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Coexistent Heteroblastic Needles of Adult *Pinus canariensis* C.Sm. ex DC. in Buch Trees Differ Structurally and Physiologically

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Abstract: Great variation in shape and size between primary (juvenile) and secondary (adult) needles, so-called leaf-heteroblasty, occurs in several *Pinus* species. Most of them lose primary needles during the juvenile-to-adult transition of the tree. An exception to this is *Pinus canariensis* (a Canary Islands endemism) in which basal resprouting twigs of adult trees frequently wear both primary and secondary needles. Taking advantage of this extraordinary study-case-species, we conducted an exhaustive comparison of both needle types through quantitative analyses of needle anatomy, photochemical performance, gas exchange, and resistance to extreme dehydration and to extreme needle temperature. We hypothesized that primary needles would show lower investment to leaf structure but higher photosynthetic efficiency. Primary needles had less stomatal density and thicker and less wettable cuticles. In cross section, primary needles showed smaller structural fraction (e.g., percent of hypodermis, endodermis and vascular tissue) and higher fraction of photosynthetic parenchyma. Significant differences between primary and secondary needles were not found in net carbon assimilation nor in their leaf mass area values. Interestingly, secondary needles showed higher electron transport rate, and they were additionally much more efficient in retaining water under severe and controlled desiccant conditions. When subjected to extreme temperatures (−10° to +50 °C), primary needles recovered better their photochemical efficiency than secondary needles, after +46° and +48 °C heat-shock treatments. Our results indicate that both needle types broaden the diversity of physiological responses against environmental constraints in basal twigs of adult *P. canariensis* trees. Considering that this is a fire-resistant and resprouting species, this advantage could be particularly useful after a drastic environmental change such as a fire or a gap opening in the forest.

Keywords: Canary Islands; heteroblasty; leaf anatomy; Mediterranean climate; photoprotection; pine; primary needle; resprouting tree; secondary needle

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1. Introduction

More than a century ago, the German botanist Karl Ritter von Goebel described the term “heteroblastic species” for the first time, to distinguish plants with abrupt changes in form and function occurring along their ontogeny from those in which no abrupt changes are perceptible [1]. Heteroblasty is thus defined as conspicuous morphological changes in stem structure, internode length or, typically, leaf form, size and arrangement throughout the lifecycle of some plants [2]. Although sometimes misused in the bibliography, and as clarified in recent reviews [2–4], leaf heteroblasty differs from heterophylly, which is a case of phenotypic plasticity exhibited as leaf form alteration in response to environmental conditions such as light intensity and quality, ambient temperature, and water availability [3,4].

Heteroblasty has evolved multiple times during plant evolution in different unrelated taxa [2,3], and some exceptional cases such as New Zealand flora particularly reach in heteroblastic plants (e.g., *Pseudopanax crassifolius*) [5], *Acacia* species, or the European Ivy (*Hedera helix*) have received deep scientific attention [2]. Another remarkable example is found within conifers as several species show a large heteroblastic modification between juvenile (primary) and adult (secondary) needles [6,7].

Foliar dimorphism has been studied in different genera such as *Juniperus* and *Taxus* [8,9], but most of the investigations, especially related to heteroblasty, have been carried out in species of *Pinus*: *P. pinea*, *P. canariensis*, *P. halepensis*, *P. brutia* and also *P. pinaster* [6,10–12]. Primary pine needles of those species are typically single and with different cross-sectional shape than secondary needles. The latter, normally arrange into multiple-needle brachyblasts, are longer, thicker and with higher dry mass per area (LMA) [7,13]. While for most *Pinus* species, primary needles are replaced by secondary needles during the first growth season, in several Mediterranean species such as *P. halepensis*, *P. pinea* and *P. canariensis*, primary needles are kept by seedlings for few years [14,15]. In the case of *P. halepensis*, this succession happens in several steps and only after the fourth year merely secondary needles are found [16]. The Canary Islands pine *P. canariensis* represents a remarkable case with extremely long-lasting juvenile stages and a prominent heteroblasty between primary and secondary needles [15,17,18].

P. canariensis C.Sm. ex DC. in Buch [19] is a fire-resistant and drought-tolerant tree endemic to the Canary Islands [20,21]. The elevation range of this pine forest spreads between 1500–2000 m on the North and 900–2200 (2300) m a.s.l. on the South of the western Islands [22]. In Tenerife, this species is responsible for the treeline formation at 2000–2100 (locally 2400) m a.s.l. [23], which corresponds with a Mediterranean climate. The capability of *P. canariensis* stands to remobilize carbon reserves from woody organs allows for resprouting and crown regeneration, after extreme loss of foliage [24]. Newly resprouted shoots, after a perturbation, have only primary needles, in first place. The remarkable tolerance to drought in adult trees of *P. canariensis* is, on the other hand, linked to its capability to tap water from deep soil when growing under semi-arid Mediterranean climate [25]. Indeed, the Canary pine dedicates an important part of its resources in this phase to the construction of a powerful radical system very different from that of its fast-growing juvenile congeners. In addition, scanning electron microscopy (SEM) studies have revealed, in secondary needles, deeply sunken stomata of an unusual type with an enlarged pre-stomatal cavity, which are very likely related to a strong resistance to water loss [18,26]. When developing under water stress, the secondary needles are additionally able to adapt their internal anatomy by rising the number of sclerenchymatic cells adjacent to the vascular bundles and by diminishing the ratio of assimilation parenchyma to vascular bundle [27]. The surface of primary needles, on the other hand, is completely covered by tubular wax crystals resulting in a glaucous appearance that increase protection against UV-radiation and also against high photon flux densities [28]. In agreement with all this previous observations, seedlings with secondary needles show better performance against drought stress than seedlings with primary needles [17].

