Stored water in the inner bark and sapwood: Atypical patterns of daily discharge and recharge, radial osmotic gradient and freezing resistance

Sandra J. Bucci[1], Luisina Carbonell Silletta[2], Agustin Cavallaro[2], Nadia S. Arias[3], Paula Campanello[4], Guillermo Goldstein[5], and Fabian G. Scholz[6]

[1]Grupo de Estudios Biofisicos y Ecofisiologicos (GEBEF), Instituto de Biociencias de la Patagonia (INBIOP, CONICET - UNPSJB)
[2]Grupo de Estudios Biofisicos y Ecofisiiologicos (GEBEF), Instituto de Biociencias de la Patagonia (INBIOP, CONICET - UNPSJB)
[3]Universidad Nacional de la Patagonia San Juan Bosco
[4]Universidad Nacional de la Patagonia San Juan Bosco - Sede Esquel
[5]Universidad de Buenos Aires
[6]CONICET

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Abstract

Stored water in inner tissues can affect plant water balance and its freezing resistance. We studied the water storages in the inner bark and sapwood of Araucaria araucana, a species with thick inner bark. Specifically, we analyzed its daily behavior, the driving force to radial water movement and its freezing resistance. The whole-stem water content and diameter and sap flow increased in the morning and decreased in the afternoon. An osmotic gradient between stem tissues was involved in the morning water storage recharge. There were no lags in the onset of sap flow between different stem positions, however sap flow at 6m height was higher than basal sap flow in the afternoon, at the time that sapwood water content started to decline followed by the inner bark. Extracellular freezing was delayed down to -6@C in the inner bark and to -8@C in the leaves. The unusual diurnal pattern of internal water use may enhance freezing resistance as a consequence of the lower water content and higher osmotic potential when the lowest temperatures occur. The contribution of stem tissues to daily water use and the pattern of ice nucleation observed make this species less susceptible to drought and very low temperatures.

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Bucci Sandra Janet[1,2]; Carbonell-Silletta Luisina[1,2]; Cavallaro Agustín[1,2]; Arias Nadia Soledad[1,2]; Campanello Paula Inés[3]; Goldstein Guillermo[4,5]; Scholz Fabián Gustavo[1,2]

[1]Grupo de Estudios Biofísicos y Ecofisiológicos (GEBEF), Instituto de Biociencias de la Patagonia (INBIOP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional de la Patagonia San Juan Bosco (UNPSJB), (9000) Comodoro Rivadavia, Argentina

[2]Facultad de Ciencias Naturales y Ciencias de la Salud, Universidad Nacional de la Patagonia San Juan Bosco (UNPSJB), (9000) Comodoro Rivadavia, Argentina

[3]CONICET and Departamento de Ingeniería Forestal, Facultad de Ingeniería, UNPSJB, Esquel, Argentina

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[4]Instituto de Ecología, Genética y Evolución de Buenos Aires. UBA-CONICET, Buenos Aires, Argentina

[5]University of Miami, Coral Gables, Florida, USA

Author to correspondence: Sandra J. Bucci

Sj_bucci@yahoo.com

Abstract

Stored water in inner tissues can affect plant water balance and its freezing resistance. We studied the water storages in the inner bark and sapwood of *Araucaria araucana* , a species with thick inner bark. Specifically, we analyzed its daily behavior, the driving force to radial water movement and its freezing resistance. The whole-stem water content and diameter and sap flow increased in the morning and decreased in the afternoon. An osmotic gradient between stem tissues was involved in the morning water storage recharge. There were no lags in the onset of sap flow between different stem positions, however sap flow at 6m height was higher than basal sap flow in the afternoon, at the time that sapwood water content started to decline followed by the inner bark. Extracellular freezing was delayed down to -6@C in the inner bark and to -8@C in the leaves. The unusual diurnal pattern of internal water use may enhance freezing resistance as a consequence of the lower water content and higher osmotic potential when the lowest temperatures occur. The contribution of stem tissues to daily water use and the pattern of ice nucleation observed make this species less susceptible to drought and very low temperatures.

Key words: Capacitance, ice nucleation temperature, osmotic potential, radial water flow, sap flow, stem contraction and expansion, thermal lethal damage.

Introduction

Internal water storage in trees, particularly in the main stem, is known to have an important role in the plant water balance by acting as an intermediate pool of water between soil and transpiring leaves (e.g., Blackman et al., 2016; Christoffersen et al., 2016; Gleason, Blackman, Cook, Laws, & Westoby, 2014; Goldstein et al., 1998; Goldstein, Meinzer, & Monasterio, 1984; Meinzer, Goldstein, & Andrade, 2001; Scholz et al., 2008).The internal water storage helps to relax the xylem tension during the period of maximum leaf water demand and can contribute with 10 to 50% of the daily water use (Goldstein et al., 1998; Holbrook & Sinclair, 1992; Loustau et al., 1996; Scholz, Phillips, Bucci, Meinzer, & Goldstein, 2011; Scholz et al., 2007, 2008; Steppe & Lemeur, 2004). Lags between crown transpiration and basal sap flow have been used to estimate whole-plant water storage (Goldstein et al., 1998; Scholz, Bucci, & Goldstein, 2014; Scholz et al., 2008) although there are controversies related to this method (Burgess & Dawson, 2008; Phillips et al., 2009) due to the absence of lags in some species (Burgess & Dawson, 2008; Chapotin et al., 2006; Kume et al., 2008; Pfautsch et al., 2011). Changes in stem diameter (Čermák, Kučera, Bauerle, Phillips, & Hinckley, 2007; Scholz et al., 2008; Zweifel, Item, & Häsler, 2001) and in stem water content (Hao, James, Holbrook, & Goldstein, 2013; Oliva Carrasco et al., 2014) have also been used to estimate the discharge and recharge of the internal storages. Stem tissues shrinking generally occurs in the morning as water in the storage tissue is exported to the leaves to supply the transpiration demand and subsequent nocturnal expansion and water recharge takes place when transpiration is minimal (Daudet, Améglio, Cochard, Archilla, & Lacointe, 2005; Scholz et al., 2008; Steppe, De Pauw, Lemeur, & Vanrolleghem, 2006; Zweifel et al., 2001). However, in some species a different diel pattern in the expansion and contraction of the stem diameter related to osmotic gradients or changes in environmental conditions has been observed (Vandegehuchte et al., 2014).

Stems can store water within the sapwood, cell walls and in inactive vessels of the xylem as well as in the elastic tissue of the bark (Pfautsch, Hölttä, & Mencuccini, 2015; Scholz et al., 2007; Zweifel, Item, & Häsler, 2000). Bark remained less studied from the point of view of its contribution to plant water balance compared to other tree tissues. Even though it is known that the inner bark can supply water to the transpiration stream through radial water movement, the small thickness of this tissues make it difficult to experimentally assess its contribution to the tree water economy. In general, water reservoirs in sapwood and inner bark have been studied as independent systems without hydraulic interactions, with some exceptions (e.g., Hölttä,

Vesala, Sevanto, Perämäki, & Nikinmaa, 2006; Hölttä, Cochard, Nikinmaa, & Mencuccini, 2009; Pfautsch, Hölttä, & Mencuccini, 2015; Sevanto, Hölttä, & Holbrook, 2011; Sevanto, Mcdowell, Dickman, Pangle, & Pockman, 2014). Small-scale mechanisms of water movement between internal water reservoirs and the long distance water transport system have been studied taking into account the amount and distribution of water, osmotic adjustments and changes in cell turgor in stem tissues (Borchert & Pockman, 2005; Pfautsch, Renard, Tjoelker, & Salih, 2015; Scholz et al., 2008; Steppe, Cochard, Lacointe, & Améglio, 2012). However, these studies provides relatively little information in terms of the total amount and the pattern of water moving from the inner bark to the sapwood and then to the transpiring leaves. In general, the studies on internal water reservoirs reflect the water behavior of the whole-stem tissues. Some authors have evaluated indirectly the water recharge and discharge of the inner bark monitoring whole-stem and sapwood diameter or water content fluctuations (di Francescantonio et al., 2018; Scholz et al., 2008; Sevanto et al., 2011). An unusually thick inner bark of up to 20 cm in the main stem of the conifer *Araucaria araucana* (Burns, 1993; Montaldo, 1974; Veblen, 1982) can be used as a model system to assess experimentally the role that living bark tissues can play in the water economy of trees. Thus, the thickness of the tissues in the stem of *A. araucana* can provide an ideal opportunity to study in vivo the daily dynamic of water stored in the inner bark and sapwood and their interactive pattern of water flow.

