ISSN 2226-3063 e-ISSN 2227-9555
Modern Phytomorphology 13: 47–53, 2019

Modern Phytomorphology

RESEARCH ARTICLE

Age-related changes in anatomical and morphological leaf traits of *Wollemia nobilis*

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Received: 03.06.2019 | **Accepted:** 28.06.2019 | **Published:** 05.07.2019

Abstract

The results highlight significant variations of *Wollemia nobilis* leaf traits which reflect age-related changes of the subsequent growth units along the branches. Age-related changes appear in a gradual increase of leaf size from young leaves to old leaves. The LMA increasing from 13.75 g/cm^2 in current year leaves to 24.84 g/cm^2 in 7 year leaves is associated with an increment of the number of lignified elements (vascular tissues, astrosclereids), of hypodermal and epidermal-cuticle structures (cuticle, wax layer) and of oil bodies abundance, which may increase resistance to stress factors. These characteristics highlight that *W. nobilis* leaves can adapt to variable environmental conditions with a return rate on a larger time-scale since leaves on a branch stay alive for a long time until the branch dies.

Keywords: Leaf anatomy, LMA, age-related changes, *Wollemia nobilis*

Introduction

The Wollemi pine (*Wollemia nobilis* W. G. Jones, K. Hill & J. M. Allen (Jones *et al.* 1995) is a critically endangered species included in the IUCN Red List (*Status* CR) (Thomas 2011). At the present *W. nobilis* is restricted to one population scattered across four sites in the Wollemi National Park, Australia (Jones *et al.* 1995; Benson & Allen 2007; Zimmer *et al.* 2014). A commercialization strategy has been developed by the Wollemi Pine Recovery Plan to manage the release of the plants into cultivation (DEC 2006; Trueman *et al.* 2007). Today plants of *W. nobilis* grow in the Botanical Garden of Rome (Gratani 2017).

Wollemia is a monotypic genus of Araucariaceae that according to phylogenetic analysis closely related to *Agathis* Salisb., forms a clade sister to Araucaria Juss. (Escapa *et al.* 2013). Some vegetative and reproductive traits of *Wollemia* are intermediate between *Agathis* and *Araucaria* spp. (Chambers *et al.* 1998; Offord *et al.* 1999). One of the remarkable features of *W. nobilis* is its architectural model (Hill 1997). The trunk produces plagiotropic first-order branches by monopodial growth, which remain unbranched and terminate by male or female strobili (Hill 1997). When a strobilus fall the branch can produce two new shoots at the top. The first-order branches relatively short-lived (on average 5-11 years) cleanly abscised with all leaves still attached and have a branch-based xylem constriction to facilitate branch abscission (Burrows *et al.* 2007; Tomlinson & Murch 2009). These characteristics are probably unique in extant woody plants (Burrows *et al.* 2007). The branches are characterized by rhythmic seasons growth distinguished by a gradual increase in leaf length at the start of the season and a decrease at the end forming, as a result, growth units (Offord *et al.* 1999), which are called "growth increments" (Chambers *et al.* 1998) or "leaf cohorts" (Lusk *et al.* 2012). This pattern of leaves development is also typical for some Araucariaceae and Podocarpaceae species (Lusk *et al.* 2012). Every unit represents one year's growth, and the abscised *in situ* branches are 70–120 cm long and have 6-13 growth units (Burrows *et al.* 2007).

The adult leaves of *W. nobilis* are opposite, decussate and typically twisted to give the appearance of four ranks phyllotaxis (Chambers *et al.* 1998). The leaf morphological and anatomical features of *W. nobilis* leaves as described (Chambers *et al.* 1998; Burrows & Bullock 1999) focused on the prominent heterophylly between leaves of adult and juvenile trees. Another type of foliage variation typical for *W. nobilis* is the appearance of leaves with different morphological traits on the same plagiotropic branch. This type of variability is represented by well-distinguished growth units that reflect age-relative changes (Chambers *et al.* 1998). Nevertheless, today

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differences among leaves of subsequent units associated with the formation of the entire falling branch and their age-related changes have not been described.

In this context, the aim of this research was to analyze anatomical and morphological differences of leaves developing in subsequent growth units along plagiotropic branches of *W. nobilis* growing in the Botanical Garden of Rome to clarify the age-related changes.

