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ORIGINAL ARTICLE

Water storage and transport in leaves of vesselless trees in the temperate rainforest of south-central Chile

Almacenamiento y transporte de agua en hojas de árboles sin vasos del bosque templado lluvioso del centro-sur de Chile

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ABSTRACT

According to a common hypothesis, some of the epidermal structural features in the leaves of tracheid-bearing plants "offset" low specific conductivity of vesselless wood. The data concerning this issue is contradictory, which can be explained by the fact that leaf water relations depend not only on the epidermis structure, but also on the structure of other leaf tissues. In the current study we aimed to evaluate the diversity of water transport systems in the leaves of tracheid-bearing woody plants in the temperate rainforest of south-central Chile. For this purpose, we collected leaves of four Podocarpaceae and two Winteraceae species in natural habitats, examined their leaf anatomy using light and transmission electron microscopy, measured the quantitative characters and analyzed the data using principal component analysis. Leaves of the studied species differ in the mesophyll and xylem anatomy. Four species have features that accelerate water transport through the leaf tissues via the apoplast (*Prumnopitys andina*), accessory transfusion tissue (*Podocarpus saligna*) and a network of veins (*Drimys* species). On the contrary, the leaves of *Saxegothaea conspicua* and *Podocarpus nubigena* accumulate water in water-storage tissue (hydrenchyma), but their ecology suggest that hydrenchyma is not an adaptation to environmental conditions. The obtained data indicate the existence of different ways of water delivery to the photosynthetic tissue in the leaves of vesselless plants. In the case of insufficient water supply through the tracheids, hydrenchyma is likely to maintain hydration of the leaves.

Keywords: accessory transfusion tissue, hydrenchyma, leaf structural traits, *Podocarpus*, *Saxegothaea*.

RESUMEN

De acuerdo con una hipótesis común, algunos rasgos estructurales en las hojas de plantas portadoras de traqueidas "compensan" la baja conductividad específica del leño sin vasos. La información sobre este tema es contradictoria, lo que puede explicarse por el hecho de que las relaciones hídricas en las hojas no dependen solo de los rasgos estructurales, sino también de la estructura de otros tejidos foliares. En este estudio nuestro objetivo fue evaluar la diversidad de los sistemas de transporte de agua en las hojas de plantas leñosas portadoras de traqueidas en especies del bosque templado lluvioso del centro sur de Chile. Para esto recolectamos hojas de cuatro especies de Podocarpaceae y dos Winteraceae en hábitats naturales, estudiamos su anatomía foliar mediante microscopía de luz y electrónica de

transmisión, determinamos caracteres anatómicos cuantitativos y analizamos los datos usando análisis de componentes principales. Las hojas de las especies analizadas se diferencian en la anatomía del mesófilo y xilema. Cuatro especies tienen rasgos que aceleran el transporte de agua a través de los tejidos foliares mediante el apoplasto (*Prumnopitys andina*), el tejido de transfusión accesorio (*Podocarpus saligna*) y la red de venas (especies de *Drimys*). Por el contrario, las hojas de *Saxegothaea conspicua* y *Podocarpus nubigena* acumulan agua en el tejido de almacenamiento de esta (hidrénquima), pero su ecología sugiere que el hidrénquima no es una adaptación a las condiciones ambientales. Los datos obtenidos indican la existencia de diferentes formas de suministro de agua al tejido fotosintético en las hojas de plantas sin vasos. En el caso de que el suministro de agua a través de traqueidas sea insuficiente, es posible que el hidrénquima mantenga la hidratación de las hojas.

Palabras clave: hidrénquima, *Podocarpus*, rasgos estructurales de la hoja, *Saxegothaea*, tejido de transfusión accesorio.

INTRODUCTION

Vessel members are thought to have evolved through the modification of the developmental program giving rise to tracheids (Frost 1930; Bailey 1953; Carlquist 1975). The fundamental difference between vessels and tracheids is that the transport of aqueous solutions occurs through perforations in the ends of each vessel elements of the vessel and not laterally through the pit membranes of punctuations as in tracheids (Mohl 1851; Carlquist 1975). This results in that vessel-based wood has a higher specific conductivity than tracheid-based wood (Tyree & Ewers 1996; Brodribb & Feild 2000; Sperry *et al.* 2006). Such facts formed the basis for the idea that vessels are one of the key innovations that allowed plants to move from wet habitats to dry ones, maintaining sufficiently large leaves to keep a high gas exchange rate (Takhtajan 1969; Carlquist 1975; Bond 1989; Sperry 2003).

At the same time, the concept emerged that the leaves of tracheid-bearing plants have structural adaptations that reduce water loss and thereby compensate for the low water transport efficiency of vesselless wood (Bailey 1944; Carlquist 1975; Carlquist 1996; Axsmith *et al.* 2004). Among them are thick cuticle, more epicuticular waxes, sunken stomata and stomatal plugs, which have long been regarded as anti-transpiration (xeromorphic) (e. g. Strasburger 1891; Haberlandt 1904; Fahn 1982; Hill 1998). More recent research showed that the involvement of these structures in limiting water loss is likely to be overestimated, but they did not completely rule it out (Brodribb & Hill 1997; Mohammadian *et al.* 2007; Jordan *et al.* 2008; Roth-Nebelsick *et al.* 2009). It was suggested that they may not be associated with xeromorphosis (Becker *et al.* 1986; Feild *et al.* 1998; Riederer & Schreiber 2001; Mohammadian *et al.* 2009; Pautov *et al.* 2017; Pautov *et al.* 2019). Regardless of the outcome of the discussion about the adaptive value of the aforementioned epidermal traits one would have to admit that they frequently occur in families with tracheid-based wood (Florin 1931; Stockey & Atkinson 1993; Stockey & Frevel 1997; Mill & Schilling 2009).

It is known that the water relations of leaves in angiosperms depend not only on the structure of their epidermis, but also on other tissues, in particular, on the structure of the veins and the presence of cells accumulating water (Haberlandt 1904; Willert *et al.* 1990; Willert *et al.* 1992; Gamalei 2004; Ogburn & Edwards 2010). The leaves of some conifers are divided into a petiole and a lamina like dicotyledonous leaves. Our hypothesis is that the morphological convergence of leaves of angiosperms and conifers is associated with histological convergence. It could result in the occurrence of features in the leaves of some conifers, influencing leaf water regime, which can compensate for the low efficiency of the vesselless wood. In the current study we aimed to evaluate the diversity of water transport systems in the leaves of tracheid-bearing woody plants in the temperate rainforest of south-central Chile. We (1) examined the leaf anatomy of some Podocarpaceae and Winteraceae species; (2) compared the leaf structural types; (3) identified specific structural features of each species that can affect the leaf water relations.

MATERIALS AND METHODS

Plant material

Plants were collected in November 2015 in temperate rainforest in Parque Nacional Nahuelbuta and Parque Nacional Puyehue (South-Central Chile). Study sites were

within the natural range of species (Debreczy & Rácz 2012; Jara-Arancio *et al.* 2012; Rodriguez *et al.* 2018). We studied four Podocarpaceae Endl. members (*Podocarpus nubigena* Lindl., *Podocarpus saligna* D.Don., *Prumnopitys andina* (Poepp. ex Endl.) de Laub., *Saxegothaea conspicua* Lindl.) and two Winteraceae R.BR. ex Lindl. members (*Drimys andina* (Reiche) R.A.Rodr. & Quezada and *Drimys winteri* J.R.Forst. & G.Forst.). Nomenclature for the species was based on The Plant List database and Rodriguez *et al.* (2018). *D. andina* was an alpine shrub; all the other species were subcanopy to canopy trees. Nine leaves for each species were randomly collected from the outer part of the crown on the height reachable by hand. Completely expanded but not senescent leaves were used. Plants in pre-reproductive and reproductive ontogenetic stages were used for sampling.

