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Leaf anatomy and biomineralization in *Empetrum rubrum* Valh ex Willd (*Ericaceae*)

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Abstract: Fernández Pepi, M. G.; Arriaga, M. O. & Zucol, A. F. 2012. Leaf anatomy and biomineralization in *Empetrum rubrum* Valh ex Willd (*Ericaceae*). *Bot. Complut.* 36: 113-121.

The present paper describes and illustrates features of *Empetrum rubrum* epidermis and transverse leaf section. It also describes and quantifies siliceous and calcium phytoliths.Biomineralization quantification is expressed as the media biomineral content (% from dry weight) with values of 1.58% ± 0.51 in silica phytoliths and 0.82% in calcicum phytoliths. *E. rubrum* is a native species from the austral end of South America. We here compare features of its leaf anatomy and biomineralization quali-quantifications with those from *E. nigrum*, a native species from the northern hemisphere. The comparison between the characters described for this species and those from macroremains and phytoliths recovered from surface soils provides valuable tools for taxonomical, ecological, paleontological and paleoecological studies. Knowledge of said characters further provides valuable information to infer changes in plant communities in the recent past as well as insight into the environmental and soil conditions at the time when such changes took place.

Key words: biomineralizations, *Empetrum* heathlands, *Empetrum rubrum*, leaf anatomy.

Resumen: Fernández Pepi, M. G.; Arriaga, M. O. & Zucol, A. F. 2012. Anatomía de las hojas y biomineralización en *Empetrum rubrum* Valh ex Willd (*Ericaceae*). *Bot. Complut.* 36: 113-121.

En este trabajo se describe e ilustra las características anatómicas de la epidermis y transcorte de hoja de *Empetrum rubrum*. Se han descrito y cuantificado a los cuerpos silíceos y cálcicos. La cuantificación de biomineralizaciones se expresa en valores medios del contenido de biominerales (% en peso seco) obteniéndose valores de 1,58% ± 0,51 correspondientes a silicofitolitos (biomineralizaciones silíceas) y 0,82% de calciofitolitos (biomineralizaciones cálcicas). La especie *E. rubrum* es nativa del sur de América del Sur, sus características anatómico foliares y los caracteres cuali-cuantitativos de sus biomineralizaciones han sido comparados con los de *E. nigrum* nativa del Hemisferio Norte. Los caracteres descriptos para esta especie, al compararlos con macrorestos o silicofitolitos recuperados en suelos recientes, constituyen herramientas de gran valor en estudios de taxonomía, de ecología, paleontología y paleoecología, aportando datos importantes para determinar las modificaciones pasadas de estas comunidades vegetales, como así también, las condiciones ambientales y del suelo en que se produjeron dichas modificaciones.

Palabras claves: biomineralizaciones, murtillar, *Empetrum rubrum*, anatomía foliar.

INTRODUCTION

Genus *Empetrum* L. originally belonged to the family *Empetraceae*, and is in fact still considered as a member of this family by some authors (Zuloaga *et al*. 2008). However, according to the circumscription based on morphological and molecular evidence (Judd *et al.* 2002) this genus is currently placed within *Ericaceae*, in the monophyletic clade of Empetroideae, which is composed by three genera: *Empetrum* (consisting of two circumboreal and one austral species), *Ceratiola* Michx. (with a North American species), and *Corema* D. Don (with a North American and an European species). These three genera share strong synapomorphies such as expanded and deeply lobed stigma, small corolla, drupaceous fruit and wind pollination. *Empetrum rubrum* Valh ex Willd ("murtilla") lives in Patagonia (from Neuquén to Tierra del Fuego provinces, Argentina), Chile, the Malvinas/Falkland and the Tristan da Cunha Islands (Good 1927, Moore 1983, León *et al.* 1988, Correa 1999). This species occurs

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as prostrate or cushion subshrub forms, developing the so-called “murtillares”, in plains on the austral slopes extremely exposed to winds, where winter snow can remain for several months (Roig 1998). It is a heliophilous plant that lives in acid soils, usually in areas that in the past used to be covered by “coirón” (*Festuca gracillima* Hook. f.). When sheeps were introduced into these regions, the scarce availability of forage grass forced animals to overgraze “coirón” causing its slow retreat, with a resulting landscape dominated mainly by “murtilla” (Correa1999, Anchorena *et al.* 2001). Recent studies indicate that sheep-grazing and steady land activity over the last 100 years has turned many grass communities into heathlands (Collantes *et al.* 1989). Given the low value of murtilla as sheep forage, many attemps have been made to substitute eroded heath with sown pasture. The recurrent failure of establishing pasture species (Serra 1969) was ascribed to the alellopathic effect of *Empetrum* leaf litter. In many places, however, deterioration by overgrazing continued and large areas are currently dominated by very poor and eroded “murtillar”, with scarce soil development (Anchorena *et al.* 2001).

