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Trichomes with crystals in the *Cephalocereus* Pfeiff. areoles

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Abstract: This research describes the trichomes produced in the old vegetative areoles of the five species of *Cephalocereus* Pfeiff. Light and scanning electron microscopy and energy dispersive X-ray analysis were used to characterize the crystals present in the stem vegetative areoles far from the apical meristem. We are reporting for the first time for the Cactaceae family capitated trichomes with crystals. Crystals had diverse shapes (prismatic, styloid, sandy) and they all contain C as their principal element, as well as Na, Cl, Si, Mg, S, and Ca in different concentrations. Trichome and crystal characteristics allowed distinguishing the species of *Cephalocereus*. For example, capitate trichomes are shared by *C. columna-trajani* and *C. senilis* but differ because in *C. columna-trajani* there is one prismatic crystal per cell and sandy crystals in *C. senilis*. The other three species share non-capitated trichomes with prismatic crystals in the apical cell and styloids in the other cells. The species of *Cephalocereus* share the ontogenetic pathway of producing trichomes and spines in the areoles far from the apical meristem, suggesting that trichomes with crystals may be protecting the spines meristems.
Key words: biominerals, *Cephalocereus apicicephalium*, *Cephalocereus columna-trajani*, *Cephalocereus nizandensis*, EDAX, scanning electron microscopy.

Resumen: Esta investigación describe los tricomas de las areolas vegetativas de las cinco especies de *Cephalocereus*. La microscopía fotónica y electrónica de barrido, así como el análisis dispersivo de energía de rayos X, se utilizaron para caracterizar los cristales presentes en las areolas vegetativas de los tallos. Se reporta por primera vez para la familia Cactaceae tricomas capitados con cristales. Los cristales tuvieron diversas formas (prismas, estiloides, arena) y todos ellos contienen C como su elemento principal, así como Na, Cl, Si, Mg, S y Ca en diferentes concentraciones. Las características de los tricomas y de los cristales permitieron distinguir las especies de *Cephalocereus*. Por ejemplo, los tricomas capitados son compartidos por *C. columna-trajani* y *C. senilis*, pero se diferencian porque en *C. columna-trajani* hay un cristal prismático por célula y cristales de arena en *C. senilis*. Las otras tres especies comparten los tricomas no capitados con cristales prismáticos en la célula apical y estiloides en las otras células. Las especies de *Cephalocereus* comparten la vía ontogenética de producir tricomas y espinas en las areolas lejos del meristemo apical, lo que sugiere que los tricomas con cristales pueden estar protegiendo los meristemos.
Palabras clave: biominerales, *Cephalocereus apicicephalium*, *Cephalocereus columna-trajani*, *Cephalocereus nizandensis*, EDAX, microscopio electrónico de barrido.

Calcium oxalate crystals can be found in different plant organs and tissues (Franceschi and Horner, 1980; Franceschi and Nakata, 2005). Members of the Cactaceae family are not an exception, as crystals have been observed in the stems, roots, and flowers, mainly in the cells of the fundamental tissue (Terrazas and Mauseth, 2002; Hartl *et al*., 2007). Crystals have been documented in the hypodermis of *Opuntia* species (Conde, 1975; Monje and Baran, 2002; Tovar-Puente *et al*., 2007) and in wood the crystals are rare (Gibson, 1973). There are also reports of crystals in stem epidermal cells of various genera of the subfamily Cactoideae, where they vary in shape and number. For example, the solitary prismatic crystals have been described in species of *Acanthocereus*, *Armatocereus*, and *Peniocereus* (Mauseth *et al*., 1998), the lenticular crystals in *Lophocereus gatesii* (Arias and Terrazas, 2006), and more than two prismatic crystals per cell in several species of *Cephalocereus*, *Neobuxbaumia*, and *Pseudomitrocereus* (Gibson and