Water stored and the consequent changes in water potentials in the tissues not only affect the plant responses to changes in soil and atmospheric water availability but also to internal freezing temperatures (Cavender-Bares, 2005; Goldstein et al., 1984). Bark is involved in mechanical, defenses and physiological functions but a less known role of bark in the plant functioning it is the insulation capacity to mitigate the damaging effects of air subzero temperatures on more internal tissues (Arco Molina et al., 2016; Burke et al., 1976). The capacity of thermal insulation of the bark is derived from its morphology and composition (De Antonio et al., 2020; Jönsson et al., 2001). A thick layer of bark with a large amount of water with low thermal conductivity and high heat capacity surrounding the cambium and parenchyma cells within the sapwood can help to prevent freezing in more sensitive tissues. Dimitri (1968) studied the thermal conductivity of bark in beech and found that the most important factor to freezing resistance is the moisture content. Although under field conditions air temperature is often considered as a proxy of tissue temperature, tissues can be buffered of low temperatures due to thermal inertia and insulating layers such as bark (Charrier et al., 2017). Therefore, stem could exhibit a radial variation of temperature. A thick bark can delay the change of temperature in the vascular tissue or decrease the duration of unfavorable temperatures (Lawes et al., 2011), thus avoiding the ice nucleation and ice propagation to other plant tissues such as leaves.

Araucaria araucana (Molina) Koch (Araucariaceae) is an evergreen tree native from Southern Andean forests in Chile and Argentina. The physiological behavior and particularly the mechanisms to cope with summer drought and late sub-zero temperature events is scarcely known for this species. In the last years the crown of several araucaria trees across their range of distribution had exhibited partial desiccation; however there is no consensus about the causes. Several hypotheses about the leading causes to this phenomenon, including drought, were mentioned (Cifuentes et al., 2020). Mundo, Roig Juñent, Villalba, Kitzberger, & Barrera (2012) found a positive association between ring width and precipitation during spring and summer in the past millennium, suggesting high sensitivity of *A. araucana* to water availability. On the other hand, dendroecological studies have detected damage in the early-wood portion of the growth rings of *A. araucana* caused by subzero temperatures occurring during the growing season and a decrease of the injury with increasing bark thickness (Arco Molina et al., 2016; Hadad et al., 2019). While predictable frost events occurring during winter generally are not critical for plants as cold hardening provide resistance against freezing injury, sub-zero temperature during the growing season could result in severe frost damage (Körner, 2003). Consistent with this, the frost rings are more frequently observed during drier years (Hadad et al., 2019). According to climate change prediction, higher temperatures and less precipitation are expected for the temperate Andean forests (Barros et al., 2015). Thus, this species could be expose to higher water deficit during the dry season and to higher freezing damage as the growing season could start earlier. The main objective of this research was to study the stem water reservoirs de *A. araucana* distinguishing the behavior of the inner bark and sapwood. Specifically we analyzed the dynamics of water storage discharge

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and recharge in the inner bark and the sapwood of the main stem. Moreover, we determined the daily changes of the osmotic potential and the contribution of both tissues to daily tree water use. Finally we studied the how sub-zero temperatures affect the freezing resistance of water storages compartments in different tissues (inner bark, sapwood and leaves) during the active growth season (spring). We hypothesized that the inner bark and sapwood contributes with water to the transpiration stream during the daytime when leaf water demand increase. Thus, we expect that inner bark and sapwood water content and diameter will decrease when sap flow begins to increase abruptly in the morning. In addition, we hypothesized that the thick inner bark protects the xylem from freezing, and predicted that ice nucleation will occur at higher temperature in the inner bark than in the sapwood and leaves. The thickness of inner bark and sapwood is large enough to accommodate available sensors to estimate non-destructible fine temporal scale of diurnal variations in water content and other physiological traits. Thus, we installed sensors of volumetric water content in both stem tissues, electronic dendrometer on inner bark and sapwood and sap flow probes a two different height in the main stem. In addition, we determined the diurnal pattern of osmotic potential gradient between both tissues as the driven force for radial water movement. The ice nucleation temperature measured through thermal analysis and lethal temperatures were determined in stem tissues and leaves to evaluate the freezing resistance of tree tissues.

Materials and Methods

Study site and species

This study was carried out in a forest stand of Los Alerces National Park (PNA) in the east flanks of the Andean range, southern Argentina (42°50' 40.3"S and 71deg50'17.4"W). Mean annual temperature is 8°C. Precipitation falls mainly from April to October and decrease abruptly from west to east, from 3000 to 800 mm (APN, 1997). Snow falls from June to September. The vegetation is a temperate forest dominated by the conifers *Austrocedrus chilensis* (D. Don) Pic. Serm. & Bizzarri and *Fitzroya cupressoides* (Molina) I.M. Johnst, and the beech species, *Nothofagus dombeyi* (Mirb.) Oerst., *Nothofagus pumilio* (Poepp. & Endl.) Krasser and *Nothofagus Antarctica* (G. Forst.) Oerst.. The native bamboo *Chusquea culeou* E. Desv. and shrubs species dominate the understory.

For this study we selected five canopy trees planted more than 60 years ago in the PNA near the Chucao small port in the Menendez Lake coast. *Araucaria araucana* is a millenary conifer distributed in the temperate Sub-antarctic forests of Chile and Argentina between 37deg20' and 40deg20' S covering an area of 450000 ha. It does not occur naturally in the study site (42deg50' S). In Argentina this species grows along a wide ecological range from the wet Andes foothills with 2000 mm year^{-1} to the ecotone of the forest-patagonian steppe in Neuquen Province with 500 mm year^{-1}, between 600-1800 m elevation (Roig & Villalba, 2008). *Araucaria araucana* grows in monospecific or mixed forests stands mainly with *N. pumilio and N. antarctica* (Veblen, 1982) under a cold temperate climate with winter temperatures dropping to -5 and -10°C (Goth et al., 2014) and frost occurring until the end of the spring. This species has a straight and cylindrical bole with regular horizontal whorls of branches. Its leaves are very coriaceous, pointed and overlap along branches. The crown is restricted to the top of the tree due to the abscission of lower branches which give it the typical shape of an umbrella. *Araucaria araucana* has a very thick bark with cracks on its surface forming regular polygons. Although *A. araucana* is a tree species of local economic (Rechene et al., 2003), cultural (Sedrez dos Reis et al., 2014) and conservation importance (Goth et al., 2014) and their ecology is relatively well known (Burns, 1993; Sanguinetti & Kitzberger, 2009; Schmidt, 1977; Veblen, 1982) but their physiology is poorly understood.

Five canopy trees of *A. araucana* were selected in the PNA. The water relations study was done from the end of January to the middle of February (summer) 2017. The thermal analysis and freezing tissue damage determinations were performed at the end of the spring season (December 2019).

Environmental variables

Air temperature and relative humidity were monitored with HOBO Pro series sensors (Onset Computer Corporation, Pocasset, MA, USA). Air saturation deficit was calculated using relative humidity and air

temperature measurements.

Sap flow

Sap flow was measured with the heat-dissipation method (Granier, 1987) installed in the sapwood near the base of the main stem (0.5 m height) and at 6 m height on the main stem in two individuals of approximately 80 cm diameter during January of 2017. The upper (downstream) probe was continuously heated at a constant power by the Joule effect, while the unheated upstream probe served as a temperature reference. Both probes were installed in the same azimuthal position and connected to a data logger (CR 1000, Campbell Scientific, USA). The temperature differences between the upstream and downstream probes were recorded every 10 sec and 10 min averages were stored in the data logger. Sap flux density was calculated from the temperature difference between the two probes using an empirical calibration (Granier, 1987). Mass flow per tree was obtained by multiplying flux density by the active sapwood cross-sectional area. The time course differences between the 6 m height sap flow and the basal sap flow were used to estimate periods of discharge (positive values) and recharge (negative values) of the water storage tissue (Goldstein et al., 1998a).

Volumetric stem water content

We used FDR technology (Model GS3, Decagon Devices, Inc., Pullman, WA, USA) to monitor changes in volumetric water content. Moisture sensors were installed at the base of the main stem and at 6 m height, before the main stem bifurcation, during the dry season. Two sensors per tree were installed inside the sapwood and two inside the inner bark. We were able to install these sensors directly into the inner bark due to the substantial bark thickness (Table 1 and Fig. 1). The sensors were connected to an EM50 data logger (Decagon Devices, Inc.) and the data were stored every 10 min. We plotted the relative changes in volumetric water content (VWC, m^3 m^{-3}) for each tissue as:

Changes in VWC_i = VWC_i–VCW_o

Where VCW_o is the VWC at 0:00 h the next day after the sensor installation and VCW_i is the VCW at different time intervals during the daytime.

We also determined the total water content (m^3) of the sapwood and the inner bark in a stem segment of 6 m length (between the base of the trunk and the height at which sap flow and VWC sensors were installed), by using the following equation:

Tissue water content (m^3) = VWC ($m^3 m^{-3}$) x 6 (m) x A (m^2)

Where A is the tissue area assuming that the stem section was a cylinder with a diameter equal to the mean diameter between the base and at 6 m in height. The VWC used was the average value between the heights at which the sensors were installed. Inner bark area was determined by the total stem area without taken into account the rhytidome and the sapwood area.