Phytoliths are a valuable tool for studying ancient vegetation and landscape (Piperno 2006). Most palaeoecologically oriented phytolith studies have focused on the reconstruction of grassland composition and tree/grass cover ratios, yielding important general information on the development and changes of large-scale vegetation communities (Alexandre *et al.* 1997; Barboni *et al.* 1999, 2007; Scott 2002; Abrantes 2003, Bremond *et al.* 2005a, b, 2008a, b; Barboni & Bremond 2009). The general analytic approach considers all morphotypes and size fractions of a phytolith assemblage (Strömberg 2004, Neumannn *et al.* 2009). Although most phytoliths in the assemblages are non-specific and can be used only to reconstruct very broad ecological groups, this approach anyway allows the detection of unique morphotypes, giving rise to diagnosis at low taxonomic levels and much more precise palaeoecological interpretations (Eichhorn *et al.* 1996). Our work thus contributes to the knowledge of *E. rubrum* anatomy, especially from the point of view of its phytolith production, enabling its use as a tool in further ecological, paleoecological, paleontological and taxonomic studies, since leaf epidermis bears relevant identification features, in addition to its property of resisting digestive processes of herbivores given its strong cuticularization and type of cell wall. Phytolith contents can be identified and quantified when present in soil strata, helping to determine the composition of plant communities on these soils in the recent past.

MATERIALS AND METHODS

To analyze the phytolith production considering the tissular origin of each form, 10 specimens of *Empetrum rubrum,* collected in the Tierra del Fuego ecotone, were sampled (Appendix I), sampling sites are affected by different charge of herbivory and /or environmental conditions (Table 1, Fig. 1).

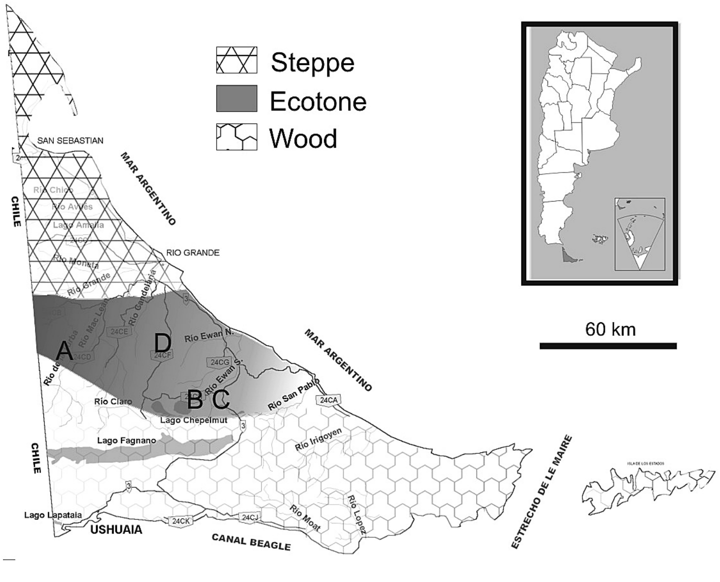


Fig. 1– Study area. Fueguian ecotone, Tierra del Fuego province (Argentina). **A:** Ea. San José. **B:** Ea. Ushuaia. **C:** Reserva Corazón de la Isla. **D:** Ea. Buenos Aires.

