



Original Article

Changes in the Concentration and Characteristics of Calcium Oxalate Crystals during Development Stages of *Agave atrovirens*

**Bernardino-Nicanor A¹, Teniente-Martínez G¹, Juarez-Goiz JMS, Filardo-Kerstupp S²,
Montañez-Soto JL³, González-Cruz L^{1*}**

¹ Departamento de Ingeniería Bioquímica-Instituto Tecnológico de Celaya –

Av. Tecnológico y A. García Cubas S/N A. P. 57 C. P. 38010, Celaya, Guanajuato, México

² Centro de Investigaciones Químicas de la Universidad Autónoma del Estado de Hidalgo (CIQ-UAEH).
Carretera Pachuca-Tulancingo Km. 6.5 Ciudad Universitaria.

³ Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional del Instituto Politécnico
Nacional, Unidad Michoacán. Justo Sierra N°28 Jiquilpan, Mich. México. C.P. 59510.

*Corresponding Author: Email: lgonzalezcruz@yahoo.com

ABSTRACT

The Agave atrovirens is an important plant in semiarid regions of Mexico that stored high levels of non-structural carbohydrates, however too contain high levels of calcium oxalate crystals which can cause irritation, for this reason the aim of this study was investigate the changes in the calcium oxalate content in leaves of different development stages of Agave atrovirens. The results showed that in Agave atrovirens the raphide crystals are the main form (Type I and III) with length ranged between 340 and 410 µm, the calcium oxalate content is not influenced by the plant age, and however the minerals content is higher in leave of young plants than in leaves of mature plants. The concentration of calcium oxalate in Agave atrovirens is in the range of 6 to 15 mg kg⁻¹ of fresh sample while the mineral content in the leaves are in a concentration of 5.3 to 13.7% in dry basis. The calcium oxalate content is 20% highest in the chlorenchyma than in the parenchyma. In conclusion the leaf positions have longer influence on the calcium oxalate content than the development stage

KEYWORDS: *Agave atrovirens, calcium oxalate, druses, raphides, styloids*

INTRODUCTION

Agave atrovirens, a non-cultivated plant, is the main source of sugars to produce “pulque”, a traditional Mexican alcoholic fermented beverage produced from the sap known as “aguamiel” [1] obtained of the *Agave atrovirens* pine that contain large amounts of non-structural carbohydrates (about 700 g per kg in dry basis) principally sucrose [2] however too contains calcium oxalate crystals, acrid oils, saponins, and other compounds known as irritants [3] that caused the agave worker’s sickness [4]. Sakai et al., [5] found that raphide crystals longer than 180 microns in length caused irritation.

Recent studies with the electron microscope have demonstrated the influence of the age on the types of calcium oxalate crystals being the 6-yrs-old agaves which present all three types of crystals (druses, raphides and styloids) reported for monocotyledons [6]. They are generally formed inside cells (called crystal idioblast) and may have various functions (plant’s need to keep up an ionic equilibrium; to regulate its osmotic pressure). It had been demonstrated on one hand, that oxalic acid is a by-product of various cellular metabolic processes; on the other hand, the plant could hardly survive with elevated amounts of calcium [7], in addition the formation of calcium oxalate crystals in plants may also be linked to the evaporation of water [8].

The difference in total calcium concentration associated with crystal age class is largely a reflection of the means of calcium transport and its lack mobility in the phloem [9]. However, few studies have examined the influence of plant age on the calcium oxalate content for example, has been observed that in the cassava leaf meal the lowest content of calcium oxalate were found in the 12-months old plants whereas that the highest content was found in the 17-months old plant [10], on the other hand in concordance with this results, some investigators found calcium oxalate

increased with plant age, however others reported spinach calcium oxalate concentrations decreased with age [11]. With relation to leaf position Okutani and Sugiyama [12] also found that the higher the leaf position from the plant base, the lower calcium oxalate concentration; in addition Pandey and Kalloo [13] reported that the oxalic acid contents were lower in stems and petioles than in leaf blades.

The present research was conducted to determine the changes in the morphology, length and concentration of calcium oxalate crystals in leaves of *Agave atrovirens* by development stage and rosette position.