Net changes of stem, inner bark or sapwood water content ($l\ d^{-1}$) were determined as the difference between the water content at 24:00 h and the water content at 24:00 h of the following day. Negative values of net changes in tissue water content indicate net discharge and positive values indicate recharge of the main stem water storage. These values were compared to the differences between sap flow at 6 m height and the sap flow at the base of the stem measured with heat dissipation sensors.

Stem diameter changes

Daily variations in sapwood and inner bark were continuously determined with point electronic dendrometers which have very low temperature sensitivity (0.27 μm °C^{-1}) (ZN11-T-WP; Zweifel Consulting, Hombrechtikon, Switzerland). The electronic displacement-sensor (linear motion potentiometer) was in contact with the sapwood or the inner bark and consequently diurnal radius variations reflected changes in the sapwood and inner bark + sapwood respectively. To install the dendrometer in contact with the inner bark only

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a small portion of the outer bark was removed. For installing the dendrometer in contact with the sapwood, the whole bark (inner and outer) of a stem portion was removed (Fig. 1). The dendrometers were installed near the base of the main stem of a tree and connected to a CR10X data logger (Campbell Scientific). Data were obtained every 10 s, and 10-min averages were recorded with a data logger (CR10X, Campbell Scientific). Changes in diameter measurements were converted to areas. The changes of the inner bark area were calculated as the changes in the total area (inner bark + sapwood) determined with the dendrometer in contact with the inner bark minus the changes in the sapwood area determined with the dendrometer in contact with the sapwood.

Water and osmotic potential

Predawn and minimum leaf water potentials were measured with a pressure chamber (PMS; Albany, Oregon) during January 2017. Minimum leaf water potential was determined approximately at 15 h when air saturation deficit reach its maximum value. Leaf water potentials of ten leaves from five different trees were sampled. Sapwood and inner bark osmotic potential were determined on 3 tissue samples at 9, 12, 14, 18 and 20 h during one clear day in January 2017, simultaneously with sap flow, VWC and stem area variations measurements. Three cores were obtained from the main stem with an increment borer. Immediately after stem extraction the samples were placed in plastic bag and refrigerated, and then transported to the laboratory to perform the determinations within 1 h of extraction. To obtain sap for osmotic potential determinations, samples were pressed with a vise. Then, 10 µl of sap was collected from the end of the core with an automatic pipette, placed on a filter paper and introduced in the chamber of a vapour-pressure osmometer (Wescor 5600, Wescor, Inc, Logan, USA). Sap osmolality was converted to osmotic potential using the Van´t Hoff equation.

Tissue density and saturated water content

Inner bark and sapwood density was determined by the water-displacement method on samples collected from the main stem of 5 trees. Each sample was extracted with a 5 mm increment borer and sealed in glass vials to be transported to the laboratory. After removal of the outer bark and separated the inner bark from the sapwood, the densities of both tissues were determined as:

Tissue density = dry mass / fresh volume density (g cm^{-3})

Dry mass was obtained after oven dried at 80 °C for 72 h. Volume was estimated by submerging the sample in a container with distilled water placed on a digital balance with a 0.0001 g precision. The sample was kept submerged during measurements with the help of a very small needle, without touching the walls of the container. Samples were hydrated overnight and then saturated water content was estimated dividing the difference between saturated and dry mass by the dry mass.

Hydraulic capacitance

Cylinders of sapwood and inner bark were obtained with a 5-mm increment borer in the main stem of 5 trees, sealed in glass vials and transported to the laboratory where they were allowed to hydrate in distilled water for less than 2 h to avoid oversaturation. Then samples were blotted to remove excess water, placed in the caps of thermocouple psychrometer chambers (JRD Merrill Specialty Equipment, Logan, UT, USA), weighed, and then sealed inside the chamber for determination of water potential isotherms. After 3 h water potential was determined with a dew point microvoltmeter (HR-33T, Wescor, Logan, UT, USA). Measurements were repeated at frequent intervals until the water potential values stabilized. Samples were allowed to dehydrate for different time intervals, re-weighed in the psychrometer caps, resealed inside the psychrometer chambers and allowed to equilibrate for another determination of water potential. Then, water potential was plotted against relative water content. The relationships between water potential and relative water content were used to calculate sapwood and inner bark capacitance as described in Meinzer, James, Goldstein, & Woodruff (2003). Capacitance was normalized by the tissue volume to compare the absolute amounts of water released per unit decline in water potential. Tissue capacitance (C ; kg m^{-3}MPa^{-1}) was calculated as:

$C = \mathrm{d}W / \mathrm{d}\Psi$

where W is weight of water per unit volume of tissue and Ψ is the water potential of the tissue (Scholz et al., 2007).

Thermal analysis and tissue damage

Leaf, sapwood and inner bark ice nucleation temperatures (INT) were determined by thermal analyses (Arias, Bucci, Scholz, & Goldstein, 2015; Goldstein & Nobel, 1994; Rada et al., 2001). Three branches from three different trees were collected in the early morning at the end of the spring (December). The branches were wrapped in plastic bags to avoid evaporation and brought to the laboratory. Then eight entire leaves and 16 stem segments were cut from branches. Inner bark and sapwood tissue samples were obtained from eight stem segments. Each sample was placed in a glass tube or eppendorf tube to avoid further dehydration and immediately placed within a programmable thermal bath (Thermo Fisher Scientific, USA) and temperatures were lowered at a rate of 3 °C h^{-1} from 5°C to -30 °C. We recorded tissue and chamber temperatures with copper-constantan thermocouples placed in close contact with the tissue. The thermocouples were connected to a datalogger (CR10X, Campbell Scientific) with a multiplexer AM16x32 (Campbell Scientific), and temperatures were recorded at 4s intervals. The tissue ice nucleation temperatures were assessed from the temperature kinetics. A rapid increase in the temperature indicated heat release from the samples due to the phase change from water to ice (Burke et al., 1976).

The electrolyte leakage method was used to determine low temperature effects on tissue damage (Arias et al., 2015). Leaves and stem samples were cut from the same branches used in the thermal analysis. Twenty leaves, inner bark and sapwood segments were placed into separated sealed test tubes and incubated in the thermal bath. The bath was then cooled down at a rate of 3°C h^{-1} to reach different target temperatures (0, -5, -10,-18, -28°C). At each temperature, samples were kept for 15 minutes and then four tubes sample type were removed from the thermal bath and thawed while kept at 4 °C. Ten ml of deionized water was then added to each tube. The solution with the samples was kept overnight at 4°C in a shaker. Electric conductivity (EC) of the solution was then measured with an electrical conductivity meter (Hanna HI 98311, Hanna instruments). The tubes with the samples were moved to an autoclave during 15 min to obtain the maximum damage and thus maximum ion leakage. After samples were taken out from the autoclave they were shaken for 24 h before EC measurement. The relative EC, as an indicator of membrane damage by ion leakage, was calculated for each sample as:

Relative EC = (EC after the temperature treatment /EC autoclave) × 100

The temperature at the 50% relative EC was defined as the lethal temperature of tissues (LT$_{50}$) (Lipp et al., 1994; Sierra-Almeida et al., 2018; Zhang et al., 2016).

Results

Sap flow and inner bark water content and expansion and contraction of stem tissues

The inner bark of *A. araucana* is uniquely thick. In the study trees it was at least 6 cm thick. The proportion of the inner bark to sapwood at the base of the tree was about 0.32. Figure 1 shows a section of the stem with a small portion of the excavated bark. A sap flow sensor and an electronic point dendrometer can be seen inserted in the sapwood of the stem (Fig. 1A and B respectively). The inner bark is not only thick but its density was relatively lower (0.48 g cm^{-3}) and its capacitance higher (348 kg MPa^{-1} m^{-3}) than the sapwood (Table 1). The dead stem tissue located at the surface of the stem bark has a grey color (Fig. 1).

During the dry season the predawn leaf water potential was close to 0 MPa and the minimum leaf water potential was also relatively high (-1.1 MPa) (Table 1). Sap flow at the base of the main stem was very low during the nighttime, increased slightly at about 9:00 h and more rapidly until midday (Fig. 2A). The peak of the flow occurred in the afternoon (15:00 - 17:00 h), coupled with the maximum air saturation deficit, and declined close to null flow at midnight. The volumetric water content in both the inner bark and the sapwood determined at the base of the main stem exhibited a similar pattern of variation than the sap flow, increasing from midmorning to sunset and decreasing during the night period (Fig. 2B). As expected, the daily pattern of expansion and contraction (changes in inner bark cross sectional area) (Fig. 2C) was also

similar to the sap flow pattern and volumetric water content observed at the base of the trunk. The variation of the sapwood cross sectional area was very small.

