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Full Length Research Paper

Microstructural differences in *Agave atrovirens* Karw leaves and pine by age effect

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We evaluated the effects of the leaves, "pine" or "head" (plants without leaves) age of *Agave atrovirens* on the epidermal, parenchyma and fibrillar microstructure as possible maturity indicators, since plant age is a determinant factor of the concentration of inulin. Samples were taken from three, six and nine year-old plants, and observations were made with scanning electronic microscopy (SEM) and light microscopy (LM). The results showed that the isolated cuticular membrane (ICM) microstructure in the *Agave* plant changes considerably with the age, becoming harder and allowing the mature plant to avoid excessive water loss, on the other hand, the increased size of the suprastomal cavity makes the respiration process more efficient in older plants. The *Agaves* present the three main types of calcium oxalate crystals reported for monocotyledons (druses, raphides and styloids). The plant age is an important factor in the identification of calcium oxalate crystals because it is only in six year-old plant that all three types were identified. However, the druses are present only in the cuticular membrane (CM), while the styloids and raphides are distributed on the parenchyma and chlorenchyma. The fibrillar structures in *Agave* are modified with age such that *Agave* can support severe hydric stress and the storage of soluble carbohydrates.

Key words: Crystals, calcium oxalate, fibrillar, druses, raphides, styloids.

INTRODUCTION

Most species of *Agave* genus are characteristic of the arid zones and are exposed to adverse environmental changes; for this reason, these plants have developed diverse biochemical and biophysical mechanisms at the cellular and structural level (Lüttge, 2004). The cuticular membrane is the most important protection mechanism and is a characteristic interface in this genus. In addition, these plants possess certain adaptation mechanisms for stress protection (drought and cold), of which increased water use efficiency is considered the most important (Szarek and Ting, 1975; Holthe and Szarek, 1985).

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Cuticular characteristics of *Agave* have been described in the literature as a complex structure formed of six layers with cutin being the principal component (Wattendorff, and Holloway, 1980, 1982).

The presence of calcium oxalate crystals has been reported as a possible protection mechanism of *Agave* plants against insects and foraging animals. These crystals commonly occur in suprastomatal cavity and cell wall in diverse forms (druses, styloids or raphides), for this reason, these compounds are the cause of the irritant contact dermatitis in the workers in tequila distilleries (Salinas et al., 2001). However, the biological role of these crystals in the plant is important because they provide high turgescence and may represent storage form of calcium and oxalic acid (Ilarslan et al., 2001). The concentration of these crystals in *Agave* plants is higher

Table 1. Physical characteristics of *Agave atrovirens* Karw leaves (mean ±standard deviation).

Variable	3 Years-old leaf	6 Years-old leaf	9 Years-old leaf
Internal length(cm)	51 ± 2.5[b]	117 ± 6.3[a]	129 ± 5.2[a]
External length (cm)	53 ± 3.1[c]	118 ± 6.5[b]	132 ± 4.5[a]
Base leaf perimeter (cm)	26 ± 1.4[c]	58 ± 4.1[b]	81 ± 5.9[a]
Weight (g)	540.70 ± 35[c]	4160 ± 228[b]	6499 ± 400[a]

Means followed by the same letter in the same row are not significantly different ($P < 0.05$).

than in agricultural plants (Nobel, 1983; Nobel and Berry, 1985), being that the age is an important factor in crystal identification because it is easier to detect calcium oxalate crystals in the first developmental stages of the plants (Ilarslan et al., 2001).

Plant age also generates changes in the fibrillar structure; in *Agave* genus and these differences are more evident because the fibers associated with conducting bundles are 40 to 70 times longer than the meristematic cells from which they originate (Chernova and Gorshkova, 2007). However, differences in fibrillar structure are also associated with the plant organ evaluated. For example, in Jerusalem artichoke bulbs, age is the determinant factor in structural changes (Jordan and Chapman, 1971), while in wheat, the differences originate in the aging primary leaves (Hurkman, 1979). Considering the diverse microstructural differences that can occur in a plant, the objective of this study was to determine differences in the epidermal, parenchymal and fibrillar microstructure in *Agave* organs (leaves or "pine") to establish a plant age marker.

MATERIALS AND METHODS

Vegetal material

Pine and leaves of *A. atrovirens* Karw were collected in summer 2007 in Singuilucan, Hidalgo State, Mexico (altitude 2640 m; 19° 59' 20" N, 98° 27' 52" W). To avoid the high acid concentrations caused by metabolic processes, samples were obtained at 16:00 ± 1 h.

Sample preparation

Leaves and pine of the plants collected were separated. From the leaves, were obtained three samples (cuticle, parenchyma and chlorenchyma), while from the pine only parenchyma was used. Samples with a dimension of 10 by 10 by 1 mm were cut from the parenchyma and chlorenchyma using a razor blade. Isolated cuticular membrane (ICM) was manually removed from *Agave* leaves. The ICM, parenchyma and chlorenchyma samples were dried in controlled conditions (22°C; 72 h; about 6% moisture) without the utilization of solvent used in the traditional process of dehydration for scanning electron microscopy (SEM) analysis.

Physical and chemical characteristics

Physical characteristics of fifteen *Agave* leaves of each group age were measured: weight, internal and external length leaf and perimeter base leaf. The sun exposed side was considered the internal length, while the opposite side was considered the external length. The chemical analysis was performed by AOAC (1995) methods: protein content N × 6.25 (Method 955.04), raw fiber (Method 962.09), crude fat (Method 920.39), moisture (Method 934.01), ash (Method 923.03) (AOAC, 1995).