MATERIALS AND METHODS

Vegetal material

The leaves of *Agave atrovirens* were collected in the summer of 2007 in Singuilucan, Hidalgo State, Mexico. All samples were taken at 16:00 ± 1h. After separation from the plant, the leaves were cut into approximately 3 cm cubes and 250 g lots were placed in polyvinyl chloride (PVC) bags and stored in a freezer (LG, Model GR-452SH) at -4°C for no more than five days. Leaves were taken from plants with 8-, 9-, 10- and 11-year-old at different leaf position on the plant (outer, middle and inner).

Calcium oxalate extraction

Calcium oxalate was extracted by suspending 3 g of frozen *Agave atrovirens* leaf from each age group in 30 mL of 3N HCl and blended it in an Ultra-Turrax (620 rpm) for 2 min, then turrax-probe was washed into the sample by using 15 mL of 3N HCl, this solution was then centrifuged at 3000 x g for 5 min at 4°C, the supernatant collected in a 75-mL volumetric flask. The extraction procedure was repeated using approximately 24 mL of 3N HCl and the supernatant was poured into the same 75-mL volumetric flask and the volume made up to 75 mL with 3N HCl for analysis [14].

Determination of oxalate

10 mL of calcium oxalate solution were poured into 40-mL plastic centrifuge tubes, and then were added 15 mL of water distilled and 5 mL of tungstophosphoric acid reagent. This solution was mixed thoroughly, and it was allowed to stand for 5 h, was then centrifuged at 3000 x g for 5 min, and supernatant was collected. Then 20 mL of supernatant was poured into a 50-mL conical centrifuge tube and adjusted to pH 4.5 with NH₄OH (12.5% w/v), to this solution was added 5 mL of calcium-containing acetate buffer solution, stirred and stored overnight at room temperature. Was then centrifuged at 3000 x g for 15 min, the precipitate was washed with 20 mL of cold wash liquid (12.5 mL acetic acid, 250 mL distilled water, 5 g calcium oxalate) and 5 mL of H₂SO₄ was added to the precipitate. This solution was titrated (50-60°C) with 0.01N KMnO₄. The oxalic acid content was quantified using the equation [14]:

$$\text{Oxalic acid (mg / 100g product)} = [(\text{mililiter of } 0.01\text{N KMnO}_4 \text{ used} \times 0.45\text{mg} \times (25/10)) / \text{Weigh of sample(g)}] \times 100$$

Where:

0.45 = mg anhydrous oxalic acid (COOH)₂ equivalent to 1 mL of 0.01N KMnO₄.

25/10 is the dilution factor

100 is the conversion factor for oxalic acid content per 100g sample.

Minerals content

Ash content was assayed by mineralization at 550-600°C and weightining the residues following mineralization to constant weight [15].

Scanning Electron Microscopy/Energy Dispersive X-Ray Analysis (SEM/EDX)

The samples were mounted in a double-sided carbon tape and covered with roughly 10 nm of gold in a Denton sputter coater. Parenchyma and chlorenchyma samples were analyzed by SEM/EDX using a JEOL (JEOL, type EX-1200, Japan) scanning electron microscope fitted with a Kevex Si(Li) x-ray detector. The analyses were performed under vacuum at an accelerating voltage of 15 kV.

RESULTS AND DISCUSSION

The calcium oxalate in *Agave atrovirens* leaves are in the concentration range of 6-15 mg·kg⁻¹ of fresh sample (Table 1), leaves of 9-yrs-old plant have the higher concentration of calcium oxalate while the lower concentration was obtained of leaves of 11-yrs-old plant. However the age effects

on the calcium oxalate content are not consistent, due probably to the active metabolism present in *Agave atrovirens* [16].

In relation with the leaf position on the *Agave atrovirens*, apparently the higher concentration it is located in the inner leaves of the basal rosette and decreases as the leaves become mature (middle and outer leaves) (Figure 1). Apparently in the unfolding of new leaves from the central spike of the basal rosette the metabolism of Ca is regulated via the formation of calcium oxalate, for this reason the concentration of calcium oxalate is higher in the central leaves of the basal rosette which will allow the plant to develop the flower stalk.

