Environmental effect on the leaf morphology and anatomy of *Berberis microphylla* G. Forst

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Abstract

Berberis microphylla G. Forst is a fruit shrub native from Patagonia, considered as a non-timber forest product. In recent years, there has been an increased demand for its fruits, both for fresh and industrialized consumption, being the establishment of commercial orchards in different sites a need to meet this demand. *B. microphylla* cloned plants have been introduced from Ushuaia, Tierra del Fuego to Buenos Aires province in order to evaluate its phenotypic plasticity and the possibility of fruit production. At the same time, a comparative study on the morphology and anatomy of the mature leaves of *B. microphylla* grown in two different environmental conditions was carried out. Moreno leaves were significantly larger than Ushuaia leaves in all the morphological parameters registered, while Ushuaia leaves were more circular than Moreno leaves with the highest roundness and elongation indexes. Nevertheless, histological sections showed that Ushuaia leaves have one more layer of palisade cells respect to Moreno leaves. Ushuaia leaves showed higher palisade cells, larger abaxial epidermal cells and thicker cuticles than Moreno leaves. The stomatal density was superior on Moreno leaves. Scanning Electron Microscope of abaxial epidermis showed a surface with numerous ridges of different forms that prevent the layout of epidermal cells on Moreno leaves. Appearance of this surface is glossy and oily. On the contrary, epidermal cells are well recognized on Ushuaia leaves. Stomata of anomocytic type were observed and surface looks waxy. Auto-fluorescence on leaf cross sections were observed on the vascular bundles and partially on the epidermis cells. *B. microphylla* leaves showed a high phenotypic plasticity between the two sites of cultivation. The changes in the leaf morphology and structure observed in Moreno leaves could indicate that the plants are trying to adjust its morphology to the new culture conditions *i.e.* higher temperatures and lower irradiance.

Introduction

Berberis microphylla G. Forst (in the past *Berberis buxifolia* Lam.) is an evergreen shrub that may be semi-evergreen where winters are particularly cold and harsh, as it occurs in Tierra del Fuego. It is a spiny and erect shrub up to 4 m high, often growing in the magellanic subpolar forest Eco region,[1] in coastal scrub, *Nothofagus* forest margins and clearings, moister areas in grass steppes, and along streams and rivers.[2] It is one of the understory species in timber quality and associated non-timber quality stands of *Nothofagus* forests in Tierra del Fuego,[3] being considered as a non-timber forest product. In recent years, there has been an increased demand for the fruits of these shrubs and particularly for *B. microphylla*, both for fresh consumption and for the production of various products such as candies and jellies, pulps for making ice cream, beverages without alcohol and they are used in cosmetic products too. Also, in *B. microphylla* as in most of the species of the genus are assigned medicinal properties due to the presence of the alkaloids called berberine and berbamine.[4-8] Moreover, phenolics like as anthocyanins in the fruits,[9-13] in the leaves[14] and in the roots[15,16] were found, which give a medicinal and tinctorial application.

Floral biology, fruit development and quality, and the assessment of morphological variability were studied in natural populations of *B. microphylla*,[12,17-20] as well as the phenological stages and the annual cycle of growth and development.[21,22] However, nothing has been yet said about the leaf morphology and structure of *B. microphylla*. The leaf can be considered as a micro copy of the plant,[23] and the variations of leaf morphology can reflect the plant capacity to acquire, use and conserve resources. Under abiotic stress, plants alter their physiology, morphology and development in response to environmental changes.[24]

Some studies have been performed in the genus *Berberis* on this subject. Arambarri *et al.*,[25] studied the leaf anatomy of *B. ruscifolia*. Histological description related to the pharmacological property was noticed on *B. aristata*, *B. lyceum* and *B. asiatica*.[26,27] Other antecedents have given greater importance to the study of the leaf compounds produced for their therapeutics action.[14,28-32]

In the last two years, a plot of *B. microphylla* cloned plants has been introduced to the Buenos Aires province in order to carry out experimental studies, *i.e.* evaluate its phenotypic plasticity and the possibility of fruit production. These plants have shown a good vegetative growth; flower differentiation was observed but until now fruits have not been formed. Leaves are photosynthetic organs thus their shapes, sizes and structure are important factors influencing the success of the plants. Therefore the aim of this work was to study the morphology and anatomy of the mature leaves of *B. microphylla* plants grown in two different environmental conditions, on the origin site where they grow spontaneously and in a site where it is interesting to introduce them.