Microscopy

Light microscopy (LM)

LM was carried out according to Clarke's method (1960). The leaf and pine samples were placed in a tube filled with 88% lactic acid and kept hot in a boiling water bath for about 30 to 40 min whereas the cuticle was removed and was not treated. The qualitative micro-morphological characteristics were observed using LM. Micro-histological micrographs were taken with a Nikon (FX-35) camera-equipped light microscope.

Scanning electron microscopy (SEM)

A JEOL (JEOL, type EX-1200, Japan) scanning electron microscope operated at 15 kV was used to visualize the microstructure in the cuticle, parenchyma and chlorenchyma samples. The samples were mounted in a double-sided carbon tape and covered with roughly 10 nm of gold in a Denton sputter coater.

Observation of microstructural differences

Pine and leaf samples, differences in calcium oxalate crystals, fibrillar structure, ICM and spandrels were examined in three, six and nine year-old *Agave* plants. In ICM, the stomata distribution and density, suprastomal cavity, stomata complex and epicuticular characteristics were examined.

RESULTS

Physical and chemical characteristics

Significant differences were observed in physical characteristics of *A. atrovirens* leaves (Table 1). High internal length of 129 cm was observed for nine year-old leaf against low internal length of 51 cm for three year-old leaf, similar results were observed for external length. Perimeter base leaves ranged between 26 and 81 cm, lowest for three year-old leaf and highest for nine year-old leaf. Higher differences were observed for weight

Table 2. Chemical analysis of *A. atrovirens* Karw (mean ± standard deviation).

Variable	3 Year-old	6 Year-old	9 Year-old
Moisture[1]	80.20 ± 2.05	88.00 ± 1.87	79.54 ± 1.58
Ash[2]	8.98 ± 1.03[a]	11.33 ± 1.65[b]	13.26 ± 1.05[c]
Protein[2]	3.09 ± 0.18[a]	6.26 ± 0.28[b]	5.70 ± 0.67[b]
Crude fat[2]	1.30±0.30[a]	1.26 ± 0.92[a]	0.87 ± 0.08[b]
Raw fiber[2]	74.14 ± 3.07[a]	58.33 ± 2.45[b]	28.02 ± 0.97[c]
Carbohydrates[2,3]	12.49 ± 2.60[a]	22.82 ± 1.66[b]	52.15 ± 2.41[c]

Values are expressed as the mean and standard deviation of three replicates, dry weight basis; [1] Percentage; [2] g/100g of dry matter; [3]Obtained by difference; Means followed by the same letter in the same row are not significantly different (P<0.05).

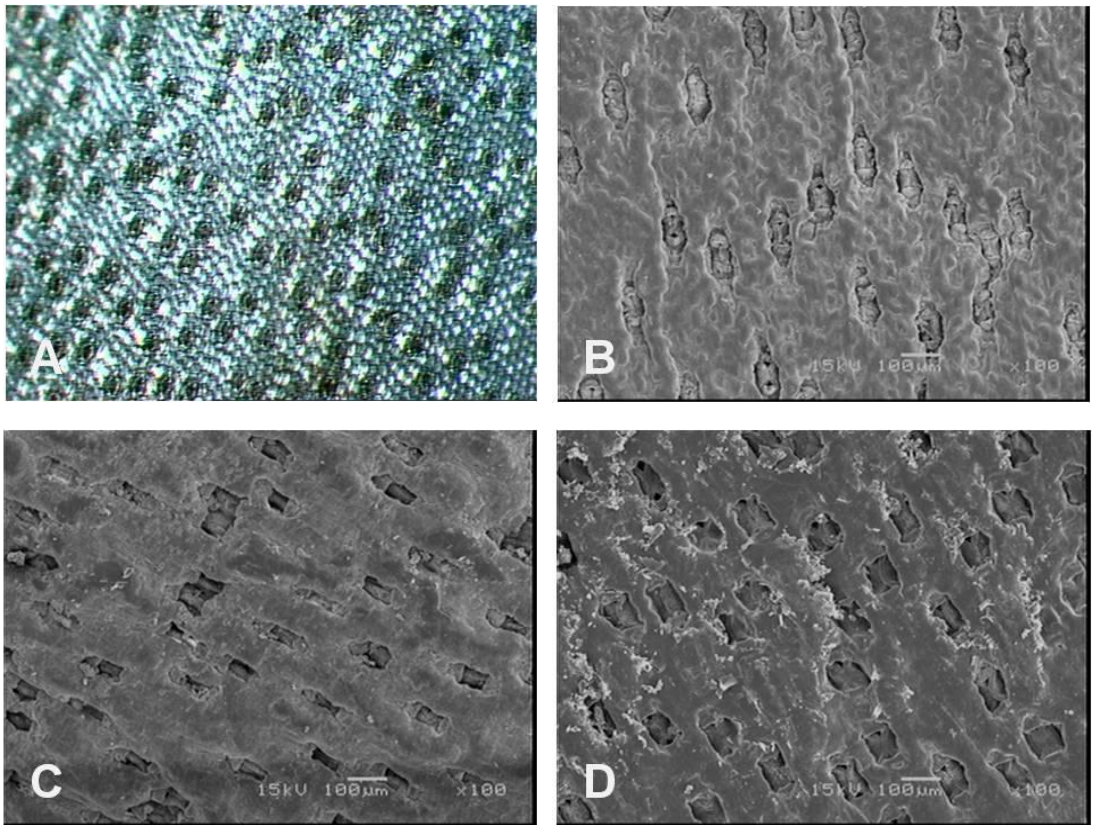

Figure 1. Surface morphology of the isolated cuticular membrane (ICM) from *Agave* leaves. (A) Stomata density and distribution observed with light microscopy. (B, C and D) Stomata density and distribution observed with SEM from 3-, 6- and 9-year-old *Agave*, respectively.

leaves, the nine year-old leaf weight is 12 time more high than 3 year-old leaf.

