and ultra-structure of the leaf epidermis and mesophyll

Stomatal densities were higher ($P < 0.05$) in *N. betuloides* than in *N. antarctica* leaves at 400 ppm CO₂, with no difference between species ($P > 0.05$) being recorded when plants were grown at 800 ppm CO₂. In general, all parameters related to stomatal anatomy in *N. betuloides* were higher at 400 ppm CO₂ compared to plants grown under enriched CO₂, while the opposite trend was recorded for *N. antarctica* leaves which had

Table 2

Total surface free energy (γ_s). Lifshitz van der Waals component (γ^{LW}), Acid-base component (γ^{AB}) with the contribution of electron donor (γ^-) and electron acceptor (γ^+) interactions, total surface free energy (γ_s) and polarity ($\gamma_s^{AB} \gamma_s^{-1}$) of *Nothofagus antarctica* and *N. betuloides* collected from plants grown at two levels of CO₂ (400 and 800 ppm). Values are mean \pm SD. For a same species and leaf side, lowercase letters indicate significant differences ($P < 0.05$) between CO₂ levels. For a same CO₂ level and leaf side, capital letters indicate significant differences ($P < 0.05$) between species.

Species	Leaf side	[CO ₂] (ppm)	γ^{LW} (mJ m ⁻²)	γ^{AB} (mJ m ⁻²)	γ_s (mJ m ⁻²)	Polarity (%)	Δ (MJ ^{1/2} m ^{-3/2})
<i>N. betuloides</i>	Adaxial	400	28.4 \pm 2.9 aA	7.1 \pm 0.6bA	35.5 \pm 3.6 aA	20.0 \pm 1.9 aA	18.0 \pm 1.9 aA
		800	20.6 \pm 2.7bA	14.5 \pm 1.9 aB	35.1 \pm 4.6 aB	41.3 \pm 5.4 aB	17.9 \pm 2.3 aB
	Abaxial	400	18.4 \pm 1.7 aB	9.5 \pm 1.0bA	27.9 \pm 2.5bA	34.1 \pm 3.3 bA	15.1 \pm 1.4bA
		800	14.7 \pm 1.0bB	34.1 \pm 2.4 aA	48.8 \pm 3.4 aA	69.9 \pm 4.9 aA	22.9 \pm 1.6 aA
<i>N. antarctica</i>	Adaxial	400	23.9 \pm 2.2 aB	2.4 \pm 0.2bB	26.3 \pm 2.4bB	9.1 \pm 0.8 bB	14.4 \pm 1.3bB
		800	23.3 \pm 2.4 aA	34.4 \pm 3.5 aA	57.7 \pm 5.8 aA	59.6 \pm 6.0 aA	26.0 \pm 2.7 aA
	Abaxial	400	23.3 \pm 1.4 aA	0.8 \pm 0.1 aB	24.1 \pm 1.4 aA	3.3 \pm 0.3 aA	13.5 \pm 0.8 aA
		800	21.5 \pm 1.7 aA	0.5 \pm 0.1bB	21.9 \pm 1.8 aB	2.3 \pm 0.3 aB	12.6 \pm 1.0 aB

Table 3

Soluble cuticular lipid (wax) concentrations and specific leaf area (SLA) of *N. antarctica* and *N. betuloides* collected from plants grown at two levels of CO₂ (400 y 800 ppm). Values are means \pm SD. For a same species, lower case letters indicate significant differences ($P < 0.05$) between CO₂ levels. For a same CO₂ level capital letters indicate significant differences ($P < 0.05$) between species.

Species	[CO ₂] (ppm)	[Wax] (μ g cm ⁻²)	SLA (m ² kg ⁻¹)
<i>N. betuloides</i>	400	131 \pm 15bA	11.8 \pm 1.3 aB
	800	172 \pm 18 aA	10.8 \pm 1.1 aB
<i>N. antarctica</i>	400	27 \pm 3bB	21.0 \pm 2.0 aA
	800	39 \pm 5 aB	22.8 \pm 2.1 aA

larger stomata and higher stomatal parameter values at the highest CO₂ concentration ($P < 0.05$) (Table 4). In the case of stomatal density, the variation in air CO₂ did not induce changes in *N. betuloides*. However, in *N. antarctica* leaves of plants grown under a CO₂ enriched environment, had a significantly higher stomatal density (Table 4). When comparing between CO₂ levels for the same species, *N. betuloides* plants had reduced guard cell and stomatal pore sizes in association with increased CO₂ (Table 4), while *N. antarctica* plants had bigger guard cells when grown at a higher atmospheric CO₂ level (Table 4).