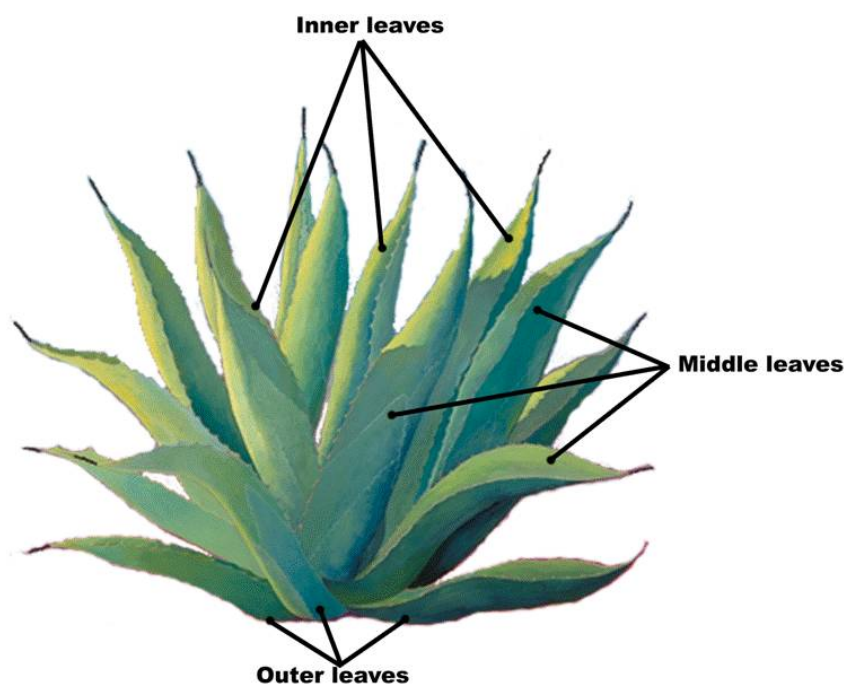


Figure1. Schematic representation of leaves position on basal rosette in *Agave atrovirens*

Table 1: Calcium oxalate concentration in *Agave atrovirens* leaves (mg·kg⁻¹ of fresh sample)

Plant age (years)	Leaf position			
	Outer	Middle	Inner	Central spike
8	13.75±1.20 ^{Aa}	10.06±1.13 ^{Bb}	6.88±0.73 ^{Dc}	ND
9	8.81±0.95 ^{Cc}	9.50±1.05 ^{Cb}	15.38±0.165 ^{Aa}	ND
10	13.44±1.12 ^{Ba}	6.63±0.57 ^{Dc}	11.94±1.25 ^{Bb}	ND
11	6.38±0.54 ^{Dc}	12.56±1.18 ^{Aa}	11.50±1.09 ^{Cb}	8.69±0.92 ^c

ND: Non-development structure. Number followed by the same letter in each row or capital letter in each column are not significantly different ($P < 0.05$) according to Tukey's test

In the chlorenchyma and parenchyma the calcium oxalate content are about 31 and 20 mg·kg⁻¹ of fresh sample respectively (Table 2). In general in all leaves of one plant, the content of calcium oxalate is up to 20-30% in the chlorenchyma; however equilibrium is present between the calcium oxalate content on the chlorenchyma and the parenchyma. The calcium oxalate content of *Agave atrovirens* leaves was higher 30% in the leaf base compared to the content in terminal spike, because probably in this leaf part, metabolic processes of calcium oxalate are implicated as important factor in the creation of new structures, development and maintenance.

Influence of plant age on the chemical composition of minerals in *Agave atrovirens* leaves.

The mineral content of *Agave atrovirens* leaves are presented in the table 3 and show a concentration range of 5.3-13.7 % in dry basis, these data show that, in *Agave atrovirens* the age is a determinant factor on the mineral content, being the 11-yrs-old plant with the lowest values and the young plants (eighth-yrs-old) with the highest values. These results suggest that in the first development stages of the leaves, the plants accumulating high levels of minerals in young leaves

to increase its size and maintenance while mature leaves exhibited a significant decline in minerals content, this may be because at this development stage all nutrients are used for the development and maintenance of flower stalk [17, 18].