Table 1
Studied area characteristics. [(1)] Coronel *et al.* 2009; [(2)] Moretto, *Pers. Com.*

	Ea. San José	**Ea. Ushuaia**	**Reserva Corazón de la Isla**	**Ea. Buenos Aires**
Charge of herbivory	Exclusive “guanacos” grazing during more than 5 years	“Guanacos” and domestic livestock grazing	Exclusive “guanacos” grazing during less than 5 years	“Guanacos”, geese and sheep grazing
Soil features	pH= 4.51 (acid) 26.4% organic matter[(1)]	pH = 4.71(acid) 17.4% organic matter[(1)]	pH = 4.71(acid) 27.6% organic matter[(1)]	pH = 4.31(acid) 22-24% organic matter[(2)]
Environmental conditions	Farther north, high altitute and low humidity	Low plains, high humidity	Low plains, high humidity	Middle altitute, middle humidity

Two material processing types were implemented, one of them to anatomical analysis, where epidermal and leaf cross-sections (10 µm thick) were treated according to traditional preparation and mounting techniques for microscopic observation (Arriaga 1983, D’Ambrogio 1986). For phytolith observations the material was washed first with neutral detergent and then with distilled water, oven-dried and weighed. The phytoliths were obtained following the calcination Labouriau’s technique (Labouriau 1983), summiting each sample to a carbonization at 200 °C during 2 h. The carbonized material was chemically digested with HCl 5N to remove the remaining organic matter and $CaCO_3$, washed with distilled water and filtered with ash-free filter paper. The remaining material was calcined at 800 ºC during 2 h, and the ashes obtained were weighed before mounting for microscopic observation. In addition, 5 specimens were calcined with the Labouriau’s technique, devoided the HCl treatment, so as to obtain both the silica and calcium material. The weights of the samples obtained by both treatments were compared to establish a relationship between total biomineralizations weight and that of the silica ones. We also calculated the content of phytoliths in relation to the initial dry matter in each sample. The ashes obtained were mounted in immersion oil for microscopic observation, and the observed biomineral bodies were identified and relatively quantified, with a Leica DMLB optical microscope. For calcium biomineralizations identification in ashes, epidermis and leaf cross-sections slides we used the same optical microscope under polarized light.

For more details on the morphology of the biomineralizations, leaf anatomy observations were made with scanning electron microscopy (SEM) after metallization of the material by evaporation of a thin layer of carbon and gold-palladium, using a Phillips XL30 SEM of the Scanning Microscopy Service from the Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”. Collected specimens, remaining ashes and microscopical slides were deposited in the herbarium and repository of the Laboratory of Plant Anatomy, respectively, of the Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”.

RESULTS

Leaf anatomy. The specimens analyzed have a highly revolute leaf with a transverse section that forms an indentation in approaching blade edges (Fig. 2F). Leaves are covered by hairs that intersect (Fig. 2, J), defining an interior camera bound by the abaxial side of the lamina (Fig. 2F); where the exposed area of the blade corresponds to the adaxial epidermis (Fig. 2F). The leaf has bifacial organization, with a palisade parenchyma formed by 1-3 layers of prismatic cells in adaxial subepidermal position and a spongy parenchyma towards the abaxial epidermis (Fig. 2F). In the middle area of the transverse section, immediately below the central vascular bundle, the intercellular spaces are larger, and look like aerenchyma of stellate cells (Fig. 2D). The central vascular bundle is well developed, with 1 (–2) smaller vascular bundles on each side (Fig. 2F).

Collateral vascular bundles are surrounded by a parenchyma sheath, unrelated to sclerenchyma (Fig. 2E, H). The transverse section also shows cross connections between the vascular bundles, formed by conducting tissue surrounded by a parenchyma sheath. A layer of isodiametric parenchyma cells is observed in abaxial-subepidermal position (Fig. 2F, D).

The adaxial epidermis is composed of cells with highly thickened walls, strongly cuticularized (Fig. 2B), 19-22 µm thick in cross-section, without stomata, formed by isodiametric cells with smooth or corrugated walls (Fig. 2C), with some lanuginous uniseriate, 1-3 cells and simple-base hairs (Fig. 2K, L), in a row along the curved zone of the blade (Fig. 2G) and over both edge margins (Fig. 2F, J). The abaxial epidermis, on the other hand, consists of un-