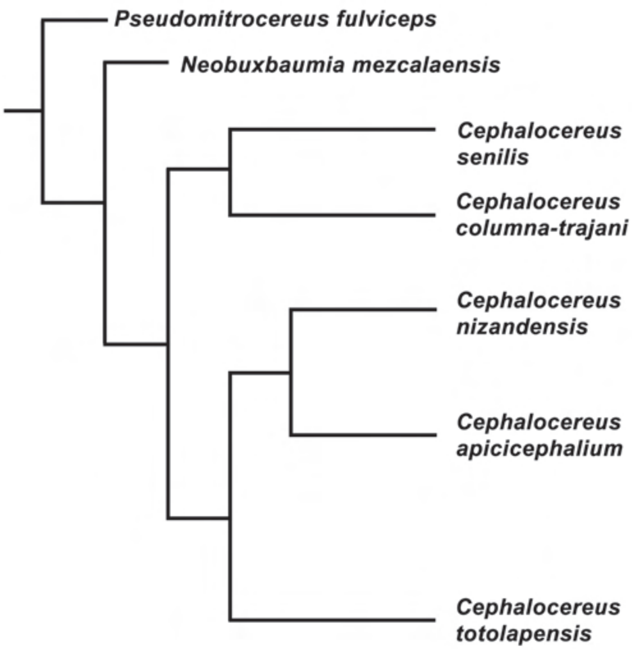

Figure 1. Phylogenetic relationships of *Cephalocereus* species (modified from Bárcenas-Argüello, 2011).

Table 1. Distribution of crystals in trichomes in angiosperms.

Species (Family)	Type of crystal	Organ	Authors
Xanthosoma sagittifolium (Araceae)	raphide	leaf (petiole)	Sakai *et al.*, 1972
Cocos (Arecaceae)	raphide		Frey, 1929
Jubaeopsis caffra (Arecaceae)	raphide	flower (petals)	Robertson, 1978
Justicia betonica, *Hygrophyla auriculata* (Acanthaceae)	needle-like, rodlike, square, prismatic	leaf	Inamdar, 1970
Nassauvia aculeata, *N. axilaris*, *N. chubutensis*, *N. darwini*, *N. fuegiana*, *N. glomerulosa*, *N. ulicina*, *N. uniflora* (Asteraceae)	prismatic, lenticular	leaf	Ragonese, 1987
Eremophila fraseri (Myoporaceae)	raphides	leaf	Dell and McComb, 1977
Conanthera campanulata (Tecophilaeaceae)	raphide	flower (tepals)	Prychid and Rudall, 1999

Horak, 1978; Loza-Cornejo and Terrazas, 2003). Cheavin (1938) reported calcium oxalate crystals in *C. senilis*, with an abundance of up to 85% of dry matter weight. According to Hartl *et al*. (2007), in Cactaceae, the occurrence of crystals is not taxonomically informative. However, several authors mentioned that crystals support groups at different taxonomic levels. For example, the monophyly of the *Cephalocereus-Neobuxbaumia-Pseudomitrocereus fulviceps* clade is supported by the presence of prismatic crystals in the epidermis (Terrazas and Loza-Cornejo, 2002; Arias *et al*., 2003). *Cephalocereus* is a monophyletic genus of five species (Figure 1) endemic to Mexico with distinctive pseudocephalium or lateral cephalium, and producing trichomes in the old vegetative areoles. However, we do not know if trichomes in older areoles are similar to those described in the young areoles and if crystals are present also in these epidermal cells.

Trichomes may exhibit crystals; however, is not common and has only been observed in fifteen species of seven families, among them Araceae and Asteraceae (Table 1). In the Cactaceae, there have been no reports of crystals deposited in trichomes produced in the areola, except for Vázquez-Sánchez *et al*. (2005), who found trichomes with crystals in the floral areoles of the lateral cephalium in *Cephalocereus senilis*. Based on this observation, one question was if the *Cephalocereus* species will have crystals in the trichomes of their old vegetative areoles. Several studies mention the importance of the trichomes in this family. Arreola-Nava (2000) indicates that a clade of seven species of *Stenocereus* is supported by the presence of glandular trichomes in the areoles; likewise, Arias and Terrazas (2006), in their study of the genus *Pachycereus* mention a group of ten species, all of which show abundant trichomes in the pericarpel and floral tube areoles. The areola is a complex region unique to members of the Cactaceae family. In this region, an axillary meristem, axillary buds or short shoot can be found, which are commonly active for a long time, or only one year or two and may be either vegetative or reproductive or both (Boke, 1944, 1951). Areoles produce uniseriate multicellular trichomes giving a woolly appearance with or without spines (Boke, 1944; Terrazas and Mauseth, 2002) developing as early as the first primordium differentiates in the flacks of the areole meristem (Boke, 1951). In this paper, we describe and compare the trichomes of the old vegetative areoles in the five species of *Cephalocereus sensu* Anderson (2001), including their shape and their mineral contents; as well as a discussion of their possible function.