The thickness of the leaf lamina, upper and lower epidermis and the percentage of spongy parenchyma were significantly higher in *N. betuloides* leaves at 400 ppm CO₂, while the percentage of palisade parenchyma was higher in *N. antarctica* (Table 5). In *N. betuloides* leaves, while the proportion of apoplastic space increased at a higher concentration of CO₂, the fraction of upper epidermis, palisade parenchyma and spongy parenchyma decreased (Table 5) (see Supplementary Material, Image 3). Other parameters such as the thickness of the lamina and epidermis (upper and lower) did not vary between levels of CO₂. In the case of *N. antarctica*, only the proportion of leaf apoplastic space increased with increasing CO₂ (see Supplementary Material, Image 4), while the thickness of lamina, thickness of lower epidermis, proportion of upper and lower epidermis and palisade parenchyma were reduced with the highest concentration of CO₂ in air (Table 5).

The cuticle thickness of the upper and the lower leaf epidermis were higher ($P < 0.05$) in *N. betuloides*, both at normal (400 ppm) and at increased CO₂ levels (800 ppm) compared with *N. antarctica*. In *N. betuloides* leaves, the high in atmospheric CO₂ level generated an increase in the thickness of the upper leaf cuticle and a decrease of the abaxial cuticle, while in *N. antarctica* no changes in cuticle thickness were detected between leaf sides (Table 6). Regarding to the area occupied by chloroplasts, no differences between species were found at 400 ppm CO₂ ($P > 0.05$). While in *N. betuloides* there were no changes in the area of chloroplasts in association with a higher CO₂ concentration, in *N. antarctica* the increase in CO₂ led to a greater area of chloroplasts (Table 6). In addition, leaf samples of *N. antarctica* and *N. betuloides* plants grown at the highest CO₂ concentration often showed chloroplast damage (see Supplementary Material, Images 5 and 6).

Table 4

Stomatal anatomy parameters of *Nothofagus antarctica* and *N. betuloides* collected from plants grown at two levels of CO₂ (400 y 800 ppm). Values are means ± SD. For the same species, lowercase letters indicate significant differences (P < 0.05) between CO₂ levels. For a same CO₂ level, capital letters indicate significant differences (P < 0.05) between species.

Species	[CO ₂] (ppm)	Stomatal density (stomata mm ⁻²)	GCL (μm)	GCW (μm)	GCA (μm ²)	SPL (μm)	SPW (μm)	SPA (μm ²)	PCI	RSPA
<i>N. betuloides</i>	400	256 ± 64 aA	27±2 aA	19±1 aA	400 ± 40 aA	12±2 aA	6±1 aA	57 ± 13 aA	19±7 aA	1.5 ± 0.6 aA
	800	222 ± 56 aA	16±2bB	9±1bB	115 ± 17bB	6±1bB	3±1bB	16±4bB	6±2bB	0.4 ± 0.1bB
<i>N. antarctica</i>	400	124 ± 25bB	17±1bB	12±1bB	155 ± 22bB	9±1bB	4±1bB	29±7bB	4±1bB	0.4 ± 0.1bB
	800	233 ± 97 aA	28±2 aA	20±1 aA	440 ± 52 aA	14±2 aA	7±1 aA	76 ± 23 aA	19 ± 10 aA	1.8 ± 1.0 aA

Abbreviations indicate: GCL: guard cell length; GCW: guard cell width; GCA: guard cell area; SPL: stomata pore length; SPW: stomata pore width; SPA: stomata pore area; PCI: potential conductance index; RSPA: relative stomata pore area.

Table 5

Size and proportion of the different leaf tissues of *Nothofagus antarctica* and *N. betuloides* collected from plants growing at two levels of CO₂ (400 y 800 ppm). Values are mean ± SD. For the same species, lowercase letters indicate significant differences (P < 0.05) between CO₂ levels. For a same CO₂ level, capital letters indicate significant differences (P < 0.05) between species. Abbreviations indicate: LT: lamina thickness; UET: upper epidermis thickness; LET: lower epidermis thickness; UE: upper epidermis; LE: lower epidermis; PP: palisade parenchyma; SP: spongy parenchyma; ES: empty spaces.

