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Cacti defeat physics: columnar chlorenchyma in tubercles of three species of *Mammillaria* Haw. (Cactaceae)

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Summary: The tubercles of three investigated *Mammillaria* spp. are specialized photosynthetic organs and not the formations to facilitate the hydration-dependent changes of volume of the plant body. There is no hydrenchyma in the tubercles. The tubercles are all-cell succulent and strikingly similar anatomically with the persistent all-cell succulent terete leaves of *Austrocylindropuntia subulata*. The so-called 'palisade cortex' does not consist of the palisade chlorenchyma. Instead, there is a particular columnar chlorenchyma therein, whose small cells are shortest cylindrical to isodiametric and arranged in oblique anticlinal rows. The columnar chlorenchyma combines advantages of the typical palisade (C_3) chlorenchyma and those of typical CAM chlorenchyma which look like absolutely incompatible. It quite fits the traits of CAM chlorenchyma at the cell-organization level and approaches the traits of genuine palisade chlorenchyma at the tissue-organization level. Accordingly, the columnar chlorenchyma is superior to the palisade (C_3) chlorenchyma, because its cells are well adapted to the CAM photosynthesis; it is superior to the typical CAM chlorenchyma in more chloroplast density and less gas diffuse resistance. The columnar chlorenchyma is concluded to have been a precondition of evolution of aphyllous stem-succulent life form in most Cactaceae, as significant as the delayed periderm development and more significant than emergence of the cortical bundle net.

Keywords: plant anatomy, cactus tubercle, palisade cortex, columnar chlorenchyma, palisade chlorenchyma, CAM chlorenchyma, stem succulent, *Mammillaria*

Leaf reduction as the most conspicuous trait of Cactaceae is a commonplace (Troll 1935; Barthlott & Hunt 1993; Mauseth 1995; Landrum 2002; Ogburn & Edwards 2009; Hernández-Hernández et al. 2011; etc.). Only a few of their representatives (Pereskioideae and Opuntioideae) have retained green photosynthesizing leaves visible to the naked eye (Britton & Rose 1919; Barthlott & Hunt 1993; Terrazas Salgado & Mauseth 2002; Wallace & Gibson 2002; Mauseth 2006) and even fewer species have the leaves which significantly contribute to the CO_2 uptake by a plant. These are the cacti with woody stems (all species of *Pereskia*, *Maihuenia*, *Pereskiopsis* and *Quiabentia*) and stem-succulent *Austrocylindropuntia subulata* (Muehl.) Backeb. (perhaps, also other species of this genus) (Nobel & Hartsock 1986; Martin & Wallace 2000; Nobel & Bobich 2002; Lüttge 2008). All other cacti take up CO_2 mostly (Opuntioideae) or exclusively (Cactoideae) by the peripheral chlorenchyma in their succulent stems (Nobel & Hartsock 1986; Nobel & Bobich 2002; Lüttge 2008).

This stem chlorenchyma is nearly invariably referred to as the 'palisade cortex' (Darbishire 1904; Ganong 1895; Boke 1980; Sajeva & Mauseth 1991; Mauseth & Sajeva 1992; Mauseth 1995, 2005, 2006; Terrazas Salgado & Mauseth 2002; Terrazas & Arias 2003; Soffiatti & Angyalossy 2003, 2007; Pereira de Arruda & Melo-de-Pinna 2015) ('palisade-like cortex' in Nyffeler & Eggli 1997) similar with the palisade chlorenchyma in dorsiventral leaves of *Pereskia* species (Sajeva & Mauseth 1991; Terrazas Salgado & Mauseth 2002), but constituted by multi-celled columns (Darbishire 1904; Nobel 1988; Terrazas Salgado & Mauseth 2002;

Mauseth 2006). Only few Rhipsalideae were shown to have a cortical chlorenchyma of staggered isodiametric cells (Calvente et al. 2008; Torres-Boeger et al. 2010) and Schumann (1897) was mistakenly attributed to by Preston (1901) that he considered chlorenchyma cells in cactus cortex isodiametric.