Material and methods

Between five and ten active vegetative areoles were collected from the middle region of the branches in three to six individuals per species of *Cephalocereus* (Table 2). Since no evidence of trichomes was observed in the middle region of the stems of *Neobuxbaumia* and *Pseudomitrocereus*, in comparison with those of *Cephalocereus* species, their areoles were not collected (Figure 2). The areoles were fixed

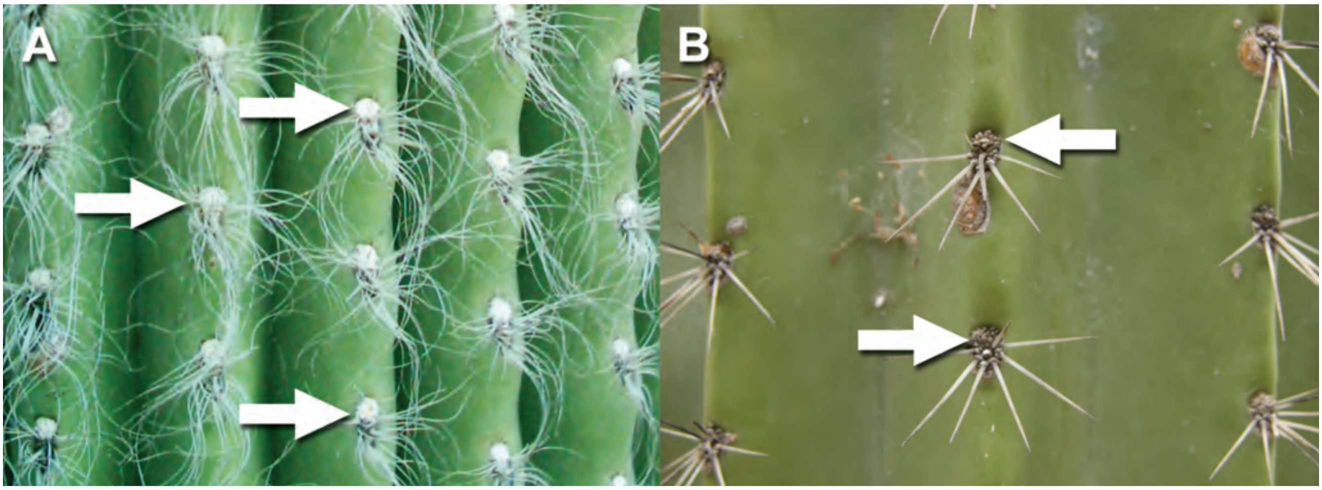

Figure 2. Areoles of the middle stem of *Cephalocereus senilis* (A) and *Neobuxbaumia mezcalaensis* (B), showing active meristems (arrows) or inactive meristems (arrow).

in formaldehyde-acetic acid-ethanol (Johansen, 1940). In the laboratory, they were stored in glycerin-ethanol-water (1:1:1) until they were studied. The areoles were observed under a stereoscopic microscope and the trichomes were separated with a needle. The complete and turgid ones were selected, placed on slides and mounted with water for immediate observation performed with an image analyzer Image-Pro-Plus, version 3.1 (Media Cybernetics, 1997) using a video camera Hitachi KPD51 and an Olympus BX-50 microscope with polarized system. Also wall thickness of apical trichome cells was quantified and trichome number of cells counted. Crystal morphology follows Franceschi and Horner (1980).

For scanning electron microscopy (SEM), the areoles were also dehydrated, cleaned with ultrasound (Cole-Parmer 8851) and critical-point dried (Samdri-780a). Dry samples were fixed to aluminium specimen holders with double-sided conductive carbon tape and coated with gold in a JEOL-JFC-1100 sputter coater. Morphological observations and photographs were carried out in a JEOL JSM-35C. In addition, the trichomes of two species (*Cephalocereus columna-trajani* and *C. nizandensis*) were fragmented. We studied only these two species based on *Cephalocereus* cladogram (Figure 1), arguing that other members of each clade will share the same traits. The fragments, in distilled water, were centrifuged in a Beckman TJ-6 at 1,000 rpm for 5 min and the supernatant was discarded with a pipette. Drops from the liquid were placed on slides and left to dry. Once dried, the crystals were removed toward the sample holders with a brush for evaluation using an energy dispersive X-ray microanalysis (EDAX) with a JEOL6300.