The daily pattern of sap flow at the base and at 6 m height of the main stem is displayed in Fig. 3A. They followed a relatively similar pattern, with exception of time periods in which above sap flow was higher (Fig 3A). Both flows were well coupled and there were no lags in the timing of increase or decrease of sap flow. Upward long distance water transport peaks at similar time in the late afternoon (17:00 – 1710 h respectively), and were consistently low during the morning of the four study days. Similarly, the inner bark volumetric water content mimicked well the daily pattern of variation in the sap flow at the base and at 6 m height (Fig. 3 B).

Differences in the time course of the basal and at 6m height sap flow were also used to determine periods of discharge and recharge of water from the storage tissues (Fig. 4A). Higher sap flow at the base of the stem than at 6 m height (negative values on the Y-Axis indicate water recharge of reservoirs) was observed mainly during the night and the morning while during the rest of the daytime there was a higher sap flow at 6 m height than at the base (positive values on the Y-axis indicate water discharge of reservoirs. The use of water storage started between 11:10 and 12:50 depending on the day. The amount of water discharged from the internal reservoirs determined by using sap flow measurements, represented 5.2%, 10.2%, 11% and 6.9% of the total sap flow measured at the base of the tree for each the four days. The total amount of water stored in the stem tissues from the base to 6 m height taking into account tissue volume (Table 1) varied between 0.96 and 0.98 m^3 (Fig. 4B), with a higher amount stored in the sapwood (0.655 to 0.675 m^3; Fig. 4D) than in the inner bark (0.305 to 0.312 m^3; Fig. 4C). The daily decrease in the water content from the base to 6 m height of the trees varied between 3.27 and 5.5 l d^{-1} in the inner bark and between 2.5 and 7.5 l d^{-1} in the sapwood (values showed in panels C and D of Fig. 4). The water discharged from the volume of inner bark between the base of the tree and 6 m height represented about 46% of the total water released from the stem tissues (inner bark + sapwood; values displayed in Fig. 4B). The water discharge started earlier in the sapwood (between 11:00 and 12:30 h) than in the inner bark, approximately at time in which sap flow at 6 m height was higher than at the base of the trees. There was a strong agreement between the amounts of water discharged from reservoirs estimated by independent methods (Fig. 5 A).

The daily accumulated sap flow for the four days showed was 242, 164, 174 and 222 liters at the base and 239, 169, 181 and 232 liters at 6 m height (Fig. 3A). Except for the first day, there was a daily net water discharge of stem reservoirs (sap flow was higher at the base of the trunk than at 6 m height). Consistent with those differences in total sap flow measured at different heights in the stem, daily patterns of total water content in the whole stem tissues showed that stored water was not replaced during a 24 h period in three last days (Fig. 4 B, C, D). The net recharge of stem reservoirs observed during the first day was consequence only of a net gain of water (1.3 liters) in the inner bark, while sapwood maintained a constant water content (Fig. 4C). However, when there was a daily net loss of water, a higher discharge was observed in the sapwood (3.2, 6.3 and 7.9 liters) than in the inner bark (1.0, 1.0 and 1.62 liters). The daily net gain or loss of water from the stem tissues was negatively correlated with the daily net difference in sap flow at different tree heights (Fig. 5B). When there was a net loss of water from reservoirs (negative values on the Y-Axis), a higher sap flow was observed at 6 m height (positive values on the X-Axis) while sap flow was higher at the base of the tree when there was a net gain of water in the storage compartments.

Osmotic potential gradients between sapwood and inner bark

The osmotic potential in the inner bark varied significantly throughout the day (F-value: 7.66, p<0.01). It was substantially low (more negative) at 9:00 h, increased by about 0.4 MPa until 18:00 h and started to decrease thereafter (Fig 6). The sapwood osmotic potential diurnal changes (F-value: 8.57, p<0.01) were the mirror image of the inner bark osmotic potential variations, decreasing during most of the daytime. The inner bark osmotic potential remained always below the sapwood osmotic potential (p<0.01), establishing a gradient favorable for water movement from the inner bark to the sapwood, particularly during the nighttime (Fig. 6).

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Relationships between daily variations in water contents and osmotic potentials, in both the inner bark and the sapwood, over the course of a daytime are depicted in Fig. 7A. Daily changes in volumetric water content were lower in the inner bark compared to the sapwood between the base of the tree and at 6 m height. The changes in water content and osmotic potential in the inner bark were linearly correlated indicating that a decrease in the amount of water content resulted in a more negative osmotic potential. There was not statistical significant relationship between these two variables for the sapwood (Fig. 7A). The relationship between the changes in volumetric water content of the inner bark tissue and the osmotic potential gradient was marginally significant ($p = 0.06$) (Fig. 7B) but the trend has a clear physiological meaning: when the water content in the inner bark decreased, the driving force for water moving from the sapwood into the inner bark (osmotic potential gradient between inner bark and sapwood) tended to increase (Fig. 7B).

Ice nucleation and sub-zero lethal temperature of stem and leaf tissues

The ice nucleation temperature occurred in the leaves at -8.16°C (Fig.8A) and in the sapwood and inner bark at -6.92 and -6.08°C, respectively (Fig. B and C). Ice formation is indicated by an exothermic event in the thermal analysis depicted in Fig.8. However, the ice nucleation can change according to variations in both water content and osmotic potential occurring during the daytime. Tissue damage occurred after ice seeding (Fig. 8D,E,F). The temperature at which 50% of membrane leakage occurs is considered to be the irreversible tissue temperature damage. The leaves were hardier compared to sapwood and inner bark because tissue damage occurred at -25.3 °C in the leaves while in the other two tissues irreversible tissue damage occurred at -20.0 °C in the sapwood and -10.3 °C in the inner bark (Fig. 8).

Discussion

Patterns of water discharge and recharge and driving force for water movement between the inner bark and sapwood

In this study an atypical pattern of water use and stem swelling was observed. Contrasting with other species in which maximum stem contraction and depletion of stem water storage are typically observed during the morning (Daudet et al., 2005; Oliva Carrasco et al., 2014; Scholz et al., 2014; Scholz et al., 2008; Steppe et al., 2006), in *A. araucana* the minimum stem diameter and water volume within the stems were observed in the late afternoon. This intriguing pattern in stem swelling was consistent with the high sap flow rates reached at 17:00 – 18:00 h. Changes in stem diameters of trees have been related to the daily sap flow dynamic consistent with water uptake and transpiration (Oliva Carrasco et al., 2014; Scholz et al., 2008; Steppe et al., 2006; Steppe & Lemeur, 2004). In those cases, the water potential was the driving force linking changes in stem diameter with stored water. However, studies in mangrove species have highlighted patterns in stem diameter variations opposite to typical behavior, which were related with osmotic changes affecting the radial water movement into storage tissues when transpiration is high (Vandegehuchte et al., 2014). Similarly, Donnellan Barraclough, Zweifel, Cusens, & Leuzinger (2018, 2019) found that daytime bark swelling is decoupled from transpiration in a mangrove species. Stems water recharge during the day and water discharge at night also has been associated to daily air temperatures fluctuations (Vilas et al. 2019). Although thermal sources of stem expansion and shrinking could have affected our results, the simultaneous determinations of volumetric water content and sap flow with independent methods, indicate that the apparent anomalous daily fluctuations in stem diameters and thus in the stored water in *A. araucana,* were not an artifact of air temperature variations.

We consider that the osmotic gradient observed between the sapwood and the inner bark could be partially involved in the daily dynamic of radial water movement between the stem tissues of *A.araucana* . In the morning, when the water transport system is assumed to be relatively free from embolisms and there was an osmotic gradient favoring the water influx from sapwood to inner bark, the magnitude of the basal sap flow was high enough to recharge simultaneously the stem water storage compartments and to supply water to the leaves sink. Then, in the afternoon, recharged reservoirs delivered water to leaves. A possible explanation for this pattern could be related to the sugar phloem influx from the photosynthetic tissues and the hydraulic efficiency of the long distance water transport system. According to the results for other temperate Andean

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tree species (Bucci et al., 2019), it is probably that *A. araucana* has higher photosynthesis rates in the afternoon and sugars loading into phloem until midnight. The photosynthates moving into the phloem would increase osmotic potential and generate the turgor signal for stem swelling (Donnellan Barraclough et al., 2019; Pfautsch, Hölttä, et al., 2015; Sevanto et al., 2011). Consistent with this mechanism, we found a correlation between the osmotic potential and tissue water content changes. However, while high inner bark osmolality was observed to occur during the nighttime period, the inner bark water recharge only occurred in the early morning together with increasing axial water flow supplying the leaves.