Materials and Methods

Study area and species

The study was carried out on mature (producing cones) plants of *W. nobilis* growing in the Botanical Garden of the Sapienza University of Rome (41° 53´ 53´´ N 12° 28´ 46´´ E; 53 m a.s.l.) in the period October 2017-February 2018.

The climate of the area is of Mediterranean type. The mean minimum air temperature (T_{min}) of the coldest months (January and February) is 5.1 ± 1.6°C, the mean maximum air temperature (T_{max}) of the hottest months (July and August) is 31.9 ± 1.4°C and the yearly mean air temperature (T_m) is 16.8 ± 6.7 °C. Total annual rainfall is 842.2 mm, most of which occurs in autumn and winter. The dry period is from June to August (91.6 mm of total rainfall). During the study period, T_{min} was 4.3 ± 0.4°C (December and February), T_{max} 23.4°C (October) and T_m 12.28 ± 3.7°C (Data from Arsial Meteorological Station, Lanciani Street).

Microclimate

Air temperature (T_A) and relative air humidity around *W. nobilis* were measured with a portable thermo-hygrometer (*HD 8901, Delta Ohm*, Italy), at 20 cm above the ground, from 8.00 to 15.00 h, every 30 min. Total irradiance (I_T) was measured with a quantum radiometer photometer Li-185B (*Licor*, USA). Measurements were carried out from the inside to the outside of the considered *W. nobilis* branches to evaluate the irradiance insisting on each growth unit. Total irradiance (I_T) ranged from 43.3 ± 2.6 µmol (photons) m^{-2} s^{-1} at U1 level to 2583 ± 115.8 µmol (photons) m^{-2} s^{-1} at the U7 level. The T_A did not show significant differences along with the subsequent segments.

Morphological leaf traits

The South-East exposed branches from the middle portion of the plant were considered. Ten branches, consisting of seven growth units (U) each (hereafter U1-U7, where U1 is the first proximal unit, and U7 is the last distal one) for each of the considered trees were selected. The length of each growth unit (L, cm) on the selected branches was measured from the smallest leaf at the beginning of each annual growth, according to (Lusk *et al.* 2012). The length of the branch was calculated by the sum of all the L (from U1 to U7) for each the considered branch. Leaf morphological traits were analyzed on 10 leaves for each growth unit (n=10). Leaf area (LA, cm^2), length (mm) and width (mm) were measured by an Image Analysis System (*Delta-T Devices*, UK). Leaf dry mass (DM, g) was determined after desiccation at 80°C to constant mass. LW was measured at the midpoint of the leaf, according to (Kuusk *et al.* 2017). Leaf mass per unit of leaf area (LMA, mg cm^{-2}) was calculated from the ratio between DM and LA (Reich *et al.* 1992) and leaf tissue density (LTD, mg cm^{-3}) by the ratio between LMA and total leaf thickness (LT, µm) (Wright *et al.* 2004).

Anatomical leaf traits

The same branches selected for leaf morphology were used for leaf anatomy. Measurements of leaf anatomical traits was carried out on the lamina sections from the central part of fully expanded leaves (n=10 leaves for each growth unit) and analyzed by light microscopy (*Zeiss Axiocam MRc 5* digital camera, Carl Zeiss) using an Image Analysis System (*Axiovision AC software*). The following parameters were measured in transverse leaf sections: total leaf thickness (LT, µm), abaxial and adaxial cuticle with cell wall and epicuticular wax thickness (µm), abaxial and adaxial epidermis thickness (µm), palisade parenchyma thickness (µm), spongy parenchyma thickness (µm), palisade parenchyma cell length and width (µm), spongy parenchyma cell diameter (for the maximum and the minimum diameter, µm), hypodermal cells length and width (µm), vascular bundles diameter (µm), resin canal diameter (µm), diameter of compartmented cells (µm). Stomatal features were measured from nail varnish impressions in paradermal view (*n*=10 leaves for each growth unit) of the adaxial and abaxial surfaces of the leaf lamina, according to (Sack *et al.* 2003). The following parameters were measured: abaxial and adaxial stomatal length (µm), abaxial and adaxial stomatal density (n mm^{-2}).

Data analysis

A principal component analysis (PCA) was carried out including all the considered leaf morphological and anatomical variables grouped per each growth unit. A one-way *ANOVA* on the principal components explaining the highest proportion of variance (PC1) was performed with units as a grouping variable. Multiple comparisons were analyzed by a *Tukey* test. Such an approach was used in order to reduce multiple testing, considering that the use of emerging collective properties (expressed by PC1) as a primary variable provides an equally robust approach (Giuliani 2017). A regression analysis was carried out to analyze the relationship between all variable and the extracted PCs. All analyses were run with the R library SMATR.