The minerals concentration was highest in the chlorenchymatous tissue than in the parenchymatous tissue (Table 4), this can be due to that several factors including the whole growth process, metabolic processes, enzymatic function and controlled changes in lignifications process are regulated in the chlorenchyma [18].

The relation between minerals content and calcium oxalate concentration is influenced by the leaf position and section analyzed, the near section to the pine (section 1) have the highest values of calcium oxalate with relation to the minerals content and decrease with increasing distance from the leaf base. On the other hand, the leaf position on the basal rosette is a determining factor in the relation calcium oxalate-minerals content, having highest proportion of calcium oxalate outer leaves than middle and inner leaves, this pattern as in *Tragia ramosa* have an important role in the protective or adaptative response against predators [19].

Table 2: Calcium oxalate concentration in chlorenchyma and parenchyma of *Agave atrovirens* leaves (mg·kg⁻¹ of fresh sample)

Plant age (years)	Chlorenchyma				Parenchyma			
	Outer	Middle	Inner	Central spike	Outer	Middle	Inner	Central spike
8	14.6 ± 0.0 ^b	7.1 ± 0.6 ^c	19.5 ± 1.3 ^a	ND	14.6 ± 0.0 ^a	7.5 ± 0.7 ^b	6.7 ± 0.0 ^b	ND
9	6.4 ± 0.6 ^c	12.0 ± 0.6 ^b	11.3 ± 1.3 ^a	ND	20.3 ± 0.0 ^a	12.0 ± 0.6 ^b	7.5 ± 0.6 ^c	ND
10	11.3 ± 0.6 ^b	8.3 ± 0.6 ^c	31.1 ± 0.6 ^a	ND	12.8 ± 2.3 ^a	7.5 ± 0.7 ^b	6.8 ± 0.0 ^b	ND
11	10.5 ± 1.1 ^b	11.6 ± 0.6 ^b	14.6 ± 0.0 ^b	20.6 ± 3.3 ^a	6.8 ± 0.0 ^b	6.8 ± 0.0 ^b	8.6 ± 0.6 ^a	9.0 ± 0.0 ^a

ND: Non-development structure. Number followed by the same letter in each row and age are not significantly different (P<0.05) according to Tukey's test

Table 3: Minerals content in *Agave atrovirens* leaves (g·kg⁻¹ fresh sample).

Plant age (years)	Leaf position	Section of the leaf		
		1	2	3
8	Outer	93.80 ± 1.90 ^c	10.16 ± 0.60 ^b	10.42 ± 0.56 ^c
	Middle	100.40 ± 1.10 ^b	10.07 ± 0.67 ^b	11.78 ± 0.45 ^b
	Inner	107.60 ± 3.80 ^a	11.46 ± 0.90 ^a	13.75 ± 2.22 ^a
9	Outer	74.40 ± 2.10 ^b	11.04 ± 1.40 ^a	10.29 ± 1.22 ^b
	Middle	85.80 ± 2.50 ^a	9.95 ± 1.50 ^b	12.85 ± 1.35 ^a
	Inner	83.90 ± 4.40 ^a	9.35 ± 2.5 ^c	10.10 ± 1.60 ^b
10	Outer	72.20 ± 2.60 ^b	9.80 ± 2.44 ^b	10.58 ± 1.02 ^a
	Middle	105.20 ± 6.10 ^a	11.31 ± 0.83 ^a	7.12 ± 0.57 ^b
	Inner	110.60 ± 4.00 ^a	9.84 ± 0.91 ^b	10.95 ± 1.20 ^a
11	Outer	58.90 ± 2.40 ^c	6.78 ± 0.63 ^b	7.12 ± 0.44 ^a
	Middle	72.90 ± 2.10 ^b	7.17 ± 0.95 ^b	8.01 ± 0.40 ^a
	Inner	71.30 ± 3.60 ^b	5.91 ± 0.21 ^c	7.72 ± 0.84 ^a
	Central spike	87.10 ± 2.30 ^a	7.94 ± 0.58 ^a	6.79 ± 0.10 ^a

Number followed by the same letter in each column and age are not significantly different (P<0.05) according to Tukey's test

Table 4: Minerals content in chlorenchyma and parenchyma of *Agave atrovirens* leaves (g·kg⁻¹ of fresh sample)