While few morpho-anatomical characters [18,28] and physiological traits such as the effect of drought [7,29], the response to high levels of CO₂ [29], and the effect of frost [30,31] of the primary vs. the secondary needles, have been evaluated in a couple of conifers including several *Pinus* species, the functional understanding of this heteroblastic dimorphism is rather limited. Overall it seems to be a trade-off between needle toughness and photochemical efficiency, which is biased towards toughness in secondary needles and towards higher photosynthetic efficiency in primary needles. Nevertheless, some evidences are contradictory and inconclusive. In addition, most previous studies have been performed in seedlings and/or immature young trees [30]. In this scenario, *P. canariensis* provides a unique case-study as primary needles constitute the photosynthetic organs not only in immature trees but also in adult tree resprouts after disturbances. Moreover, gaining knowledge on primary needle anatomy and physiology will improve our understanding

on the capacity of this species to endure forest fires that are expected to increase their frequency in future Mediterranean climate conditions [32]. *P. canariensis* is one of the five in the genus known to resprout in adult stages together with *P. rigida*, *P. serotina*, *P. leiophylla* and *P. patula* [20,33,34]. In addition, the frequent resprouting basal twigs on low density stand individuals provided us the opportunity to compare same-age primary vs. secondary needles from 60–70 y old trees, in morpho-anatomical and physiological terms. All things considered, we aimed at elucidating whether and how morpho-anatomical differences may relate with photosynthetic performance and response to abiotic stress in secondary vs. primary coexistent needles of adult *P. canariensis* trees. Specifically, we wanted to (i) characterise morpho-anatomical traits, (ii) compare photosynthetic performance and its acclimation at day/seasonal time-scales, (iii) assess tolerance to severe dehydration, and (iv) evaluate tolerance to extreme temperatures of primary vs. coexistent secondary needles.

2. Materials and Methods

2.1. Plant Material and Experimental Design

This study was conducted in a treeline pine forest located in Las Cañadas of Teide National Park, Tenerife (28°18'21.5" N, 16°34'5.8" W; Canary Islands, Spain). The study plot was at an elevation of 2070 m above sea level, where the dominant species is *Pinus canariensis* C.Sm. ex DC. in Buch., with an almost non-existent understory. The site is characterized by a semiarid Mediterranean climate with a mean annual precipitation of 368 mm, where the drought period lasts from June to August, and precipitation is concentrated in late autumn and winter. The drought period coincides with the highest temperatures, while the minimum temperatures (subzero) are reached in the rainy period [21]. At the time of study (2019–2020) the trees were 60–70 years old. All measurements were conducted in individual twigs containing same year juvenile and adult needles (Figure 1), from ten selected trees. Most measurements were conducted either in autumn (November 2019, 2020) or summer (July 2020) (specific dates, and replicates per each type of measurement are depicted in Table S1). All needles measured were, approximately 1 year old. A single experiment (to check for frost tolerance) was conducted with spring needles (April 2019). When measurements were not directly performed in the field, twigs containing both types of needles were placed in hermetically sealed bags at 100% relative humidity, to prevent desiccation, transferred to the laboratory and incubated in darkness for 16 h before experiments.

2.2. Leaf Anatomy

For morpho-anatomical analyses a total of 20 needles per type were studied: 5 twigs were selected (from a different tree each). From each branch, four representative primary and four representative secondary needles were taken. Several parameters were measured on intact needles or branches (Table 1). Finally, one cross-section per needle was analysed. A 2–3 mm length piece of the middle part of the needles was cut and fixed in FAA (100 mL FAA = 90 mL 70% ethanol + 5 mL acetic acid 96% + 5 mL formaldehyde solution 37%) for 48 h. Samples were then rinsed with water and dehydrated through a series of 50%, 70%, 85%, 96% and 100% ethanol. Needle pieces were then embedded in paraffin and sectioned at 6 µm thickness with a rotary microtome MT.5505 (PCE Iberica S.L., Albacete, Spain). All sections were dewaxed by a xylene series, stained with safranin and counterstained with fast green (Gerlach, 1984). Finally, needle samples were preserved using the EUKITT mounting medium (O. Kindler GmbH). All sections were examined with a light microscope (AmScope, CA, USA), coupled with a Dual Pixel camera (SM-G955, Samsung). Image analysis was conducted in ImageJ 1.5 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The number of hypodermis cell layers was measured considering the entire perimeter of the leaf (Figures 2 and 3). Stomatal density ($N^{\circ} \text{mm}^{-2}$) was assessed under the microscope on a square of 1 mm side carefully marked over the central part of each needle. All measured features are described on Table 2.