Table 2. List of taxa investigated in this study. All voucher collections are T. Terrazas, vouchers deposited at CHAPA. *Specimen used in SEM.

Species	State and collection number
C. apicicephalium E.Y.Dawson	Oaxaca 508, 512, 509, 513, 704*, 711
C. columna-trajani (Karw. Ex Pfeiff) K.Schum.	Puebla, Oaxaca 443, 444, 681*, 690
C. nizandensis (Bravo & T.MacDoug.) Buxb.	Oaxaca 699, 700*, 703
C. senilis (Haworth) Pfeiff.	Hidalgo 640*, 930
C. totolapensis (Bravo & T.MacDoug.) Buxb.	Oaxaca 516, 572, 574*, 628

Results

Trichome description. The five species of *Cephalocereus* showed uniseriate, multicellular capitated and non-capitated trichomes (Figures 3, 4), and they are common in the old areoles in the middle and basal part of the stems, but never present in *Neobuxbaumia* (Figure 2) and *Pseudomitrocereus*. The non-capitated are large (> 30 cells per trichome) and all the cells are of equal size and equal wall-thickness. The capitated trichomes have less than 20 cells long per trichome in four of the species, and more than 20 cells in *C. senilis*. The number of apical cells that expand varies among the species, in *C. columna-trajani*, *C. senilis* and *C. totolapensis* it is three to five cells (Figures 3B-D, 4A-C), while in *C. nizandensis* and *C. apicicephalium* it is only one cell (Figure 3A, E). However, in *C. apicicephalium* cell expansion is so slight that it is only observed when these trichomes are compared to non-capitated ones, which have very thin and large cells (Figure 3E). The apical cells acquire different shapes, in *C. columna-trajani* and *C. senilis* they are round (Figure 3D, F), and in *C. apicicephalium*, *C. totolapensis* and *C. nizandensis* they are apiculate (Figure 3A, C, G). Apical cells