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Materials and Methods

Plant material and growing conditions

Berberis microphylla plants were obtained through clonal propagation from a natural population and cultivated in pots (n=40) in the experimental field of the Centro Austral de Investigaciones Científicas, Ushuaia, Tierra del Fuego, Argentina (54°48' SL, 68°19' WL, 30 m a.s.l) for five years. At the beginning of 2013, twenty plants were taken to Moreno (Buenos Aires) and cultivated in the experimental field of the Faculty of Agriculture, University of Morón (34° 39' SL, 58° 47' WL, 19 m a.s.l).

Moreno climate is temperate sub humid with 1200 mm rainfalls per year and extreme temperatures are 40 and –1°C. On the other hand, Ushuaia climate is classified as sub polar oceanic with mild temperatures, *i.e.* not

Table 1. ***Berberis microphylla*** **morphological characteristics and measures according to the cultivation site.**

Cultivation site	Area, mm^2	Perimeter, mm	Leaf length, mm	Leaf width, mm			Petiole length
				Middle fraction	Bottom fraction	Top fraction	
Moreno	123.46^a	60.86^a	23.66^a	9.63^a	7.92^a	8.32^a	3.18^a
Ushuaia	46.64^b	30.94^b	12.06^b	5.73^b	4.71^b	4.51^b	2.27^b

Means in the same column followed by different letters are significantly different at P≤0.01 (Tukey's test).

extreme and abundant rainfalls throughout the year like the English weather and the extreme temperatures are 15 and –5°C. Climatic data were collected for mean, maxima and minima air daily temperatures (ºC), and rainfall (mm) by the Meteorological Station located at the Centro Austral de Investigaciones Científicas, and the Meteorological Station located at the Moreno experimental field, from January 2013 to February 2014 (Figure 1).

Leaf morphology and anatomy

One year after their establishment (February 2014), mature leaves (n=20) were taken from the plants of the two sites of culture and immediately they were scanned. The leaf area, leaf perimeter, leaf major and minor axis were registered. Then, the index of elongation (leaf major axis/leaf minor axis), roundness [$(4 \times \pi \times \text{area}) / \text{perimeter}^2$] and compactness [sqrt ($4 \times \text{area} / \pi$) / major axis length] were calculated. Then, the leaves were fixed on FAA solution. Some of them were employed for histological section using Spurr's resin technique and the others were studied by SEM (Scanning Electron Microscope).

Light microscopy: leaves were dehydrated in an ethanol series and embedded in Spurr's resin. Thin sections (75-90 nm thick) were stained with uranyl acetate and lead citrate. Histological sections were used to study the mesophyll structure. Cells of each tissue were measured in height and width and the volume was calculated. Leaf cross sections without stain were observed by UV filter BP 340-380.

Scanning electron microscopy: leaves were dehydrated in an ethanol series and critical point drying technique was employed. Samples were sputter coated with 20 nm gold and observed with a SEM (Philips XL-30; Philips, Amsterdam, The Netherlands). Stomata of abaxial epidermis were measured and then density (stomata number/mm^2) was calculated.