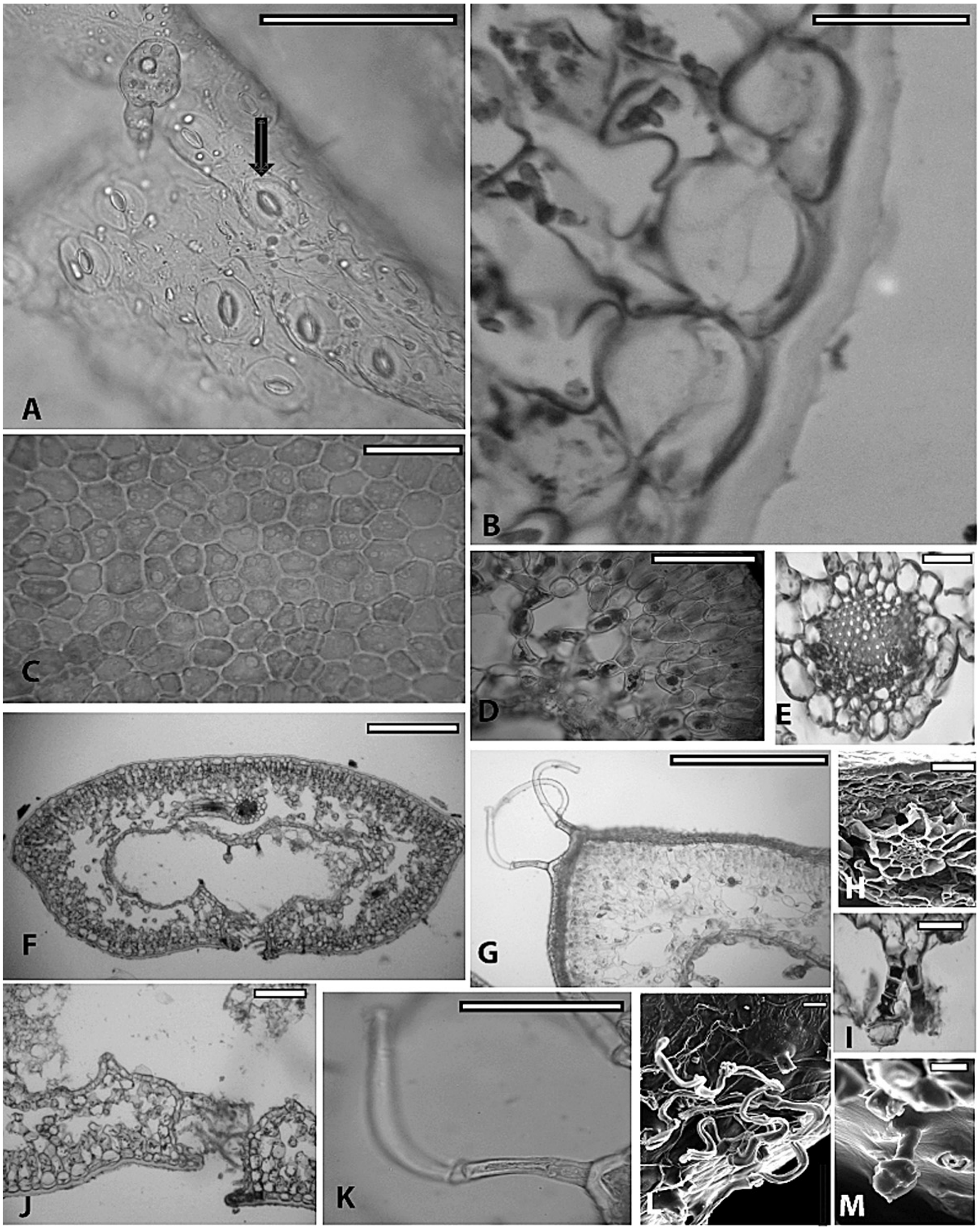
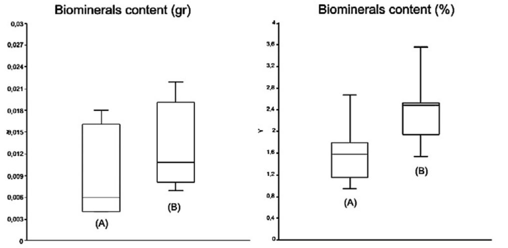


Fig. 2– *Empetrum rubrum* leaf anatomy. **H, L** and **M**, Scanning Electrone Microscopy photomicrographies. **A-G** and **I-K**, Optical Microscopy photomicrographies. **A:** Abaxial epidermis overview, stomata can be observed. **B:** Adaxial epidermis transection, very thick cuticule can be observed. **C:** Adaxial epidermis overview devoided of stomata. **D:** Section through palisade and spongy parenchyma. **E:** Vascular bundle section. **F:** General leaf transaction. **G:** Hairs over leaf bending area. **H:** Vascular bundle section. **I:** Uniseriate multicellular gland hairs. **J:** Woolen hairs crossing over leaf proximal extremes. **K:** Uniseriate multicellular wollen hair. **L:** group of uniseriate multicellular hairs showing wollen aspect. **M:** Uniseriate multicellular glandular hair. Scale bars: 20 µm.