Plant Age (years)	Chlorenchyma				Parenchyma			
	Outer	Middle	Inner	Central spike	Outer	Middle	Inner	Central spike
8	67.9 ± 5.80 ^c	125.3 ± 15.0 ^a	110.4 ± 9.11 ^b	ND	119.7 ± 9.21 ^a	75.5 ± 6.25 ^c	104.7 ± 10.88 ^b	ND
9	73.7 ± 6.10 ^c	112.5 ± 9.34 ^a	92.8 ± 8.03 ^b	ND	75.0 ± 7.42 ^a	59.1 ± 7.16 ^b	75.0 ± 6.90 ^a	ND
10	81.7 ± 5.00 ^b	107.4 ± 8.69 ^a	108.4 ± 9.15 ^a	ND	62.7 ± 6.35 ^b	102.9 ± 9.62 ^a	112.8 ± 10.22 ^a	ND
11	66.9 ± 4.50 ^b	77.9 ± 6.58 ^{a,b}	75.5 ± 6.42 ^{a,b}	85.9 ± 5.45 ^a	50.9 ± 5.11 ^c	67.8 ± 7.32 ^b	67.1 ± 7.66 ^b	88.2 ± 7.63 ^a

ND: Non-development structure. Number followed by the same letter in each row and plant part are not significantly different (P<0.05) according to Tukey's test

Calcium and calcium oxalate content in *Agave atrovirens* leaves by SEM/EDX

On the other hand using Scanning Electron Microscopy/Energy Dispersive X-Ray Analysis (SEM/EDX) the leaf of the 8- and 9-yr-plant have the higher calcium content in form of calcium oxalate crystals, however in the last development stage during scape elongation and inflorescence development the relation between calcium oxalate form and total calcium decreases. This suggest that during the formation of reproductive structures in *Agave atrovirens* the calcium oxalate is probably laid down in the scape during the formation of the pole and inflorescence.

The Calcium content of the *Agave atrovirens* leaves ranges from 1.70 to 3.94 % of total minerals content (Table 5). It can to observe that the calcium concentration in *Agave atrovirens* is influenced by leaf position in the rosette; the highest values of calcium content were obtained from outer and inner leaves of *Agave atrovirens*. Apparently during rosette development the absorption and fixation of calcium is active in the outer leaves due to that the photosynthetic capacity and efficiency in this position are enhanced, on the other hand, the inner leaves act as reservoirs for calcium oxalate and for this reason highest values are observed.

Morphological characteristics of calcium oxalate crystals

In *Agave atrovirens*, raphides of four-, six- or eight-sides single crystals that have two symmetrical pointed ends were observed and according Horner and Wagner [20] are classified as type I and III

Table 5: Calcium concentration in *Agave atrovirens* leaves (% of total mineral content)

Plant age (years)	Leaf position		
	Outer	Middle	Inner
8	1.75±0.05	2.11±0.15	2.80±0.15
9	1.74±0.08	3.75±0.19	1.70±0.09
10	2.67±0.12	2.12±0.01	3.94±0.21
11	2.03±0.11	3.31±0.14	2.04±0.10

Number followed by the same letter in each row are not significantly different ($P < 0.05$) according to Tukey's test