However, all stem-succulent Cactaceae are known to be constitutive 'strong' (*sensu* Nelson & Sage 2008) CAM plants (Nobel & Hartsock 1986; Sajeva & Mauseth 1991; Terrazas Salgado & Mauseth 2002; Nobel & Bobich 2002; Lüttge 2008; Ogburn & Edwards 2009; Niechayev et al. 2019) combined with CAM–C_3 switching at higher humidity in Opuntioideae and C_3 photosynthesis in their leaves and young cladodes or juvenile plants (Nobel & Hartsock 1986; Nobel & Bobich 2002; Griffith 2008; Lüttge 2008).

The CAM photosynthesis demands isodiametric chlorenchyma cells, not the palisade ones (Sage 2002; Nelson et al. 2005; Niechayev et al. 2019). Thus, the 'palisade cortex' attributed to the stem succulent Cactaceae seems completely incompatible with CAM photosynthesis revealed in their stems (Sage 2002; Nelson et al. 2005; Nelson & Sage 2008; etc.). Therefore, the chlorenchyma in cactus stems is worth being re-investigated anatomically and physiologically.

We have chosen species of *Mammillaria* as the type genus of Cactaceae and Cactoideae for our anatomical investigation. All species of this genus have tuberculate stems. The tubercles seem to be an ancestral trait of Cactoideae, but they are considered evolutionary derived formations in Mammillarieae (Higgins 1936; Buxbaum 1958; Butterworth et al. 2002). The tubercles increase the photosynthetic area and potential net photosynthesis of a plant (Gibson 1996). They are rather long and resemble the terete leaf in some *Mammillaria* species. Such leaf-like tubercles are certainly specialized photosynthetic organs of these plants. We expected that the chlorenchyma of *Mammillaria*'s tubercles would show best the anatomical function-based specificity of this tissue.

Tubercles of *Mammillaria* spp. are attributed either to firm-textured or to soft-textured (flaccid) ones (Hunt 1981; Butterworth & Wallace 2004). Both types are present in investigated species. *M. vetula* ssp. *gracilis* (Pfeiff.) D.R. Hunt has the smallest firm-textured tubercles up to 6 mm long and 3 mm in diameter (Fig. 1A). *M. decipiens* ssp. *albescens* (Tiegel) D.R. Hunt has medium-sized soft-textured tubercles up to 20 mm in length and 3 mm in diameter which look like short terete leaves (Fig. 1B). *M. longimamma* DC. has the largest soft-textured tubercles up to 25 mm long and 8 mm in diameter, very similar with the terete leaves (Fig. 1C).

Materials and methods

Material was taken from living plants grown in stock greenhouse of Tsitsin Main Botanical Garden of Russian Academy of Sciences, Moscow. The plants were fixed with 70% ethyl alcohol according to Prosina (1960). The voucher specimens are deposited at the Herbarium of Tsitsin Main Botanical Garden of Russian Academy of Sciences [MHA], Nos. Ozerova 20-2 [MHA] (*M. vetula* ssp. *gracilis*), Ozerova 20-3 [MHA] (*M. decipiens* ssp. *albescens*) and Ozerova 20-4 [MHA] (*M. longimamma*), respectively.

The tubercles were detached and dehydrated in rising alcohol series. Some specimens were paraffinized through xylol for microtome sectioning in accordance to Prosina's (1960) manual. 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 the microscope Nikon H550L, equipped with the digital camera DS-Vi1. Other specimens were hand-razored either longitudinally or transversely, kept in HCl for 15 minutes to dissolve slimes and 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.