	Biominerals content (gr)	Biominerals content (%)
(A) With chloridric treatment	0.004 (0.0092 +/- 0.0055) 0.0018	0.9400 (1.5800 +/- 0.5090) 2.6700
(B) Devoided chloridric treatment	0.007 (0.0134 +/- 0.0097) 0.0219	1.5330 (2.4040 +/- 0.7590) 33.5500

Fig. 3– Biominerals distribution contents in *Empetrum rubrum* analyzed samples, according to with (A) or devoided chloridic (B) treatment. Graphic values are expressed in grams (graphic on the right) and porcentuals (graphic on the left), maximum value (media –/+ standard deviation) minimum value on statistical results.

thickened, scarcely cuticularized cells, 10-14 µm thick in cross-section (Fig. 2J). Also observed are abundant stomata, most of them anisocytic and some anomocytic (Fig. 2A). Multicellular glandular hairs are also present with uniseriate 2-3 cell stalk and a multicellular ellipsiodal apical head (Fig. 2I, M), where cell divisions take place transversally to hair length (Fig. 2M).

Biomineral content. The comparative analysis of biomineralization percentages taken from samples from *E. rubrum* specimens is based on different life conditions from the Fuegian ecotone, such as humidity degrees, topography, soil, exposure to sun and grazing rates, and was carried out by comparing the confidence intervals developed with the average values and deviation standards. These intervals overlap do not show significant differences either in total or partial biomineralization percentages. Mean values of biomineral contents in relation to the dry weight obtained in both carbonization treatments (*i.e.* either with or without HCl) showed higher amounts of total biomineralizations than those of silica (Fig. 3). From an average of 0.007 g (1.533% dry matter) of biomineral content, the average of silica elements was 0.004 g (0.94% dry matter, the remaining material (0.003 g, or 0.590%) being estimated as biomineralizations of another chemical nature (Fig. 3).

Biomineralization characteristics. Amorphous structures (silica phytoliths). The main silica biomineralizations were found in polyhedrical elements, some with a constriction in their middle transversal section, with regular or irregular ends (Fig. 4A-F, N-Q, S, T). Prismatic, elongated, smooth-edged morphotypes were less abundant (Fig. 4G-L) so were globular ones, which range from spherical to irregular (Fig. 4K, M, O, P, R), and silicified