Transmission electron microscopy (TEM)

Pieces of leaf laminas (4 x 4 mm) were fixed in 2.5% paraformaldehyde (Serva, Germany) and 2% glutaraldehyde (Serva, Germany) in potassium phosphate buffer (20 mM KH_2PO_4, 80 mM Na_2HPO_4, pH 7.4) at 4 °C for 1–3 days and post-fixed overnight in 2% osmium tetroxide in the same buffer at 4 °C. The tissue was dehydrated in an ethanol-acetone series and embedded in Epon812-Araldite M epoxy resin (Fluka, Switzerland). Ultrathin sections (60–75 nm) were cut with glass knives on a Leica EM UC6 (Leica Microsystems CMS GmbH, Germany) ultratome, contrasted with lead citrate on grids according to a modified method by Reynolds (1963) and viewed and photographed with a JEM-1400 high resolution electron microscope (JEOL Ltd., Japan).

Light microscopy

Semi-thin (3000–4000 nm) sections obtained during TEM preparation were stained with 1% toluidine blue (Serva, Germany) in 1% sodium borate, embedded in Entellan mounting medium (Merck KGaA, Germany) on a microscope slide, and preserved under a coverslip. Plant material fixed in 70% ethanol was used to make thick sections by hand using a razor blade. For epidermis preparation pieces of leaf laminas were macerated in a mixture of aqueous solution of potassium chlorate and concentrated nitric acid (Schultze reagent) (Nautiyal *et al.* 1976; Meyen 1987; Barykina *et al.* 2004). The sections were stained with a combination of alcian blue and safranin. All specimens were embedded in a glycerin-gelatin medium on a microscope slide and preserved under a cover glass. Sections were examined with a manual inverted microscope Leica DMI3000 B (Leica Microsystems CMS GmbH, Germany). Images were captured using a Leica DMC 2900 digital camera (Leica Microsystems CMS GmbH, Germany) and Leica Application Suite X image-analytical software (Leica Microsystems Ltd, Switzerland). All images were cropped and contrasted using Adobe Photoshop CS 5.1 software (Adobe Inc., USA).

Leaf dry weight

To calculate leaf dry weight, the leaves were dried in paper bags in a thermostat at 60 °C until constant weight and then were weighed. Nine leaves of each species were sampled.

Leaf measurements

Quantitative measurements of leaf morphological and anatomical traits were made using digital images processed with ImageJ software (National Institutes of Health, USA). We made 10–20 measurements per each leaf depending on the trait and the leaf tissue. Then the values obtained from one leaf were averaged and used for statistical analysis. In total, 20 traits were examined, describing leaf morphology and the structure of the epidermis, mesophyll and petiole tissues (Table 1, Supplementary Material).

The number of stomata per unit of dry leaf weight was determined as the ratio of the number of stomata per unit of leaf area to leaf mass per unit of leaf area. The ratio of palisade mesophyll area in the transverse section of the lamina to total mesophyll area (palisade index) was determined as Spal/(Spal+Sspon+Swat), where Spal, Sspon, Swat were areas of palisade mesophyll cells, spongy mesophyll cells and water-storage tissue respectively. We defined water-storage tissue (water-storing parenchyma, hydrenchyma) as living mesophyll cells with large vacuoles, which were fully or partially chlorophyll-free (Shields 1950; Willert *et al.* 1992; Evert & Eichhorn 2006; Ogburn & Edwards 2010; Jura-Morawiec & Marcinkiewicz 2020; Heyduk 2021). The ratio of total mesophyll area to intercellular spaces area in the transverse section of the lamina (mesophyll density) was determined as (Sint+Spal+Sspon+Swat)/Sint, where Sint was the area of intercellular spaces and the other abbreviations were the same as in the previous equation. Stomatal density was determined as number of stomata per 1 mm^2. The number of cell generations (how many times has the cell pool divided) in abaxial and adaxial epidermis was determined as $\log_{10}(Ncell \times Slam)/\log_{10}2$, where Ncell was cell number per 1 mm^2 and Slam was lamina area. Stomatal index was determined as 2Nstom/(2Nstom+Ncell_low), where Nstom was the number of stomata per 1 mm^2 and Ncell_low was abaxial epidermis cell number per 1 mm^2. We defined transfusion tissue as living cells of irregular isodiametric shape with secondary cell wall thickenings adjoining midvein (Frank 1864; Griffith 1957; Hu & Yao 1981). We defined accessory transfusion tissue (ATT) as elongated lignified dead cells with bordered pits which were perpendicular to the midvein

(Worsdell 1897; Buchholz & Gray 1948; Lee 1952; Griffith 1957). Relative conducting surface was determined as ratio of lamina area to xylem area in petiole transverse section. Vein density was calculated as the total length of all veins (mm) in an area of 1 cm^2. We did not determine this index for single-veined leaves. In the case of *P. saligna*, a vertical stack of ATT tracheids in contact with each other was taken as a vein equivalent. Maximum mesophyll hydraulic path length was measured according to Brodribb *et al.* (2007) with an exception of *P. saligna*. For this species, we took the stack of ATT tracheids as the unit of the conductive element of the leaf (vein equivalent). Also for *P. saligna*, the horizontal apoplastic path length was taken to be zero. Leaf size classes were determined according to Raunkiaer's (1934) classification. The size of starch grains in chloroplasts of *P. nubigena* leaves was determined as starch area in TEM images. Qualitative assessment of trait values was adapted from Vasiliev (1988) and Ash *et al.* (1999).

Data analysis

For each of the quantitative traits, descriptive statistics (mean, minimum, maximum, standard error and standard deviation) were calculated. The average size of starch grains in the chloroplasts of palisade, spongy and water-storage tissue of *P. nubigena* leaves was compared using Mann-Whitney U-test in STATISTICA 10.0 software (Tibco Software, USA). For preliminary assessment of leaf structural traits correlations, the Pearson correlation coefficient was used. After that, the most informative traits were chosen and analyzed using principal component analysis (Kendall & Stuart 1977; Jolliffe 2002) using STATISTICA 10.0 software (Tibco Software, USA).

RESULTS

Leaf structure

P. nubigena leaves (Fig. 1A) had the multilayered, dorsoventral mesophyll (Fig. 2A). Palisade mesophyll contained well-developed chloroplasts with small starch grains (average area was 3.05±0.22 μm^2, n = 94) (Fig. 2B). There were occasional small crystals on spongy mesophyll cell surface. A significant part of the mesophyll volume in *P. nubigena* leaves was taken up by water-storage tissue. It was located in the central part of the leaf and was accompanied from above and below by chlorenchyma cells (Fig. 2A). Water-storing cells were larger than the spongy mesophyll cells and were connected with them by numerous plasmodesmata (Fig. 2C). This tissue was poorly specialized and contained chloroplasts (Fig. 2D). They differed from spongy mesophyll chloroplasts (Fig. 2E) in that they had a weaker thylakoid system and larger starch grains (average area of starch grains in chloroplasts of spongy mesophyll cells was 4.90±0.42 μm^2, n = 94 vs 9.09±0.43 μm^2, n = 94 in water-storing cells; $P < 0.05$ in Mann-Whitney U-test). Occasional sclereids occurred in the mesophyll. They were similar in shape and size to spongy mesophyll cells. Their lumens were usually small. One-layer hypodermis lay beneath the adaxial epidermis and above the abaxial epidermis, discontinuous near the stomata. Hypodermal