The results of chemical analysis are presented in Table 2. The main components of the *Agave* plant are the water and the fiber. The highest raw fiber and crude fat content was obtained in three year-old plant, an inverse correlation was observed between age plant and both raw fiber and crude fat content, while direct correlation was observed between age plant and both ash and carbohydrates content. The highest ash and carbohydrates content were obtained in nine year-old plant, on the other hand, the protein and moisture content show a maximum value at physiological maturity plant (six year-old).

Microstructural differences related to plant age in the ICM

The ICM (Figure 1A) undergoes micromorphological differences; three-year-old *Agave* cuticle possesses cavities on the superficial wax, generating an irregular

Table 3. Stomata characteristics of *Agave* leaves average values (Mean ± Standard deviation).

Agave age (years)	Stomata complex length (μm)	Stomata complex thickness (μm)	Stomata area (μm^2)	Stomata density (stomata*mm^{-2})
3	140.51 ±13	45.40 ± 7	6428.00 ± 1447	22
6	53.84 ± 6	33.07 ± 4	1761.69 ±199	30
9	70.77 ± 7	63.83 ±11	4524.00 ± 946	29

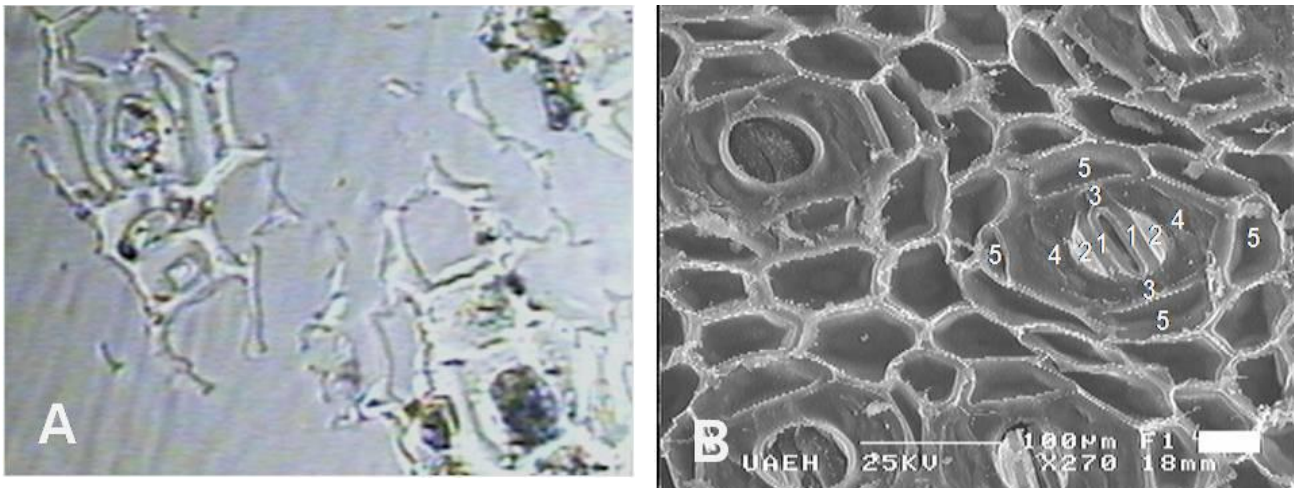

Figure 2. Epidermal cells from *A. atrovirens*. (A) LM, (B) SEM. 1. Guard cells. 2. Pair of inner lateral subsidiary cells. 3. Pair of terminal subsidiary cells. 4. Pair of outer lateral subsidiary cells. 5. "Normal" epidermal cells.

structure (Figure 1B). In contrast, these cavities are covered in older plants (six and nine years); for this reason, the ICM microstructure is more regular (Figure 1C and D) due to the formation of a second layer in the cuticular membrane (Wattendorff and Holloway, 1980).

The stomata complex area on the leaf surface in *A. atrovirens* is modified with plant age (Table 3), the six-year-old plant have minor area; this difference can be attributed to variations in the thickness and distribution of the cuticular wax (Garcia, 2007). Stomata complex area obtained for the six-year-old plant (1761 μm^{-2}) is similar to that reported for five-year-old *Agave tequilana* (1848 μm^{-2}, Hernández et al., 2003); these results indicate similarity within the *Agave* genus.

Stomata density differences are present only in the first developmental stages (three years) because at this age,the plant has only 22 stomata mm^{-2}, whereas at six and nine years, stomata density reaches 30 stomata*mm^{-2}; these results are in concordance with those reported for diverse *Agave* species whose stomata density is in the range of 18 (*Agave promontorii*) to 34 (*Agave deserti*) stomata*mm^{-2} (Gentry and Sauck, 1978). In *A. atrovirens*, the most precise identification of each structure in the stomata complex requires SEM (Figure 2B) to permit the perfect identification of stomata complex components (guard cells, subsidiary cells and normal epidermal cells), that is difficult with LM (Figure 2A).

Age-related differences in the adhering cell walls

ICM showed differences in the structure of the adhering cell wall, because it tends to become more rigid with age, and thus, there are differences in the structure and morphology of the three-dimensional cavities (spandrels) (Figure 4A). At three year-old, the adhering cell walls form a translucent and elastic layer on the spandrels (Figure 4B), while at six year-old (Figure 4C), the adhering cell wall apparently forms a more compact layer with the cuticular layer and the cutin bodies, permitting the best isolation of the cuticular membrane. At nine year-old, a greater proportion of spandrels are covered with an amorphous and opaque layer because of the rigidity of the adhering cell walls (Figure 4D).