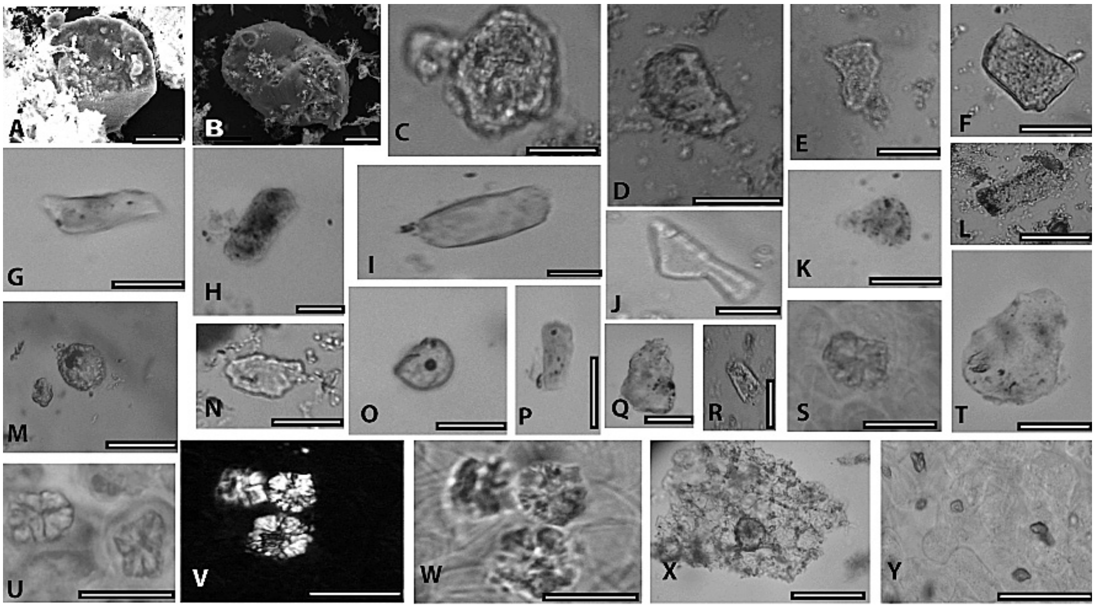


Fig. 4– Biomineralization in leaf of *Empetrum rubrum*. **A-T:** Silicophytoliths in ashes from chloridric treatment. **U-W** and **Y:** Calcium druse in leaf diafanization. **V:** The same observed in W, under polarizated light. **X:** Druse in ashes from non chloridic treatment. **Y:** cristals, druses and silicophytoliths in leaf diafanization. Scale bars: 10 µm.

unicellular trichomes. These silica bodies almost completely fill the cell lumina, mostly on the abaxial epidermis. They are present in the entire cell layer, constituting a complete siliceous coating.

Crystal structures (calcium phytoliths). As regards crystals, we observed the presence of simple, square and prismatic crystals 0.50-1 µm long (Fig. 4Y) distributed in cells of both parenchyma. We also observed larger druses with a diameter of 1-2.5 µm, formed by the concrescence of simple crystals located in the spongy parenchyma (Fig. 4U-Y). All crystal structures show refringence when observed under polarized light (Fig. 4, V)

DISCUSSION AND CONCLUSIONS

Previous studies of *Empetrum rubrum* (Rossow, in Correa 1999) described their leaves as narrow and longitudinally ridged on their abaxial surface. The present analysis shows that these leaves are strongly revolute and that their groove edges are simply leaf margins facing each other, with hair intercrossing between both margins. According to Gibelli (1876) and Gruber (1882) (*sensu* Solereder 1908) leaf folding is due to the fact that the lower epidermal cells, located in both lateral portions of the central vascular bundle, grow faster than the rest of the cells of both epidermis. In cross-section, the leaf is seen as a hollow structure, with the lower epidermis located towards the inner part, presenting abundant glandular hairs. The adaxial epidermis that forms the periphery of the cross-section lacks stomata and has a very thick cuticle. Uniseriate multicellular hairs are common in young leaves, especially along leaf margins.

Although *Empetrum* is represented by only three species worldwide, and has medicinal and food value (Altan & Özdemir 2004), only the anatomical structure of *E. nigrum* L. has been described so far (Solereder 1908, Metcalfe & Chalk 1950). We are thus here contributing to the knowledge of the anatomical structure of the austral species used as forage by native and domestic herbivores, especially in seasons when trophic overlapping is present in Patagonian grasslands (Fernández Pepi *et al*. 2009a, Alvarenga *et al*. 2009).

The present observations on *E. rubrum* leaf anatomy agree with the type, tissue position, presence and location of simple or compound crystals of *E. nigrum* described by Solereder (1908) and Metcafe & Chalk (1950). Observations on Patagonian *Gaultheria* L., a genus from the same family placed within another group, namely within subfamily *Vaccinioideae* (*sensu* Kron 1997, Kron *et al.* in Judd *et al.* 2002), also showed druses placed in special idioblasts or in lumen of chlorenchymatic cells. In *G. phyllyreifolia* (Pers.) Sleumer, druses were also observed on the hypodermis (Hermann & Pérez Cuadra 2011).

Concerning the biomineral content in *Ericaceae*, in a phytolith analysis of alpine tundra flora Carnelli *et al.* (2001) reported the presence of 0.16% silica in relation to leaf dry matter of *E. nigrum*. Hodson *et al.* (2005) reported silica content in several species of *Ericaceae*, where that of *E. nigrum* was between the mean values of those mentioned for this family, and equivalent to 0.39% of dry mass.

The data obtained from our analysis show 0.94% of silica biomineralizations and 0.59% of other types of biomineralizations, higher than the ones described above. Although these differences in biomineralization percentages can be attributed to the characteristics of each species (*E. rubrum* vs *E. nigrum*) they can also derive from their different environmental conditions or phenological stage (Bertoldi de Pomar 1975, Zucol 2001). Indeed, Carnelli *et al.* (2001) studied subalpine-alpine species in a transitional zone of heathland, stunde trees and meadows above the timberline of the central Swiss Alps at 2000-2300 m a.s.l. Our studies, on the other hand, were carried out regarding the Fuegian ecotone, between the steppe and the southern forest area covering the central part of the island where the relief is more pronounced than that of the steppe and is characterized by a mosaic of small patches of open forest of *Nothofagus antarctica* alternating with bunch of grasses in the higher parts and herbaceous communities in the depressions (Cassola *et al*. 1975). The marked different environmental conditions of these two regions could account for the differences in biomineralization percentages mentioned above. Indeed, according to Piperno (1988) there are environmental factors such as climate, soil, moisture and plant age, which affect the development of silica bodies, regulating the concentration of dissolved silica available in plants.

