

Structure of the chlorenchyma in the persistent terete leaves of *Austrocylindropuntia subulata* (Muehl.) Backeb. (Cactaceae): anatomy beyond functional limitation

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Summary: The persistent leaves of *Austrocylindropuntia subulata*, which are the largest in opuntias, are terete, though they are not strictly radial symmetric in their anatomy. They are all-cell succulent, but differ from the most common early deciduous, terete all-cell succulent leaves of Opuntioideae in the outer palisade and the inner non-palisade chlorenchyma. The former consists of short and wide to nearly isodiametric cells arranged in mostly oblique anticlinal rows. The latter consists of tightly packed, isodiametric cells in longitudinal rows. These leaves were hypothesized earlier to be evolutionary derivatives of the persistent, flat, fleshy *Pereskopsis*-/*Quiabentia*-like leaves or of small persistent, terete, storage succulent, *Maibuenia*-like leaves or of small, early deciduous, terete, all-cell succulent leaves typical of most opuntias, respectively. Any of these hypotheses is hardly compatible with molecular phylogeny of Opuntioideae or with anatomical specifics of the leaves or with mainstream leaf evolution in the family Cactaceae including subfamily Opuntioideae. The leaf chlorenchyma of *A. subulata* is suitable for both water storing (all-cell succulence) and crassulacean acid metabolism (CAM). It is anatomically intermediate between the typical chlorenchyma of C₃-plant leaves and typical chlorenchyma of CAM-plant leaves. Reversible C₃-CAM switching of photosynthesis might actually take place in the leaves of this species. But if so, the chlorenchyma thickness in the leaves of *A. subulata* highly exceeds the revealed limitations of the chlorenchyma thickness in both C₃ and CAM all-cell succulent leaves.

Keywords: functional plant anatomy, terete leaf, all-cell succulent leaf, palisade chlorenchyma, non-palisade chlorenchyma, C₃ chlorenchyma, CAM chlorenchyma, *Austrocylindropuntia subulata*

Opuntioideae have the most diverse foliage leaves among Cactaceae (GRIFFITH 2009). However, anatomy of these leaves is still the least explored and it is usually completely ignored in characteristics of the subfamily (BOKE 1980; BARTHLOTT & HUNT 1993; WALLACE & GIBSON 2002; GRIFFITH 2004; MAUSETH 2005; GRIFFITH & PORTER 2009; HERNÁNDEZ-HERNÁNDEZ et al. 2011). Even the extremely reduced foliage leaves of Cactoideae turned out to be studied anatomically better than the conspicuous leaves of Opuntioideae (BOKE 1952; MAUSETH 2007).

The reason might be that the persistent, fleshy, flat leaves of *Pereskopsis* and *Quiabentia* are interpreted as only a narrowed variant of the persistent, fleshy, flat leaves of *Pereskia* (BAILEY 1960, 1968; BARTHLOTT & HUNT 1993); the anatomy of the latter having been repeatedly described (MAUSETH & LANDRUM 1997; TERRAZAS SALGADO & MAUSETH 2002; FARAGO et al. 2004; MAUSETH 2006, 2007; OGBURN & EDWARDS 2009). More typical terete leaves of Opuntioideae in their turn have generally been considered as vestigial structures on the way of vanishing (BARTHLOTT & HUNT 1993; WALLACE & GIBSON 2002; EDWARDS & DONOGHUE 2006) which are functionally insignificant (NOBEL & BOBICH 2002; MAUSETH 2006). Therefore, they are thought to have no traits that would be systematically or diagnostically important (e.g. not even



Figure 1. *Austrocyllindropuntia subulata* in the greenhouse of Tsitsin Main Botanical Garden, RAS.

mentioned in CONDE 1975; LABRA et al. 2003; RITZ et al. 2012; etc.) or to demonstrate some functional specialization of a foliage leaf.

However, the terete leaves of species of Opuntioideae are assumed to be photosynthetic active (LÜTTGE 2008). The succulent leaves of *Austrocyllindropuntia subulata* (Muehl.) Backeb. (Fig. 1) are the largest in opuntias (BRITTON & ROSE 1919; BOKE 1980), up to 12 cm long and 0.6 cm in diameter. They contribute up to 88% of net CO₂ uptake of the plant (NOBEL & HARTSOCK 1986), which is only slightly lower than the persistent, fleshy, flat leaves of leafy *Pereskia* and *Quiabentia* (MARTIN & WALLACE 2000; NOBEL & BOBICH 2002).