Results and Discussion

Branch and leaf morphology

For the investigated plants, well-developed plagiotropic branches of the first order consisted of eight units (U), were unbranched and sometimes ended with a male or female strobilus (Fig. 1). The total length of the branches (L_{tot}) was 93.2 ± 3.3 cm. Every U was distinguished by a gradual increase in leaf length, with a maximum in the central part (Fig. 1). The length of each U increased from the most distal (young) to the most proximal (old) and it was due to the increase in the length of the internodes (Tab. 1). The highest U length was 27.8 ± 3.7 cm for the oldest (U1) part of the branch, and the lowest length for the youngest one (U7, current year, 5.5 ± 0.7 cm).

The largest leaf size (length and width), LA, and DM (9.7 ± 0.5 × 0.58 ± 0.02 cm, 4.6 ± 0.2 cm^2, and 0.11 ± 0.01 g, respectively) were measured for leaves of U2 which were significantly different

from all other units. The lowest values for specified parameters were measured for the youngest unit (U7) (4.1 ± 1.2 cm × 0.34 ± 0.04 cm, 1.1 ± 0.1 cm^2, and 0.015 ± 0.001 g, respectively).

The LMA decreased by 44% from U1 to U7, while LTD did not show significant differences in the considered units (Tab. 1).

Anatomical leaf traits

The general histological aspects of leaves for all the units were similar, except for some details, described below. Leaves were amphystomatic, with a well-developed cuticle and the mesophyll differentiated into palisade and spongy tissues (Fig. 2A and 2B). The total leaf thickness increased by 42% with advancing of leaf age and it was the lowest for U7 (327.7 ± 9.6) and the highest for U1 (565.8 ± 23.5 µm).

Abaxial epidermis consisted of rectangular or polygonal epidermal cells with thick outer periclinal walls, covered by cuticle and waxes layer (from 7.3 ± 0.7 to 8.1 ± 0.8 µm), and stomata arranged in discontinuous rows (Fig. 2G). Stomata were sunken and guard cells had lignified periclinal walls with thickened polar ventral wall ends, confirmed by the phloroglucinol reaction (Fig. 2B, 2G, 2H), and four to six subsidiary cells. Stomatal length increased not significantly for the adaxial surface and significantly for abaxial surface from younger to older units, reaching 50.2 ± 1.9 µm and 48.8 ± 1.7 µm, respectively (Tab. 2).

Stomatal density was higher for the abaxial surface (from 120.5 ± 10.5 n mm^{-2} to 154.1 ± 11.2 n mm^{-2}) than for the adaxial surface (from 19.0 ± 2.6 n mm^{-2} (U1) to 28.0 ± 3.3 n mm^{-2} (U4)) for leaves of all growth units. The thickness of adaxial cuticle was the highest for U1 (12.6 ± 1.1 µm) decreasing for the subsequent growth units and reaching 10.3 ± 0.7 µm for U7. The hypodermis was characterized by one or two layers of thick-walled cells with a narrow lumen under the epidermis (Fig. 2F). These cells formed continuous lignified fibers between the rows of stomata (Fig. 2G). In transverse section, hypodermal cells were elliptic or polygonal (12.3 ± 2.3 µm × 22.9 ± 5.3 µm) with not significant differences between the different growth units (Fig. 2F).

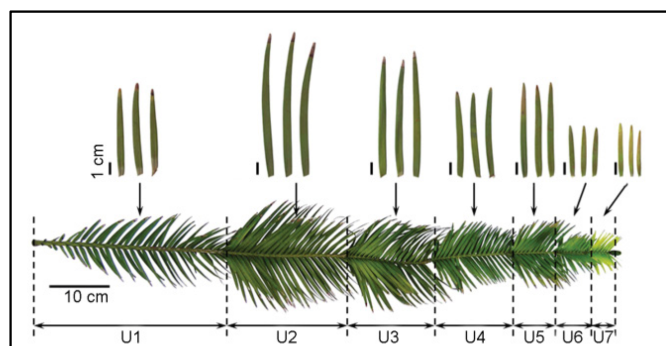

Figure 1. Morphology of *Wollemia nobilis* branch (growth units from proximal to distal part, U1: growth unit 1; U2: growth unit 2; U3: growth unit 3; U4: growth unit 4; U5: growth unit 5; U6: growth unit 6; U7: growth unit 7).