Species	[CO ₂] (ppm)	LT (μm)	UET (μm)	LET (μm)	UE (%)	LE (%)	PP (%)	SP (%)	ES (%)
<i>N. betuloides</i>	400	288±8 aA	33±7 aA	23±7 aA	11±1 aA	8±2bA	18±3 aB	22±4 aA	42±6bB
	800	294±7 aA	32±5 aA	26±5 aA	9±1bA	7±1bA	13±5bB	14±6bB	56±8 aA
<i>N. antarctica</i>	400	186±9 aB	24±4 aB	19±4 aB	12±2 aA	10±2 aA	23±4 aA	14±4bB	41±5bB
	800	165 ± 11bB	20±3 aB	13±2bB	10±3bA	8±2bA	18±7bA	15±5bB	49±8 aB

Table 6

Leaf cuticle thickness and chloroplast area of *N. antarctica* and *N. betuloides* grown at 400 or 800 ppm CO₂. Values are means ± SD. For a same species, lowercase letters indicate significant differences (P < 0.05) between CO₂ levels. For a same CO₂ level, capital letters indicate significant differences (P < 0.05) between species.

Species	[CO ₂] (ppm)	Adaxial cuticle thickness (μm)	Abaxial cuticle thickness (μm)	Chloroplast area (μm ²)
<i>N. betuloides</i>	400	1.07 ± 0.09bA	1.12 ± 0.18 aA	7.6 ± 3.2 aB
	800	1.7 ± 0.31 aA	0.95 ± 0.04bA	6.6 ± 2.7 aB
<i>N. antarctica</i>	400	0.26 ± 0.02 aB	0.29 ± 0.12 aB	7.5 ± 3.2bB
	800	0.28 ± 0.04 aB	0.28 ± 0.06 aB	12.2 ± 5.5 aA

4. Discussion

In this investigation, we evaluated the effect of ambient CO₂ concentration on the foliar wettability and anatomy of a deciduous (*N. antarctica*) and an evergreen (*N. betuloides*) species, being the first study of such nature performed with native forest species of Patagonia. The results obtained are relevant because leaf surfaces play a crucial role in protecting plants against various biotic and abiotic stress factors (Riederer, 2006) and this will be key for survival under the harsh growing conditions of Patagonia. In addition, the ecophysiological importance of foliar water uptake has been recognised as an important factor for plant water economy in different natural ecosystems worldwide (Dawson and Goldsmith, 2018; Berry et al., 2014; Binks et al., 2019; Guzmán-Delgado et al., 2016). The wettability of leaf surfaces and the possible adherence or repellence of water drops from rain, dew or fog may facilitate or impede the foliar water absorption process (Fernández et al., 2017; Bahamonde et al., 2018). In our study, it was observed that the leaf surfaces of the *N. antarctica* leaves generally had higher water contact angles (i.e., they are less wettable) than those of *N. betuloides*. This implies that *N. betuloides* leaves are prone to get wet and retain water on their surface. The values obtained for the adaxial leaf side were similar to those found in other surfaces with low roughness such as the olive (Fernández et al., 2017). The slightly higher angles measured for *N. antarctica* leaves are in a similar range to those obtained for the *Ficus elastica* upper side (Guzmán et al., 2014). In the case of both *Nothofagus* species, the lower side is somewhat more wettable than for

example, *Fagus sylvatica* leaves (Bahamonde et al., 2018).

Despite the mentioned differences, both species presented values considered in the range of wettable surfaces (<90°), with the exception of the lower side of the *N. antarctica* leaf which is over 90° (Fernández et al., 2017). Working with Northern Patagonian spp. (Argentina), Brewer and Nunez (2007) observed that the leaves of *N. dombeyi* had water contact angles of 50 and 61° in its upper and lower side, respectively. In this same study, Brewer and Nunez (2007) measured the water contact angles of 37 species growing from the steppe to Andean forest, and found that species growing in more arid places had higher water contact angles. This would be consistent with the higher values of water contact angles measured for *N. antarctica* in our study. However, it should be noted that the information published by these authors was gathered from plants growing under field conditions, unlike our data which stem from *Nothofagus* seedlings grown under controlled conditions.