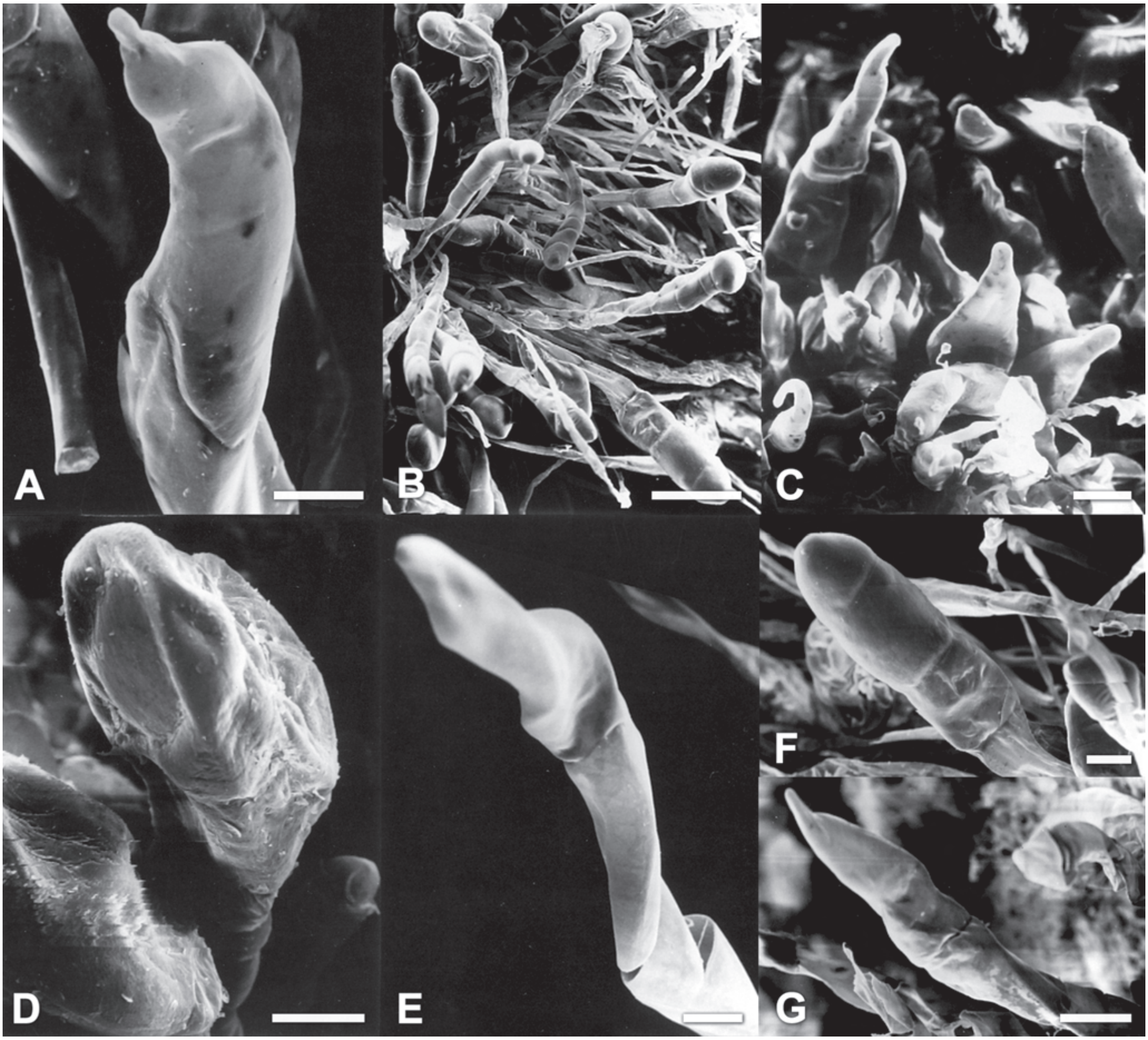

Figure 3. Trichomes in *Cephalocereus* species. **A.** *C. nizandensis (Terrazas 700)*. **B.** *C. columna-trajani (Terrazas 681)*. **C.** *C. totolapensis (Terrazas 574)*. **D.** *C. senilis (Terrazas 640)*. **E.** *C. apicicephalium (Terrazas 704)*. **F.** *C. columna-trajani (Terrazas 681)*. **G.** *C. nizandensis (Terrazas 700)*. Scale 50 µm.

wall thickness varies from 1.68 ± 0.08 µm thick in *C. apicicephalium* to 3.18 ± 0.17 µm thick in *C. senilis* (Table 3). The trichome cells remain alive with cytoplasm and nucleus, the latter more evident in trichome basal cells. Also dark contents in different proportions were observed; in *C. columna-trajani* these contents were reddish-brown and moderately abundant, while in *C. totolapensis* contents were orange and very abundant, in *C. nizandensis* they were greenish-yellow and scarce, and absent in the two remaining species.

Crystal description. The crystals were found in the cells of both types of trichomes, near the walls of adjacent cells, apparently in the vacuole. In the cells of capitated trichomes, prismatic, styloid, and sandy crystals were observed, with one to more-than- five crystals per cell (Table 4, Figure 4). For example, in *Cephalocereus columna-trajani*, it is common to observe only one prismatic crystal per cell (Figure 4D, E); and in *C. senilis*, sandy crystals occurred in every cell (Figure 4F). In *C. apicicephalium*, *C. nizandensis* and *C. totolapensis*, several prismatic crystals can be seen in apical cells and in the other cells exclusively styloids (Figure 4G-J). In *C. columna-trajani* and *C. nizandensis*, for which crystal biomineral composition was evaluated, we found that both prismatic and styloid crystals contained C, Na, Cl, and O,

Table 3. Wall thickness (mean ± standard error) of the apical cell of the capitated trichomes present in the vegetative areolae in *Cephalocereus*. Different letters indicate statistical differences (Tukey, $P < 0.05$).

Species	Thickness (μm)
C. apicicephalium	1.68 ± 0.08^a
C. columna-trajani	2.81 ± 0.16^b
C. nizandensis	1.77 ± 0.09^a
C. senilis	3.18 ± 0.17^b
C. totolapensis	1.77 ± 0.08^a

as well as traces of Si and Ca in their mineral composition (Figure 5A), while they were more diverse in *C. columna-trajani*, containing O, Si, C, Na, Ca, Mg, Cl, and traces of K and S in their composition (Figure 5B).