Effect of age on calcium oxalate crystals

In comparison to *Agave tequilana*, which possesses calcium oxalate crystals on the parenchyma in styloid form, *A. atrovirens* has all three main types of crystals reported for monocotyledons. The three developmental stages show crystals on the epidermal cells in the *A. atrovirens* cuticle (Figure 5B, C and D). However, the amount apparently is determined by the calcium regulation process in the parenchyma, and for this

reason, the three- and six-year-old plants have a small amount of crystal around the suprastomatal cavities.

The druses are on the cuticular surface surrounding the stomata complex (Figure 5A); while the calcium oxalate crystal present on the parenchyma and chlorenchyma are raphides and styloids (Figure 6). This results shows a characteristic of the *Agave* plant that resembles some species in the Araceae family, which also have all three types of crystals reported for monocotyledons (druses, styloids and raphides). However, age is a determinant factor on the calcium oxalate crystal types presence because at the first development stages in *Agave* (three year-old) only styloids were found around the vascular strands (Figure 6A) or in cells adjacent to the endodermis in the leaves.

Raphides crystals present in *A. atrovirens* form bundles that appear elliptical or circular in cross-section (Figure 6B and C) and have been defined as type III (Wattendorff, 1976b). On the other hand, in *A. atrovirens*, calcium oxalate crystal composition differs depending on analyzed plant organ because in the pine, only styloid crystals were detected (Figure 6D).

Age-related differences in the fibrillar structure

Fibrillar structures in *Agave* are bundles (Figure 7A) which permit maintenance of turgor pressure in the leaf; age is an important factor in bundles morphology. At three year-old, the fibrous bundles are homogeneous structures composed mainly of cellulose, lignin and hemicellulose with a low content of waxes and ash. These bundles are compact and parallel to the leaf longitudinal section (Figure 7B). Also, at three year-old, the water storage tissues (hydrenchyma) are reduced, when the plant reaches physiological maturity (six year-old) there is an increase in hydrenchyma volume. In the last developmental stage (9 year-old), storage area is increased, permitting the accumulation of water and carbohydrates (Figure 7D).

DISCUSSION

Physical and chemical characteristics

Results obtained show that *A. atrovirens* at early development stages (three year-old) exhibit a lowest volume occupied by hydrenchyma and for this reason, the three year-old plant is more severely affected by hydric stress than the six and nine year-old plants. In relative terms, hydrenchyma increased while chlorenchyma and epidermis proportions decreased with age; this behavior is characteristics of CAM plants, in which the oldest leaves had higher hydrenchyma volume than the youngest leaves (Nobel and Meyer, 1985). On the other hand, at six and nine year-old plants, the higher parenchyma volume permit storage of large amount of non-structural carbohydrates (500 g kg dry basis), used in alcoholic beverages production (Wang and Nobel, 1998; Pinos-Rodriguez et al., 2006, 2008).

Results showed the influence of plant age on chemical composition. These are similar to previous results reported by Pinos-Rodríguez et al. (2008) in *A. salmiana* at 12, 14 and 16 year-old, which is attributed to changes in concentration of enzymes involved in carbohydrates metabolism. Same behavior was observed in this study since *A. atrovirens* showed a lower concentration of protein in young plants, associated with the low carbohydrates concentration, while in oldest plants, highest non-structural carbohydrates concentration were obtained. The non-structural carbohydrate storage in the hydrenchyma generates a reduction in the fiber content (Van Soest, 1994) as protective mechanism against hydric stress.

Microstructural differences in the ICM

In *Agave* plants, age is a determinant of ICM micromorphology, mainly in stomata density and distribution; these differences diminish the effects of hydric stress because in *Agave* cuticle, the primary function is to act as a barrier to impede water loss (Garcia and Fernández, 1991). On the other hand, stomata complex is also an important structure in water regulation process. However, in addition, differences resulting from maturity and the climatic conditions also contribute to process adaptation. For this reason, flexibility of these plants to adapt to prolonged hydric stress can be great. Unfortunately, there are few published works about microstructural differences during plant development, and as such, this work is the first study with data on age effects on the ICM microstructure. Stomata density is also modified with increasing age. Additionally, this is evidence of adaptation process to diverse stress conditions because in other monocotyledons and dicotyledons, stomata density can oscillate from 100 to 300 stomata mm^{-2}, ten times more stomata than *A. atrovirens*.

Suprastomatal cavity in *Agave* is a xeromorphic adaptation for desert survival, and stomata complex proper lies at the bottom of this cavity, where the guard cells are surrounded by four epidermal cells. Because stomata complex is morphologically tetracytic, these four epidermal cells are considered subsidiary cells, which are structurally specialized cells that are distinct from other epidermal cells associated with the guard cells of the mature stomata (Gentry and Sauck, 1978). On the other hand, the occlusions in cuticular membrane observed in the earlier developmental stages in *A. atrovirens* tend to disappear with plant maturity. This phenomenon can be due to accumulation of the epicuticular wax on the ICM to reduce the amount of water evaporation as a mechanism