In our study, no significant differences were found in biomineralization percentages (total and partial) of samples from different Fuegian ecotonal sites which differed in humidity, topography, soil, exposition and grazing rates. Collantes *et al.* (1989) reported that the presence of "*murtillares*" of *Empetrum* in the steppe of Tierra del Fuego is probably associated with anthropogenic causes, particularly sheep overgrazing. The scarcity of other plant communities associated with *Empetrum* sp. could be due either to the fact that *Empetrum* has a very low organic matter degradation rate, thus inhibiting the growth of other species (Monteith 1970), or to soil acidification resulting from the blockade of N mineralization, low levels of

Ca^{++} and high content of Al^{+++}, which shows an alteration in soil evolution (Collantes *et al.* 1989). Although environmental conditions and herbivore load are different from those in the steppe, in our ecotone there has been an increase of "*murtillar*" in overgrazed places. The different sampling areas where organic matter and charge were studied did not seem to affect the rates or types of biomineralization observed. Their respective environmental parameters did not influence leaf biomineralization of the species studied. While environmental factors are considered to influence biomineralization rates, ecotone conditions do not affect the incorporation of minerals for phytolith formation. Thus, no significant differences were found among biomineralization values. Soil acidity was the most constant of all factors in our ecotone sampling sites, allowing the availability of the same quantities of silice. Fernández Honaine *et al*. (2011) found no significantly different silical contents in plants from different sampling sites, unlike the case of young and scenescent individuals, which showed higher biomineralization percentages. The material we studied was all at fruit stage, so maturity differences do not apply.

Because biomineralizations last for so long without any degradation (Rovner 1983, Piperno 1988), further knowledge of this process may allow its future application in paleontological and paleoecological studies to infer land cover development in the recent past in a particular area, and to look into the kind of soil and the chemical conditions that affected it at a given time. The description of the types of silica phytoliths and of leaf anatomy will thus contribute to the identification of diet macroremains in the region where this species develops and to soil researches thereon. Paleoecological studies on peatlands of Tierra del Fuego (a different type of plant community) used phytoliths as a tool for paleoenvironmental reconstruction (Benvenuto *et al.* 2008, 2009), concluding that certain levels reflect a stream pattern decrease or decrease of environmental moisture which resulted in a thicker plant cover, where they found some dicotyledons such as Asteraceae and Ranunculaceae. Previous phytolith descriptive studies on this ecotone were carried out on *Gunnera magellanica* Lam. (Acosta Ricci *et al.* 2011) and on dominant grass species *Poa pratensis* L., *Festuca magellanica* Lam. (Fernández Pepi *et al*. 2009b, 2010) and *Phleum alpina* L. (Fernández Pepi *et al.* 2011a), and constitute reference material for studies on changes in these communities in the recent past as a result of overgrazing. The first profiles analyzed show high abundance of grasses in a land very seldom used for grazing (Fernández Pepi *et al.* 2011b). We believe that the data we are here providing on *Empetrum* will definitely add to the study of ecotone changes in communities of Tierra del Fuego.

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Appendix I

Specimens examined

ARGENTINA. TIERRA DEL FUEGO. Dpto. Ushuaia: Ea. Buenos Aires, March 2008, *Fernández Pepi et al.,* BA 92194; *Ibidem,* Feb. 2009, BA 92195; Ea. San José, Nov. 2007, *Fernández Pepi et al.,* BA 92197 & BA 92190; *Ibidem,* March 2008 *Fernández Pepi et al.,* BA 92198; *Ibidem,* Ea. Ushuaia, Nov. 2007, *Fernández Pepi et al.,* BA 92197, BA 92192, BA 92193; Reserva Corazón de la Isla, Nov. 2007, *Fernández Pepi et al.,* BA 92191; *Ibidem*, March 2008, *Fernández Pepi et al.,* BA 92199.