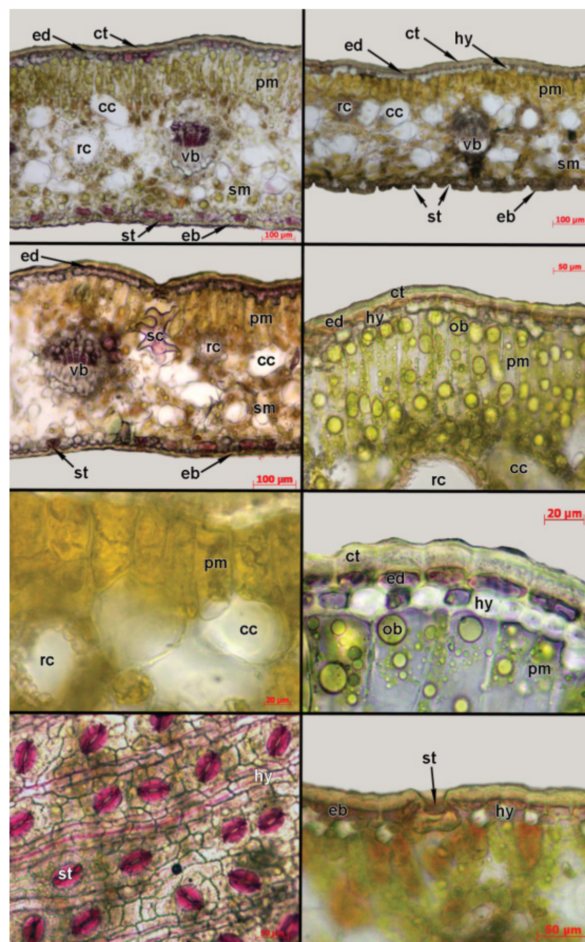

Figure 2. Transverse (A-F, H) and paradermal (G) sections of *Wollemia nobilis* leaves of different growth units: **A**: growth unit 1; **B**: growth unit 7; **C**: growth unit 3; **D**: growth unit 1; **E**: growth unit 7; **F**: growth unit 1; **G**: growth unit 6; **H**: growth unit 6 (**cc**: compartmented cell, **ct**: cuticle with epicuticular wax layer; **eb**: abaxial epidermis; **ed**: adaxial epidermis; **hy**: hypodermal cells; **ob**: oil body; **pm**: palisade mesophyll; **rc**: resin canals; **sc**: sclereid; **sm**: spongy mesophyll; **st**: stoma; **vb**: vascular bundle).

Table 1. Morphological leaf traits of *Wollemia nobilis* in subsequent growth units along a branch (from proximal to distal: U1, U2, U3, U4, U5, U6, U7): leaf mass per unit of leaf area (LMA), Leaf Tissue Density (LTD). Each value denotes the mean (± SD) of ten leaves (n=10). Different letters indicate significant differences between leaves of different growth units, ns indicates no significant difference (One way ANOVA; p ≤ 0.05).

	U1	U2	U3	U4	U5	U6	U7
Growth unit length (cm)	27.8 ± 3.7^{a}	20.4 ± 1.9^{b}	13.6 ± 1.4^{c}	10.1 ± 2.1^{d}	8.9 ± 1.5^{de}	6.9 ± 1.3^{ef}	5.5 ± 0.7^{f}
Leaf length (cm)	6.5 ± 1.7^{a}	9.7 ± 0.5^{b}	8.2 ± 0.3^{c}	6.8 ± 0.4^{ad}	6.4 ± 0.7^{ade}	5.6 ± 0.6^{ae}	4.1 ± 1.2^{f}
Leaf wigth (cm)	0.55 ± 0.08^{a}	0.58 ± 0.02^{ab}	0.51 ± 0.04^{ac}	0.48 ± 0.02^{cd}	0.48 ± 0.03^{cd}	0.42 ± 0.04^{e}	0.34 ± 0.04^{f}
Leaf area (cm^2)	3.3 ± 0.3^{a}	4.6 ± 0.2^{b}	4.0 ± 0.3^{c}	2.2 ± 0.2^{d}	2.4 ± 0.1^{d}	1.2 ± 0.1^{e}	1.1 ± 0.1^{e}
Leaf dry mass (g)	0.08 ± 0.01^{a}	0.11 ± 0.01^{b}	0.09 ± 0.01^{a}	0.04 ± 0.01^{c}	0.043 ± 0.003^{c}	0.020 ± 0.001^{d}	0.015 ± 0.001^{d}
LMA (mg cm^{-2})	24.8 ± 1.5^{a}	23.1 ± 3.0^{ab}	21.4 ± 3.0^{b}	17.9 ± 2.4^{c}	17.7 ± 1.0^{c}	17.4 ± 1.8^{c}	13.9 ± 0.7^{d}
LTD (mg cm^{-3})	438.6 ± 18.8^{ns}	443.4 ± 57.7^{ns}	457.6 ± 69.5^{ns}	493.8 ± 73.2^{ns}	478.4 ± 34.8^{ns}	465.8 ± 47.9^{ns}	425.4 ± 23.8^{ns}
Leaf water content (%)	44.0 ± 0.9^{a}	42.9 ± 2.1^{a}	43.7 ± 2.2^{a}	46.4 ± 1.8^{b}	49.3 ± 1.1^{c}	51.1 ± 0.4^{cd}	53.8 ± 0.6^{d}