Results

Leaf morphology

Berberis microphylla leaf morphology was significantly different between the cultivation sites. In effect, Moreno leaves were significantly larger than Ushuaia leaves in all the parameters registered (Table 1). Leaf length, leaf width and leaf perimeter of Moreno site were about twice than those of Ushuaia leaves, while leaf area was three times higher than Ushuaia leaves. Petiole length of Moreno leaves was significantly larger than Ushuaia leaves too (Table 1). Conversely, values of index calculated were higher for Ushuaia leaves (Table 2). Ushuaia leaves were more circular than Moreno leaves with the highest roundness and elongation indexes.

Table 2. Elongation, roundness and compactness indexes of ***B. microphylla*** **leaf according to the cultivation site.**

Cultivation site	Elongation	Roundness	Compactness
Moreno	0.43^b	0.43^b	0.54^b
Ushuaia	0.48^a	0.61^a	0.64^a

Means in the same column followed by different letters are significantly different at P≤0.01 (Tukey's test).

Light microscopy

Leaves of *B. microphylla* are exhibited dorsiventrality. Leaves are formed by a mesophyll differentiated into a palisade and a spongy mesophyll. Palisade cells are arranged on the adaxial surface. Cells of the palisade are elongated in the transverse plane of the leaf, with many chloroplasts (Figure 2A,B) and they are densely packed together into two layers on Moreno leaves (Figure 2A) and into three layers on Ushuaia leaves (Figure 2B). Sclerenchyma cells are present in adaxial sub epidermical position only on Ushuaia leaves (Figure 2B). The spongy mesophyll is present with irregular branching cells containing chloroplasts and separated by large air spaces. The spongy mesophyll is arranged on the abaxial side (Figure 2A,B,G,H). Vascular bundles which are part of the venation can be observed into the mesophyll. Phloem and xylem tissues are in abaxial and adaxial positions, respectively (Figure 2A). Parenchyma cells and some sclerenchyma cells are present surrounded the vascular bundle on Ushuaia leaves (Figure 2B). Nevertheless, parenchyma cells dominate Moreno leaves. Auto-fluorescence on leaf cross sections was observed. In effect, on the vascular bundles there was possible to detect cells with fluorescence (Figure 3A,B). Some parts of the epidermis present fluorescence too (Figure 3C). This confirms that there are laticifer-idioblast cells.

All the mesophyll is surrounded by the epidermis and cover by the cuticle (Figure 2E,F,I,J). The adaxial epidermis has larger cells than abaxial epidermis (Figure 2C-F). No significant differences were observed between the cell measures of adaxial epidermis of the two cultivation sites (Table 3). However, the height and volume of the abaxial epidermis cells and the cuticle was larger on the Ushuaia leaves. In effect, the cuticle of Moreno leaves were 2 µm wide while Ushuaia leaves were 5-6 µm wide (Figure 2E,F). On the other hand, the same differences were observed on the abaxial epidermis. Cuticle of Ushuaia leaves were 2.5 - 3 µm width, while Moreno leaves were 1-1.5 µm width, (Figure 2I,J). The abaxial epidermis cells on Ushuaia leaves were significant higher than those of Moreno leaves (Table 3). Finally, Moreno leaves were thinner than Ushuaia leaves, being the leaf thickness of 350 µm and 470 µm, respectively (Figure 2A,B).

Scanning electron microscopy

The abaxial epidermis appearance is different depending on the cultivation site. In effect, the leaves taken from Moreno site show an irregular surface due to the molecular architecture of the films of the epi-cuticle wax. The surface presents numerous ridges of different forms and closely placed near to the stomata that don't allow the differentiation of the arrangement of epidermal cells (Figure 3E,F).The appearance of this surface is glossy and oily. Numerous particles coating the surface of the guard cells flow from the stomatal pore (Figure 3G). On the other hand, epidermal cells are well recognized on abaxial epidermis from Ushuaia leaves (Figure 3H,I). Stomata of anomocytic type are observed, being the pair of guard cells surrounded by 5-6 unspecialized epidermal cells, and surface looks waxy (Figure 3G,I). Venation with its ramification is well recognized on diaphanized leaves (Figure 3K-M). Adaxial epidermal cell arrangement is isodiametric (Figure 3L) and

the stomata surrounded by generic format of epidermal cells are noticed on the abaxial epidermis (Figure 3M). Stomatal density for leaves collected on Moreno site varied between 330 and 450 per mm^2, while in leaves from Ushuaia site the values recorded were 210-330 stomata/mm^2 (data not shown).