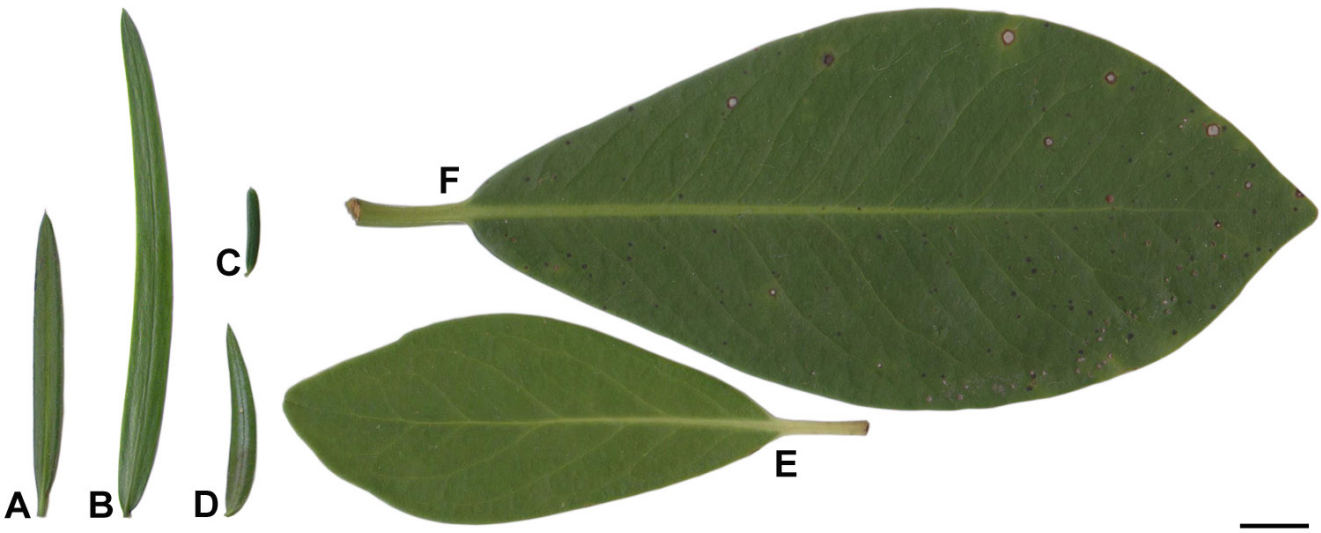

Figure 1. General view of the leaves of the studied species. A, *Podocarpus nubigena*, B, *P. saligna*, C, *Prumnopitys andina*, D, *Saxegothaea conspicua*, E, *Drimys andina*, F, *D. winteri*. Scale bar = 1 cm. / Visión general de las hojas de las especies estudiadas. A, *Podocarpus nubigena*, B, *P. saligna*, C, *Prumnopitys andina*, D, *Saxegothaea conspicua*, E, *Drimys andina*, F, *D. winteri*.

cells were fibres. There was fibre aggregation near the leaf margin. The water conduction system in the leaves of *P. nubigena* included the midvein with scarce transfusion tissue located on either side (Fig. 2F). The midvein included sieve cells, parenchyma cells and tracheids. Transfusion tissue consisted of isodiametric tracheids (Figs. 2G-2I) of irregular shape bearing scalariform and reticulate thickenings, which were occasionally located in the same cell. Tori were absent. Transfusion tissue of *P. nubigena* belonged to the *Taxus*-type according to classification of Hu & Yao (1981).