Discussion

Trichomes in the Cactaceae family have been mostly studied in developing areoles near the apical meristem (Gibson and Nobel, 1986; Arreola-Nava, 2000). Other authors indicate

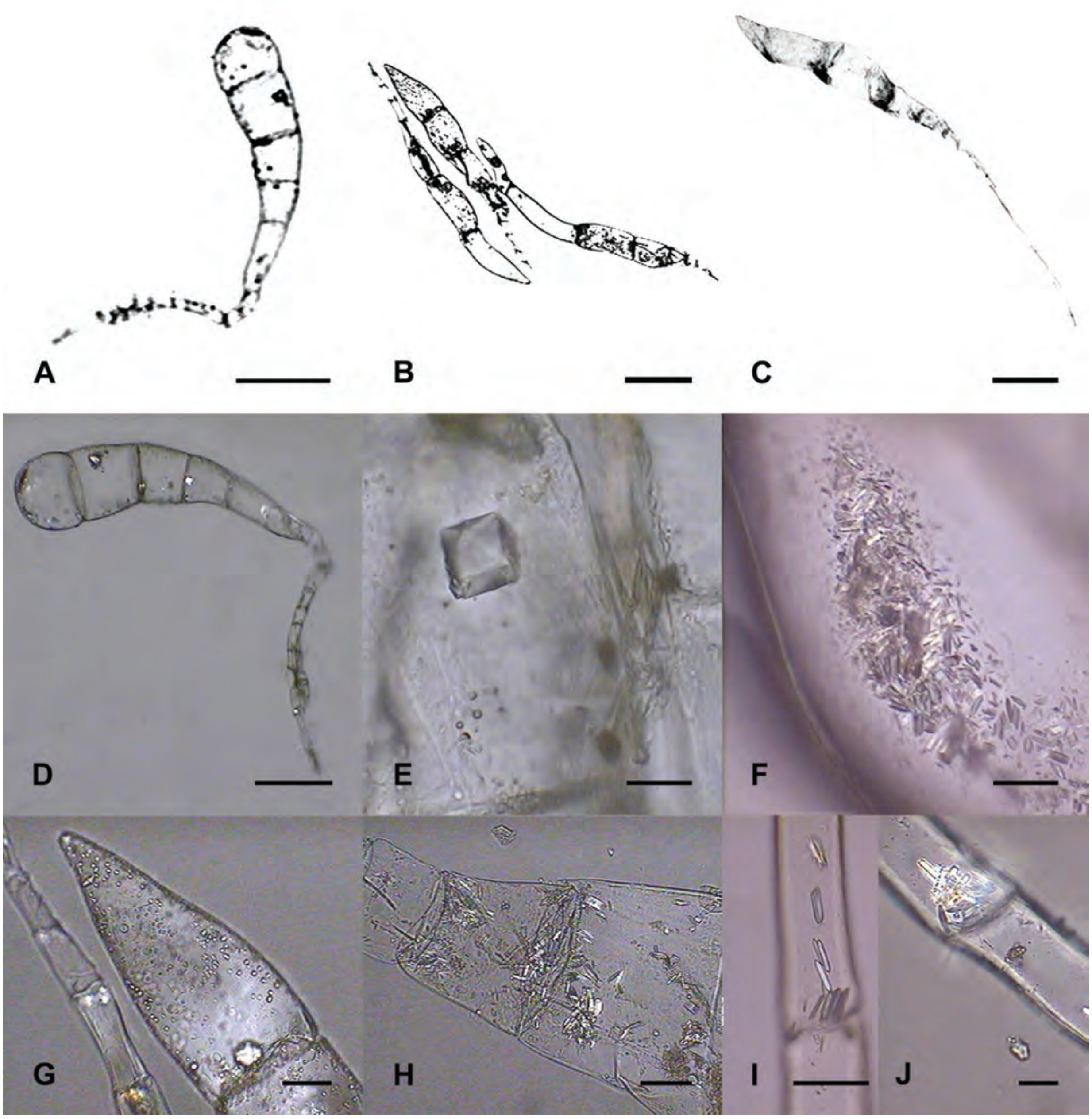

Figure 4. Number of apical cells and crystals in trichomes *Cephalocereus* species. **A, D-E.** *C. columna-trajani (Terrazas 681).* **B, G-H.** *C. totolapensis (Terrazas 516).* **C, F.** *C. senilis (Terrazas 640).* **I.** *C. apicicephalium (Terrazas 508).* **J.** *C. nizandensis (Terrazas 703).* Scales A = 350 μm; B, C = 300 μm; D = 150 μm; E, J = 20 μm; F-H = 50 μm.