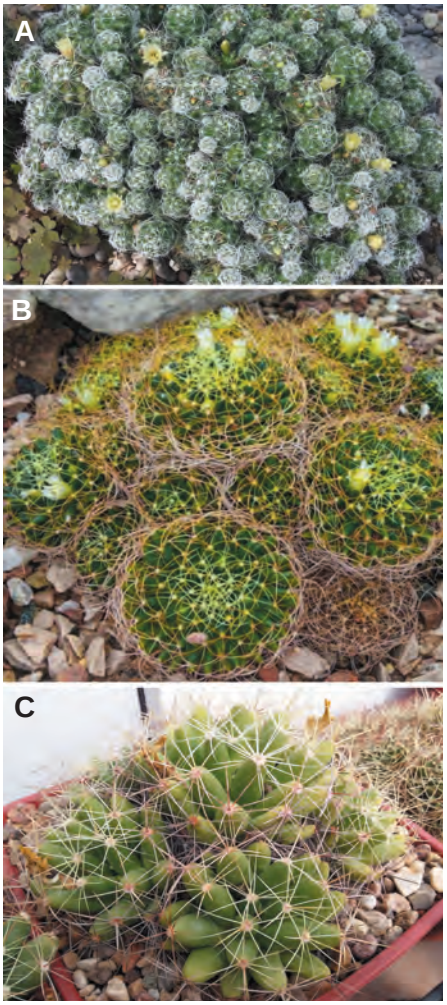

Figure 1. Investigated cacti. A – *Mammillaria vetula* ssp. *gracilis*; B – *M. decipiens* ssp. *albescens*; C – *M. longimamma*.

Results

Tubercles of all investigated species differ only quantitatively in accordance with their sizes despite the fact that they belong to different types.

The tubercles are round in cross section (Figs 2A; 3A). Their vasculature consists of larger eustele bundles with wide primary rays in between and of vascular plexus of smaller cortical bundles in the inner third of cortex. The chlorenchyma of uniform isodiametric cells fills the tubercle around the vascular bundles.

The longitudinal section of the tubercle (Figs 2B; 3B) shows that there are really two chlorenchyma masses therein which clearly differ in cell shape and arrangement. The cells of the outer chlorenchyma are isodiametric (Fig. 2A) in cross sections and isodiametric to slightly elongated in the longitudinal sections (Fig. 2B) and arranged in 4–5-membered ascending oblique anticlinal rows. The cells of the inner chlorenchyma are clearly elongated (1:2–1:3) and arranged in distinct longitudinal rows (Figs 2B; 3B).

Discussion

Mauseth (2006) described the outer palisade cortex jacketing the hydrenchyma of inner cortex and stele in cactus tubercles just as in their succulent stems. All three investigated species of *Mammillaria* do not have hydrenchyma, but the chlorenchyma throughout the cortex and stele of their tubercles. This indirectly confirms that the tubercles of *Mammillaria* are specialized photosynthetic organs (Ganong 1895) and not the organs for safe swelling of the plant body during wet seasons as they were interpreted by Mauseth (l.c.).