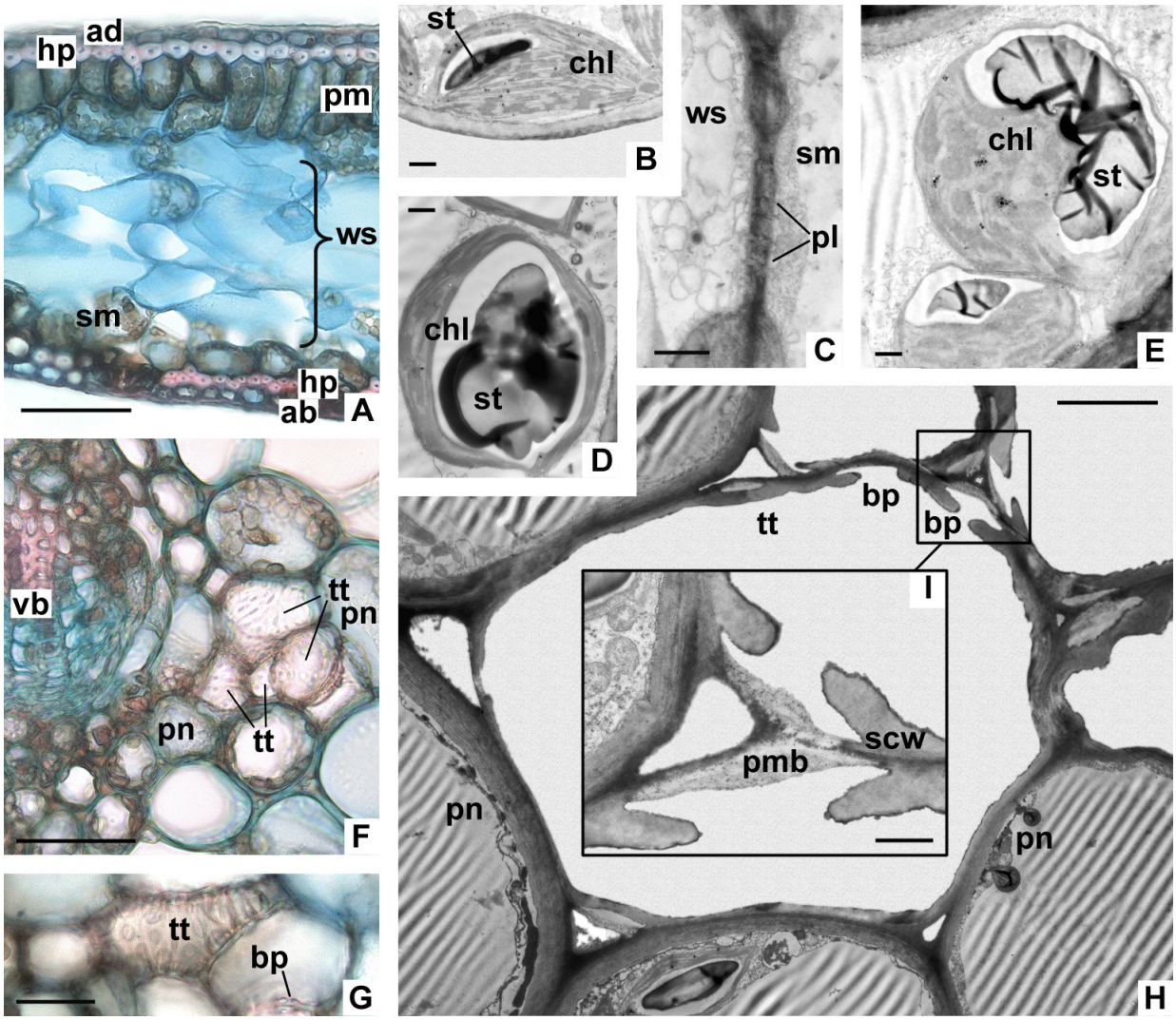

Figure 2. *Podocarpus nubigena* leaf structure. Light (A, F, G) and TEM (B-E, H, I) micrographs of transverse sections. A, leaf lamina, B, chloroplast in palisade mesophyll cell, C, plasmodesmata between spongy mesophyll cell and water-storing cell, D, chloroplast in water-storing parenchyma, E, chloroplast in spongy mesophyll cell, F, water conduction tissues in leaf lamina, G, H, transfusion tracheid, I, bordered pit in transfusion tracheid. Scale bar = 1 µm (B-E, I), 5 µm (H), 20 µm (G), 50 µm (F), 100 µm (A). ad, adaxial epidermis; ab, abaxial epidermis; bp, bordered pit; chl, chloroplast; hp, hypodermis; pl, plasmodesmata; pmb, pit membrane; pm, palisade mesophyll; pn, parenchyma; scw, secondary cell wall; sm, spongy mesophyll; st, starch; tt, transfusion tracheid; vb, vascular bundle; ws, water-storing parenchyma. / Estructura foliar de *Podocarpus nubigena*. Micrografías de luz (A, F, G) y del TEM (B-E, H, I) de secciones transversales. A, lámina de la hoja, B, cloroplasto en una célula del mesófilo de empalizada, C, plasmodesmos entre células del mesófilo esponjoso y células que almacenan el agua, D, cloroplasto en parénquima de almacenamiento de agua, E, cloroplasto en células del mesófilo esponjoso, F, tejidos conductores de agua a la lámina de la hoja, G, H, traqueida de transfusión, I, punteadura rebordeada en una traqueida de transfusión. Escala = 1 µm (B-E, I), 5 µm (H), 20 µm (G), 50 µm (F), 100 µm (A). ad, epidermis adaxial; ab, epidermis abaxial; bp, punteadura rebordeada; chl, cloroplasto; hp, hipodermis; pl, plasmodesmos; pmb, membrana de punteadura; pm, mesófilo empalizada; pn, parénquima; scw, membrana secundaria; sm, mesófilo esponjoso; st, almidón; tt, traqueida de transfusión; vb, haz vascular; ws, parénquima de almacenamiento de agua.

P. saligna leaves (Fig. 1B) had the multilayered, dorsoventral mesophyll (Fig. 3A). One or two layers of hypodermal fibres lay beneath the adaxial epidermis and above the abaxial epidermis, discontinuous near the stomata. The number of fibres increased near the midvein. The water conduction system in the leaves of *P. saligna* included the midvein, conspicuous transfusion tissue and ATT (Fig. 3A). The midvein included sieve cells, parenchyma cells and tracheids. Transfusion tracheids lay on both sides of the midvein and had an irregular isodiametric shape, sometimes elongate. They bore scalariform thickenings or circular bordered pits (Fig. 3B). Pit apertures were circular or slitlike. The border was pronounced (Fig. 3C and 3D). Tori were absent. One might describe the transfusion tracheids in the leaves of *P. saligna* as specialized. They differed from wood tracheids in the disordered pit arrangement that could not be classified as either alternate or opposite. The sheath of transfusion tissue consisted of one layer of cells with vacuoles filled with electron-dense material. ATT was composed of long tracheids, which extended perpendicularly to the midvein and almost reached the edge of leaf lamina (Figs. 3A and 3E). Tracheids were in close contact with each other in vertical stacks (Fig. 3F). The density of this stacks was 4 times higher than vein density of *Drimys* species (Table 1, Supplementary Material). ATT tracheids had conspicuous cavities (Fig. 3G) and slitlike bordered pits (Fig. 3H). Transfusion tissues of *P. saligna* belonged to the *Cycas*-type according to classification of Hu & Yao (1981). There were many prismatic or irregular crystals on the cell surface and in the primary cell wall of ATT tracheids (Fig. 3I). Parenchyma cells between ATT and mesophyll also bore numerous crystals on the walls contacting with intercellular spaces (Fig. 3J).

P. andina leaves (Fig. 1C) had the multilayered and isolateral mesophyll (Fig. 4A). Abaxial palisade mesophyll was prominent near leaf margins and included one discontinuous layer of cells. Hypodermis was absent. The water conduction system in the leaves of *P. andina* included the midvein with well-developed transfusion tissue located on either side (Fig. 4B). The midvein included sieve cells, parenchyma cells and tracheids. Transfusion tissue consisted of circular-elongated

Table 1. Factor loadings and percentage of variance for the three principal components from the 16 traits analysed. Zero and point before values are omitted. Loadings with the absolute value of the Pearson correlation coefficient greater than or equal 0.5 are marked bold. / Factores de carga y porcentaje de la varianza para tres componentes principales de 16 rasgos analizados. Cero y punto antepuesto a los valores son omitidos. Las cargas con valor absoluto del coeficiente de correlación de Pearson que son mayores o iguales a 0,5 aparecen en negrita.

Characters	Principal components		
	1	2	3
Lamina area	**-950**	-141	-003
Lamina thickness	002	-300	**-880**
Palisade index	**-567**	**662**	161
Mesophyll density	-195	**515**	**612**
Number of palisade mesophyll layers	**-755**	310	018
Number of spongy mesophyll layers	**-610**	-431	246
Number of abaxial epidermis cells per 1 mm^2	**-691**	474	-309
Stomatal density	**-816**	-499	025
Number of stomata per 1 mg of dry leaf weight	-058	**-907**	-244
Stomatal index	-117	**-880**	291
Number of cell generations in abaxial epidermis	**-947**	-113	155
Xylem area in petiole transverse section	**-931**	-039	-107
Phloem area in petiole transverse section	**-940**	-034	-089
Average area of tracheid lumens	**-822**	143	-254
Area of tracheid lumens in bundle transverse section	-392	194	-401
Relative conducting surface	-271	-357	**750**
Variation explained, %	43.2	21.0	14.4

tracheids with reticulate thickenings with a prominent border (Figs. 4C-4E). Tori were absent. Transfusion tissue of *P. andina* belonged to the *Taxus*-type according to classification of Hu & Yao. In the transfusion tissue sheath cells, there were occasional large prismatic crystals in the middle lamella (Fig. 4F). Sometimes they could be found on the walls of mesophyll cells contacting with intercellular spaces (Fig. 4G).