Table 2. Anatomical leaf traits of *Wollemia nobilis* in subsequent growth units (from proximal to distal: U1, U2, U3, U4, U5, U6, U7) along a branch. Each value denotes the mean (± SD) of ten leaves (n=10). Different letters indicate significant differences between leaves of different segment, ns indicate no significant difference (One way ANOVA; $p \leq 0.05$).

Trait	U1	U2	U3	U4	U5	U6	U7
Total leaf thickness (µm)	565.8 ± 23.5^{a}	520.7 ± 13.3^{b}	469.4 ± 16.1^{c}	363.6 ± 9.4^{d}	369.6 ± 12.4^{de}	381.3 ± 9.2^{de}	327.7 ± 9.6^{f}
Palisade parenchyma thickness (µm)	146.1 ± 11.2^{a}	137.9 ± 10.9^{a}	102.1 ± 9.2^{b}	66.0 ± 6.3^{d}	68.9 ± 3.9^{d}	75.9 ± 8.4^{d}	65.0 ± 4.2^{d}
Spongy parenchyma thickness (µm)	351.3 ± 22.3^{a}	300.5 ± 11.6^{b}	281.8 ± 19.5^{b}	227.7 ± 21.0^{c}	235.6 ± 17.6^{c}	243.1 ± 17.9^{c}	201.3 ± 8.7^{d}
Vascular bundless diameter (µm)	177.8 ± 13.4^{a}	177.3 ± 11.7^{a}	175.2 ± 6.9^{a}	136.3 ± 9.5^{b}	128.1 ± 4.9^{b}	138.1 ± 6.1^{b}	115.1 ± 6.3^{c}
Resin canals diameter (µm)	149.9 ± 12.2^{a}	131.4 ± 8.3^{b}	109.6 ± 9.4^{c}	89.3 ± 8.8^{d}	82.5 ± 8.0^{d}	99.9 ± 10.5^{cd}	68.5 ± 6.2^{e}
Adaxial cuticle thickness (µm)	12.6 ± 1.1^{a}	11.4 ± 0.7^{ab}	11.0 ± 0.6^{b}	9.2 ± 1.0^{c}	8.5 ± 1.2^{c}	10.5 ± 1.3^{bcd}	10.3 ± 0.7^{bc}
Adaxial epidermis thickness (µm)	9.5 ± 1.4^{a}	10.2 ± 0.9^{ab}	9.8 ± 0.9^{ab}	8.3 ± 0.8^{ac}	8.6 ±1.1^{ac}	8.8 ± 1.0^{abc}	7.6 ± 0.9^{c}
Hypodermal cells length (µm)	11.3 ± 1.3^{ns}	12.3 ± 2.3^{ns}	10.7 ± 1.1^{ns}	10.4 ± 1.4^{ns}	10.5 ± 1.1^{ns}	10.5 ± 1.7^{ns}	10.8 ± 1.8^{ns}
Hypodermal cells width (µm)	20.0 ± 1.0^{ns}	22.9 ± 5.3^{ns}	19.6 ± 4.0^{ns}	19.5 ± 2.0^{ns}	20.7 ± 2.9^{ns}	19.1 ± 3.2^{ns}	18.8 ± 1.7^{ns}
Palisade parenchyma cells length (µm)	115.0 ± 10.4 ^{a}	129.1 ± 9.1^{b}	92.1 ± 9.3^{c}	62.3 ± 4.4^{d}	67.9 ± 5.6^{de}	77.4 ± 6.1^{ef}	65.3 ± 5.1^{de}
Palisade parenchyma cells width (µm)	26.9 ± 1.7^{a}	28.7 ± 2.5^{ab}	24.8 ± 2.7^{ac}	26.4 ± 2.3^{abc}	26.2 ± 3.0^{abc}	29.9 ± 2.5^{abd}	26.6 ± 2.1^{abc}