Figure 1. General aspect of the studied needles: (a) Intact resprouting twigs; (b) Detail of a detached twig with primary needles in the base and secondary needles in the tip. (c) Detail of a detached primary needle (left) and a brachyblast containing three secondary needles (right). Scale bars are: (b) 5 cm; (c) 2 cm.

Figure 2. Fresh cross-sections of *Pinus canariensis* needles. (a) secondary (adult) needle. (b) primary (juvenile) needle. Abbreviations: end, endodermis; ep, epidermis; hyp, hypodermis; rd, resin duct; schp, spongy chlorophyll parenchyma; st, stomata; tti, transfusion tissue; vt, vascular tissue. Scale bars are 50 µm.

Figure 3. Stained cross-sections of *Pinus canariensis* secondary (adult) needles. (a) Whole view of the needle cross-section. (b) Detail of the stomata and outer tissues of the needle. (c) Detail of the vascular bundle and all the conductive elements. (d) Detail of a resin duct. Abbreviations: end, endodermis; ep, epidermis; epc, epithelial cells; gc, guard cells; hyp, hypodermis; ph, phloem; rd, resin duct; sc, subsidiary cells; schp, spongy chlorophyll parenchyma; sec, substomatal chamber; shc, sheath cell; st, stomata; tp, transfusion parenchyma; tt, transfusion tracheid; tti, transfusion tissue; vt, vascular tissue; xy, xylem. Scale bars (a), 50 µm, (b–d), 20 µm. Safranin—fast green staining.

Table 1. Morphological traits of secondary (adult) and primary (juvenile) needles in *P. canariensis*. Values are mean $\pm$ SE ($n = 20$, except for leaf water content, dry weight/fresh weight (DW/FW) ratio and leaf mas area (LMA) where $n = 9$, and contact angle (CA) where $n = 16$ – 20). Values highlighted in bold with different letters depict significant differences between secondary (adult) and primary (juvenile) needles ($p < 0.05$).

Needle Part	Parameter	Secondary Needles	Primary Needles
General traits	Needles per brachyblast (N°)	3	1
	Internode length among needle insertions/brachyblasts (cm)	0.13 $\pm$ 0.01^b	0.22 $\pm$ 0.01^a
	Leaf length (cm)	11.5 $\pm$ 0.4^b	4.36 $\pm$ 0.10^a
	Leaf width (mm)	0.408 $\pm$ 0.002^b	0.573 $\pm$ 0.006^a
	Leaf thickness (mm)	0.231 $\pm$ 0.002 ^a	0.232 $\pm$ 0.001 ^a
	Leaf water content at turgor (gH ₂ O g ^{−1} DW)	1.39 $\pm$ 0.03 ^a	1.36 $\pm$ 0.04 ^a
	Leaf DW/FW ratio	0.418 $\pm$ 0.005 ^a	0.425 $\pm$ 0.008 ^a
	LMA (g m ^{−2})	181.7 $\pm$ 5.9 ^a	164.7 $\pm$ 5.7 ^a
	CA (°)	114.2 $\pm$ 2.7^b	137.8 $\pm$ 2.1^a
Stomata	Number of rows on adaxial surface (N°)	3 to 5	2 to 5
	Number of rows on abaxial surface (N°)	4 to 5	2 to 4
	Density on the adaxial surface (N° mm ^{−2})	39.8 $\pm$ 1.2 ^a	41.5 $\pm$ 1.7 ^a
	Density on the abaxial surface (N° mm ^{−2})	51.4 $\pm$ 1.4^b	36.9 $\pm$ 1.9^a

Table 2. Anatomical traits in secondary (adult) and primary (juvenile) needles estimated under the light microscope in stained cross-sections. Values are mean $\pm$ SE ($n = 20$). Units are specified in brackets for each parameter. Percentages refer to total cross-section area. Values highlighted in bold with different letters depict significant differences between secondary (adult) and primary (juvenile) needles ($p < 0.05$).

Needle Part	Parameter	Secondary Needles	Primary Needles
Whole needle	Cross-section area (cm ²)	0.32 $\pm$ 0.01^b	0.25 $\pm$ 0.01^a
Cuticle	Thickness (adaxial side) (μm)	0.532 $\pm$ 0.034^b	0.906 $\pm$ 0.058^a
Hypodermis	Layers (N°)	1 to 5	1 to 2
	Cells diameter (μm)	6.13 $\pm$ 0.18^b	9.20 $\pm$ 0.40^a
	Hypodermis (%)	22.9 $\pm$ 0.4^b	16.5 $\pm$ 0.2^a
Mesophyll	Spongy parenchyma (%)	33.9 $\pm$ 0.4^b	46.9 $\pm$ 0.4^a
Endodermis	Cell diameter (μm)	16.9 $\pm$ 0.5^b	21.1 $\pm$ 0.9^a
Vascular bundle	Transfusion tissue (%)	8.63 $\pm$ 0.12^b	13.9 $\pm$ 0.2^a
	Vascular bundles (N°)	2	1
	Vascular tissue (%)	4.63 $\pm$ 0.07^b	2.14 $\pm$ 0.03^a
	Xylem (%)	3.28 $\pm$ 0.06^b	1.34 $\pm$ 0.02^a
	Phloem (%)	1.35 $\pm$ 0.03^b	0.806 $\pm$ 0.014^a
Resin ducts	Number per needle	2	2
	Position	under hypodermis	under hypodermis
	Resin ducts lumen area (μm ²)	327 $\pm$ 9^b	669 $\pm$ 10^a
	Resin ducts diameter (μm)	40.5 $\pm$ 0.7^b	47.7 $\pm$ 0.7^a
	Sheath-cell layers (N°)	2	1
	Sheath-cell diameter (μm)	9.47 $\pm$ 0.54^b	12.97 $\pm$ 0.38^a