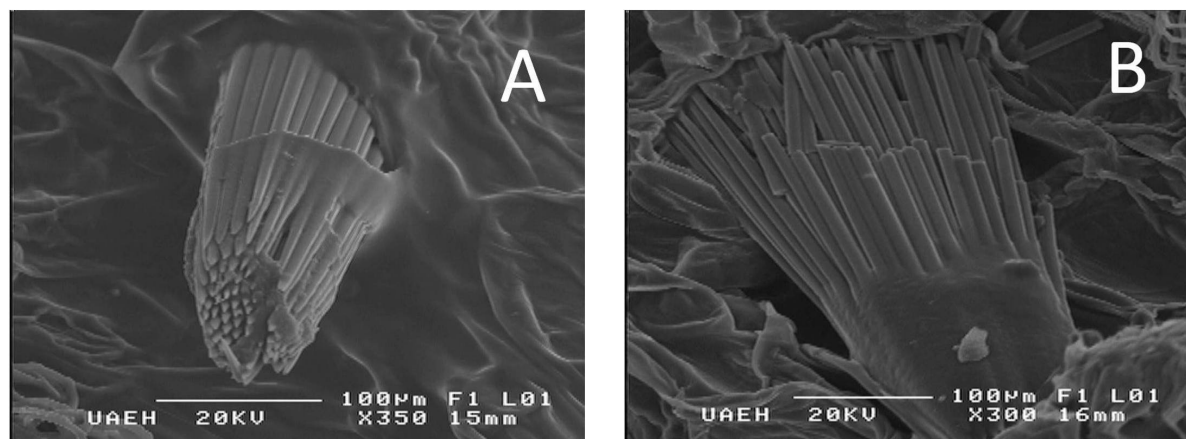


Figure 2. Type of calcium oxalate crystals in *Agave atrovirens*, A) Type I, B) Type III

(Figure 2A; 2B respectively), length of raphides in general ranged from 340 to 410 μm . Results showed that the plant age is determinant for the length of crystals, which were longer in leaves of eight-yrs-old plants (410 μm) than in leaves of 11-yrs-old plants (374 μm). The length of raphide crystals of *Agave atrovirens* is 100% longer in comparison with those obtained from *Agave americana*, and probably and probably for this reason, people who works in pulque industry have more health damage that workers of mezcal or tequila industry.

On the other hand the leaf position too is determinant in the crystal's morphological characteristics because only in the inner leaves of *Agave atrovirens*, crystals idioblasts were observed (Figure 3A). In inner leaves the crystals are in the first development stage and for this reason the raphide crystals are still covered by a protein matrix that enable the leaf to regulate the concentration of oxalic acid and calcium [19]. Apparently during leaf development (middle and outer leaves), the

crystals are released from the protein matrix and allow act as an effective defense against predators (Figure 3B). In the middle leaves the raphide crystals are still pooled, whereas in the outer leaves displayed disaggregation of raphide crystals.

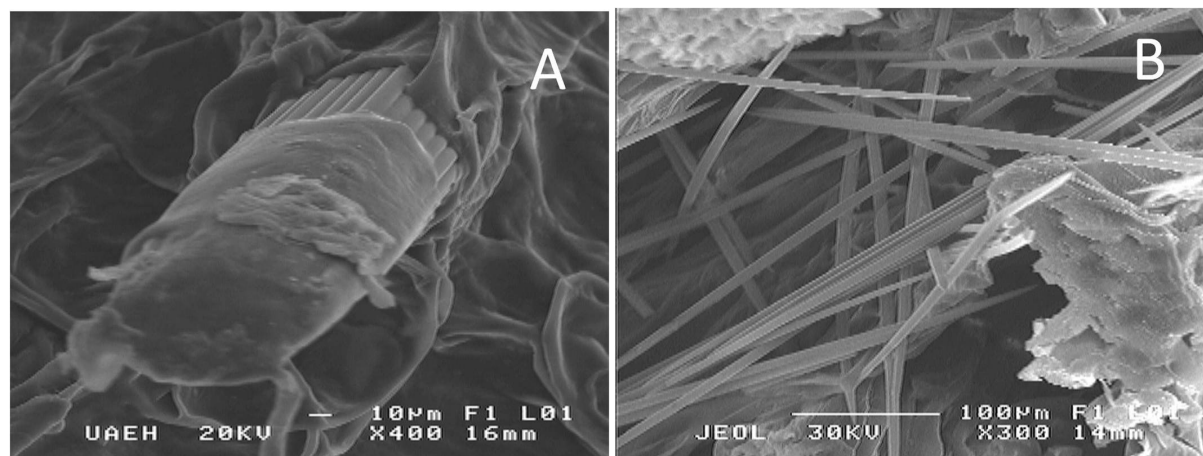


Figure 3. Calcium oxalate crystals in *Agave atrovirens* A) Crystals idioblast, B) Crystals disaggregated.