Discussion

Leaves are important organs for photosynthesis and play a crucial role in survival and growth of a plant. The leaf can be considered as a microcopy of the plant, and the variations of leaf morphology can reflect the plant capacity to acquire, use and conserve resources.[33] Leaf morphology like as specific leaf area changes with the plant growth conditions *i.e.* temperature.[34] Light availability can modify leaf anatomy and therefore affect plant growth.[35] Climatic conditions of the two selected sites for this study are quite different so it is expected to be one of the causes of morphological differences. Furthermore, many species have acquired plasticity for the leaf shape giving responses to changes in the environmental conditions. Plants were grown in Moreno site surrounded by trees to reduce high temperatures, so that the presence of neighboring vegetation could have modified the light environment. It has been demonstrated in *Arabidopsis thaliana* that changes in environmental light generate signals that are perceived by phytochromes and cryptochromes.[36] In consequence, an elongation of the petiole and an increase of the leaf blade area is a typical example of leaf shape plasticity called shade-avoidance syndrome. Ushuaia leaves have a higher roundness and elongation indexes. A correlation between leaf roundness and irradiance was found in *Nothofagus solandri*, decreasing with increasing irradiance; however, a non-significant correlation was found in *N. fusca*.[37]

As well knows on others species, external changes in *B. microphylla* were reflected in an increase in the volume of each cell when a decrease in cell proliferation was observed as a compensation phenomenon.[38] Another differ-