The large capacity of *A. araucana* to fulfill simultaneously the leaf water demand and the recharge of water storage compartments is also consistent with an extended root system tapping to abundant soil water (Veblen, 1982) and with high hydraulic conductivity of vascular system. The high predawn and minimum water potentials found in our study support those hydraulic traits. In previous studies (Bucci et al., 2012, 2019) we observed that some tree species co-occurring with *A. araucana*have also high water potentials have and an efficient water transport system. The identical sap flow increase timing in the morning (without time lags) between the base and 6 m height of the main stem indicate a very low hydraulic resistance in the vascular pathway. However, high hydraulic efficiency can result in high vulnerability. In line with this, Zimmer, Brodribb, Delzon, & Baker (2015) found that Australian araucarias have a high level of hydraulic vulnerability. Consequently, when atmospheric evaporative demand and xylem tension are relatively high (i.e. in the afternoon), the roots, which are generally less resistant to drought based in the theory of hydraulic segmentation (Tyree & Ewers, 1991), could strongly cavitate, reducing its ability to supply water to leaves. This may explain why stem contraction and water release from internal water storages occur in the late afternoon, indicating that stem reservoirs are delivering water to the transpiring leaves. Although the osmotic gradient between inner bark and sapwood persist throughout the day, during the afternoon it takes relatively high values (0.2 to 0.4 MPa), which may not be capable to compensate the drop of xylem water potential. A decline in sapwood water content was observed at the time that sap flow was higher closer to transpiring leaves than at the base of the tree, indicating that the water discharge occur firstly in the tissue proximal to the conductive vessels. Inner bark water decline lagged always behind the sapwood (in average 2.5 h). This suggests the water flow pathway between xylem conduits and xylem parenchyma would have higher hydraulic conductance allowing a faster water exchange than between sapwood and inner bark.

If the axial water transport system loses its hydraulic capacity during the afternoon, then it is plausible that the nighttime water recharge of storage tissues will be reduce. However, at nigh embolisms recovery possibly occurs (Brodersen et al., 2018; Trifilò et al., 2014) such that in the next morning the hydraulic conductance return to high values. A slight decrease of water content in the inner bark and an increase in the sapwood at night suggests that water needed for xylem refilling could be delivered from the inner bark. Based in the very low osmotic potentials in the inner bark at night, a tissue pressure contained within stem by external bark could cause a transient tissue pressure to squeeze water into xylem vessels (Bucci, Scholz, Goldstein, Meinzer, & Sternberg, 2003; Canny, 1997; Salleo, Lo Gullo, Trifilò, & Nardini, 2004). Then, in the next morning the root water uptake could compensate the crown water demand as well as the radial water flow from sapwood to inner bark driven by the osmotic gradient. Further studies are needed to evaluate this hypothesis of embolism refilling.

Contributions of water storages to daily water use

The water storage is an important component of whole-plant water balance, and in *A. araucana* allows for the maintenance of relatively high values of minimum leaf water potentials during the dry season (about 1 MPa). According to a study by Zimmer et al. (2015) with Australian araucarias, this genus is highly isohydric, maintaining its leaf water potential relatively constant even under large decreases in the soil moisture. A large capacity to store and release water to the transpiration stream is also observed at leaf level in *A. araucana* (1.3 mol m^{-2} MPa^{-1}, Bucci et al. unpublished data; Brodribb, Holbrook, Zwieniecki, & Palma (2005)) and in Australian araucarias species (Zimmer et al., 2015). At stem level, the duration of the lags between the sap flow onsets at different heights in the trees has been considered a proxy for the capacity of stem water storage to supply water to transpiration stream (Scholz et al. 2007; Phillips et al. 2009; Chen et al 2015).

Therefore, the lack of the time lags would suggest a low contribution from water storages to the daily water use. However, in this study, despite both basal and 6 m height sap flow had a similar pattern throughout the day, the small differences in sap flow that were mainly observed in the afternoon suggesting that between 5 and 11% of the daily water use is derived from internal water storages. These values are within the range observed in several tree species of different ecosystems (Goldstein et al., 1998b; Meinzer et al., 2004; Oliva Carrasco et al., 2015; . Scholz et al., 2007, 2014). Although sap flow determinations at different tree positions overestimated the stored water use (32% to 54%) respect to the values calculated with the decline in tissue water content, there was a strong agreement between values obtained by both methods.

The exceptionally thick bark of *A. araucana* helped to quantify explicitly the relative contribution of the sapwood and inner bark to the transpiration stream. Tissue density is a good predictor of the amount of water that can be stored in a reservoir (Bucci et al., 2004; Oliva Carrasco et al., 2014; Stratton, Goldstein, & Meinzer, 2000) and released (Scholz et al., 2007; Ziemińska et al., 2019). In *A. araucana* we found that the inner bark has lower density than the sapwood, consistent with its higher saturated water content, higher volumetric water content and capacitance. Despite the lower amount of tissue in relation to sapwood, the inner bark had almost a contribution as important as the sapwood to the transpiration stream (46 and 54% respectively), which possibly results from its high capacitance and water content. The inner bark had higher capacity per volume tissue unit to discharge water than the sapwood as this tissue exhibited larger fluctuations in the volumetric water content than the sapwood. These results contrast partially with our previous studies (Scholz et al., 2008) where we found that the inner bark of the savanna trees has lower capacity to release water compared to the sapwood and it contributes little to the plant water balance despite the larger proportion of this tissue in relation to sapwood in those species (up to 2.3) compared to *A. araucana* (0.32). This highlights different functional significance of the inner bark depending on species and ecosystem (e.g. fire protection, mechanical stability, water storage; Scholz et al. 2007; 2008).

Freezing resistance in water storage tissues and the relationship with the daily changes in water content and osmotic potential

We assessed the freezing resistance in late spring due to common occurrence of frosts in this period when trees exhibit high physiological activity. Frost injuries in *A. araucana* are mostly restricted to the middle section of the growth rings (Hadad et al. 2019), suggesting substantial damage during late spring frosts. Results from others studies with conifers (Gurskaya, 2007; Kidd et al., 2014; Payette et al., 2010) including *A. araucana* (Arco Molina et al., 2016) indicate lower freezing damage with tree age. Old trees have a thick bark and thus it may provide higher thermal insulation.

Result in our study indicate that the inner bark, sapwood and leaves of *A. araucana* are not exposed to the effects of freezing until temperatures reach thresholds of -6 or -8 @C depending on tissue. This mechanism prevents the extracellular freezing and the cellular dehydration (Cavender-Bares, 2005) and thus the tissue can maintain the metabolic activity to below zero temperatures. Below those, we observed that extracellular ice nucleation began first in the inner bark and then spread to the sapwood. The ice nucleation temperature depends on osmotic potential (Burke et al., 1976), tissue anatomy (Lintunen et al., 2013), and water content (Arias et al., 2017; Goldstein et al., 1985). A low osmotic potential in the tissues can balance the drop of water potential during the intracellular dehydration and can contribute to the decrease of the ice nucleation temperature (Cavender-Bares, 2005). In addition it is know that tissue water content influence on the capacity to avoid the ice nucleation (Arias et al., 2015; Goldstein, Meinzer, & Monasterio, 1985). Thus, the atypical daily patterns of recharge and discharge of water storages compartments observed in *A. araucana* can reflect a strategy of freezing resistance. Lower water content in the stem tissues and more negative osmotic potential in the inner bark during the nighttime period, when the lowest temperatures are observed, may result in avoidance of freezing damage.

Ice formation in the inner bark could lead to strong water potential gradient between the inner bark and sapwood causing water to move into the inner bark from the sapwood. This water movement may delay the extracellular ice formation in the sapwood due to the decreasing in water content in this tissue and thus protecting it from freezing damage. However, the effect of osmotic potential in the decrease of ice nucleation

temperature was not observed between tissues. Although the inner bark had always more negative osmotic potentials the ice nucleation was observed at higher temperature than the sapwood. The higher ice nucleation temperature observed in the inner bark might be caused by the larger volumetric water content compared to the sapwood.

Earlier freezing in the inner bark can be beneficial for the sapwood by providing temporary cold protection due to heat release during ice formation (Zachariassen & Kristiansen, 2000). This effect should be stronger in thicker barks. Inner bark in the main stem of *A. araucana* stored about 0.3 m^3 of water up to 6m height and released 0.1 MJ of energy (latent heat of fusion of water: 335kJ/liter) to surrounding tissues during freezing. Previously, thick bark of *A. araucaria* was related mainly with fire protection (Burns, 1993; Montaldo, 1974). Our study provides evidence than in addition to the role delivering water to leaves, the bark protects the inner stem tissues from freezing. The presence of thermal insulating tissues that can maintain the cambium and water transport function of stems has also been observed in other plant species (Goldstein & Meinzer, 1983; Rada et al., 1985).

At branch level, leaves can delay the ice seeding as they are radially arranged on branches. These insulating layers can contribute with maintaining tissue temperature above air temperature. Leaf visual (Sakai et al., 1981) and exothermal analysis of frozen tissues (Offord, 2011) of Australian Araucariaceae indicate that this plant family may withstand minimum temperatures in the range of -5 to -25 @C without damage. Our results show that *A. araucana* tolerates extracellular freezing in the leaves, in the sapwood and in the inner bark. Leaves had higher safety margin between the temperature of ice nucleation and lethal injury (17.2 @C) than sapwood (12.7 @C) and inner bark (4.2 @C). Cell properties, like cell wall elasticity and membrane composition could be involved in the decrease of lethal temperature in these tissues (Alonso-Amelot, 2008; Zhang et al., 2016). Although stem xylem water delivery to leaves could be partially blocked due to frozen xylem, the high leaf water content and capacitance could supply water to photosynthetic tissue maintaining the functionality. *Araucaria araucana* has extreme leaf longevity (about 24 years; Lusk, 2001) with which the high degree of freezing tolerance found in their leaves would allow to amortize their high cost of construction (leaf dry mass ranged between 270-370 g cm^{-2}; Lusk, 2001).