These persistent succulent leaves of *A. subulata* are usually considered terete (NOBEL & HARTSOCK 1986; BARTHLOTT & HUNT 1993; BERCU 2005; MAUSETH 2006), but BAILEY (1968) disputed this because these leaves differed from the genuine terete leaves of opuntias in their flattened shape, palmate-derived venation pattern and mesophyll structure (BAILEY 1968). Genuine terete leaves have either homogeneous chlorenchyma of isodiametric cells throughout the mesophyll (BOKE 1944; FREEMAN 1970; HAMILTON 1970) or continuous subepidermal 'jacket' palisade chlorenchyma (BAILEY 1968). The subepidermal 'jacket' palisade chlorenchyma is also revealed in the leaves of *A. subulata*, but it is abaxially discontinuous (BAILEY 1968; BERCU 2005).

The inner ground tissue of the leaf of *A. subulata* was differently interpreted. BAILEY (1968) considered these leaves transitional from the persistent, fleshy, flat leaves of *Pereskia* to the early deciduous, succulent, genuine terete leaves of most opuntias. Neither the former nor the latter ones have a specialized hydrenchyma (BOKE 1944; BAILEY 1968; FREEMAN 1970; HAMILTON 1970; MAUSETH & LANDRUM 1997; TERRAZAS SALGADO & MAUSETH 2002; FARAGO et al. 2004; MAUSETH 2006, 2007; OGBURN & EDWARDS 2009), then, the inner mesophyll tissue of the leaves of *A. subulata* should logically be equaled to the spongy chlorenchyma. However, the inner mesophyll tissue of these leaves was attributed to the water-storage parenchyma by BERCU (2004), but presented data were inconclusive. Thus, the leaves of this species cannot confidently be attributed to either of two types of succulent leaves of IHLENFELDT (1985).

The presented work is aimed to clarify and detail the information on the anatomical structure of chlorenchyma in the leaves of *A. subulata*.

Materials and methods

The definitive leaves were taken from the living plants grown in stock greenhouse of Tsitsin Main Botanical Garden of Russian Academy of Sciences, Moscow. Some unprocessed leaves were razor-cut for detecting hydrenchyma. Other leaves were fixed with 70% ethyl alcohol. The voucher specimen was deposited at the Herbarium of Tsitsin Main Botanical Garden of Russian Academy of Sciences [MHA], No. Ozerova 20-1 [MHA].

For light microscopy, the fixed material was dehydrated in rising alcohol series, then impregnated with xylol and paraffinized for microtome sectioning according to PROSINA (1960). Longitudinal and transverse 10 µm thick sections were deparaffinized, rehydrated and successively stained with carbol fuchsin and Delafield's haematoxylin according to BARYKINA et al. (2004), then they were dehydrated again and embedded in Canada Balm. The preparations were analyzed and photographed under microscope Nikon H550L, equipped with the digital camera DS-Vi1.

For scanning electron microscopy, the fixed leaves were razor-cut either longitudinally or transversely, kept in HCl for 15 minutes to dissolve slimes and then rinsed in water. The rinsed sections were dehydrated in rising alcohol and acetone series and critical-point dried using the Hitachi HCP-2 Critical Point Dryer, mounted on stabs, coated with Au and Pd using Eiko IB-3 Ion-coater and observed and photographed under CamScan 4DV at Laboratory of Electron Microscopy, Faculty of Biology, Lomonosov Moscow State University.

Results

The leaves are roundish in cross section (Fig. 2). The chlorenchyma fills the inner of the leaf; no hydrenchyma was detected in the available material. The mucilaginous idioblasts are scattered in this chlorenchyma, some of them are giant. The vasculature consists of 1 or 2 midvein bundle(s) in the leaf median plane accompanied by two sets of variable number of lateral veins which anastomose with each other and midvein ones. If paired, the midvein bundles also anastomose. The midvein bundles are the deepest, but adaxially eccentric. The lateral veins are arranged about half the length of the leaf radius, in the shape of abaxially opened crescent in cross section.