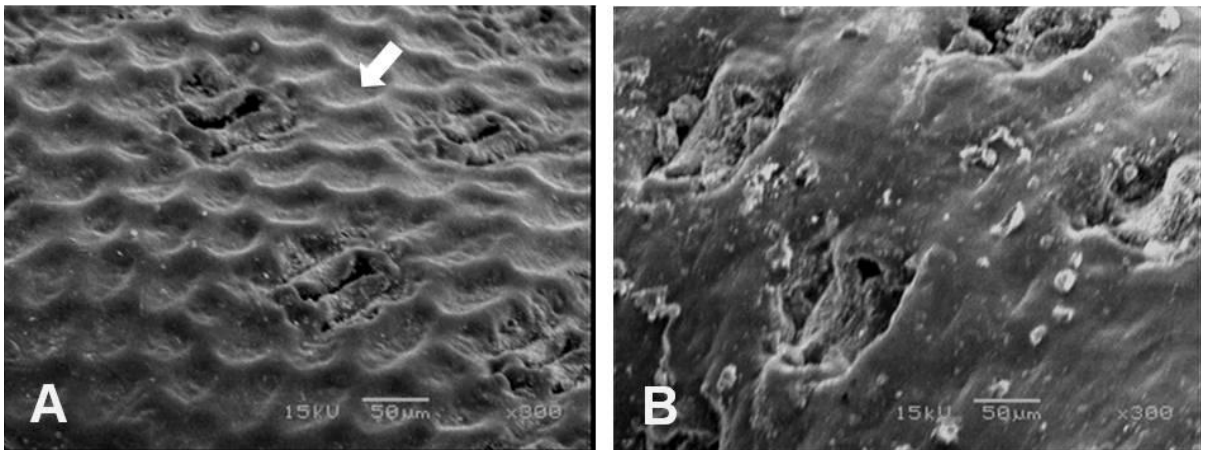

Figure 3. Surface morphology differences of isolated cuticular membrane (ICM) from *A. atrovirens*. (A) 3 years. (B) 9 years. Epicuticular crystalline wax deposits (arrow).

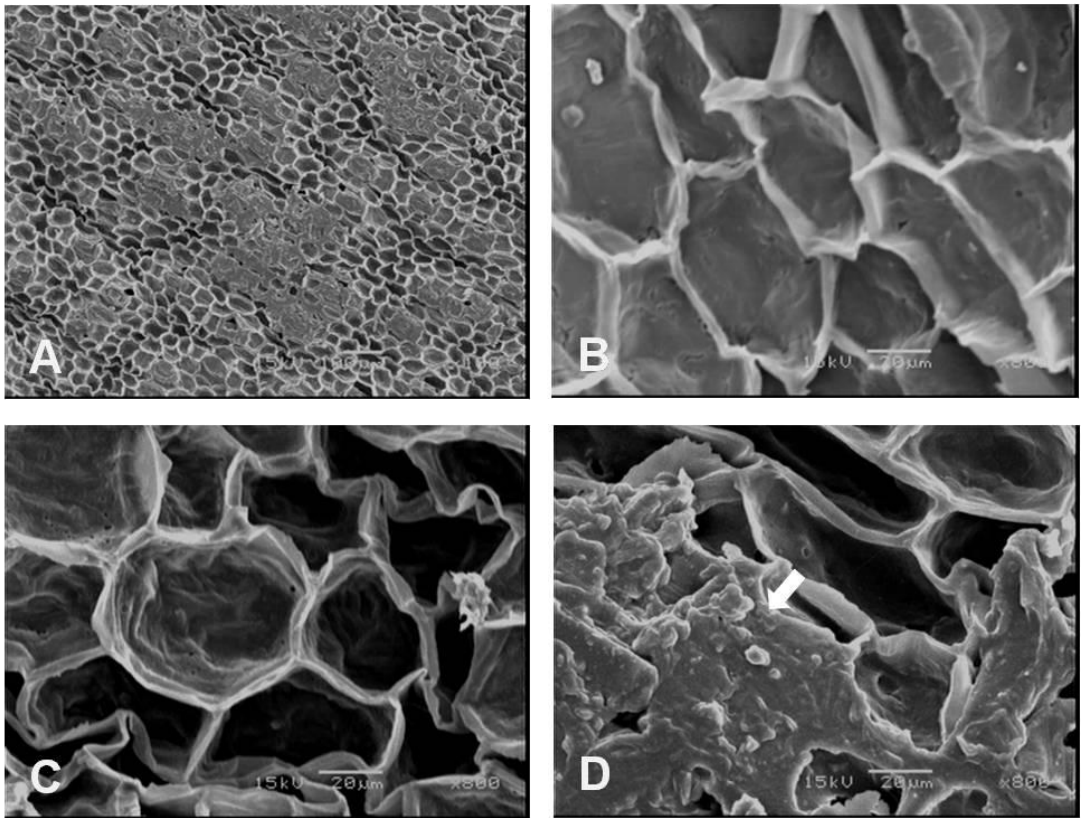

Figure 4. Spandrel characteristics in *A. atrovirens*. (A) Irregular distribution of adhering cell walls on the spandrels (B) Adhering cell walls form a translucent layer on the spandrels in three-year-old plants. (C) Partial isolation of the adhering cell wall in six-year-old plants. (D) Adhering cell walls are opaque in nine-year-old plants. Arrow indicates the rigid adhering cell wall.

for survival on an extremely small free-water intake. For this reason, in the last developmental stage (nine years), the ICM was practically a smooth surface.