2.3. Chlorophyll Fluorescence Analyses

Rapid Light Curves (RLC) were developed in situ with a portable modulated fluorometer (miniPAM, Walz GmbH, Effeltrich, Germany). Measurements were carried out in four healthy needles exposed to light intensity below 40 μmol m^{−2} s^{−1} in the early morning. Then, nine increasing light intensities (from 10 to 1856 μmol m^{−2} s^{−1}) were continuously applied on the naturally exposed surface of needles at intervals of 15 s. After each interval,

a saturation pulse was applied in order to determinate steady fluorescence value under illumination (F') and maximum fluorescence value under illumination (F_m'), in order to calculate ϕPSII , and the electron transport rate (ETR), by the following formula [35–37]:

$$\text{ETR} = \phi\text{PSII} \times \text{PPFD} \times 0.5 \times 0.84 \quad (1)$$

where ϕPSII is the photochemical yield of the PSII in the light, PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 is a factor that assume equal excitation of both PSII and PSI, and 0.84 takes into account that only a fraction of incident light is really absorbed by photosystems.

The analysis of ETR/PPFD curve allows the calculation of the maximum ETR (ETR_{max}) and the maximum PPFD at which the maximum ETR is maintained (PPFD_{sat}). Both parameters are derived from a polynomial equation adjusted to the final stages of the ETR curve (Solver, Microsoft Excel, 2010). Following [38], the apparent quantum efficiency (AQE, potentially transported $\mu\text{mol electrons m}^{-2} \text{ s}^{-1}$ per each absorbed $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) was calculated directly by averaging $\phi\text{PSII} \cdot 0.5$ at lower irradiances. The minimum absorbed saturating photosynthetic photon flux density (EK , $\mu\text{mol m}^{-2} \text{ s}^{-1}$) was calculated as $\text{ETR}_{\text{max}} / \text{AQE}$ [39].

2.4. Gas Exchange Measurements

Net CO_2 assimilation (A_N) and stomatal conductance (g_s) were measured, at approximately 9.00 to 13.00 h solar time, with a portable photosynthesis system (LI-6400, Li-Cor, Lincoln, NE, USA) equipped with a 2 cm^2 leaf chamber fluorometer (6400-40, Li-Cor). Because this chamber is designated for flat leaves, needles were spread over the chamber forming a one-needle-deep layer (as previously described by Niinemets et al., e.g., [40]). The chamber was completely filled with needles preventing overlapping and wholes between needles. Healthy needles were measured at $400 \mu\text{mol CO}_2 \text{ mol}^{-1}$ air (controlled with the 6400-01 CO_2 mixer), $1200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of PPFD [41], ambient relative humidity and ambient temperature (using the cuvette cooler to avoid overheating during measurements).

2.5. Analysis of Photosynthetic Pigments

Chlorophylls and carotenoids were quantified in needles sampled the same days as gas exchange and fluorescence measurements were performed. The middle part of the needles (both types) was collected in the field at noon, frozen in liquid N_2 and stored at -80°C until analysis. Additional branches were maintained under an atmosphere saturated with H_2O , in darkness and at $+20^\circ\text{C}$ overnight (for approximately 16 h) in the laboratory. Both needle types were taken the next day and accounted as artificial (dark-acclimated) conditions and also frozen in liquid N_2 . Samples were pulverized in a mortar with liquid nitrogen and extracted with 100% acetone with CaCO_3 in order to avoid acid traces that modify the pigment composition. They were centrifuged for 15 min at 4°C and syringe-filtered through $0.45 \mu\text{m}$ Millipore filter. Pigments were quantified with a double beam spectrophotometer (Shimadzu, UV-160 A) following the method proposed by Lichtenthaler (1987) [42].

2.6. Estimation of Needle Wettability through Contact Angle

The contact angle (CA) of droplets of distilled water was measured over the surface of the needles, following [43] with small modifications. Briefly, sessile droplets of $5 \mu\text{L}$ were placed on the adaxial side of the central part of each needle. Measurements were conducted with a video-based Optical Contact Angle measuring instrument (OCA 15EC, from DataPhysics Instruments GmbH, Filderstadt, Germany). The contact angle was estimated, at one second after contact, with the SCA software (DataPhysics Instruments GmbH, Filderstadt, Germany) for optical contact angle, v.4.4.3. The measuring precision was of $\pm 0.1^\circ$. Typically, hydrophobic surfaces show water static contact angles $>90^\circ$, while hydrophilic surfaces show water static contact angles $<90^\circ$ [44]. Measurements were

performed in needles collected from 6 different trees. Each value was calculated as the mean from the optically left and right margins of the water droplet. Two to four measuring replicates were conducted over each needle. Needles were handled with caution to prevent epicuticular wax removal.