Table 4. Characteristics of cells and crystals in the capitated trichomes of the old vegetative areoles in *Cephalocereus*, *Neobuxbaumia mezcalaensis* and *Pseudomitrocereus fulviceps*. - = lacking.

Species	Apical cel shape	Number of wider apical cells	Crystal types in the apical cell	Cyrstal types in the foot cells
C. nizandensis	apiculate	one	prismatic	styloids
C. apicicephalium	apiculate	one	prismatic	styloids
C. totolapensis	apiculate	3-5	prismatic	styloids
C. columna-trajani	botuliforme	3-5	prismatic	prismatic
C. senilis	botuliforme	3-5	sandy	sandy
Neobuxbaumia mezcalaensis	-			
Pseudomitrocereus fulviceps	-			

that areoles located in branches far from the apical meristem lose their trichomes and are usually inactive (Benson, 1982). This is not the case in *Cephalocereus*, whose species display the presence of uniseriate, multicellular, capitated and non-capitated trichomes with crystals in the areoles far from the apical meristem. The ability of *Cephalocereus* to produce these trichomes contrasts with their sister taxa *Neobuxbaumia* and *Pseudomitrocereus* that do not have them.

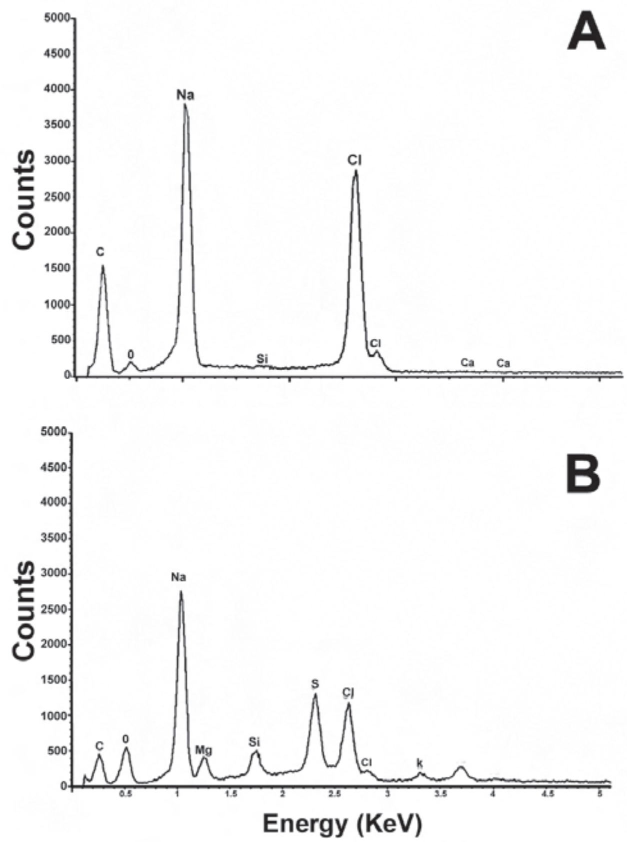

Figure 5. EDAX spectra on the isolated biominerals from trichomes. **A.** *C. nizandensis (Terrazas 681)*. **B.** *C. columna-trajani (Terrazas 699).*

The shape, number, and distribution of crystals within the plant's body may be distinctive for certain species (Wu and Kuo-Huang, 1997; Prychid and Rudall, 1999; Lersten and Horner, 2008). In the Cactaceae family, crystals have contributed to the delimitation of subfamilies and genera; for example, Conde (1975) mentions that druses located in the *Opuntia* hypodermis define the Opuntioideae subfamily, an asseveration supported by their type of biominerals present (Monje and Baran, 2002). Calcium oxalate monohydrate crystals located in epidermal or cortical cells distinguish species of the *Rhipsalis* genus, while calcium oxalate dihydrate crystals characterize species of the *Lepismium*, *Hatioria*, and *Schlumbergera* genera (Hartl *et al*., 2003). Terrazas and Loza-Cornejo (2002) found prismatic crystals in the epidermis of the species of *Neobuxbaumia*, *Cephalocereus*, and *Pseudomitrocereus*. Thus, the diversity of crystal number in trichome cells of the *Cephalocereus* species is not surprising.