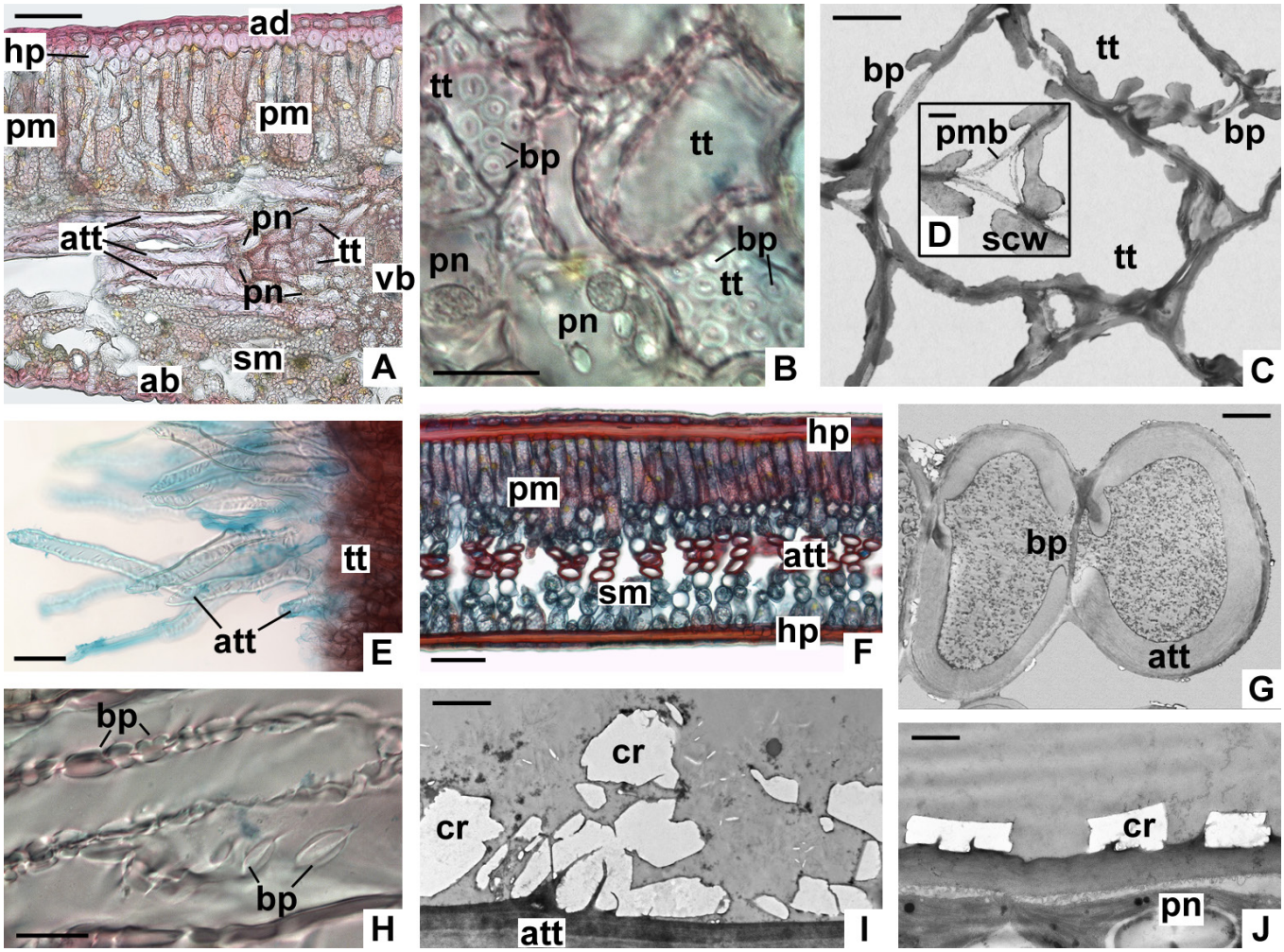

Figure 3. *Podocarpus saligna* leaf structure. Light (A, B, E, F, H) and TEM (C, D, G, I, J) micrographs of transverse sections (A-D, H-J) and longitudinal sections (F, G), with respect to the long axis of the leaf. A, water conduction tissues in leaf lamina, B, C, transfusion tracheids, D, bordered pit in transfusion tracheid, E, fragment of the leaf conductive tissues separated from the mesophyll, stacked (F) accessory transfusion tracheids (G, H), I, crystals in intercellular space on the accessory transfusion tracheid, J, crystals in intercellular space on parenchyma cell. Scale bar = 1 µm (D, J), 2 µm (I), 5 µm (C, G), 20 µm (B, H), 100 µm (A, E, F). ad, adaxial epidermis; ab, abaxial epidermis; att, accessory transfusion tracheid; bp, bordered pit; cr, crystal; hp, hypodermis; pmb, pit membrane; pm, palisade mesophyll; pn, parenchyma; scw, secondary cell wall; sm, spongy mesophyll; tt, transfusion tracheid; vb, vascular bundle. / Estructura foliar de *Podocarpus saligna*. Micrografías de luz (A, B, E, F, H) y del TEM (C, D, G, I, J) de secciones transversales (A-D, H-J) y secciones longitudinales (F, G), en relación con el eje largo de la hoja. A, tejidos conductores de agua en la lámina de la hoja, B, C, traqueidas de transfusión, D, punteadura rebordeada en una traqueida de transfusión, E, fragmento de los tejidos conductores de la hoja separados del mesófilo, traqueidas de transfusión accesoria (G, H) apiladas (F), I, cristales en espacio intercelular sobre la traqueida de transfusión accesoria, J, cristales en espacio intercelular sobre la célula de parénquima. Escala = 1 µm (D, J), 2 µm (I), 5 µm (C, G), 20 µm (B, H), 100 µm (A, E, F). ad, epidermis adaxial; ab, epidermis abaxial; att, traqueida de transfusión accesoria; bp, punteadura rebordeada; cr, cristal; hp, hipodermis; pmb, membrana de punteadura; pm, mesófilo empalizada; pn, parénquima; scw, membrana secundaria; sm, mesófilo esponjoso; tt, traqueida de transfusión; vb, haz vascular.

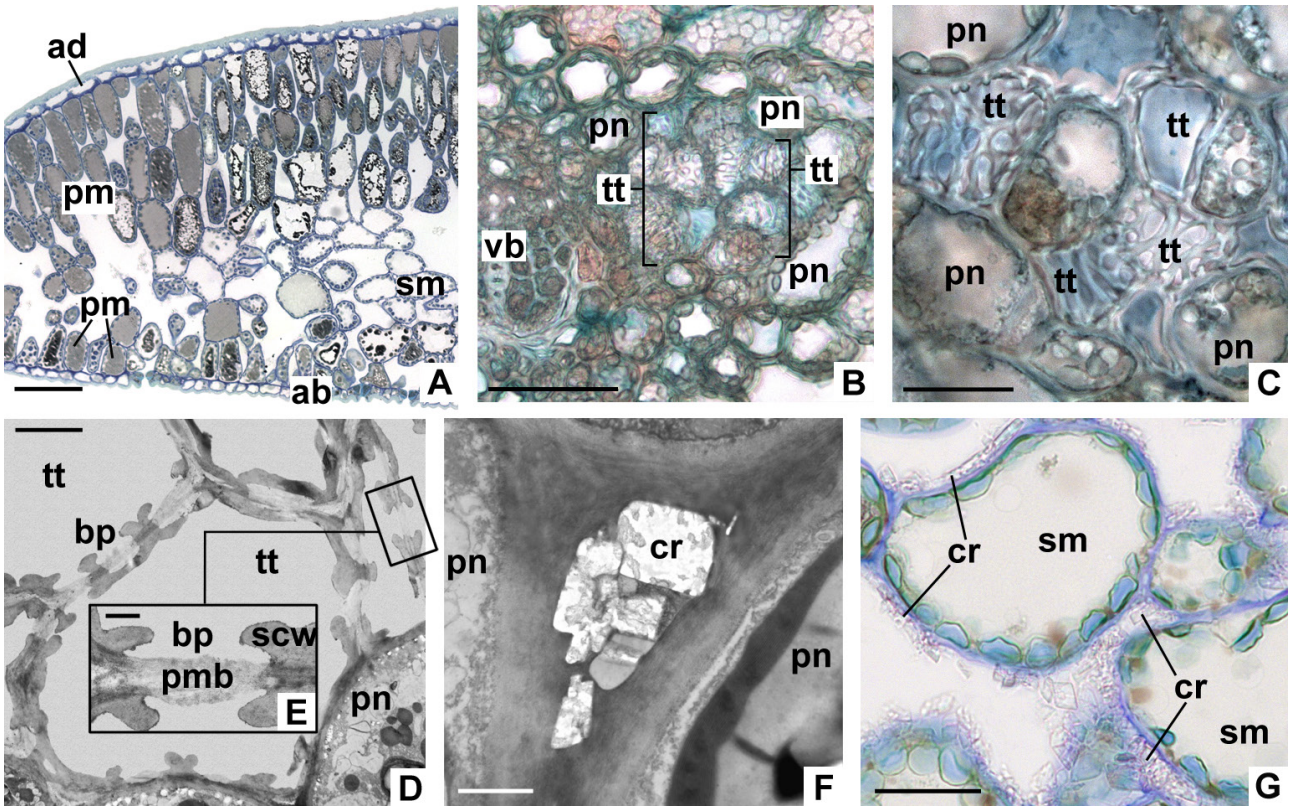

Figure 4. *Prumnopitys andina* leaf structure. Light (A-C, G) and TEM (D-F) micrographs of transverse sections. A, leaf lamina, B, water conduction tissues in leaf lamina, C, D, transfusion tracheids, E, bordered pit, F, crystals in the cell wall of transfusion tissue sheath cells, G, crystals in the intercellular spaces of spongy parenchyma. Scale bar = 1 µm (E, F), 5 µm (D), 20 µm (C, G), 50 µm (B), 100 µm (A). ad, adaxial epidermis; ab, abaxial epidermis; bp, bordered pit; cr, crystal; pmb, pit membrane; pm, palisade mesophyll; pn, parenchyma; scw, secondary cell wall; sm, spongy mesophyll; tt, transfusion tracheid; vb, vascular bundle. / Estructura foliar de *Prumnopitys andina*. Micrografías de luz (A-C, G) y del TEM (D-F) de secciones transversales. A, lamina de la hoja, B, tejidos conductores de agua en la lámina de la hoja, C, D, traqueidas de transfusion, E, punteadura rebordeada, F, cristales en la pared celular de las células de la vaina del tejido de transfusión, G, cristales en los espacios intercelulares del parénquima esponjoso. Escala = 1 µm (E, F), 5 µm (D), 20 µm (C, G), 50 µm (B), 100 µm (A). ad, epidermis adaxial; ab, epidermis abaxial; bp, punteadura rebordeada; cr, cristal; pmb, membrana de punteadura; pm, mesófilo empalizada; pn, parénquima; scw, membrana secundaria; sm, mesófilo esponjoso; tt, traqueida de transfusión; vb, haz vascular.