2.7. Desiccation Experiments

Two desiccation experiments were performed. In a first experiment, a modification of the “Falcon method”, described in López-Pozo et al. (2019) [45], was used to compare the tolerance to severe dehydration in both needle types [45]. Briefly, approximately 120 mg of needle pieces were desiccated at ca. 75%, 50% or 10% relative humidity (RH). In the first experiment, the relative water content (RWC) was estimated at the beginning, after 48 h of desiccation and after 24 h of rehydration as:

$$(FW - DW)/(TW - DW) \times 100 \quad (2)$$

with TW (turgor weight) corresponding with the weight measured at full turgor, FW the weight of samples at a given time during the treatment, and DW the dry weight of samples after 24 h at 70 °C in the oven. The maximal photochemical efficiency of PSII (Fv/Fm) was used as fitness estimator and measured at the beginning of the experiment, after desiccation and after rehydration treatments, and then expressed in percent as relative values to that of the control.

In a second experiment a detailed monitoring of water loss, during long dehydrations (300 h) under the two most contrasting conditions (75% and <10% RHs) was conducted in intact needles. Needle base was covered with Vaseline to prevent massive water loss from the abscission area and to evaluate, instead, desiccation kinetics through intact needle surfaces. Both WC and Fv/Fm were monitored during the long desiccation kinetic and expressed as percent of the control.

2.8. Thermo-Tolerance Assessment

Two separate experiments were conducted. To evaluate tolerance to extreme low temperature, needles collected in April (winter to spring transition) were subjected to −10 °C for 30 min and maximal photochemical efficiency of PSII (Fv/Fm) used as estimation of needle fitness. For this Fv/Fm values obtained immediately after the treatment and after 24 h of recovery (+20 °C and darkness) were compared with control values (prior treatment). To evaluate tolerance to high temperatures, needles collected in September (end of summer) were subjected to 30, 32, 34, 36, 38, 40, 42, 44, 46, 48 or 50 °C for 30 min and Fv/Fm checked immediately after the treatment and after 24 h of recovery. Five replicates per needle type were used per each treatment. For temperature treatments we followed the protocol described in [46].

2.9. Statistical Analyses

One-way ANOVA with Tukey test as *post hoc*, when necessary, was conducted to evaluate statistical differences among treatments and/or needle types after checking homoscedasticity. All tests were conducted with SPSS 2019 (IBM Corp.) and with an $\alpha = 0.05$.

3. Results

3.1. Morpho-Anatomical Features of Needles

Morphological and anatomical study of *P. canariensis* needle pairs taken from the same resprouting twigs of adult (60–70 y old) trees, led us to identify and quantify main differences between primary and secondary needles. Some of them were already apparent to the bare eye (Figure 1). Thus, the organization in single needles in macroblasts (primary) vs. three needles per brachyblast (secondary), the longer internode length among needle insertion/brachyblasts in the twig, the shorter leaf length and the wider leaf width, were all statistically significant between primary and secondary needles (Table 1). Unexpectedly, LMA did not differ significantly between needle types although was lower in primary

needles (Figure 2, Table 1). Interestingly, primary needles had much lower stomatal density in the abaxial side compared to secondary needles (Figures 2 and 3, Table 1). Their bluish color agreed with a significantly thicker cuticle (almost 2-fold), and hydrophobicity (higher contact angle) when compared to secondary needles (Figure 1, Table 1). Major intratissular differences included (i) smaller structural fraction (e.g., % of hypodermis, endodermis and vascular tissue to cross-section area), (ii) higher fraction of spongy parenchyma, (iii) higher cell diameter in dermal tissues and (iv) wider resin ducts, in primary needles (Figures 2–4 and Table 2). Frequency distribution of tracheid diameter followed a similar pattern in both needle types with tracheids of 3–4 μm ϕ being responsible for ca. 50% of the total conductive area (Figure S1).

Figure 4. Stained cross-sections of *Pinus canariensis* primary (juvenile) needles. (a) Whole view of the needle cross-section. (b) Detail of the stomata and outer tissues of the needle. (c) Detail of the vascular bundle and all the conductive elements. (d) Detail of a resin duct. Abbreviations: end, endodermis; ep, epidermis; epc, epithelial cells; gc, guard cells; hyp, hypodermis; ph, phloem; rd, resin duct; sc, subsidiary cells; schp, spongy chlorophyll parenchyma; sec, substomatal chamber; shc, sheath cell; st, stoma; tp, transfusion parenchyma; tt, transfusion tracheid; tti, transfusion tissue; vt, vascular tissue; xy, xylem. Scale bars (a), 50 μm , (b–d), 20 μm . Safranin—fast green staining.