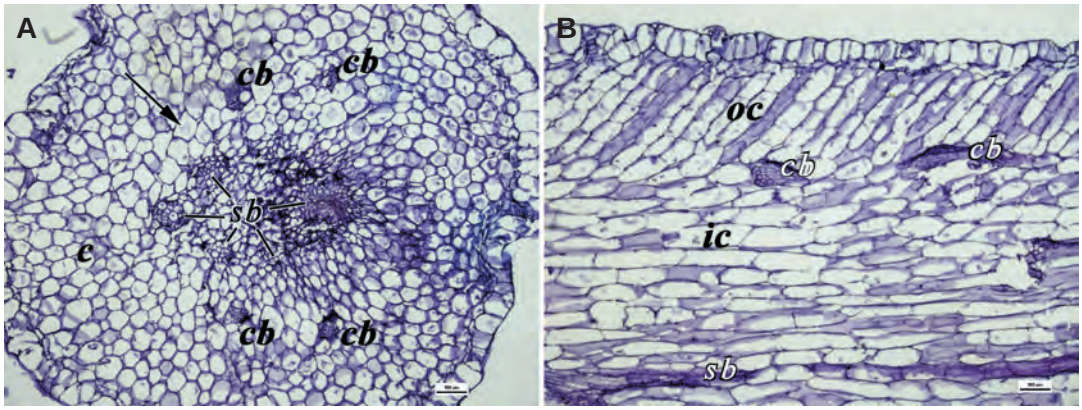

Figure 2. Tubercle of *M. vetula* ssp. *gracilis*, light microscopy. A – cross section; B – longitudinal section. *c* – chlorenchyma; *cb* – cortical bundle; *ic* – inner chlorenchyma; *oc* – outer chlorenchyma; *sb* – stele bundle; arrow – approximate border between the outer and inner chlorenchyma. Scale bars = 100 µm.

We have revealed that not only *M. elongata* DC. (Darbishire 1904) but other species of this genus have peripheral cortical chlorenchyma of nearly isodiametric cells arranged in multi-membered, oblique, anticlinal rows. However, these cell rows are only discernible in the longitudinal sections of the tubercle, whereas total chlorenchyma appears homogeneous of staggered isodiametric cells in the cross sections. Quite the same chlorenchyma was attributed to the stems of Rhipsalideae (Calvente et al. 2008; Torres-Boeger et al. 2010). These plants might also have outer chlorenchyma cells in rows, but the latter were not identified, since the researchers limited themselves to studying the chlorenchyma only in cross sections. The true structure of the cortical chlorenchyma in cactus stems can certainly be understood only by studying it in transverse and longitudinal sections combined. The (outer) cortical chlorenchyma of cells in more or less anticlinal rows is most likely inherent in all succulent stems of cacti as it is usually attributed to them (Ganong 1895; Boke 1980; Sajeva & Mauseth 1991; Mauseth 2006; Terrazas Salgado & Mauseth 2002; Terrazas & Arias 2003; etc.).

Such a peripheral cortical parenchyma is traditionally referred to as palisade cortex (Ganong 1895; Boke 1980; Sajeva & Mauseth 1991; Mauseth & Sajeva 1992; Mauseth 1995, 2005, 2006; Terrazas Salgado & Mauseth 2002; Terrazas & Arias 2003; etc.). Indeed, more or less anticlinal rows of chlorenchyma cells give this tissue some resemblance to palisade chlorenchyma. The latter one typically consists of narrow rod-like cells (Meyer 1962; Napp-Zinn 1973). The cell shapes of the palisade cortex are usually neglected, but they were reported to be variable (Preston 1901). There are very few published images of these cells. Only single species have chlorenchyma cells which would be compared to the very short and wide palisade cells (Soffiatti & Angyalossy 2007: Fig. 8; Pereira de Arruda & Melo-de-Pinna 2015: Fig. 4A, D). However, the isodiametric chlorenchyma cells are visible in Nyffeler & Eggli (1997: Figs 4–6), Soffiatti & Angyalossy (2003: Fig. 7) and Pereira de Arruda & Melo-de-Pinna (2015: Fig. 4C). The cells of palisade cortex of the tubercles in *Mammillaria* species are also nearly isodiametric (see also Darbishire 1904: Figs 15, 20).

The term 'palisade' is hardly applicable to such cells and tissue they constitute. At the same time, it seems objectionable to discriminate two types of peripheral cortex chlorenchyma in cacti. The shortest and widest cells of palisade cortex are much more similar with the isodiametric