The incidence of environmental factors on the interaction between leaf surfaces and different liquids was detected at an intra-specific level (Bahamonde et al., 2018). Values of total surface free energy calculated for the upper and lower leaf side of the *N. antarctica* and *N. betuloides* leaves, were within the average range of 23–35 mJ m⁻² estimated for synthetic paraffin surfaces with low roughness (Jańczuk and Białopiotrowicz, 1988) and leaves with rather smooth surfaces (Fernández et al., 2017; 2021). The solubility parameter values obtained for *N. betuloides* leaves are within the theoretical range described for epicuticular waxes (around 16–17 MJ^{1/2} m^{-3/2}, Khayet and Fernández, 2012). The abaxial (lower side) surfaces of the *N. antarctica* leaves showed lower values due to the effect of roughness conferred by the cuticular folds (see Supplementary Material, image 7), minor venation (extensions of the upper side sheath) and probably due to the presence of non-glandular trichomes (Supplementary Material 1, Image 8). The presence of trichomes and pubescence influence leaf wetting and drop adherence or repellence of water droplets (Brewer et al., 1991; Bickford, 2016). It is remarkable the increase in total surface free energy and solubility parameter of the *N. betuloides* lower leaf surface of plants grown at 400 ppm CO₂ and of the *N. antarctica* upper leaf side of seedlings grown at 800 ppm CO₂, due to the increase in the surface free energy polar component (γ^{AB}). This suggests the presence of polar material on both surfaces, which may be associated with the more pronounced topography of upper side bundle

sheath extensions in both species, and/or the presence of trichomes irregularly covered with lipids, being non-glandular in the *N. antarctica* and glandular in *N. betuloides*. The occurrence of nano-scale polar areas in the surface of a plant organ has been recently shown by atomic force microscopy (Almonte et al., 2022) and future studies should be carried out for characterising the chemical heterogeneity of plant surfaces because they may ultimately influence wettability and foliar permeability processes.

5. Effect of ambient CO₂ concentration on leaf cuticular lipids, SLA and stomatal density

The presence of soluble cuticular lipids (waxes) is crucial for preserving the water balance of plants, as it is considered as the main barrier for cuticular transpiration (Riederer and Schreiber, 2001) and foliar permeability to water and other substances (e.g., hydrophilic or lipophilic chemicals) (Schreiber and Schönherr, 2009; Fernández et al., 2021). In the present study, *N. betuloides* was found to have a significantly higher concentration of waxes and a greater cuticle thickness than *N. antarctica*, at both CO₂ levels. There are no data on these parameters measured in other *Nothofagus* species, but the values estimated for *N. antarctica* were similar to those reported for a northern hemisphere Fagaceae (i.e., *Fagus sylvatica*) (Bahamonde et al., 2018).

Considering the potential barrier effect of such cuticular waxes for water permeability, it can be hypothesised that owing to their lower wax amounts the leaves of *N. antarctica* are more likely to absorb water than those of *N. betuloides*. Furthermore, when attempting to enzymatically isolate *N. antarctica* leaves in 2% cellulose and 2% pectinate (Guzmán et al., 2014), leaves disintegrated after one day in the extraction medium suggesting their high permeability rate which enables the easy access of polysaccharide degrading enzymes to target areas in the leaf surface (data not shown). In both species, the increased wax concentrations under elevated CO₂ conditions provides evidence for the effect of this environmental factor on cuticular structure and composition. Although there are no other studies relating cuticular lipid amounts to different levels of CO₂ in the air, it has been shown that environmental factors such as radiation, temperature and humidity can significantly modify this leaf cuticle (e.g., Bahamonde et al., 2018; Shepherd and Wynne Griffiths, 2006). The higher SLA observed in *N. antarctica* was maintained at both CO₂ levels. In general terms, several studies reported higher values of SLA in deciduous than evergreen woody species from a same genus (Escudero et al., 2017; Onoda et al., 2017; Sancho-Knapik et al., 2021).