The chlorenchyma cells are very similar in cross section and vary from isodiametric to elongated (Figs 3A; 4A; 5A–C). They seem to be tightly packed. Two masses of the chlorenchyma are recognizable, and they are indistinctly delimited in cross sections (Fig. 3A), but clearly

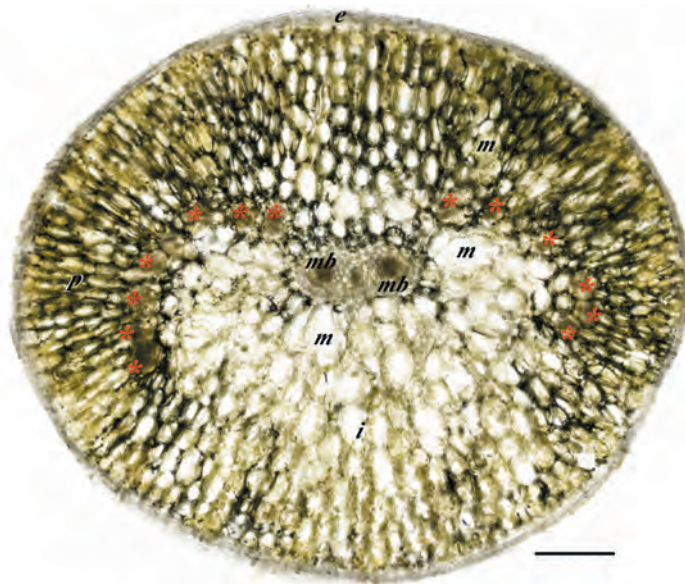


Figure 2. *Austrocylindropuntia subulata*. Leaf cross section, light microscopy. *e* – epidermis; *i* – non-palisade chlorenchyma; *m* – mucilaginous cell; *mb* – midvein bundle; *p* – palisade chlorenchyma; *red asterisks* – lateral vein bundles. Scale bar = 0.5 mm.