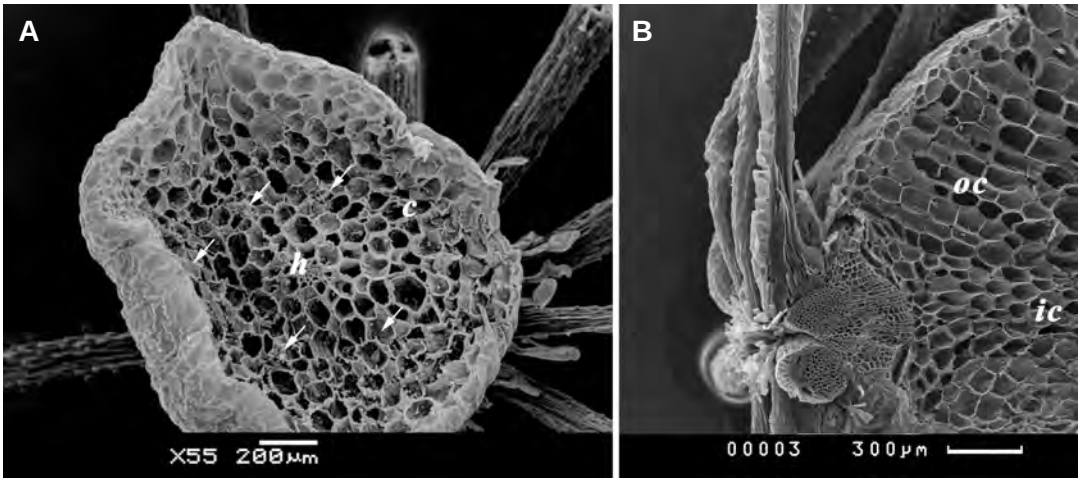

Figure 3. Tubercle of *M. vetula* ssp. *gracilis*, SEM microscopy. A – cross section; B – longitudinal section. *c* – chlorenchyma; *ic* – inner chlorenchyma; *h* – hydrocytic body which terminates all stele bundles; *oc* – outer chlorenchyma; arrows – cortical bundles/hydrocytic continuation of cortical bundle.

chlorenchyma cells than with the typical palisade cells. The arrangement of chlorenchyma cells in long, multi-membered (oblique), anticlinal rows drastically differs from the arrangement of cells in any other type of chlorenchyma. Such an arrangement of the constituting cells is surely the most striking character of the outer cortical chlorenchyma in succulent stems of cacti. The cell rows were once termed 'columns' by Mauseth (1995) and Mauseth et al. (1998). So we think this tissue would be the best termed '*columnar chlorenchyma*'.

The thickness of the chlorenchyma in succulent organs is limited by the gas conductivity of its intercellular air spaces (Ripley et al. 2013). The columnar chlorenchyma has nearly straight intercellular air spaces between the oblique anticlinal rows of its cells. The diffusion resistance of straight intercellular air spaces to gases is reduced, so the inter-row intercellular air spaces of this chlorenchyma permit intensive supply of gaseous CO_2 deep into the tissue (Mauseth 2004a). The columnar chlorenchyma in tubercles of *Mammillaria* species in all likelihood has enabled the substitution of the cortical and stele hydrenchyma by the inner chlorenchyma. Such a hydrenchyma-to-chlorenchyma substitution shows that the *Mammillaria*'s tubercles are specialized in photosynthesis, not in facilitation of the plant body's swelling/contraction in accordance with its fluctuating water content as they were interpreted by Mauseth (2006).

The columnar chlorenchyma uniquely combines traits of palisade C_3 chlorenchyma and typical CAM chlorenchyma which seem to be mutually exclusive.

Typical palisade chlorenchyma consists of narrow rod-like cells whose 90–95 % cell wall area is exposed into the intercellular air space (Gibson 1996). Numerous chloroplasts are located next to these exposed cell walls to minimize the path which CO_2 has to run through the highly resistant aqueous medium from the intercellular air space to the chloroplasts (Bolhàr-Nordenkampf & Draxler 1993; Gibson 1996). The palisade chlorenchyma thus has maximal specific chloroplast number per unit volume of the tissue (Meyer 1962; Napp-Zinn 1973). As the diffusion resistance of straight intercellular air spaces along the palisade cells is reduced (Parkhurst 1994), it limits the CO_2 uptake if only the intercellular air spaces exceed 300 µm length (Gibson l.c.). Accordingly, the palisade chlorenchyma can relevantly be considered the most efficient

photosynthetic tissue. However, this chlorenchyma is characterized by low water-use efficiency and has insufficient vacuole volume per cell to accumulate bulky malate. Therefore, the palisade chlorenchyma is able to maintain only C_3 photosynthesis and it is inefficient for succulent plants.