S. conspicua leaves (Fig. 1D) had the multilayered, dorsoventral mesophyll (Fig. 5A). A significant part of the mesophyll volume in *S. conspicua* leaves was taken up by water-storage tissue. It was located in the central part of the leaf and was accompanied from above and below by one or two layers of spongy mesophyll cells. Its cells were much larger than chlorenchyma cells. No plasmodesmata were found between water-storing cells and spongy mesophyll cells. Plastids in water-storing cells occurred very rarely. They were small and had extremely weak thylakoid system and contain virtually no starch (Fig. 5B). One layer of hypodermal fibres lay beneath the adaxial epidermis and above abaxial epidermis, discontinuous near the stomata and midvein. The water conduction system in the leaves of *S. conspicua* included the midvein with transfusion tissue located on either side (Fig. 5C). The midvein included sieve cells, parenchyma cells and tracheids. Transfusion tissue consisted of isodiametric tracheids of irregular shape with reticulate cell wall thickenings. The border was not prominent (Fig. 5D). Tori were absent. Unlike other species, the transfusion tissue of *S. conspicua* was interspersed with parenchyma cells. It belonged to the *Taxus*-type according to classification of Hu & Yao (1981).

D. andina leaves (Fig. 1E) had the multilayered, dorsoventral mesophyll (Fig. 6A). Palisade mesophyll contained occasional oil idioblasts (Fig. 6A). Hypodermis was absent. The water conduction system in the leaves of *D. andina* included the system of reticulate brochidodromous veins. Minor veins consist of sieve tubes, parenchyma cells and tracheids.

D. winteri leaves (Fig. 1F) had the multilayered, dorsoventral mesophyll (Fig. 6B). Palisade mesophyll contained occasional oil idioblasts. Hypodermis lay beneath the adaxial epidermis and consisted of one layer of isodiametric cells with contents similar to palisade mesophyll cells. The walls of hypodermis cells were not thickened (Fig. 6B). The water conduction system in the leaves of *D. winteri* included the system of reticulate brochidodromous veins. Minor veins consisted of sieve tubes, parenchyma cells and tracheids.

Comparison of the leaf structure of the studied species

Petiole and lamina structure of the studied species was collated using principal component analysis (for the quantitative values of traits see Table 1, Supplementary Material). The analysis extracted five factors. The first three axes accounted for 78.6% of the total variance (Table 1). The first component accounted for 43.2% of total variance and was weighted heavily for lamina area, xylem and phloem area, average area of tracheid lumens, number of layers of palisade and spongy mesophyll, palisade index, number of cell generations in abaxial epidermis, stomatal density and number of ordinary epidermal cells in abaxial epidermis. The indicator trait was lamina area (r = -0.95). This component mainly characterized leaf size and degree of development of structural elements responsible for water conduction. Considering the correlation of traits with the first component, we could conclude that larger leaves have more vascular tissue in the petiole and larger tracheid lumens. The mesophyll in large leaves consisted of multiple layers with conspicuous palisade parenchyma. There were numerous anticlinal divisions in abaxial epidermis, which led to a higher number of cells per unit of area. At the same time, stomata in the abaxial epidermis were arranged relatively densely. The combination of features listed above was the most pronounced in *D. winteri*, which stood separately from other species in the factor space (Fig. 7). On the other hand, small leaves had less xylem and phloem area, and their tracheid lumens in the petiole were small. There were also fewer mesophyll layers than in the previous case, palisade parenchyma was weakly developed. The cells of the abaxial epidermis divided less frequently and their number per unit of epidermis area was small, the stomatal density was also low. The listed structural features were first of all characteristic of *P. nubigena* and *S. conspicua*, the mesophyll of which was partly transformed into water-storage tissue. *D. andina*, *P. andina*, and *P. saligna* stood in the intermediate position between the extreme values of the F1 trait complex (Fig. 7). It should be noted that species distribution in the F1 axis did not replicate leaf size ranking. In particular, *P. andina* (the smallest average lamina size) tended towards an intermediate position and grouped with *P. saligna* (the area of the lamina was by an order of magnitude larger). This indicated the dominant influence of the complex of water relations-associated traits on the distribution of species in the factor space.

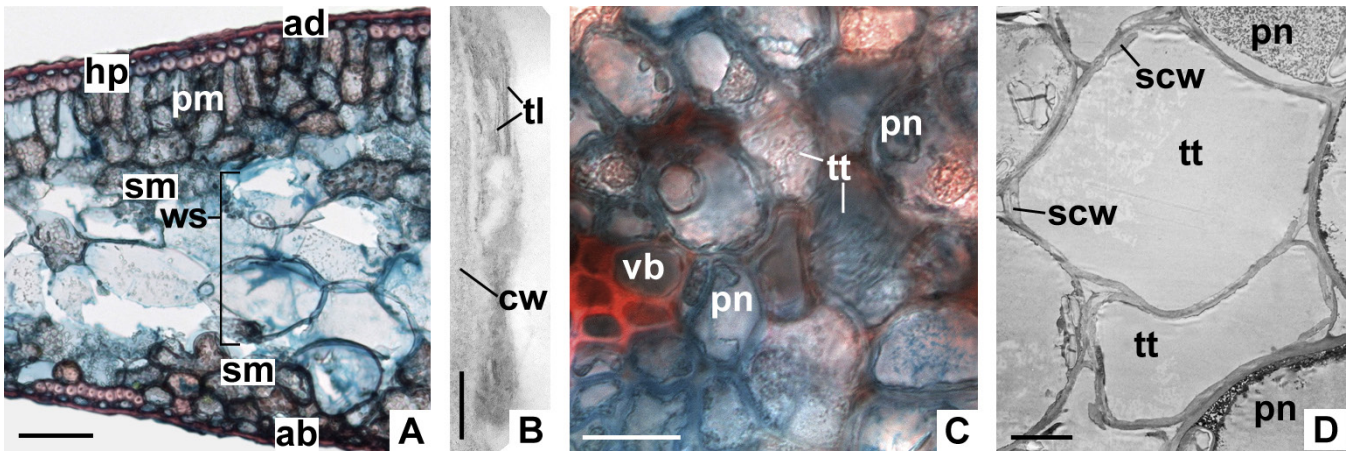

Figure 5. *Saxegothaea conspicua* leaf structure. Light (A, C) and TEM (B, D) micrographs of transverse sections. A, water conduction tissues in leaf lamina, B, plastid in water-storing parenchyma cell, C, D, transfusion tracheids. Scale bar = 100 nm (B), 5 µm (D), 20 µm (C), 100 µm (A). ad, adaxial epidermis; ab, abaxial epidermis; cw, cell wall; hp, hypodermis; pm, palisade mesophyll; pn, parenchyma; scw, secondary cell wall; sm, spongy mesophyll; tl, thylakoid; tt, transfusion tracheid; vb, vascular bundle; ws, water-storing parenchyma. / Estructura foliar de *Saxegothaea conspicua*. Micrografías de luz (A, C) y del TEM (B, D) de secciones transversales. A, tejidos conductores de agua en la lámina de la hoja, B, plastos en una célula de parénquima de almacenamiento de agua, C, D, traqueidas de transfusion. Escala = 100 nm (B), 5 µm (D), 20 µm (C), 100 µm (A). ad, epidermis adaxial; ab, epidermis abaxial; cw, pared celular; hp, hipodermis; pm, mesófilo empalizada; pn, parénquima; scw, membrana secundaria; sm, mesófilo esponjoso; tl, tilacoide; tt, traqueida de transfusión; vb, haz vascular; ws, parénquima de almacenamiento de agua.