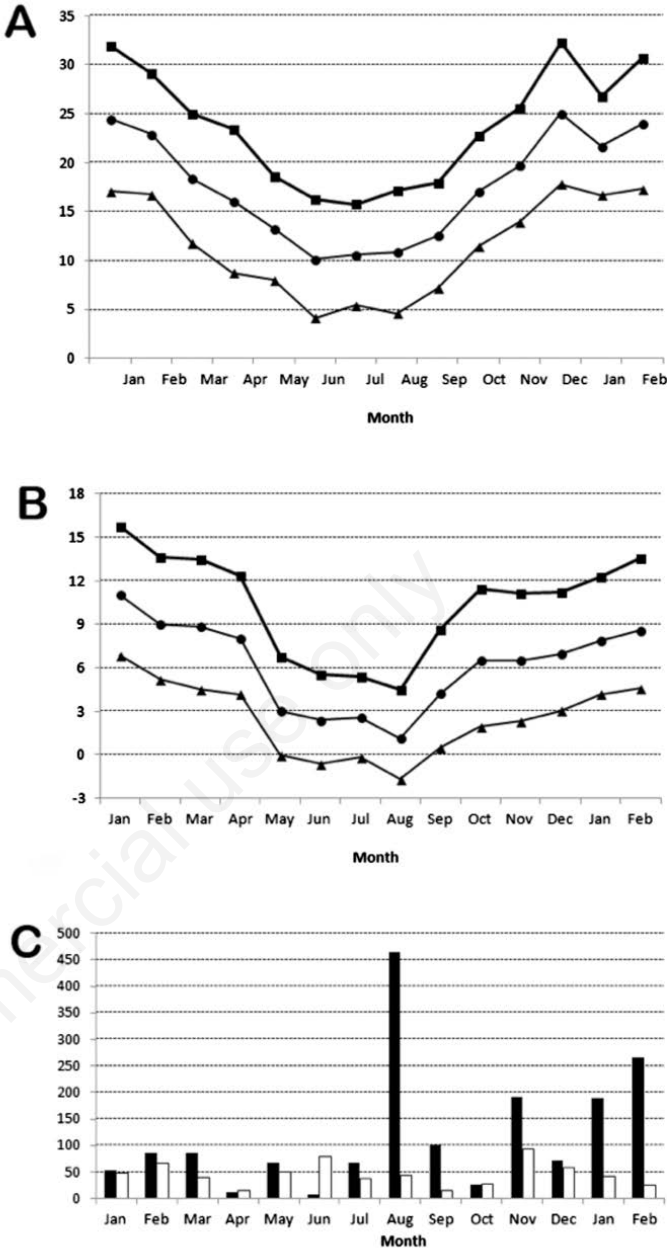

Figure 1. Temperature (A-B) and rainfall (C) registered by the Meteorological Station located at the Centro Austral de Investigaciones Científicas (Ushuaia, Tierra del Fuego, Argentina; B-C) and the Meteorological Station located at the Moreno experimental field (Buenos Aires, Argentina; A-C) from January 2013 to February 2014. A-B) Mean (circle), maxima (square) and minima (triangle) air daily temperatures (°C); C, rainfall (mm), Moreno (black), Ushuaia (white).

Table 3. *Berberis microphylla* leaf cell measures studied by light microscopy according to the place of cultivation.

Tissue	Height µm		Width µm		Volume $µm^3$	
	Moreno	Ushuaia	Moreno	Ushuaia	Moreno	Ushuaia
Palisade cells	47.33[b]	72.17[a]	19.10a	19.80[a]	912.00[b]	1435.00[a]
Spongy cells	19.40[a]	21.25[a]	25.00b	30.35[a]	512.00[b]	635.00[a]
Adaxial epidermis cells	19.04[a]	18.5[a]	38.50a	37.80[a]	746.15[a]	702.50[a]
Abaxial epidermis cells	11.20[b]	14.75[a]	21.80a	23.30[a]	248.00[b]	340.00[a]
Stomata	3.24[a]	2.10[b]	3.53a	2.07[b]	-	-

Means in the same line and for each variable followed by different letters are significantly different at P≤0.01 (Tukey's test).

ence found between these leaves was the thickness of the cuticle. The cuticle has two main components: cutin and waxes. Cutin is a tough, cross-linked polyester matrix primarily composed of C16 and C18 oxygenated fatty acids and glycerol. Wax is a heterogenous mixture, primarily composed of very-long-chain fatty acid derivatives.[39] The cuticular wax in *Berberis* is crystalloid usually as clustered tubuli, chemically dominated by nonacosane-10-ol.[40] Little is known about how waxes are trafficked within the cell from their site of synthesis at the endoplasmatic reticulum to the plasma membrane.[41] Many authors considered that wax extruded from epidermal cells.[41-43] Epicuticular waxes are modified by changes in plant growth conditions such as temperature, relative humidity, irradiance, and wind, or acid rain.[40] Climatic conditions of the two selected sites for this study are quite different so it is expected to be one of the causes of morphological differences. Ushuaia leaves had thicker cuticle probably for the most extreme weather conditions that plants undergo. On the other hand, Ushuaia site has a greater intensity of visible and UV light and this factor is positively correlated with the enlargement of cuticle thickness to create a lotus effect to reflect UV radiation and integration of protective flavonoid compounds in the cuticle. These results are in agreement with Davi *et al.*[23] who determined that plants grown in high light generally have thick leaves caused by extra layers of palisade mesophyll or longer palisade cells to protect them from high-light damage.