Compartmented cells maximum diameter (µm)	63.3 ± 21.8^{ns}	69.5 ± 12.5^{ns}	77.9 ± 16.2^{ns}	75.0 ± 18.9^{ns}	72.2 ± 13.6^{ns}	72.5 ± 9.9^{ns}	72.6 ± 12.7^{ns}
Compartmented cells minimum diameter (µm)	49.9 ± 17.5^{ns}	50.8 ± 5.4^{ns}	52.6 ± 9.3^{ns}	52.0 ± 7.3^{ns}	50.5 ± 9.9^{ns}	54.4 ± 4.4^{ns}	50.2 ± 7.1^{ns}
Spongy parenchyma cells maximum diameter (µm)	52.8 ± 8.3^{a}	57.1 ± 5.5^{a}	64.5 ± 9.3^{a}	54.4 ± 11.9^{ab}	50.8 ± 9.3^{b}	54.9 ± 6.9^{ab}	56.0 ± 6.8^{ab}
Spongy parenchyma cells minimum diameter (µm)	37.8 ± 4.5^{ns}	37.4 ± 5.9^{ns}	41.0 ± 8.0^{ns}	38.4 ± 7.6^{ns}	33.4 ± 5.7^{ns}	36.0 ± 3.2^{ns}	38.5 ± 4.8^{ns}
Abaxial epidermis thickness (µm)	10.3 ± 1.1^{a}	11.6 ± 1.3^{ab}	11.2 ± 1.0^{ab}	9.6 ± 1.1^{ad}	9.4 ± 0.6^{ad}	8.8 ± 1.3^{d}	9.6 ± 0.7^{ad}
Abaxial cuticle thickness (µm)	7.6 ± 0.5^{ns}	7.5 ± 0.8^{ns}	7.3 ± 0.7^{ns}	7.7 ± 1.2^{ns}	7.5 ± 0.7^{ns}	8.1 ± 0.8^{ns}	7.9 ± 0.7^{ns}
Abaxial stoma length (µm)	48.8 ± 1.7^{a}	46.8 ± 2.3^{ab}	45.6 ± 1.9^{b}	46.4 ± 2.7^{abc}	47.5 ± 2.4^{abc}	49.2 ± 2.0^{ac}	48.3 ± 1.5^{abc}
Adaxial stoma length (µm)	50.2 ±1.9^{ns}	50.2 ± 1.9^{ns}	49.1 ± 1.2 ^{ns}	48.7 ± 1.8^{ns}	48.3 ± 1.7^{ns}	48.2 ± 2.6^{ns}	49.1 ± 2.1^{ns}
Abaxial stomatal density (n mm^{-2})	120.5 ± 10.5^{a}	154.1 ± 11.2^{b}	153.4 ± 8.1^{b}	154.3 ± 5.5^{b}	139.7 ± 6.9^{c}	127.3 ± 8.9 ^{ad}	126.6 ± 7.5^{ad}
Adaxial stomatal density (n mm^{-2})	19.0 ± 2.6^{a}	26.3 ± 3.5^{b}	25.2 ± 3.6^{bc}	28.0 ± 3.3^{bcd}	21.0 ± 2.0^{ace}	23.9 ± 3.9^{bcde}	19.8 ± 2.5^{ae}

The mesophyll was differentiated into palisade and spongy tissues. The palisade parenchyma was better developed in the proximal growth units (U1-U2) occupying about 23% of the mesophyll. In particular, the size of palisade cells ranged from 129.1 ± 9.1 µm × 28.7 ± 2.5 µm (U2) to 65.3 ± 5.1 µm × 26.6 ± 2.1 µm (U7). There was not palisade tissue near the abaxial surface. The spongy parenchyma consisted of polygonal cells in transverse sections whose size ranging from 64.5 ± 9.3 µm (U3) to 50.8 ± 9.3 µm (U5). Differences among the subsequent units were not significant.