Every CAM plant temporally accumulates dissolved malate in the vacuoles of photosynthesizing cells to use it during daylight for the synthesis of hexoses in the Calvin cycle (von Caemmerer & Quick 2000). Regular accumulation of large amount of the dissolved malate in cell vacuoles needs the chlorenchyma of rather large, uniform, regular polyhedral (= 'isodiametric') cells which tend to be tightly packed to gain the bulkiest specific volume of vacuoles per unit of CAM chlorenchyma volume (Ting 1985; Gibson 1996; Sage 2002; Nelson et al. 2005; Nelson & Sage 2008; Bräutigam et al. 2017; Griffith & Males 2017; Niechayev et al. 2019; Grace 2019). The intercellular air spaces are very narrow and the specific exposed cell surface is accordingly small in the typical CAM chlorenchyma because of its tightly packed cells (Sage 2002; Nelson et al. 2005; Nelson & Sage 2008; Earles et al. 2018; Niechayev et al. 2019). The chloroplasts in CAM chlorenchyma also tend to be located next to the cell walls exposed into the intercellular air spaces (Kondo et al. 1998). Therefore, there is a little room for chloroplasts in the tissue. The specific number of chloroplasts per unit of tissue volume and photosynthesis rate are resultantly lower than those of the palisade C_3 chlorenchyma, though the CAM chlorenchyma highly exceeds the latter in water-use efficiency and water accumulation (Gibson 1996).

Besides, high tortuosity of the narrow intercellular air spaces in the CAM chlorenchyma increases the diffusion resistance for gaseous CO_2 (and O_2) (Maxwell et al. 1997; Earles et al. 2018; Niechayev et al. 2019) and limits photosynthesis rate in CAM plants (Niechayev et al. 2019). More complete CO_2 uptake by CAM chlorenchyma and low specific number of its chloroplasts somewhat weaken the limiting effect of high diffusion resistance of the tissue's intercellular air spaces (Ripley et al. 2013). Nevertheless, the CAM chlorenchyma is rarely more than 1 mm thick and it never exceeds 3 mm (Gibson 1996).

Resultantly, the typical CAM chlorenchyma fits well the photosynthesis under water deficiency, but its photosynthesis is low productive.

The isodiametric shape of columnar chlorenchyma cells maximizes specific volume of the vacuole in a cell which is necessary for CAM photosynthesis, but unfeasible in the narrow rod-like palisade cells due to stereometric characters of our space. Small cells of the columnar chlorenchyma result in increased specific number of chloroplasts per unit of tissue volume, but also decreased specific vacuole volume therein. The columnar chlorenchyma is seemingly less efficient than the typical CAM chlorenchyma in water storage but more efficient in the photosynthesis. However, it has a less specific number of chloroplasts per cell (Mauseth 2004b) and unit of tissue volume and is less efficient than the palisade C_3 chlorenchyma. The row arrangement of cells of the columnar chlorenchyma results in straightened intercellular air spaces along these cell rows. Such cell rows can achieve a length of 20 cells (Nobel 1988) and transform the straight intercellular air spaces in between into a kind of the longest air ducts which reduce the diffusion resistance of this chlorenchyma so much that its thickness highly exceeds that of palisade chlorenchyma and even that of typical CAM chlorenchyma and reach 2 cm in extreme cases (Mauseth et al. 1998).