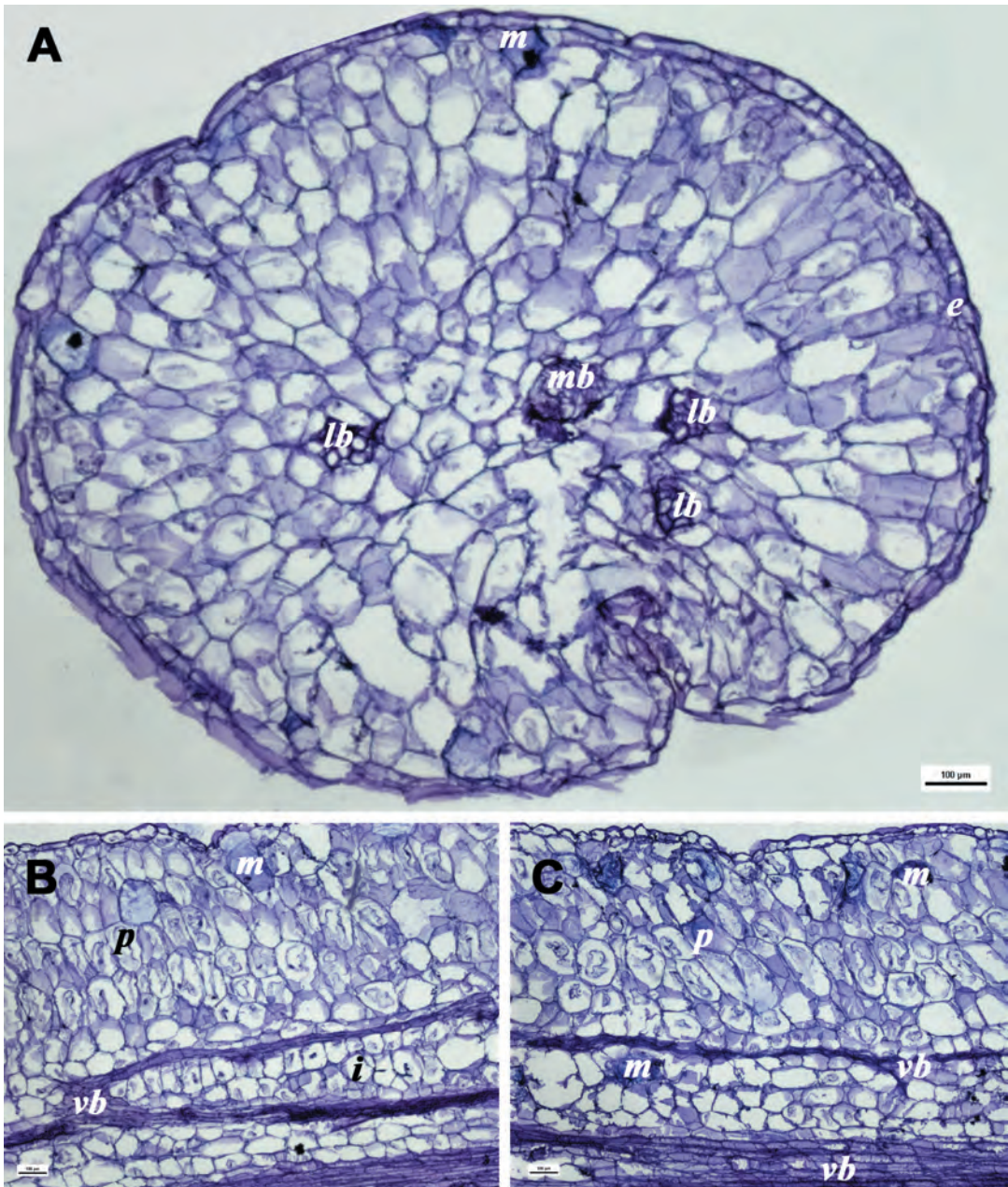


Figure 3. *Austrocyllindropuntia subulata*. Anatomy of the leaf; light microscopy. A – cross section; B, C – longitudinal sections. *e* – epidermis; *i* – non-palisade chlorenchyma; *lb* – lateral vein bundle; *m* – mucilaginous cell; *mb* – midvein bundle; *p* – palisade chlorenchyma; *vb* – vascular bundle. Scale bars = 100 μ m.

distinguishable in longitudinal sections (Figs 3B, C; 4B). The lateral veins are at the border between the inner and the outer chlorenchyma; the midvein bundle(s) are in the inner one and do not contact with the outer chlorenchyma (Fig. 2).

The outer chlorenchyma adjoins the epidermis, but does not reach the vascular bundles (Figs 3B, C; 4B; 5A). It is abaxially discontinuous in the basal part of the leaf and continuous in its distal part (Figs 2; 4A; 5B). This chlorenchyma mostly consists of elongated (1:2–1:2.5)

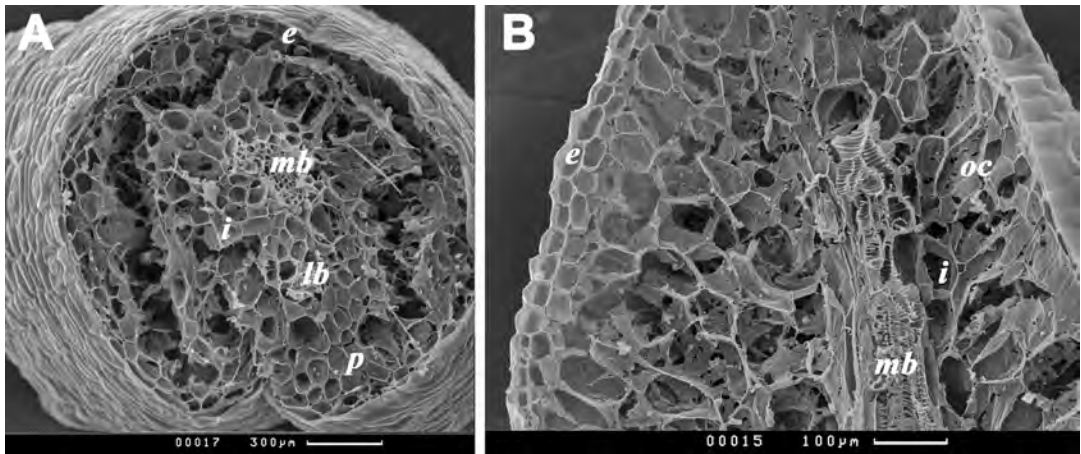


Figure 4. *Austrocylindropuntia subulata*. Anatomy of the leaf; SEM microscopy. A – cross section; B – longitudinal section. *c* – chlorenchyma; *e* – epidermis; *i* – non-palisade chlorenchyma; *lb* – lateral vein bundle; *mb* – midvein bundle; *oc* – outer chlorenchyma; *p* – palisade chlorenchyma.