3.2. Photochemistry and Gas Exchange

Rapid Light Curves analysis showed different behaviour between needle type and season (Figure 5a). The highest maximum ETR (ETR_{max}) was obtained in secondary needles in autumn, reaching $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ while, on the contrary, primary needles in summer showed the lowest ETR_{max} values ($57 \mu\text{mol m}^{-2} \text{s}^{-1}$, Figure 5b). Similar modulation was detected in the maximum PPFD at which the maximum ETR is maintained (PPFD_{sat}) and the minimum absorbed saturating photosynthetic photon flux density (EK), showing the highest values in secondary needle in autumn ($733 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $318 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively) and the lowest values in summer ($424 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $184 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively) (Figure 5c,e). No differences between needle types were observed in summer (Figure 5c,e). Finally, the apparent quantum efficiency (AQE) values did not show significant differences between needle types in autumn but these were observed in summer reaching the highest values the secondary needles (Figure 5d). Despite of being photochemically more efficient, secondary needles contained lower content of chlorophyll (Chl) as expressed per needle-projected area (Figure 6a). This difference was constant along the year. No significant differences were obtained between needle types in the ratios Chla/b or Carotenoids/Chl even if considering different daytime and seasonal samplings (Figure 6b,c).

Figure 5. Rapid Light Curves and derived parameters as estimated through chlorophyll fluorescence analyses, of *P. canariensis* needles. (a) Light curves showing electron transport rate (ETR, in $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$) achieved by the needles at each photosynthetic photon flux density (PPFD, in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). (b) Maximum electron transport rate (ETR_{max}) expressed in $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$. (c) Maximum PPFD at which the maximum ETR is maintained (PPFD_{sat}) expressed in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. (d) Apparent quantum efficiency (AQE) expressed in $\mu\text{mol photons used for photochemical processes per } \mu\text{mol photons absorbed by PSII}$. (e) Minimum saturating irradiance (E_k) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Data are mean $\pm$ SE ($n = 4$). Different letters above bars depict significant differences among needle types and seasons ($p < 0.05$). See Section 2.3 of the Materials and Methods for details on how the parameters shown in panels (b–d) were estimated.

Figure 6. Photosynthetic pigment composition of *P. canariensis* needles as measured in the field at midday, or after 16 h of dark acclimation (at $\approx +20$ °C). (a) Total chlorophyll content in $\mu\text{mol m}^{-2}$. (b) Ratio of Chl *a* to *b* in mol mol^{-1} . (c) Total carotenoids content per chlorophyll in mmol mol^{-1} . Data are mean $\pm$ SE ($n = 5$). When significant, differences among needle types, daytime and seasons are depicted with different letters above the bars ($p < 0.05$). No significant differences among needle types, daytime or seasons were found for Chl *a*/*b* ratio (b) nor for Car/Chl ratio (c).

Gas exchange results are shown in Figure 7. Contrary to our expectations, net carbon assimilation of primary and secondary needles of *P. canariensis* did not differ significantly, even if primary needles showed slightly lower values (i.e., 1.8 ± 0.5 in primary vs.

$3.5 \pm 0.6 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in secondary needles, in summer; 7.3 ± 2.4 vs. 9.5 ± 1.2 , in autumn) (Figure 7). This pattern was actually maintained across seasons, and secondary needles showed lower values than primary needles again in autumn, after the rainfalls had already reactivated photosynthetic activity (Figure 7a). A similar behaviour was observed in the stomatal conductance. Lowest values of $0.066 \pm 0.007 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ were obtained for primary needles in summer, and highest values of $0.095 \pm 0.030 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ were obtained for secondary needles in autumn (Figure 7b).

Figure 7. Gas exchange parameters measured in intact needles in the field and under natural conditions. (a) Net carbon assimilation (A_N) in $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. (b) Stomatal conductance (g_s) in $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$. Data are mean $\pm$ SE ($n = 3\text{--}5$). No significant differences were found between needle types.

3.3. Tolerance to Desiccation and Dehydration Kinetics

The “Falcon test” concluded that none of the needle types was tolerant to desiccation, i.e., none of the samples that dehydrated below 30% RWC were able to recover $\geq 75\%$ of initial F_v/F_m values (Figure S2). Interestingly, in all desiccating conditions, primary needles reached significantly lower RWCs than secondary needles (Figure S2). The results, of a second experiment, where the loss of water (dehydration kinetic) was carefully monitored under $<10\%$ and 75% RH conditions is shown in Figure 8. Notably, secondary needles were much more slower losing water even if tremendously low RH $< 10\%$ was used (Figure 8b).

Figure 8. Loss of water in *P. canariensis* needles monitored during controlled desiccation and estimated through changes in water content (WC). The WC is expressed as fresh weight to turgor weight ratio in %. (a) Dehydration conducted at 75% RH. (b) Dehydration conducted at <10% RH. Secondary needles are depicted in dark green, primary needles in light green color. Data are mean $\pm$ SE ($n = 6$). Asterisks depict significant differences between changes in water content at certain desiccation time ($p < 0.05$).

3.4. Tolerance to Extreme Temperatures

When *P. canariensis* needles were subjected to low temperatures of -10°C for 30 min, no significant differences were obtained in the maximal photochemical efficiency of PSII (F_v/F_m) (Table 3). The F_v/F_m decreased slightly but not significantly in both needle types that keep comparable values. More marked differences between needles were obtained upon high-temperature treatments (Figure 9). Overall, both needles started a decrease in F_v/F_m at temperatures higher than 40°C . This depression, however, was more reliable when evaluated 24 h after the treatment (when irreparable damage to the leaf can be more accurately estimated) (Figure 9b). In these conditions, primary needles showed higher tolerance to $+44$ and $+46^{\circ}\text{C}$ treatments.