Different appearance between the abaxial epidermis could be due to Moreno leaves were still growing while Ushuaia leaves growth had ceased. In effect, surface wax appears to be deposited only on young leaves, and essentially only during or shortly after the period of leaf expansion. Wax deposition is probably related to the development and solidification of the cuticular layer.[44]

The structural basis of cuticle is a combination of surface roughness in the micrometer range combined with a strong hydrophobicity caused by superimposed wax sculptures (epicuticular waxes) in the nanometer range.[45] Epicuticular waxes strongly influence the wet ability, self-cleaning behavior and the light reflection at the cuticle interface.[46] It is remarkable that particularly secondary alcohols as nonacosane-10-ol among others induce non-planar structures such as tubules and other irregular forms.[45] Waxes are solid, but their structures reveal analogies to those of liquid crystals. The formation of a layer structure may be inhibited by a strong variation in the chain length of the molecules or by a missing positional order, causing a nematic structure with a two-dimensional order. For the other hand, wax synthesis and extraction stopped when cell expansion ceased.[47]

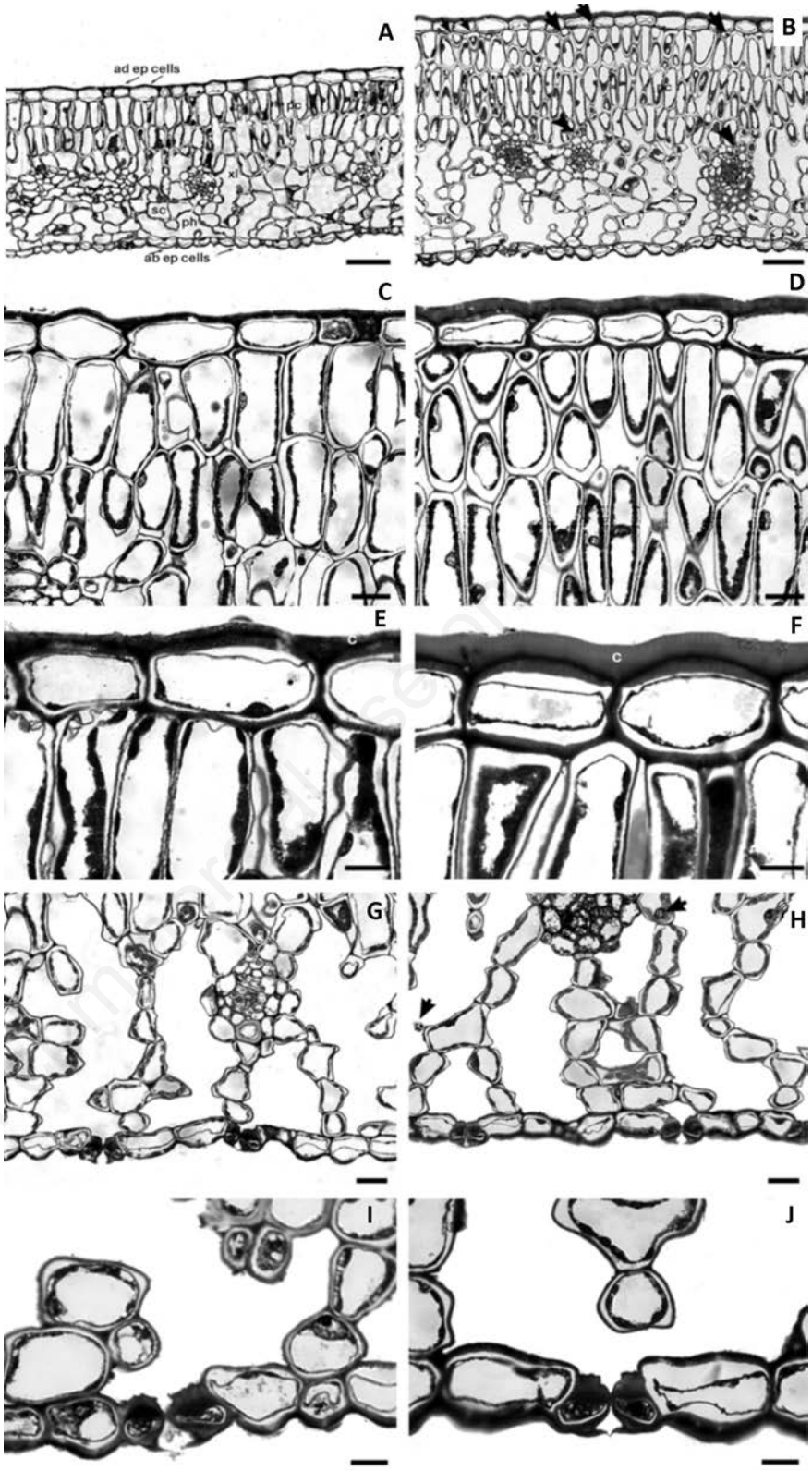

Figure 2. Micrograph of cross-section of *Berberis microphylla* leaves collected on Moreno (A,C,E) and Ushuaia sites (B, D, F). A-B, view of total cross-section leaf with adaxial epidermal cells (ad ep cells), abaxial epidermal cells (ab ep cells), palisade mesophyll cells (pc), spongy mesophyll cells (sc), phloem cells (ph), xylem cells (xl) and sclerenchyma cells (arrowhead); C-D, Detail of adaxial epidermal and palisade mesophyll tissues; E-F, Detail of abaxial epidermal cells and cuticle (c). Bars: A-B, 100 µm; C-D, 25 µm; E-F, 10 µm. G-J) Detail of the palisade mesophyll tissue and the stomata of abaxial epidermal of *Berberis microphylla* leaves collected on Moreno (G,I) and Ushuaia sites (H,J). Bars: G-I, 25 µm; H-J, 10 µm.