The lower SLA values of *N. betuloides* determined in this study are consistent with those reported by Calabria and Puntieri (2008) for evergreen *Nothofagus dombeyi* grown in Patagonia. Specific leaf area differences between deciduous and evergreen species have been associated with leaf cell parameters (Onoda et al., 2017; Sancho Knapik et al., 2021), which will be further discussed further in the section below.

Contrary to other studies reporting SLA decreases in *Betula pendula* leaves (Oksanen et al., 2005) or increases (in several forestry species, Norby et al., 1999) with increasing CO₂ air concentration, in our study the increase in CO₂ did not induce SLA modifications in any of the species analysed. At current atmospheric CO₂ concentrations (400 ppm) *N. betuloides* showed advantageous stomatal characteristics (number and size of stomata) compared to *N. antarctica*, which resulted in higher values of the potential conductance index. This would imply a higher photosynthetic rate in *N. betuloides* as reported for other species (Xu and Zhou, 2008; Van Wittenberghe et al., 2012), mainly due to an increase in stomatal conductance (Maherali et al., 2002). However, we must be careful with this assessment, because other key factors may limit photosynthesis (biochemical or mesophyll conductance), in addition to stomatal conductance (Peguero-Pina et al., 2017). In this regard, different authors suggested that mesophyll conductance may be the main constraint for photosynthesis as shown for different Mediterranean

(Galmés et al., 2007) and Antarctic plant species (Sáez et al., 2017).

Growing under an elevated CO₂ air concentration, raised the leaf stomatal density of *N. antarctica*, but induced no changes in *N. betuloides* plants. In prior studies, increases (Marchi et al., 2004), decreases (Sekiya and Yano, 2008) or no variations (Ogaya et al., 2011) on leaf stomatal densities in response to increased CO₂ concentrations have been reported. In a recent review, Klein and Ramon (2019) found lower values of stomatal conductance in some perennials in relation to increased CO₂. Furthermore, it has been shown that stomata cell formation and size is strongly influenced by hormonal modifications (Caspar et al., 2014) or genes (Bergmann and Sack, 2007). Our results suggest that in *N. betuloides* leaf genetics may control stomata formation rather than the effect of increasing ambient CO₂, but this hypothesis should be evaluated in future studies.

6. Structure and ultra-structure of the leaf epidermis and mesophyll

Absolute values related to leaf tissues parameters (lamina thickness, adaxial and abaxial epidermis thickness) were higher in *N. betuloides* under 400 ppm CO₂ conditions. This would explain the higher SLA values of the deciduous species (*N. antarctica*) as mentioned above. Higher values of lamina and epidermis thickness have been associated especially with evergreen species living in environments with short growing season periods (Alonso-Forn et al., 2020; Sancho-Knapik et al., 2021), as it would be in our case due to prevailing cold conditions during most of the year in Patagonia. However, when these parameters were calculated in relative terms, the percentages tended to be higher in *N. antarctica*. Higher values of lamina and parenchyma thickness have been related to higher photosynthetic rates in different species (Niinemets et al., 2011). In our case, although the size of chloroplasts did not vary between species when grown at 400 ppm CO₂, it is expected that, having a larger lamina and parenchyma area, *N. betuloides* should contain a greater number of chloroplasts, which could imply higher photosynthetic rates for this species. This in agreement with the density and size of stomata determined for *N. betuloides* leaves as discussed above. Nevertheless, as previously mentioned, other limitations to photosynthesis (Galmés et al., 2007; Sáez et al., 2017) must be considered for a better understanding of the implications of these anatomical differences.