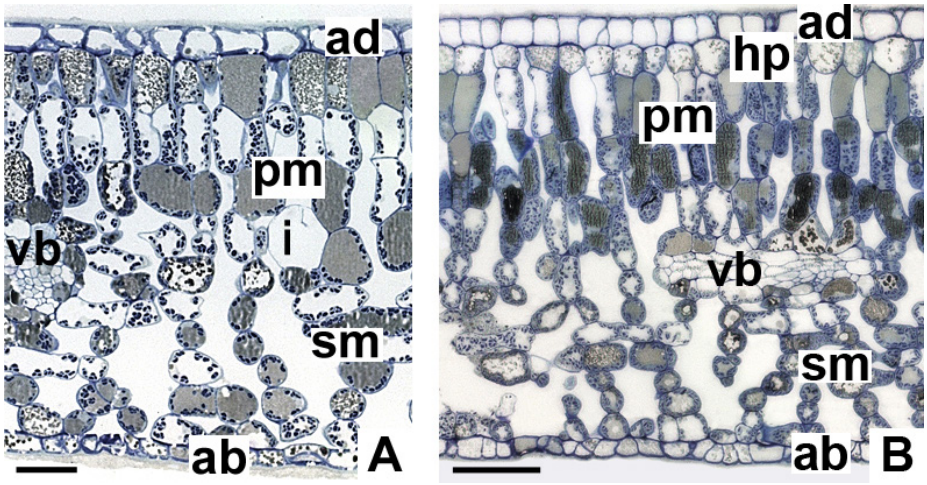

Figure 6. *Drimys* leaf structure. Light micrographs of transverse sections. A, leaf lamina of *D. andina*, B, leaf lamina of *D. winteri*. Scale bar = 50 µm (A), 100 µm (B). ad, adaxial epidermis; ab, abaxial epidermis; i, idioblast; pm, palisade mesophyll; sm, spongy mesophyll; vb, vascular bundle. / Estructura foliar de *Drimys*. Micrografías de luz de secciones transversales. A, lámina foliar de *D. andina*, B, A, lámina foliar de *D. winteri*. Escala = 50 µm (A), 100 µm (B). ad, epidermis adaxial; ab, epidermis abaxial; i, idioblasto; pm, mesófilo empalizada; sm, mesófilo esponjoso; vb, haz vascular.

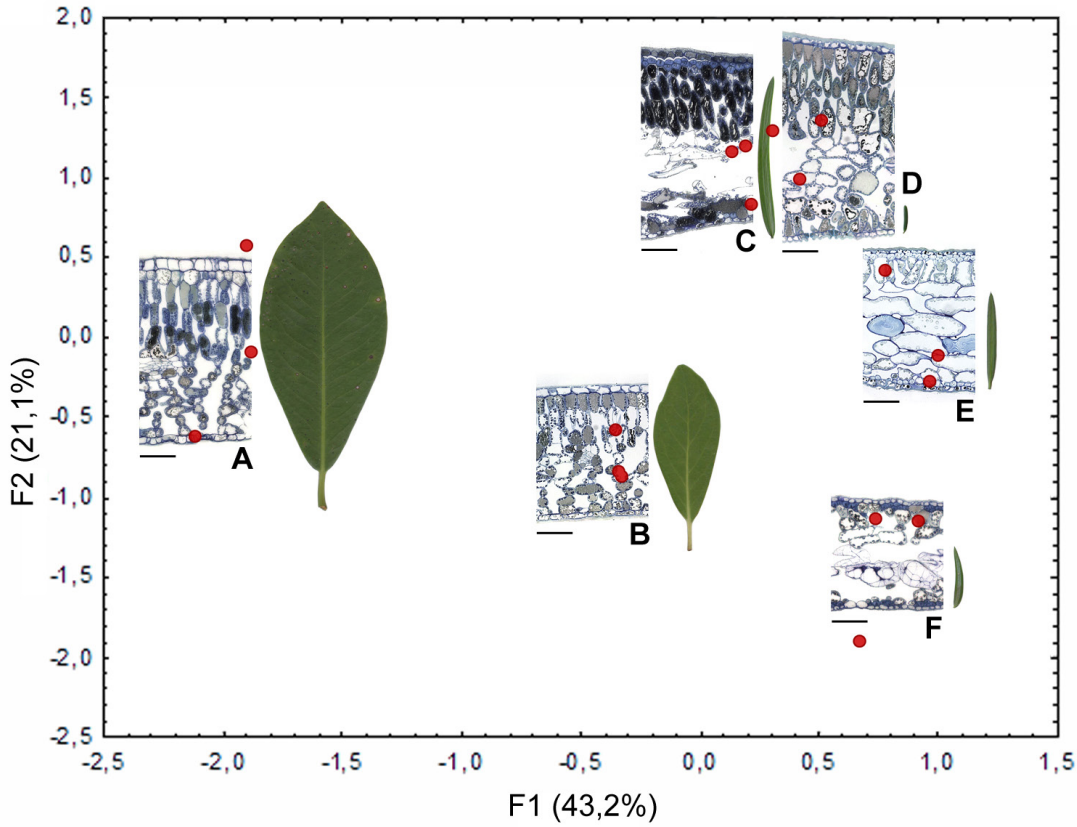

Figure 7. Principal component analysis scatterplot of the first two axes based on 16 structural traits, displaying leaf structural groups in vesselless trees in the temperate rainforest of south-central Chile. A, *Drimys winteri*, B, *D. andina*, C, *Podocarpus saligna*, D, *Prumnopitys andina*, E, *Podocarpus nubigena*, F, *Saxegothaea conspicua*. Red dots indicate individuals. / Diagrama de dispersión del análisis de componentes principales de los dos primeros ejes basados en 16 rasgos estructurales, que muestra los grupos estructurales de las hojas de especies arbóreas sin vasos del bosque templado lluvioso del centro-sur de Chile. A, *Drimys winteri*, B, *D. andina*, C, *Podocarpus saligna*, D, *Prumnopitys andina*, E, *Podocarpus nubigena*, F, *Saxegothaea conspicua*. Los puntos rojos indican individuos.

The second component accounted for 21% of total variance. It was weighted heavily for palisade index, number of stomata per unit of leaf dry weight and stomatal index. F2 was also weighted heavily for traits which showed a slightly lower level of association: stomatal density (r = -0.499) and mesophyll density (r = 0.515). The indicator trait was the number of stomata per unit of leaf dry weight (r = -0.907). F2 complex included structural traits responsible for leaf gas exchange properties. They reflected a connection between the number of stomata, the volume of intercellular spaces and the biomass of photosynthetic organs. *S. conspicua* lay separately in the F2 axis due to the weakly developed palisade parenchyma, very dense mesophyll and the highest number of stomata per unit of leaf dry weight among the studied species. *P. saligna* and *P. andina* were the most remote from *S. conspicua* in the F2 axis. These species had a small number of stomata per unit of dry leaf weight, a small stomatal index, sparsely located stomata and well-developed palisade tissue.