cells, though the isodiametric ones are also present (Fig. 3A). The chlorenchyma cells are arranged in 3–5(6)-membered rows. These cell rows are anticlinal (radial) in cross sections (Fig. 3A) and from anticlinal (Fig. 3B) to oblique-anticlinal, tilted towards the leaf tip in the longitudinal sections (Fig. 3C). Relative arrangement of the cells in neighboring rows looks irregular in cross section (Fig. 5C), but the longitudinal section shows a distinct layered arrangement of the cells of neighboring rows (Fig. 5D).

The inner chlorenchyma mostly consists of larger isodiametric cells, though the elongated ones are also visible therein (Figs 3A; 5B). They are arranged irregularly in cross section and in clear longitudinal rows in longitudinal section. The vascular bundles are imbedded in this chlorenchyma.

Discussion

Thick succulent leaves of *A. subulata* are outwardly very similar with the ordinary terete leaves, although they are somewhat flattened in the basal part. Some traits of the dorsoventral structure are also discernible in their anatomy, especially in their vasculature. Based on these traits, BAILEY (1968) did not recognize these leaves as ‘truly terete’. However, the terete leaves are not anatomically uniform (NAPP-ZINN 1973). They can be classified into at least centric and concentric anatomical types (TIMONIN & OZEROVA 1994). The radial symmetry is most expressed only in the terete, unifacial leaves of concentric anatomical type (OZEROVA & TIMONIN 2009: Fig. 2A). The reduced adaxial side gives traits of dorsoventrality and bilaterality to the terete, subunifacial leaves of the concentric (semiconcentric according to OZEROVA & TIMONIN 1993) type (OZEROVA & TIMONIN 2009: Fig. 2B). A (very) massive midvein bundle also gives traits of dorsoventrality and bilaterality to the terete leaves of both the concentric (GIBSON 1977: Figs 6, 7) and centric types (DOOSTMOHAMMADI et al. 2020: Fig. 23). The leaves of *A. subulata* do not fully fit any anatomical types described so far. They should probably be attributed to some new type or variant of leaf anatomy. In any case, we do not see sufficient reasons to exclude the leaves under consideration from terete leaves.