On the other hand, the effect of increased CO₂ concentration on leaf anatomical parameters has been studied for various plant species including trees, shrubs, grasses and legumes (Oksanen et al., 2005; Han et al., 2007; Tipping and Murray, 2011). However, no such records have been found for South American *Nothofagus*. In this study, a higher CO₂ concentration resulted in a decrease in leaf lamina, epidermis and palisade parenchyma in *N. antarctica*. While in *N. betuloides* the absolute values did not change significantly, the percentages of upper epidermis, palisade parenchyma and spongy parenchyma decreased with increasing air CO₂. In other words, in both species there was a decrease in leaf anatomical tissues, and consequently an increase in intercellular spaces, but this effect was more pronounced in *N. antarctica*. Although there is uncertainty about the mechanisms inducing the observed leaf tissue decrease with increased CO₂, some reports showed that increased ambient CO₂ modified the rates of cell division and growth in different parts of plants (Kinsman et al., 1997; Masle, 2000). Related to this, there is evidence that the consequent increase in inter-cellular spaces involves an increase in mesophyll conductance to CO₂ and photosynthesis (Scarfaro et al., 2011; Niinemets et al., 2011). On the other hand, a larger inter-cellular space may raise the susceptibility to frost damage (Beck et al., 2007; Wyka and Oleksyn, 2014). In our case this could be more critical for *N. betuloides*, which is an evergreen species having leaves also during winter season. Additionally, this increased risk of frost damage would be paradoxically enhanced by the predictable temperature increases in Patagonia (Pabón-Caicedo et al., 2020). This would happen because an increase in temperature could stimulate the early breaking of

bud dormancy, hence exposing leaves and flowers to spring frost damage (Cannell and Smith, 1986; Gu et al., 2008).

Although these leaf tissue decreases were statistically significant, they did not lead to an increase in SLA values with increasing CO₂ as previously discussed. For other species increases (Conroy et al., 1986; Radoglou and Jarvis, 1990; Pritchard et al., 1999; Oksanen et al., 2005) and decreases (Pushnik et al., 1995) in different leaf tissues with CO₂ enrichment have been reported. The increase in leaf tissues with higher CO₂ concentration may be due to the stimulation of the enzymatic activity of xyloglucan-endotransglycosylase, an enzyme closely related to cell wall expansion (Ranasinghe and Taylor, 1996). In our study, the mechanisms explaining a reduction in leaf tissues due to increased ambient CO₂ concentration cannot be ascertained and will require further investigation. The increase in chloroplast size due to the high CO₂ concentration found in *N. antarctica* has also been measured in *Betula pendula* leaves (Oksanen et al., 2005). Enlargement and damage of chloroplasts due to elevated CO₂ concentrations have been related to starch accumulation in these structures (Prior et al., 2004; Wang et al., 2004) and in some cases, due to swelling of the thylakoid membranes (Utriainen et al., 2000; Velikova et al., 2009). However, we failed to observe chloroplast starch grains in the obtained TEM images.

7. Conclusions

In this study, the effect of growing an evergreen (*N. betuloides*) and a deciduous (*N. antarctica*) species under elevated versus standard air CO₂ concentrations was evaluated on various leaf traits. Excepting for the abaxial side of *Nothofagus* when in contact with water drops, the remaining leaf surfaces analysed were wettable for the three different liquids measured. Growing at increased ambient CO₂ concentrations raised the polarity of all the leaves surfaces examined, excepting the lower side of *N. antarctica* which remained unaffected. Although *N. betuloides* was more wettable than *N. antarctica*, its high concentration of epicuticular waxes may yield it less permeable to water compared with *N. antarctica* which was also found to degrade shortly after immersion in a cellulose and pectinase enzymatic solution. However, more specific trials should be carried out to evaluate water permeability of such leaf surfaces of under field and laboratory conditions.

Exposure to a higher CO₂ concentration, led to different epidermal and mesophyll modifications which often varied upon species. When grown under elevated CO₂ conditions, the susceptibility to foliar changes of *N. antarctica* was not higher than that of *N. betuloides*. The high CO₂ concentration treatment evaluated corresponds to approximately the double concentration currently found in the earth's atmosphere which despite triggering leaf changes, only induced specific leaf damage (e.g., on chloroplast). Hence, it can be reckoned that a gradual increase in atmospheric CO₂ concentration may not involve a significant loss of leaf functional capacity which may severely compromise the growth and survival of these *Nothofagus* species. However, further trials should be carried out for further assessing the effect of elevated CO₂ on *Nothofagus* and forest species, also in combination with higher frequency of extreme climatic events climate as stress factors, such as water shortage and/or high temperature.

Author contributions

HB, PLP and IA designed the study. HA and IA carried out the experimental set up and greenhouse trial. HB and VF developed the laboratory experiments. HB, PLP, JG, IA and VF analysed and interpreted the results. The draft was largely written by HB with the collaboration of VF. All the authors contributed to improving the manuscript and approved its final version.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2022.11.020>.

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