As in epidermal cells of *Agave tequilana* and *Odyssea paucinervis* (Somaru et al., 2002), epicuticular wax in *A. atrovirens* leaf forms a characteristic depression in the ICM. However, these occlusions are most evident in the first developmental stage (3 years) (Figure 3A) because

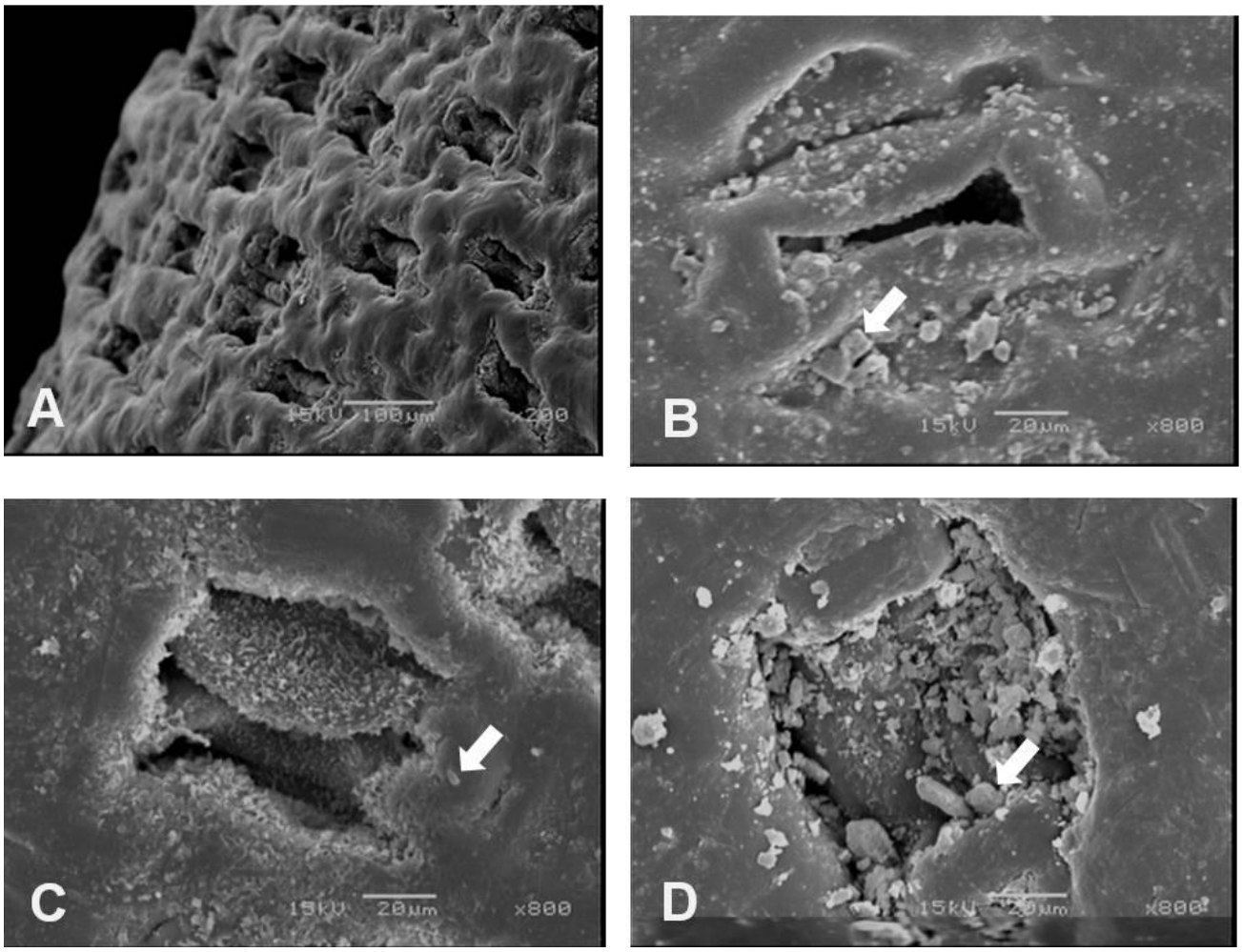

Figure 5. Distribution of calcium oxalate crystals. A) Scanning electron micrograph of the stomatal periphery that show the suprastomatal cavity. (B, C and D) Crystal distribution in the stomata periphery from ICM at 3, 6 and 9 years, respectively. Arrows indicate the calcium oxalate crystal localization.

the older plants (six and nine years) have ICMs that appears less rough than the three-year-old *Agave* (Figure 3B).

Age-dependent differences in the adhering cell walls

In earlier developmental stages, adhering cell walls form a translucent layer with elastic characteristics that permit easy isolation of cuticular membrane in *Agave* leaf and facilitate the identification of spandrels. However, withplant development adhering cell walls become more rigid, and isolation of cuticular membrane is irregular, leaving some spandrels exposed. While, at six years old, isolation of cuticular membrane is homogeneous. Six years is the best age for extraction of the “mixiote” (ICM) used in Mexican food, taking advantage of the fact that forces adhering cells to one another are stronger at this age.