Multiple oil bodies per cell were observed mostly in leaf palisade cells in all the growth units. Spongy parenchyma was more abundant in the oldest leaves (U1-U3) (Fig. 1D and 1F).

In the mesophyll of all the growth units compartment cells were observed (Fig. 2A-2C, 2E) which had the same size (64.5 ± 9.3 µm × 41.0 ± 8.0 µm) for leaves of the different units, but in terms of percentage concerned more space in young leaves (U1-U3).

In leaves of all the growth units, there were vascular bundles and resin canals (Fig. 2A-2C), decreasing in diameter by 42% and 55%, respectively from U1 to U7. The randomly arranged branched astrosclereids with lignified thick cell walls (Fig. 2C) were observed in the mesophyll only for the older growth units (U1-U4).

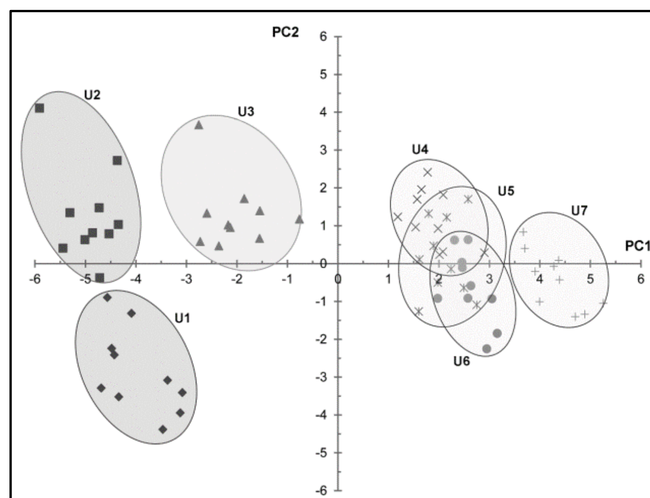

Figure 3. Principal Component Analysis (PCA) carried out using anatomical and morphological variables for seven growth units along the branch of *Wollemia nobilis* (growth units from proximal to distal part, U1: growth unit 1; U2: growth unit 2; U3: growth unit 3; U4: growth unit 4; U5: growth unit 5; U6: growth unit 6; U7: growth unit 7).

Data analysis

The PCA returned two axes explaining a cumulative variance of 46.2% (Tab. 3).

In particular, PC1 (37% of total variance) was negatively related (R>0.8) to unit length, leaf length, leaf width, leaf area, leaf

Table 3. Factor loadings for the principal component analysis (PCA) carried out on the considered traits (see Tab.1 and 2).

Variable	Variable loadings		Variable	Variable loadings	
	PC1	PC2		PC1	PC2
Length of the growth unit	-0.89645488	-0.30588591	Hypodermic cells width	-0.23017474	0.1835163
Leaf length	-0.75705414	0.44901631	Palisade parenchyma cells length	-0.92143668	-0.08139106
Leaf width	-0.83003837	0.10659392	Palisade parenchyma cells width	-0.17671688	-0.20108095
Leaf area	-0.90186501	0.24119589	Compartmented cells maximum diameter	0.05272425	0.32040473
Leaf dry mass	-0.94982074	0.15316093	Compartmented cells maximum diameter	0.07030174	0.03988491
Leaf mass per unit of leaf area	-0.85144131	-0.0790483	Spongy parenchyma cells maximum diameter	-0.2043588	0.53462444
Total leaf thickness	-0.9497731	-0.18184605	Spongy parenchyma cells minimum diameter	-0.28024157	0.36417208
Leaf tissue density	0.14271851	0.15805018	Abaxial epidermis thickness	-0.55857868	0.04839313
Palisade parenchyma thickness	-0.92285655	-0.24917106	Abaxial cuticle thickness	0.11223717	0.04826154
Spongy parenchyma thickness	-0.87630281	-0.31486704	Abaxial stomatal length	0.13591593	-0.51720306
Vascular bundles diameter	-0.92494598	-0.03722576	Abaxial stomatal width	-0.21678332	-0.25562375
Resin canals diameter	-0.88216753	-0.30769902	Adaxial stomatal length	-0.33911484	0.08068837
Adaxial cuticle thickness	-0.44027876	-0.42555438	Adaxial stomatal width	-0.40471595	0.33282974
Adaxial epidermis thickness	-0.63905217	0.25517018	Abaxial stomatal density	-0.20416087	0.76956451
Hypodermic cells length	-0.31550498	0.14156295	Adaxial stomatal density	-0.02542065	0.46582819

dry mass, LMA, total leaf thickness, palisade and spongy parenchyma thickness, the diameter of vascular bundles and resin canals. Along PC1, all segments showed significant differences (one-way ANOVA, F value=496.7, p<2e-16), with the exception of U5 with both U4 and U6, which in turn were significantly different between each other (Fig. 3).