The columnar chlorenchyma is probably efficient also in especially thick succulent leaves of C_3 plants. Thus, persistent all-cell succulent terete leaves of *Austrocylindropuntia subulata* (Muehl.) Backeb. have just similar peripheral columnar chlorenchyma constituted by the isodiametric to

the shortest cylindrical cells (Ozerova & Timonin 2020). These leaves are strikingly similar in their anatomy with the tubercles of investigated *Mammillaria* species, especially with the long flaccid leaf-like ones.

We believe that the columnar chlorenchyma, as soon as it came into being, became one of the crucial evolutionary steps to the aphyllous stem succulent habitus which is so characteristic of the vast majority of Cactaceae.

The total volume of the leaf chlorenchyma highly exceeds that of the stem chlorenchyma in typical leafy shoots. Consequently, the evolutionary loss of leaves in most Cactaceae must have inevitably reduced the total volume of chlorenchyma in a plant. Besides, the cactus leaves perform C_3 photosynthesis, whereas their stems maintain CAM (Nobel & Hartsock 1986; Lüttge 2008). The palisade C_3 chlorenchyma has the highest chloroplast density (Bolhàr-Nordenkampf & Draxler 1993) and accordingly its potential productivity seems to exceed that of its CAM counterpart. Therefore, the evolution of aphyllous stem succulent would be hardly possible without efficient compensation of photosynthesis reduction.

Any improvement of the physicochemical processes of CAM metabolism is impossible (Bräutigam et al. 2017). The increasing of specific volume of the typical CAM chlorenchyma in the stem is limited by high diffusion resistance of this tissue (Maxwell et al. 1997; Earles et al. 2018; Niechayev et al. 2019), so it is unlikely to have compensated the loss of C_3 photosynthetic leaves. Only the columnar chlorenchyma makes possible to increase significantly the chloroplast density and chlorenchyma thickness while maintaining water-use efficient CAM photosynthesis. The aphyllous stem succulents are resultantly characterized by unexpectedly high productivity of net photosynthesis (Nobel 1991; Lüttge 2008).

Conclusion

The tubercles of investigated *Mammillaria* species are specialized all-cell succulent photosynthetic organs. They do not have a genuine palisade chlorenchyma and their 'palisade cortex' is occupied by peculiar columnar chlorenchyma. This chlorenchyma consists of oblique anticlinal 4–5-membered rows of small nearly isodiametric cells.

The columnar chlorenchyma combines physically incompatible advantages of the palisade C_3 and CAM chlorenchyma. It quite fits the demands of CAM photosynthesis at the cell-organization level. It approaches the structure of C_3 palisade chlorenchyma at the tissue organization level. Its cell rows resemble the giant palisade cells and provide rather high chloroplast density and reduced gas diffusion resistance while maintaining the CAM photosynthesis. The chlorenchyma in tubercles resultantly exceeds the thickness limits of both typical palisade chlorenchyma and typical CAM chlorenchyma for more contribution to the net photosynthesis of an organ.

The evolutionary loss of leaves in cacti is proclaimed to be preconditioned by emergence of cortical bundle net and delayed development of the periderm (Sajeva & Mauseth 1991; Mauseth & Sajeva 1992; Edwards et al. 2005; Ogburn & Edwards 2009). Such a leaf loss would hardly be feasible, if the ancestral cacti had retained either palisade C_3 chlorenchyma or typical CAM chlorenchyma in their stems, because the both would seemingly have resulted in drastic reduction of plant photosynthesis. There is only the columnar chlorenchyma that could efficiently compensate loss of leaves in cactus evolution. That is why, we believe the columnar chlorenchyma to be as significant as precondition of the evolution of characteristic cactus life form

as the delayed periderm development and more significant than the emergence of the cortical bundle net. Emergence of the columnar CAM chlorenchyma in ancestral cacti could be a key innovation *sensu* Liem (1974), which enabled the cacti to become the most diversified taxon of leafless stem-succulent CAM plants.

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