Table 3. Effects of freezing assay (-10°C during 30 min) on the F_v/F_m of secondary and primary needles. Values are the average $\pm$ SE ($n = 9$). Non-significant differences between secondary and primary needles were found after one-way ANOVA ($p < 0.05$). Corresponding F and p values are shown in italics.

Needle Age	Fv/Fm Freezing Treatment (-10°C)		
	Initial	Post-Treatment	24 h Recovery Post-Treatment
Secondary	0.763 ± 0.010	0.744 ± 0.014	0.743 ± 0.013
Primary	0.785 ± 0.006	0.771 ± 0.007	0.765 ± 0.008
<i>F</i>	<i>3.59</i>	<i>3.00</i>	<i>2.15</i>
<i>p</i>	<i>0.076</i>	<i>0.103</i>	<i>0.162</i>

Figure 9. High temperature effects on *P. canariensis* needles, estimated through values of maximal photochemical efficiency of PSII (F_v/F_m). (a) Measured immediately after heat treatments. (b) Measurements 24 h after the treatment (needles incubated at +20 $^{\circ}\text{C}$ in darkness). Data are mean $\pm$ SE ($n = 5$). Asterisks depict significant differences between needle types at certain temperature treatments ($p < 0.05$). No statistical differences were found in the absolute water content between needle types used for this experiment: primary needles had $1.43 \pm 0.04 \text{ gH}_2\text{O g}^{-1} \text{ DW}$ and secondary needles $1.63 \pm 0.05 \text{ gH}_2\text{O g}^{-1} \text{ DW}$ (mean $\pm$ SE, $n = 15$).

4. Discussion

4.1. Study Frame

Leaf anatomy and function are strongly related. “Leaf economics spectrum” theory offers a solid framework to this fact across environments and species with just a couple of exceptions escaping to it [47,48]. On the other hand, both organ (leaf) and individual (tree) age can strongly affect the metabolic activity (e.g., tocopherol content rises with leaf-age [49]) and the reallocation of resources that will end in different structural traits. As an example, a recent work by Azuma et al. (2019), revealed significant differences in needle LMA of mature (ca. 100 years) vs. old (ca. 300 years) trees of *P. densiflora* [50] despite comparable photosynthetic capacity around $5 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. Thus, typically, anatomical, environmental and ontogenic factors overlap, making it difficult to disentangle the reasons behind differential physiology across needles. While leaf-heteroblasty may represent one of the most extreme evidences regarding anatomical divergence within a single plant, most often, different leaf types will occupy different environments (e.g., understorey vs. canopy in seedling vs. adult trees) [2]. Here, we present a quite unique and promising case study in which two needle types, ontogenically-driven, co-habit in the same microenvironment and sprout almost contemporarily from the same adult tree: ca. 70 years individuals of *P. canariensis*; primary and secondary needles from resprouting twigs (Figure 1).

4.2. Anatomical and Physiological Traits Related to Photosynthesis

By comparing needles from five different *Pinus* species, Wang et al. (2019), have recently concluded that needle size in pines is related to anatomical traits in a way that reflects the mechanical and physiological properties of the needle [51]. Thus, longer needles, generally encounter a trade-off between the relative fractions of support and photosynthetic tissue across *Pinus* species [51]. Secondary needles of *P. canariensis* evaluated in our study were significantly longer than their contemporary primary needles (Figure 1, Table 1). Accordingly, their intra-leaf tissue showed higher proportion of mechanically supporting tissue. Despite showing a much higher proportion of “mechanical tissue fraction” (e.g., ratio of the sum of epidermal tissues and xylem areas to needle cross-sectional area as

in [51]), and higher conductive area per needle cross section (Table 2), secondary needles of *P. canariensis*, showed similar LMA, and A_N values than primary needles (Figure 7). The obtained values of net carbon assimilation of 2–11 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ fall within the expected range for this species and habitat. As an example, data obtained in the same arid tree-line ecotone at Teide Mountain and covering a whole year and a wide variety of physiological conditions (including drought episodes) ranged between -1 and $+18 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ [52]. We did not find significant differences in A_N between primary and secondary needles. This agrees with previous studies on *P. halepensis*, *P. nigra*, and *P. pinea*, provided both needle types have the same age [53], as was also our case. The LMA values obtained in our study, are also comparable to other Mediterranean pines [7]. We did not find significant differences in LMA between primary and secondary needles. This, contrasts with data from other species of Mediterranean *Pinus*, in which primary needles typically show lower LMA [7]. However, in that study is noteworthy that primary needles of seedlings were compared with secondary needles of adult trees. This differs with our case-study in which we compare coexisting primary and secondary needles in the same twig of adult trees.