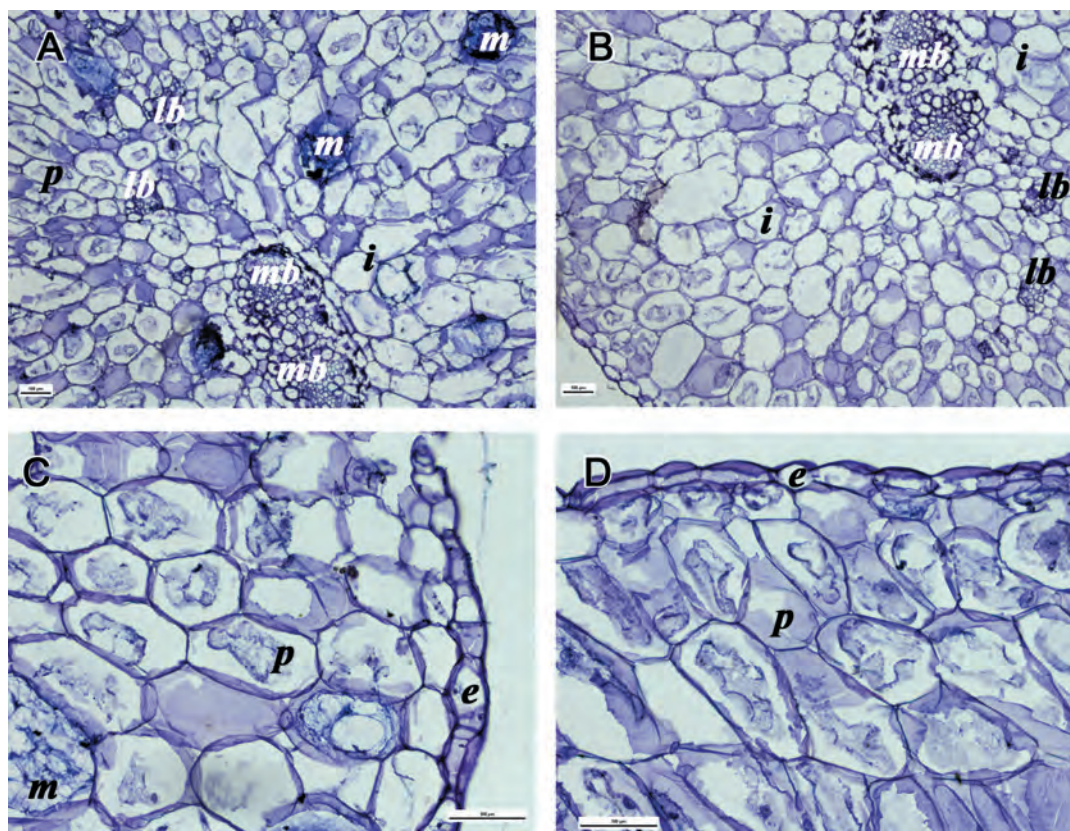


Figure 5. *Austrocyllindropuntia subulata*. Anatomy of the leaf, details; light microscopy. A – central and adaxial part, cross section; B – central and abaxial part, cross section; C – palisade chlorenchyma, cross section; D – palisade chlorenchyma, longitudinal section. *e* – epidermis; *i* – non-palisade chlorenchyma; *lb* – lateral vein bundle; *m* – mucilaginous cell; *mb* – midvein bundle; *p* – palisade chlorenchyma. Scale bars = 100 μ m.

Typical all-cell succulent leaves have a homogeneous chlorenchyma (IHLENFELDT 1985). The terete leaves of *A. subulata* are all-cell succulent, but their chlorenchyma is two-typed. The outer chlorenchyma is worth being attributed to the palisade chlorenchyma. The typical layering of the constituting cells is detectable only in longitudinal sections of leaves, whereas the cells are in radial rows but not in layers in cross sections (Figs 3–5). This peculiar arrangement of chlorenchyma cells is certainly caused by the characteristic genesis of the terete leaves in Opuntioideae (BOKE 1944; MAUSETH 2007). These leaves do not develop the (sub)marginal meristem which determines the layering of developing lamina and its palisade chlorenchyma in typical flat leaves (NAPP-ZINN 1973). Instead, the developing terete leaves have a peripheral jacket meristem, whose cells divide mostly periclinally to produce distinct radial rows of their derivative cells of the mesophyll (BOKE 1944; MAUSETH 2007). Extended intercalary growing of the leaf (BOKE 1944; MAUSETH 2007) results in layers of the outer chlorenchyma cells clearly visible in the leaf longitudinal sections.

This growing also causes distinct longitudinal rows of the inner chlorenchyma cells. The latter contrasts with the typical spongy chlorenchyma as usually described (BOLHÀR-NORDENKAMPF & DRAXLER 1993) in its tightly packed isodiametric cells. Therefore, the tissue in question can hardly be termed spongy chlorenchyma. That is why we simply name it non-palisade chlorenchyma.

The principal evolutionary trend of vegetative organs of cacti was outlined as a conversion from leafy, woody, *Pereskia*-like plants to stem succulents with small, early deciduous, terete leaves in most Opuntioideae and to 'leafless' stem succulents of the most advanced and diverse Cactoideae (TROLL 1935; DRAKE 1942; MAUSETH 1995, 2007; EDWARDS & DONOGHUE 2006; HERNÁNDEZ-HERNÁNDEZ et al. 2011). Within the framework of this outline, the persistent, large, terete leaves of *A. subulata* were pertinently considered transitional from the flat leaves of *Pereskia*- or *Quiabentia*-like ancestral Opuntioideae to the early deciduous, small, terete leaves of higher opuntias (BAILEY 1968). Then, the presence of palisade and non-palisade ('spongy') chlorenchymas in the leaves of *A. subulata* can reasonably be recognized as only a remnant of the dorsoventral anatomy of the ancestral flat leaves.