Effect of age on calcium oxalate crystals

In *A. atrovirens*, calcium oxalate crystals lend the plant a high capacity for calcium regulation and protection against herbivory (Thurston, 1976). On the other hand, this plant contains all three of the main calcium oxalate crystals reported for monocotyledons and this characteristic is unique to this species and for this reason, *A. atrovirens* is an interesting plant for an exhaustive study. Druses crystals have been identified primarily in dicotyledons but have also been reported in some species of monocotyledons (Gaiser, 1923; Sakai et al., 1972; Kausch and Horner, 1981; Sunell and Healey, 1981; Genua and Hillson, 1985). Apparently, the presence or absence of calcium oxalate crystals is due to calcium regulation in the parenchyma that permits elimination of excessive calcium and release through stomata aperture, followed by storage of this calcium around the guard cells, playing an important role in the transduction signal

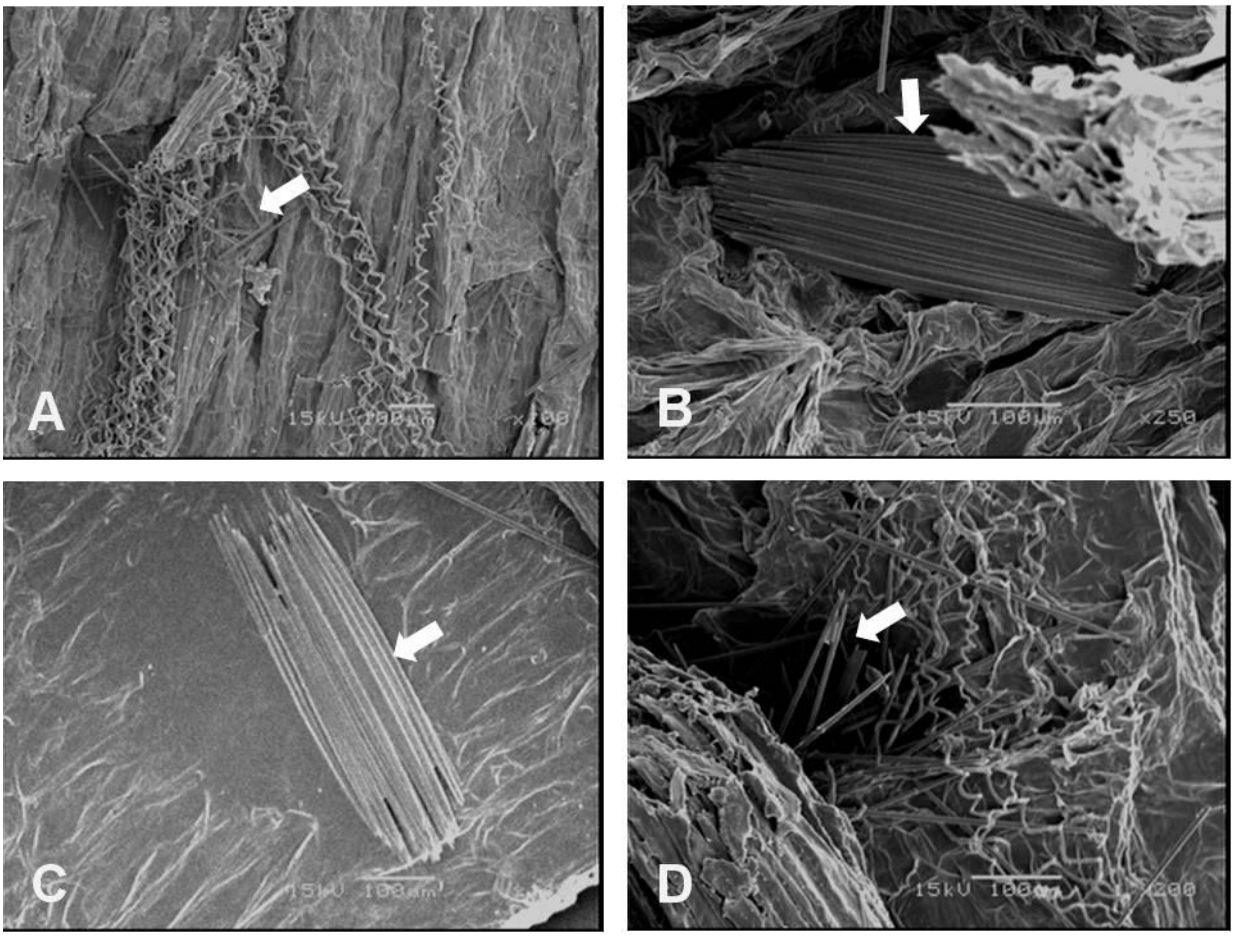

Figure 6. Calcium oxalate crystal localization in *A. atrovirens* (arrows). (A) Styloids in 3-year-old leaves (B and C) Raphides in 6- and 9-year-old leaves, respectively. (D) Styloids in the pine.

that permits maintenance of urgency in the guard cells (McAinsh et al., 1992). In addition, calcium oxalate crystals also protect the plant against calcium ion release into extracellular fluids because the crystals act as sequestering agents (Ruiz and Manfield, 1994), in which calcium is stored in styloids and raphides. Age does not influence the concentration of these compounds. Simultaneous presence of raphides and styloids has been reported in Agavaceae family (Sakai and Hanson, 1974; Wattendorff, 1976a, b; Arnot, 1981; McDougall et al., 1993). However, in *A. atrovirens* in particular, crystal types present are apparently dependent upon plant age, being raphides, the main form present at six and nine year-old plants, but raphides and styloids crystals vary in size and shape within species and for this reason cannot used for comparison within genus *Agave*.

At three and six years, styloid crystals were identified, so apparently calcium regulation during maturity was necessary. During senescence (nine years), calcium oxalate crystals are absent because pine is the main reserve organ for carbohydrates and because metabolism diminishes; for this reason, a protective system that permits calcium sequestration is not necessary. Styloids morphology in *Agave* is characteristic of this crystal type: thick; solitary within a cell; with pointed ends; the size in longitudinal section (typically long and slender) is 315 µm. This type of crystal is apparently the cause of the irritant contact dermatitis in workers in tequila distilleries and *Agave* plantations (Salinas et al., 2001).