Both needle types had a comparable thickness, but secondary needles had a significantly thinner cuticle (Tables 1 and 2). This, together with their higher stomatal density (on abaxial surface) very likely facilitates CO_2 diffusion into secondary needles (Table 2). In accordance, secondary needles tended to have higher ETR_{max} , A_N and g_s than primary needles. Primary needles had higher Chl a+b content (Figure 6), which agreed with their higher proportion of photosynthetic tissue per leaf-cross-section area (Figure 2). The content of photosynthetic pigments can change in response to internal and external factors affecting the plants [54]. At daily scale, Chl a/b and Chl a+b can oscillate in following endogenous circadian rhythms under controlled conditions in some species [55] and around summer solstice in *P. sylvestris* growing in the arctic [56]. For *P. canariensis* Tausz et al. (2001) found significant rise in Chl a+b at noon, but this results were obtained in potted seedlings [57]. We did not observe such a pattern in *P. canariensis* wild adult trees (Figure 6). This resembles the lack of diurnal fluctuations in the content of pigments described by Porcar-Castell et al. (2012) for *P. sylvestris* [58]. Seasonally, Chl a+b can slightly decrease towards winter in conifers [52,59,60] but generally remains constant between summer and autumn [61], as was also the case of *P. canariensis* in our study and in previous studies with this species [52]. Overall, higher photochemical efficiency but lower Chl content were obtained in secondary needles of *P. canariensis*. Future analyses on traits affecting intratissular CO_2 diffusion, such as cell-wall thickness and composition, and light use efficiency, such as chloroplast distribution and movements, or carotenoid composition etc. [7] would be interesting to deepen into the factors determining photosynthesis capacity and acclimation in the two needle types of *P. canariensis*.

4.3. Water Relations and Stress Tolerance

Both DW/FW ratio and water content at turgor were equal between secondary and primary needles in *P. canariensis* (Table 1), indicating a similar water holding capacity. Nevertheless, cuticle was almost two-fold thicker in primary needles, which were also less wettable, and had less stomatal density (Tables 1 and 2). The wettability of primary needles was strongly dependent on the presence of epicuticular waxes, which were easily removed upon manipulation, and we found a trend from less wettable base towards more wettable tip of the needle (data not shown). Wettability loss in parallel to loss of epicuticular waxes was already reported for *P. sylvestris* by Cape, 1983 [62]. More wettable surface at the tip than at the base of *P. radiata* needle could be related to wax composition and with higher weathering [63]. On the light of these anatomical and physical properties we would have expected a strong resistance against leaf dehydration. Nevertheless, both the “Falcon test” and the “Desiccation kinetic” experiments revealed a much slower water loss of secondary needles over the time (Figure 8). Secondary needles of *P. canariensis* have a very particular stomatal anatomy, different to any other pine, with 16 cells including uniquely

shaped polar cells that form a cover above the epistomatal chamber [26]. This features have been related with an extreme adaptation to harsh environmental conditions [26]. These morphological adaptations, together with the different structure and chemistry of epicuticular waxes that differ both needle types [28], may be the reason why secondary needles are much more efficient than primary needles preventing water loss (Figure 8). This higher efficiency under dehydrating conditions of secondary needles is in agreement with experiments comparing seedlings of *P. canariensis* with both needle types developed under very arid conditions [17]. Drought experiments under controlled conditions have shown increasing LMA and decreasing A_N in seedlings of some conifers [64] and a reduced leaf/area ratio in seedlings of *P. canariensis*, in which interaction with irradiance conditions was markedly relevant, i.e., more drastic effects of drought were obtained in a shade environment [65]. However, none of these studies has compared physiological responses of primary vs. secondary needles, which could increase our current understanding about the influence that leaf anatomical differences have on the functioning of *P. canariensis* needles.

Previous studies have indicated that primary needles are more sensitive to frost damage than secondary needles in many pine species [30,31]. In agreement with data from seedlings of *P. canariensis* [30], we did not found such a difference in secondary and primary needles from adult trees (Table 3). This is in accordance with temperature ranges on the study-ecotone, where freezing temperatures are common during several months along the year including frequent episodes of $<-5^{\circ}\text{C}$ [52]. This species seems to suitably acclimate to freezing during winter conditions; it shows a slight down-regulation of photochemical efficiency and a rearrangement of photosynthetic pigment composition that successfully prevents irreparable damage [52]. Interestingly, primary needles were more tolerant to moderately high temperatures (43 to 47°C) what could represent some advantages in low-density stands during resprouting, after fire.

5. Conclusions

Resprouting twigs with coexisting primary and secondary needles of *P. canariensis* offers a valuable case study to assess morpho-physiological trade-offs at leaf level. Under the conditions of this study, set in a semi-arid treeline, primary needles had higher fraction of photosynthetic parenchyma and lower fraction of water conducting (xylem) and mechanical tissue (xylem plus epidermal tissues), similar net carbon assimilation, slightly better tolerance to heat and lower water retention capacity than secondary needles. Considering that *P. canariensis* is a fire-resistant and resprouting species, we conclude that primary needles enhance the possibilities of physiological response against some environmental cues in resprouting twigs. These advantages could be particularly useful after a fire in a strongly defoliated tree, and they are probably less evident in an understory environment.

Supplementary Materials: The following are available online at <https://www.mdpi.com/1999-4907/12/3/341/s1>, Figure S1: Frequency distribution of tracheid diameters according to their contribution to total conductive area obtained from primary and secondary needle cross-sections of *P. canariensis*, Figure S2: Relative water content (RWC) and maximal photochemical efficiency of PSII (F_v/F_m) of *P. canariensis* needles, obtained in the “Falcon Test”. Table S1: Numbers of replicates and dates of collection for all samples used in this study.

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