However, molecular phylogeny data indicate that the large, persistent, flat, fleshy leaves of *Pereskia* could rather have evolved from highly reduced leaves and that the ancestral cacti have been similar to the modern *Maihuenia* (GRIFFITH 2004 and literature therein), which has small, persistent, terete, succulent leaves (BRITTON & ROSE 1919). The molecular phylogeny also indicates *A. subulata* to be either a deepest (MARTIN & WALLACE 2000; GRIFFITH 2004; BÁRCENAS et al. 2011) or the most advanced (GRIFFITH & PORTER 2009; RITZ et al. 2012) lineage in the subfamily Opuntioideae. Anyway, the large, persistent, flat, fleshy, *Pereskia*-like leaves cannot be a source of *A. subulata* leaves. The latter must have derived either from small, persistent, succulent, terete, *Maihuenia*-like leaves or from the small, early deciduous, succulent, terete, *Maihueniopsis*- or *Pterocactus*-like leaves (GRIFFITH 2009).

The leaves of *Maihuenia* are storage-succulent and have peripheral palisade chlorenchyma jacketing the centralized mass of water-storage tissue of large chlorophyll-free cells, either highly vacuolated in *M. poeppigii* (Otto et Pfeif.) F.A.C. Weber ex K. Schum. or mucilaginous in *M. patagonica* (Phil.) Britton et Rose (GIBSON 1977). The stems of these plants early develop periderm nearly throughout (except for small patches of remaining epidermis) (MAUSETH 1995), which prevent them from the efficient photosynthesis (NOBEL & HARTSOCK 1986; MAUSETH 1995; OGBURN & EDWARDS 2009). The stems of *Pereskia* are also incapable of efficient photosynthesis, if any (NOBEL & HARTSOCK 1986; SAJEVA & MAUSETH 1991; EDWARDS et al. 2005). The assumable evolution of ancestral, small, terete, storage-succulent, *Maihuenia*-like leaves to the large, flat, fleshy ones without hydrenchyma is therefore easily interpretable as evolutionary enhancing of net photosynthesis of a plant in more humid habitats. Similar interpretation is hardly applicable to the evolution of *A. subulata* leaves. As other opuntias, this species is characterized by the palisade cortex (NOBEL 1988) and delayed periderm formation (MAUSETH 2005; EDWARDS et al. 2005) to make the stem a noticeable contributor to the net photosynthesis of a plant (NOBEL & HARTSOCK 1986). Due to such a stem structure, evolutionary enhancing of the stem photosynthesis accompanied by the loss of leaves would be more expected in *A. subulata* lineage just as in Cactaceae in general than the evolutionary progressing of the leaves.

The evolution of large, persistent, all-cell succulent, terete leaves of *A. subulata* from the small, storage-succulent, terete leaves of ancestral Opuntioideae must additionally have been accompanied by substitution of the hydrenchyma by the non-palisade chlorenchyma and change of initial narrow columnar palisade cells into those ranging from short thick to nearly isodiametric in the species under consideration. The palisade chlorenchyma of tightly packed, narrow columnar cells is more specialized for light uptake (MEYER 1962; BOLHÀR-NORDENKAMPF & DRAXLER 1993).

Short thick palisade cells are adapted to function under reduced insolation (MEYER 1962; NAPP-ZINN 1984) or to store water (GIBSON 1996). Such cells are certainly specialized to store water in *A. subulata* leaves, but they unlikely store water more efficient than the specialized hydrenchyma cells in ancestral *Maihuenia*-like leaves.

The evolutionary origin of the large, persistent, succulent, terete leaves of *A. subulata* (if it were so), would not only be opposite to the mainstream evolution of Cactaceae, but it would have resulted in substitutions of the more efficient photosynthetic palisade chlorenchyma and hydrenchyma by the less efficient tissues. We believe such an evolution to be very unlikely.