The presence of crystals in the trichomes is not very common, though it has been observed in leaves (Inamdar, 1970; Ragonese, 1987), petioles (Sakai *et al*., 1972), tepals (Prychid and Rudall, 1999), and petals (Robertson, 1978) of different plant families. The type of crystals located in trichomes may be raphide (Inamdar, 1970; Sakai *et al*., 1972; Robertson, 1978; Prychid and Rudall, 1999), prismatic, and lenticular (Ragonese, 1987). In this study, we describe for the first time crystals present in trichomes of the vegetative areoles of *Cephalocereus* species. Moreover, their shapes were more diverse than those reported in stem epidermal cells, limited only to prismatic crystals. Vázquez-Sánchez *et al*. (2005) mention the presence of small crystals in trichomes of floral areoles in the cephalium of *C. senilis*. Our observations confirm the occurrence of crystals in the vegetative areoles in the stem of the five species.

Various functional explanations have been given to the occurrence of calcium oxalate crystals in plant tissue; e.g. to protect against damage caused by small animals (Conde, 1975; Franceschi and Nakata, 2005), because if those tissues are eaten, they cause irritation (Prychid and Rudall, 1999). They may also serve as an attraction for pollen spreading biological agents (Robertson, 1978). In addition, the sodium chloride crystals that accumulate on the leaf surface can increase reflectivity, protecting the epidermis against excessive light (Uchiyama and Sugimura, 1985; Karimi and Ungar, 1989; Rajput and Sen, 1991), which may explain the presence of Na and Cl in the crystals deposited in the trichomes that protect the areola meristems in species of *Cephalocereus*. Furthermore, several authors (Conde, 1975; Darling, 1989) point out that the crystals help to reduce evaporation or increase reflectivity, as well as to decrease transmittance. This may occur in the five species of *Cephalocereus*; growing in regions where diurnal temperature exceeds 40 °C and the crystals may contribute to protect the young tissues located in the areoles by reducing the temperature. It is outstanding that the biominerals present in the trichomes of the two studied species of *Cephalocereus*, as well as those found in the ordinary epidermal cells of three species of *Cephalocereus* (Bárcenas-Argüello *et al*., 2010) are more diverse than those reported for other Cactaceae species (Rivera and Smith, 1979; Monje and Baran, 2002). Bárcenas-Argüello (2011) have demonstrated that the availability of these minerals in the soil and their occurrence in stem of five species *Cephalocereus* is not related, further studies are needed to understand their metabolic development.

Trichome morphology has been used as an important attribute in the classification of genera and species (Heintzelman and Howard, 1948; Inamdar, 1970; Valencia and Delgado, 2003; Batterman and Lammers, 2004), and for the identification of interspecific hybrids (Rollins, 1944). Hardin (1979) indicates that trichome morphology is commonly not affected by the environment, and therefore, may contribute to understanding the phylogenetic relations of different plant groups. Trichomes can display a wide variation within a family or can be quite uniform; they are also different depending on the organ that is being studied (Rao and Ramayya, 1977; Cosa *et al*., 2002). In the Cactaceae family, papillose surfaces have been described in species of the Cactoideae subfamily (see Terrazas and Arias, 2002). However, the presence of trichomes has been restricted to epidermal derivatives associated with the areoles. Gibson y Nobel (1986) describe the ontogeny of these simple multicellular trichomes and, with the exception of the glandular trichomes reported by Arreola-Nava (2000) for the seven species of *Stenocereus*, apical cells have been described as a triangular shape, and so the capitated trichomes described herein for *Cephalocereus* are unique. Both shape and number of cells that expand in the apical region of the trichomes characterize the *Cephalocereus* species (Table 4).

The species *Cephalocereus apicicephalium*, *C. nizandensis*, and *C. totolapensis* have apical capitated trichomes with prismatic crystals and in the other trichome cells styloid crystals. The rounded capitated trichomes allow us to recognize *C. columna-trajani* and *C. senilis*, though the first is distinguished by its brown contents and prismatic crystals in all of the trichome cells and the second is characterized by the presence of sandy crystals.

In conclusion, the morphological differences in crystals and trichomes developed in the old areoles in the five species of *Cephalocereus* allow distinguishing among them and from their sister taxa (*Neobuxbaumia* and *Pseudomitrocereus*). In addition, the five species of *Cephalocereus* share the ontogenetic pathway of producing trichomes and spines in the areoles far from the apical meristem. These trichomes together with crystals constitute a high-specialization characteristic that protects the meristems located in the areoles.

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