Conclusions

We showed that stored water use estimated with sap flow sensors is consistent with the estimations from independent approaches. In addition we found that, even without time lags between sap flow at different positions in the main stem, an important amount of water is discharged first from sapwood and then from inner bark to transpiration stream during the afternoon in *A. araucana* . This species has an efficient water transport system that allows supplying the water demand from leaves while simultaneously recharging the reservoirs during the morning. At this time water movement from sapwood to inner bark is driven by a strong osmotic gradient. The late water discharge from reservoirs could sustain the leaves demand during afternoon and the resulting low water content in the tissues could contribute to decrease in the ice nucleation temperature. Besides the water storage capacity, the inner bark has a freezing protection function of more internal tissues. Our results suggest that *A. araucana* would not be strongly affected by drought due to the large volume of stored water in stems, a high leaf and stem tissue capacitance and probably also to an efficient water transport system. Climatic predictions consider increases in air temperatures for the next decades in which the growing season could occur earlier and thus the araucaria forests could be more frequently exposed to late frosts. However, a large safety margin between extracellular freezing and lethal cell damage protect the stem tissues and leaves in adult trees. These findings may rule out to the drought as a cause leading to the partial crown desiccation observed in this species and contradict studies about the effects of low temperatures and precipitation on the width and damage of growth rings. The responses and mechanisms observed at a daily or short term scale could differ from those at a seasonal scale or long term. Further ecophysiological studies are necessary to enhance our understanding on the responses of Araucaria araucana to long-term water stress and freezing temperatures.

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Table 1. Physiological and morphological characteristics of *A. araucana* trees and tissues studied during the dry season.