Discussion and Conclusion

The results of this research highlight that *W. nobilis* trees growing in the Botanical Garden of Rome, characterized by a Mediterranean type of climate, produce branches, which are comparable in size and number of growth units with the branches from trees growing in the Wollemi National Park (Burrows et al., 2007). The structure of the *W. nobilis* is characterized by subsequent growth units, with leaves having significant variations in morphological and anatomical traits. Despite the fact that each unit occupies a different position in the branch with different microclimate conditions (in particular, irradiance), all leaves are primarily formed only distally, in conditions of maximum irradiance. The differences in the leaves structure more clearly reflect age-related changes than an adaptation of the leaves to different lighting conditions, according to the results of (Niinemets & Lukjanova 2003) for *Pinus sylvestris*.

Age-related changes in the leaves of *W. nobilis* appear in a gradual increase of size from U7 to U2, indicating the long period of leaf growth (at least six-year). In comparison with other Gymnosperms, *W. nobilis* leaves have a longer period of growth, according to the results of (Gratani *et al.* 2001, 2015; Kuusk *et al.* 2017) for Mediterranean *Pinus* species. The most proximal unit (U1) differs from the following one for a smaller leaf size, which may be related to the ontogenetic development of the first segment from the apical bud of the orthotropic shoot (Tomlinson & Murch, 2009).

Age-dependent increase of LMA (Wright *et al.* 2006, Poorter *et al.* 2009; Kuusk *et al.* 2017) is due to the accumulation of carbon-rich chemicals reflecting the thickness and enhanced lignification of leaf tissues (Niinemets 1997). Accordingly, our results highlight significant differences for LMA along with the subsequent growth units. In particular, LMA is 78% higher in old (U1) than in young (U7) leaves, with values following in the range of Gymnosperms (Poorter *et al.* 2009). The LMA increase is associated with an increment of the number of the lignified elements (vascular tissues, astrosclereids) and hypodermal and epidermal-cuticle structures (outer cell walls, cuticle, wax layer) (Dragota & Riederer 2007) which improve the resistance to mechanical damage and may directly lead to the increase of leaf longevity (Niinemets & Lukjanova 2003) and resistance to stress factors (Gratani & Bombelli 1999). The presence of abundant oil bodies in the mesophyll cells of *W. nobilis* older leaves is comparable with those shown in the senescent leaves of some angiosperms (Lersten et al., 2006).

The occurrence of compartmented cells (also called mucilage cells) in the Gymnosperms is exclusive for the genera *Araucaria* and *Wollemia* and it is an important trait for the evolutionary and taxonomic interpretation (Burrows & Bullock 1999; Mastroberti & Mariath 2003). The compartmented cells are related to water storage and translocation throughout the apoplast solute pathway (Mastroberti & Mariath 2008). Our results show compartmented cells in the spongy parenchyma tissue of all the growth units. The compartmented cells did not show significant differences in cell size (71.9 ± 4.6 µm, the mean value of all the units) in leaves of different age, compared to the typical spongy cell size (55.8 ± 4.4 µm, mean value). According to (Mastroberti & Mariath 2008), compartmented cells differentiation starts in the primordial leaf and occurs simultaneously with leaf development. In our research, the size of these cells does not increase with the age, while the size of other parenchymatous cells slightly enlarged.

As a consequence, we argue that the rate of investment in leaf consistency in relation to that in carbon assimilation can be

partially decoupled in *W. nobilis* since the pay-back time for leaf construction has to be interpreted at the branch and not at the leaf scale. In other words, the variable rate of investment/cost overrides the above-mentioned trade-offs due to the branch longevity. As branches may live from 5 to 15 years before abscission, it follows that the proximal leaves on a branch are alive for this period of time (Burrows *et al.* 2007). This strategy implies that *W. nobilis*, differently from other Gymnosperms, can adapt new leaves to variable environmental conditions with a return rate on a larger time-scale.

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