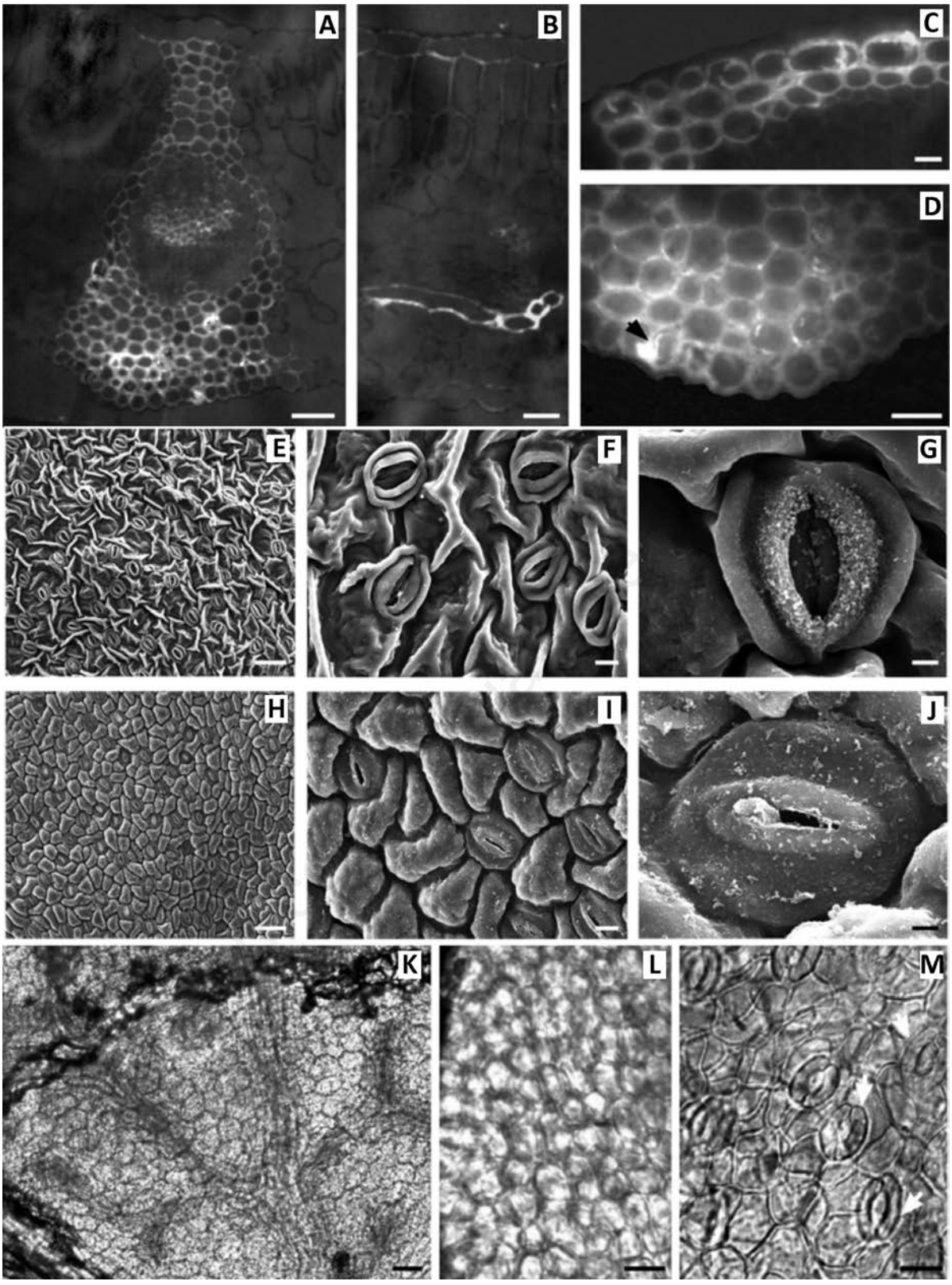

Figure 3. A-D) Micrograph of cross-section of *Berberis microphylla* leaves observed by UV filter BP 340-380. A) View of central vascular bundle and parenchyma cells with fluorescence; B) transversal vascular bundle with fluorescence; C) epidermal and sclerenchyma cells on the leaf apex with fluorescence; D) detail of stomata (arrowhead) and laticifer-idioblast cells. Bars: A-B, 10 µm; C, 25 µm; D, 5 µm. E-J) SEM micrograph of abaxial epidermis of *Berberis microphylla* leaves collected on Moreno (E-G) and Ushuaia site (H-J). E,H) General view; F,I) detail of surface of abaxial epidermis tissue; G,J) stomata detail. Bars: E,H, 50 µm; F,I, 10 µm; G,J, 5000 nm. K-M) Micrograph of diaphanized *Berberis microphylla* leaves. K, leaf venation; L, Detail of adaxial epidermal cells; L, Detail of abaxial epidermal cells with stomata (arrows). Bars: 2 µm.

Flavonoid compounds usually accumulate in the central vacuoles of guard cells and epidermal cells as well as subepidermal cells of leaves.[48] Several classes of phenolic compounds are strongly autofluorescent when irradiated with UV or blue light. Therefore, fluorescence microscopy is a powerful tool for studying tissue localization of these metabolites.[49] Berberine is a quaternary ammonium salt from the pro toberberine group of isoquinoline alkaloids that possess low fluorescence emission in an aqueous solution[50] and it is well known that berberine stain a bright yellow.[6] This property would explain the intense yellow color of diaphanized leaves (data not shown).

Conclusions

Plant plasticity is an advantageous strategy for survival in changing environmental conditions, being leaves photosynthetic organs thus their shapes, sizes and structure are important factors influencing the success of the plants. *B. microphylla* leaves showed a high phenotypic plasticity between the two sites of cultivation. The changes in the leaf morphology and structure observed in Moreno leaves could indicate that the plants are trying to adjust its morphology to the new culture conditions *i.e.* higher temperatures and lower irradiance. The correlation of the observed changes together with the plasticity on the leaf physiology, the nutrient and pigment contents will be of interest to study, as well as with the reproductive performance to apply on future genetic improvement.

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