| Plant traits	Mean $\pm$ SE
Predawn leaf water potential (MPa)	-0.083 $\pm$ 0.01
Minimum leaf water potential (MPa)	-1.1 $\pm$ 0.1
Inner bark density (g cm^{-3})	0.48 $\pm$ 0.03
Sapwood density (g cm^{-3})	0.65 $\pm$ 0.19
Inner bark saturated water content (%)	218 $\pm$ 39
Sapwood saturated water content (%)	104 $\pm$ 3
Inner bark volumetric water content (m^3 m^{-3})	0.45 $\pm$ 0.0003
Sapwood volumetric water content (m^3 m^{-3})	0.38 $\pm$ 0.001
Inner bark capacitance (Kg MPa^{-1} m^{-3})	348 $\pm$ 32
Sapwood capacitance (Kg MPa^{-1} m^{-2})	298 $\pm$ 45
Inner bark volume $_{base\ to\ 6m}$ (m^3)	0.694
Sapwood volume $_{base\ to\ 6m}$ (m^3) Mean daily basal sap flow (l d^{-1}) Mean daily 6 m height sap flow (l d^{-1})	1.722 200 $\pm$ 19 2
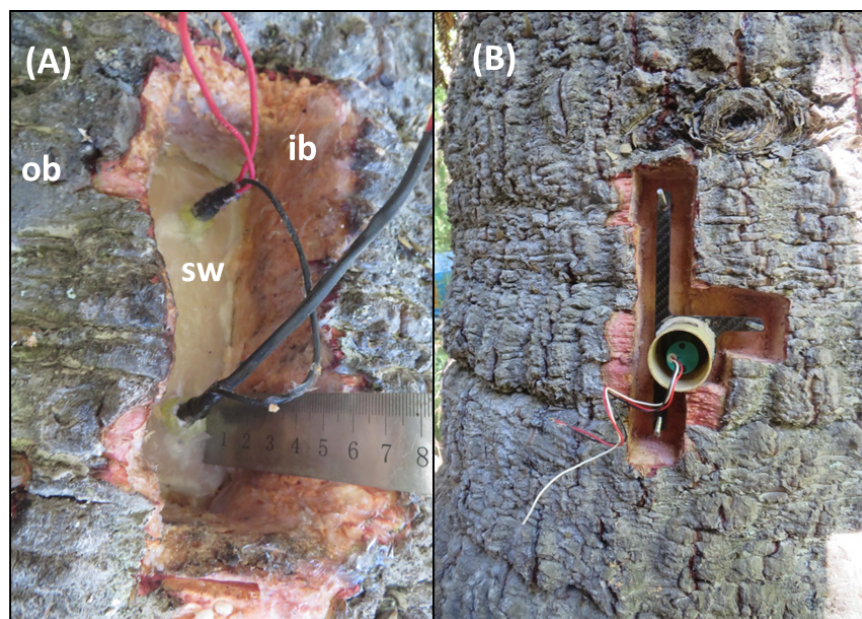
Figure 1. Image of a section of *A. araucana* trunk showing the outer bark (ob), and the thickness of the inner bark (ib). In (A) sap flow sensor installed in the sapwood (sw), the sap flow sensor has two black cables containing (i) the upper one a heated thermocouple and (ii) the lower one the reference thermocouple. In (B) a point dendrometer installed in the sapwood of the same tree can be seen.
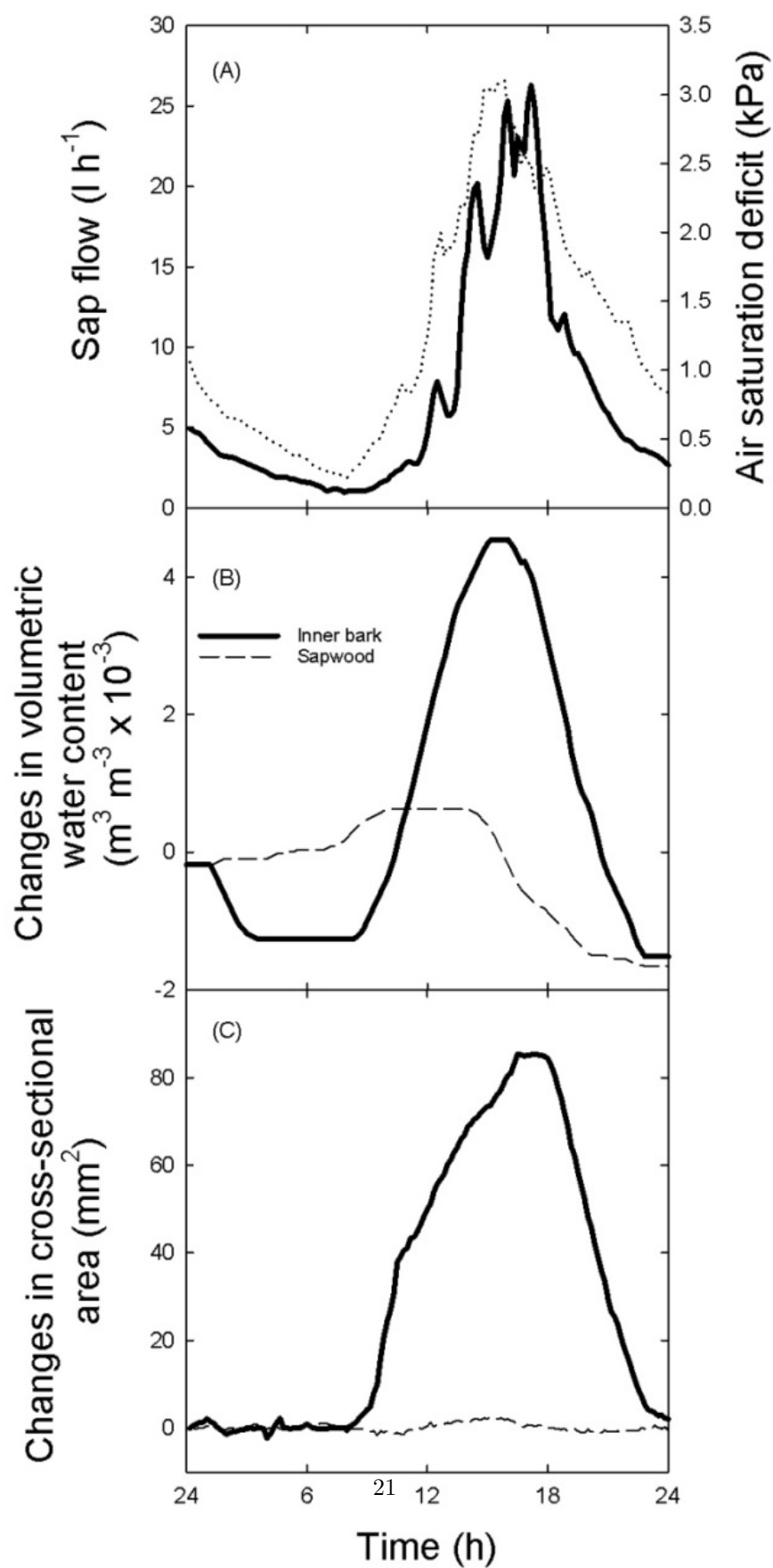
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Figure 2. Typical daily pattern of (A) sap flow and air saturation deficit, (B) variations in the sapwood and inner bark volumetric water content at the base of the main stem, and (C) variations in inner bark and sapwood cross sectional area at the base of the trunk of *A. araucana* in the dry season. Positive values in the Y- axis of panel B indicate water content increase (recharge) and negative values indicate water content decrease (discharge). Positive values in the Y-axis of panel C indicate tissue expansion and negative values indicate tissue contraction.
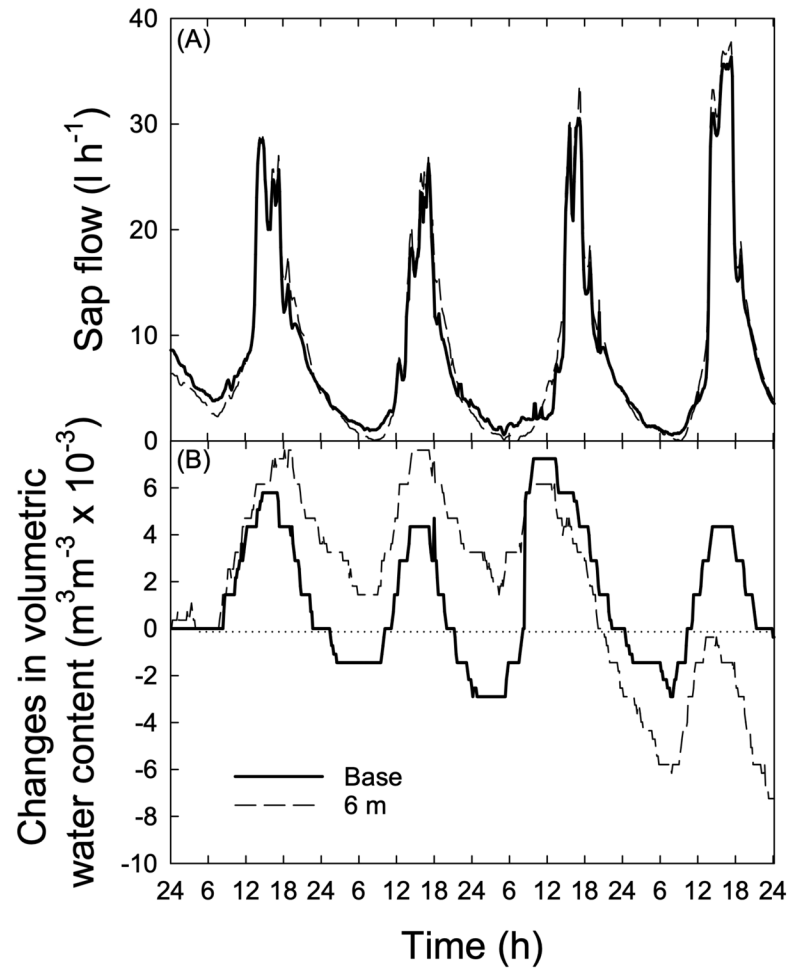
Figure 3. Daily patterns of (A) sap flow, and (B) changes in the inner bark volumetric water content at the base (solid line) and at 6 m height in the main stem (dashed line) of an individual of *A. araucana* in the dry season. Interpretations of patterns of changes are similar as in panel B of Figure 2.
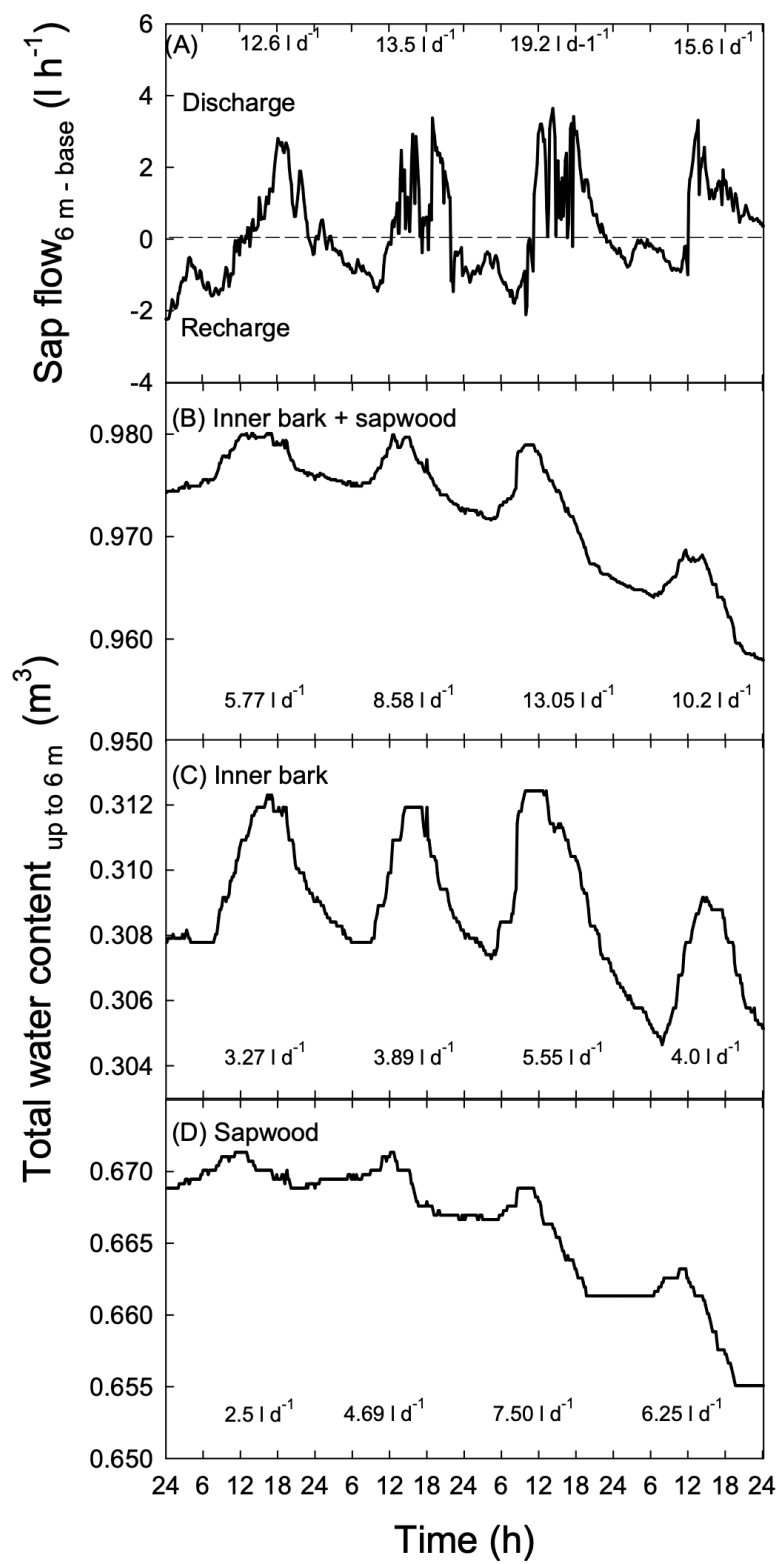
Figure 4. (A) Daily differences between sap flow measured at 6 m height and at the base of the main stem, (B, C and D) water content in the stem (inner bark + sapwood), in inner bark and in sapwood, respectively, from base up to 6 m height in the main stem. Values in liters within panel A indicate the water recharge (negative values) and water discharge from reservoirs (positives values). Values in liters within panels B, C and D indicate the amount of water discharged from tissues each day.
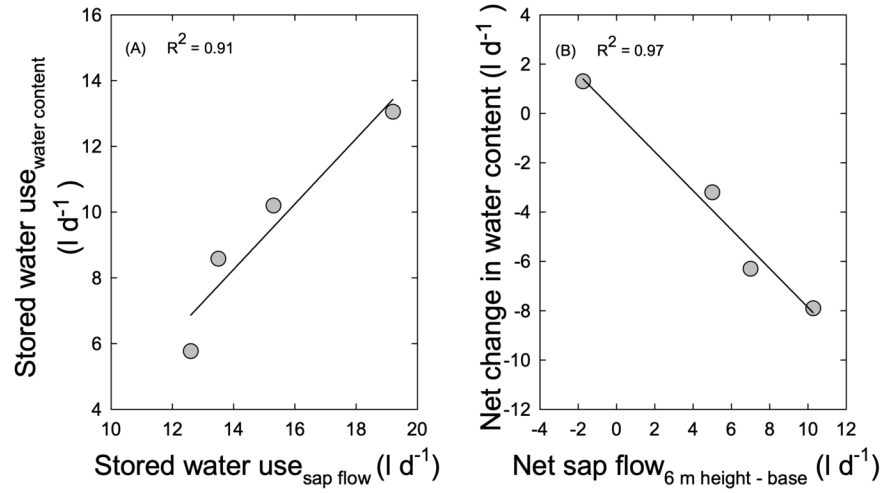