The third factor accounted for 14.4% of total variance. It was weighted heavily for lamina thickness, mesophyll density and relative conducting surface. The indicator trait was lamina thickness (r = -0.880). With respect to these traits S. *conspicua* again stood out due to extremely thick lamina, very dense mesophyll and the least relative conducting surface among all the studied species. *D. andina* stood in the opposite position in the F3 axis due to a relatively thin lamina and the largest relative conducting surface among the studied species. The fourth factor accounted for 8.5% of total variance and was weighted heavily only for the area of tracheid lumens in the transverse section of the vascular bundle. The fifth principal component accounted for 5.6% of total variance and none of the traits included in the analysis were weighted heavily for this factor.

DISCUSSION

The results confirmed the working hypothesis. The leaves of the studied species of vesselless seed plants differ significantly in their structure, including the complexes of traits involved in leaf water relations (Table 1, Supplementary Material and Figs. 2-6). Based on the leaf anatomy, we infer there are two contrasting 'water management' strategies, aimed either at increasing water movement or at water retention. These two directions of adaptation are also present in the leaves of angiosperms (Gamalei 2004; Kadereit *et al*. 2021).

Leaves of *Drimys* species have structural features creating conditions for accelerated delivery of large volume of water to the mesophyll cells with a transpiration stream, maintaining the photosynthesis. They do not only have an obviously larger leaf lamina compared to the genus *Podocarpus*, but also a larger amount of xylem in the petiole, larger tracheid lumens, reticulate venation and the highest stomatal density among the studied species (Table 1, Supplementary Material). Measured values of vein density of *Drimys* species are low compared to other angiosperms (Boyce *et al*. 2009), which is consistent with other studies (McElwain *et al*. 2016). Maximum mesophyll hydraulic path length in *Drimys* leaves is among the highest values for angiosperms (Brodribb *et al*. 2007), but is much shorter than in the studied podocarps, with an exception of *P. saligna* (Table 1, Supplementary Material).

The leaves of *P. saligna* have features that facilitate water transport through the leaf tissues. It does not have reticulate venation, however it has a well-developed transfusion tissue and ATT. Transfusion tissue accompanying the midvein is common in gymnosperms and undoubtedly facilitates water conduction to the adjacent mesophyll cells (Frank 1864; Lederer 1955; Esau 1977; Hu & Yao 1981; Dörken 2013; Dörken & Parsons 2016; Dörken *et al*. 2019; Moreau *et al*. 2021). Transfusion tissue in the leaves of *P. saligna* consists of multiple highly specialized tracheids, the pitting pattern of which is similar to wood tracheids. ATT composed of elongated tracheids additionally enhances water transport in the leaves of *P. saligna*. It was described for podocarps with broad lamina (Griffith 1957; Brodribb *et al*. 2007; Locosselli & Ceccantini 2012). Although stacks of ATT tracheids are less specialized conductive units than veins of angiosperms, this is offset by their number and extremely short maximum mesophyll hydraulic path length (Table 1, Supplementary Material and Fig. 3F). The venation density in *P. saligna* is within the angiosperm range, although it is much higher than the average (Boyce *et al*. 2009). Our data are fully consistent with earlier studies connecting ATT with increased hydraulic conductivity in areas of the leaf lamina remote from the midvein (Brodribb & Holbrook 2005; Brodribb *et al*. 2007). *P. saligna* has the widest leaves among the studied Podocarpaceae (Table 1, Supplementary Material). It is the closest to angiosperms in the factor space along the F1 axis (Fig. 7).

Tightly contacting cells of *P. andina* palisade tissue facilitate the movement of water in the lamina due to the apoplastic transport via cell walls (Fig. 4A). *P. andina* is notably close to *P. saligna* in the factor space along the axis of the first factor, which includes leaf traits, responsible for water conduction and transpiration (Fig. 7). Crystals in the leaves of *P. andina* and *P. saligna* are likely to be calcium oxalate. They are believed to result from the elevated water flow through the leaves of these species. Although the leaves of *P. andina* do not have ATT, their hydraulic conductance was reported to be about 4 mmol/(m^2sMPa) (Brodribb *et al*. 2014), which exceeds *P. saligna* leaf hydraulic conductance (2.3 mmol/

(m^2sMPa)), measured in the same study, and is equal to *D. winteri* leaf hydraulic conductance published in another work (3.9±0.9 mmol/(m^2sMPa) (Brodribb *et al.* 2005)). An inverse (compensatory) relationship between the density of venation and the mesophyll cells density in dicotyledons has long been identified (Wylie 1946). This indicates the similar functions of these two aspects of the leaf anatomy in maintaining the water supply of the leaf. Later, new instrumental methods allowed to obtain experimental evidence that the higher the thickness of the palisade mesophyll, the lower the hydraulic resistance of the leaf (Sack & Frole 2006). However, hydraulic transport through the apoplast appears to be efficient over short distances. Indeed, the leaves of *P. andina* are the narrowest among the studied podocarps (Table 1, Supplementary Material). This confirms the point of view, that univeined anatomy of leaves of most gymnosperms is a considerable limitation on overall leaf size and shape (Hill & Brodribb 1999).

The unique feature of the leaves of *P. nubigena* and *S. conspicua* is the water-storage tissue (hydrenchyma). It is much more specialized in the leaves of *S. conspicua*, representing an example of so-called storage succulence, whereas in the leaves of *P. nubigena* hydrenchyma can be related to all-cell succulence. History of these terms was reviewed by Males (2017). Hydrenchyma is generally recognized as accumulating water and occurs in a number of plants growing in arid climate (Willert *et al.* 1990; Willert *et al.* 1992; Ogburn & Edwards 2010; Males 2017). Unfortunately, we have little data on the conditions under which *P. nubigena* and *S. conspicua* evolved. Their modern habitats are not dry. If we assume evolution in arid climate in the past, *P. nubigena* and *S. conspicua* would have been adapted to full light, but they are highly shade-tolerant (Donoso 1989; Lusk 1996). Therefore, an ecology of *P. nubigena* and *S. conspicua* gives no reason to believe that hydrenchyma provides the persistence of the leaves in the dry season. However, one can suppose, that in this case hydrenchyma can increase the availability of water for mesophyll cells. In fact, water-storage tissue is the layers of cells that border both the midvein and the chlorenchyma cells. Mesophyll cells of *P. nubigena* and *S. conspicua* leaves, obviously, take part in the transport of water from the vein to the periphery of the leaf, which is inevitable at such high values of maximum mesophyll hydraulic path length (Table 1, Supplementary Material). The hydraulic conductance of *S. conspicua* leaves is reported to vary from 1.6 mmol/(m^2cMPa) (Brodribb *et al.* 2005) to 2.5 mmol/(m^2cMPa) (Brodribb *et al.* 2014), which is close to the *P. saligna* values. High degree of parenchymatization indicates a possible functional change in that part of the water transport path in *S. conspicua* leaves, which is mediated by the transfusion tissue.

In summary, leaves of Podocarpaceae species of the temperate rainforest of south-central Chile have significant convergence with flowering plants in the presence of tissues and structural features that are involved in the water conduction to the mesophyll cells. The ways of expressing this convergence are 1) ATT, which is an analogue of lateral veins in the leaf of dicotyledons, 2) tightly contacting mesophyll cells, which, like in flowering plants, are capable of providing apoplastic water transport over short distances and 3) hydrenchyma. In the first two cases, mesophyll cells take water directly from the transpiration stream, which is provided by the stomata operation. In turn, water-storage tissue receives water from the midvein and accumulates it for further use by chlorenchyma cells. It should be noted that hydrenchyma completely encompasses the leaf blade, which enhances the availability of water for chlorenchyma cells. The ecology of the studied Podocarpaceae species indicates that water-storage tissue is not an adaptation to environmental conditions. We assume that it can potentially maintain the hydration of leaves during insufficient water supply through the tracheids. The next step to understanding the problem of water-storage tissue in the leaves of podocarps should be the investigation of its development and physiology. Functional studies will show how informative anatomical structure can be when discussing physiological processes.

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