The absence of hydrenchyma in the leaves of *A. subulata* seems quite natural, if we assume that these leaves have originated from the small, early deciduous, terete, all-cell succulent leaves typical of opuntias (GRIFFITH 2009). This assumption also enables the origin of palisade chlorenchyma to be easily interpreted as evolutionary enhancing of photosynthesis of the progressing leaves. However, the evolutionary progression of the leaves in *A. subulata* lineage still retains unreasoned contrary to the mainstream evolution of Cactaceae to the 'leafless' succulence.

The thickness of all-cell succulent leaves is strictly limited by the high diffusion resistance of the intercellular airspaces (IHLENFELDT 1985; GIBSON 1996; MALES 2017; NIECHAYEV et al. 2019). Evolutionary increase in leaf thickness can be effectuated by either transformation of the all-cell succulent leaf into storage succulent one or by substitution of C_3 photosynthesis by CAM photosynthesis (RIPLEY et al. 2013). The thick, terete leaves of *A. subulata* are reported to perform C_3 photosynthesis (NOBEL & HARTSOCK 1986; LÜTTGE 2008) and 'only very weak CAM' (LÜTTGE 2008). Their mesophyll differs from its counterpart in typical C_3 plants in very short and thick palisade cells and tightly packed isodiametric cells of non-palisade chlorenchyma both manifesting a characteristic feature set of all-cell succulence (GIBSON 1996). It is noteworthy that a very similar feature set is characteristic of the CAM chlorenchyma cells (GIBSON 1996; NIECHAYEV et al. 2019) and that the succulent specialization of the chlorenchyma can be a precondition for CAM evolving (HEYDUK et al. 2016). Quite a few CAM plants are capable of reversible CAM to C_3 switching in accordance to the hydration state of their tissues (SAGE 2008; WINTER et al. 2019). The C_3 to CAM switching was also revealed in opuntia stems in relation to their hydration state (LÜTTGE 2008) and age (NOBEL & HARTSOCK 1986). It is tempting to assume that the C_3 -CAM intermediate structure of the chlorenchyma in thick, terete leaves of *A. subulata* is adopted to their reversible C_3 to CAM switching which could be hinted on by the 'only very weak CAM'. The CAM in leaves of this species could be weak, when being studied at improper physiological state or at the wrong age of leaves under experiment.

The significant facultative CAM photosynthesis in the leaves in *A. subulata* lineage could enable the latter to evolve thicker terete leaves. However, even the strict CAM photosynthesis allows the chlorenchyma to increase commonly to 1 mm and sometimes up to 3 mm thickness in exceptional cases (GIBSON 1996). Yet, the leaves of *A. subulata* highly exceed both common and 'exceptional' limitations.

Conclusion

The persistent, terete, all-cell succulent leaves of *A. subulata* differ from the common early deciduous, terete, all-cell succulent leaves of opuntias in their chlorenchyma differentiated into outer palisade and inner non-palisade tissues. The leaves under consideration still seem enigmatic

as to their evolutionary origin as well as to their functioning. Their anatomy could be interpreted as a derivative of that of persistent, flat, fleshy, *Pereskopsis*-/*Quiabentia*-like leaves or persistent, terete, storage succulent, *Maihuenia*-like leaves or early deciduous, terete, all-cell succulent ones typical of opuntias. The first interpretation fits best with leaf anatomy of *A. subulata*, but it contradicts the molecular phylogeny of Opuntioideae. The second interpretation is consistent with some molecular phylogenies of Opuntioideae, but it requires to recognize evolutionary progression of the leaf paradoxically accompanied by transformation of the efficient storage succulent mesophyll to less specialized, all-cell succulent one. The third interpretation is in best agreement with the molecular phylogeny of Opuntioideae and it is in a good agreement with the anatomical specifics of early deciduous and persistent, terete leaves, but it leaves unreasoned the evolutionary progression of the leaf in *A. subulata* lineage, which is reverse to the mainstream evolution of the leaves in Cactaceae.

The anatomy of the mesophyll of *A. subulata* is intermediate between the anatomy of typical mesophyll of a C₃ plant and mesophyll of a CAM plant, which allows to assume an actual reversible C₃–CAM switching of the photosynthesis in the leaves of this species. If even so, the leaves of *A. subulata* are too thick to be all-cell succulent. They are certainly worth being explored physiologically to solve the problems of their origin, evolution and counter-limitation functioning.

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