Figure 5. (A) Correlation between daily stored water use determined by measuring stem tissue water content and by using sap flow sensors at different positions in the main stem. (B) Correlation between daily net changes in stem water content (inner bark + sapwood) and the net difference between daily sap flow measured at different positions in the main stem of *A. araucana* . The lines are the linear functions fitted to the data ((A) y = -5.7 + 0.99x, p= 0.04; (B) y = 0.02 - 0.78x; p = 0.01)
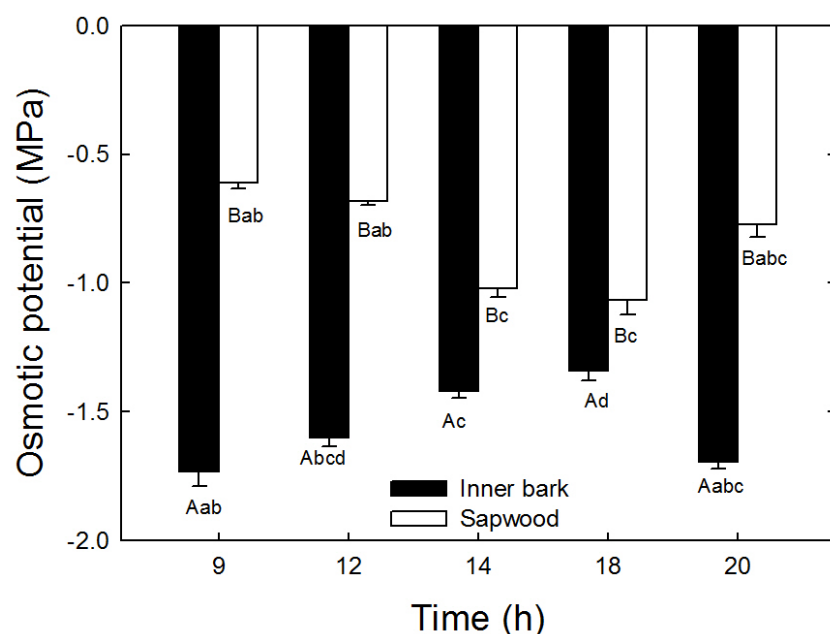
Figure 6. Time course of osmotic potential of sapwood (white) and inner bark (black) of *A. araucana* in the dry season. Bars are mean ± SE (n= 3 to 5). Different uppercase letters indicate significant differences between sapwood and cortex at a specific time, and different lowercase letters indicate significant differences within sapwood or inner bark along the course of a day.
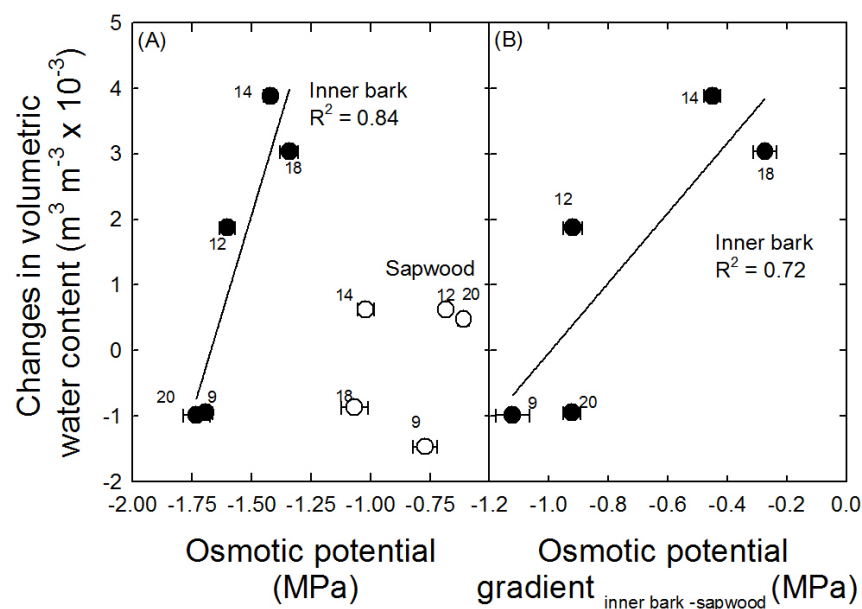

Figure 7. (A) Volumetric water content in the inner bark (black) and in the sapwood (white) versus the tissue

osmotic potential of *A. araucana* . (B) Inner bark volumetric water content versus the osmotic potential gradient between inner bark and sapwood in *A. araucana*.Values above symbols indicate the daytime. The lines are the linear regressions fitted to the data (A) y= 20.20 + 12.1x, p=0.029; (B) y = 5.3 + 5.33x; p=0.06.
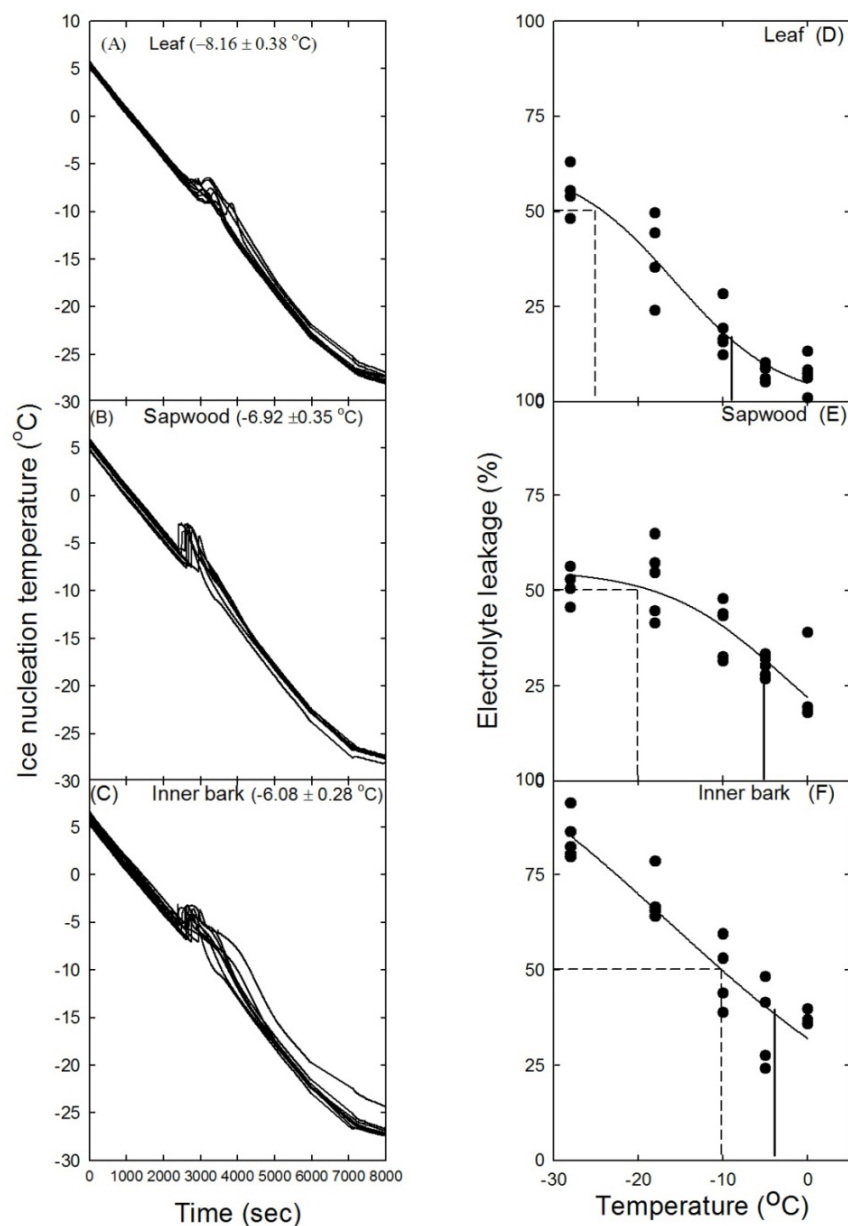

Figure 8. Ice nucleation temperature of (A) leaves, (B) sapwood, and (C) inner bark of *A. araucana* . Percentage of membrane leakage in relation to temperature in (D) leaf, (E) sapwood and (F) inner bark of *A. araucana* . In left panels, mean values ± SE (n= 8) of ice nucleation temperature are indicated. In right panels, solid lines indicate the ice nucleation temperature and dashed line the temperature at which 50% of

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tissue damage is reached.