Age-dependent differences in fibrillar structure

Fibers of *Agave* genus are commercially important, and their presence allows *Agave* leaves become several times thicker than those of other succulents. During plant maturity, there is increased formation of hydrenchyma, and thus, age is an important factor in microstructural differences that occur in fibrillar tissue. This property permits water and carbohydrates storage, allowing the plant to maintain tugour pressure in the chlorenchyma

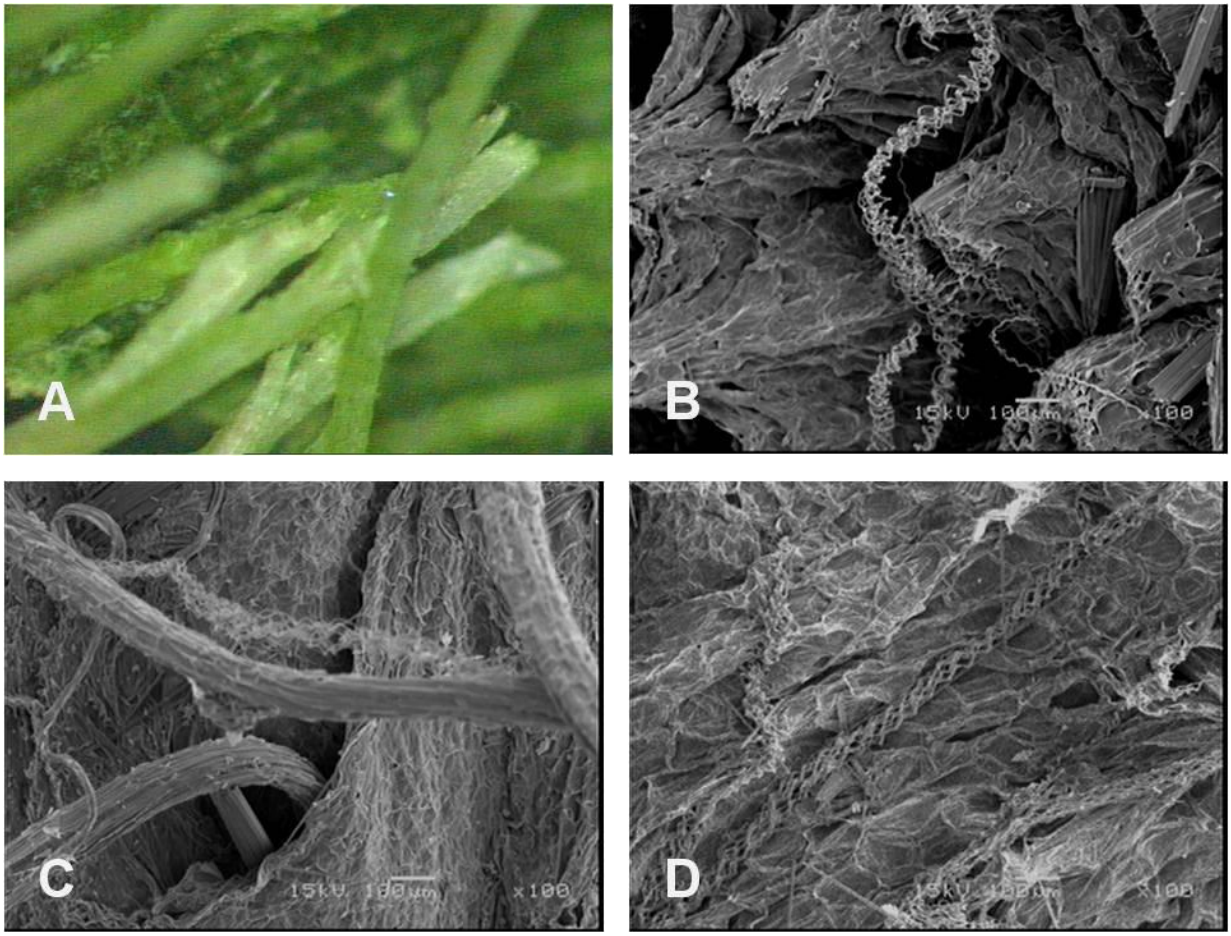

Figure 7. Fibrillar structures in *A.* atrovirens (A) Fibrillar complex observed with LM (B, C and D) Fibrillar complex observed with SEM from *Agave* plants at 3, 6 and 9 years of age, respectively.

even after eight months of drought leading to loss of about half of total leaf water content (Schulte and Nobel, 1989). At three year-old, plant metabolism is active; allowing plant to generate new structures, for this reason hydrenchyma content is reduced. However, when plant reaches physiological maturity (six year-old), there is an increase in hydrenchyma as a protective system under stress conditions, that is why relative proportions of fibrillar tissue and water storage tissue change (Figure 7C) while the number of fibrillar bundles is constant in the leaf (Gorshkova et al., 2003), this difference thus, enables plant to survive long periods of adverse conditions since water requirements are met by hydrenchyma. Similar results were observed at nine year old leaf.

Conclusion

A. atrovirens presents differences during maturity, including ICM thickness, suprastomal cavity area and stomata density, which may function as protective mechanisms allowing water utilization efficiency. In inner cuticle, adhering cell walls are most rigid in the last developmental stage, generating an irregular isolation of the cuticular membrane, and therefore, spandrels are partially covered with an opaque layer of adhering cell walls. With increasing age, there are also differences in calcium oxalate crystals present in plant. This plant contains all three crystal types present in monocotyledons (raphides, styloids and druses), with mainly raphides in *Agave* leaf. With age, reserve structures in *Agave* increase in size and generate differences in the morphology of fibrillar structures, with an increase in hydrenchyma that serve as protective covering during long periods of stress.

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