CONCLUSION

The concentration of calcium oxalate have random pattern by development stage effect in the *Agave atrovirens* plants, however the leaf position is determinant in the calcium oxalate content, apparently the inner leaves have highest concentration than middle and outer leaves. The calcium oxalate content in the chlorenchyma is approximately 20% greater than in parenchyma. Mineral content is greater in young leaves of *Agave atrovirens*, lower in mature plants (11-yrs-old); the chlorenchymatous tissue have highest minerals content than the parenchymatous tissue and the relation between minerals content and calcium oxalate content is influenced by leaf position on the plant, however development stage too have influence on this relation. *Agave atrovirens* have raphide crystals type I and III with length ranged from 340 to 410 μm.

REFERENCES

- Escalante, A., Rodríguez, M. E., Martínez, A., López-Munguia, A., Bolívar, F. & Gosset, G. (2004). Characterization of bacterial diversity in pulque, a traditional Mexican alcoholic fermented beverage, as determined by 16S rDNA analysis. *FEMS Microbiol. Lett.*, 235:273-279.
- González-Cruz, L., Jaramillo-Flores, M. E., Bernardino-Nicanor, A. & Mora-Escobedo, R. (2012). Influence of the harvest age on fructan content and fructosyltransferase activity in *Agave atrovirens* Karw pine. *Int. J. Plant Physiol. Biochem.*, 4(5):110-119.
- Ricks, M. R., Vogel, P. S., Elston, D. M. & Hivnor, C. (1999). Purpuric agave dermatitis. *J. Am. Acad. Dermatol.*, 40(2 II SUPPL.): 356-358.
- Salinas, M. L., Ogura, T. & Soffchi, L. (2001). Irritant contact dermatitis caused by needle-like calcium oxalate crystals, raphides, in *Agave tequilana* among workers in tequila distilleries and agave plantations. *Contact Dermatitis*, 44: 94-96.
- Sakai, W. S., Shiroma, S. S. & Nagao, M. A. (1984). A study of raphide microstructure in relation to irritation. *Scan. Electron Microsc.*, 11:979-986.
- Bernardino-Nicanor, A., Mora-Escobedo, R., Montañez-Soto, J. L., Filardo-Kerstupp, S. & González-Cruz, L. (2012). Microstructural differences in *Agave atrovirens* Karw leaves and pine by age effect. *Afr. J. Agric. Res.*, 7(24):3550-3559.
- Malainine, M. E., Dufresne, A., Dupeyre, D., Vignon, M. R. & Mahrouz, M. (2003). First evidence for the presence of weddellite crystallites in *Opuntia ficus indica* parenchyma. *Z. Naturforsch. C.*, 58:812-816.
- Wu, C. C. & Kuo-Hang, L. L. (1997). Calcium crystals in the leaves of some species of Moraceae. *Bot Bull Acad Sin.*, 38:97-104.
- DeHayes, D. H., Schaberg, P. G., Hawley, G. J., Borer, C. H., Cumming, J. R. & Strimbeck, G. R. (1997). Physiological implications of seasonal variation in membrane-associated calcium in red spruce mesophyll cells. *Tree physiol.*, 17:687-695.
- Wobeto, C., Corrêa, A. D., de Abreu, C. M. P., dos Santos, D. & Pereira, H. V. (2007). Antinutrients in the cassava (*Manihot esculenta* Crantz) leaf powder at three ages of the plant. *Ciênc. Tecnol. Aliment.*, 27(1): 108-112.
- Mou, B. (2008). Evaluation of oxalate concentration in the U. S. spinach germplasm collection. *HortScience*, 43(6):1690-1693.

12. Okutani, I. & Sugiyama, N. (1994). Relationship between oxalate concentration and leaf position in various spinach cultivars. *HortScience*, 29:1019-1021.
13. Pandey, S.C. & Kalloo, G. (1993) Spinach *Spinacia oleracea* L (Eds. Kalloo, G. and Bergh, B. O.) Genetic improvement of vegetable crops. Pergamon Press, Oxford, UK. pp. 325-336.
14. AOAC. (1990). Association of Official Analytical Chemists. Official Methods of Analysis (15th Ed), 974.24, Arlington, Virginia. pp. 993-994
15. Szczesna, T. (2007). Concentration of selected elements in honeybee-collected pollen. *Journal Apicult. Sci.*, 51(1):5-13.
16. Webb, M.A., Cavaletto, J. M., Carpita, N. C., Lopez, L. E. & Arnett, H. J. (1995). The intravacuolar organic matrix associated with calcium oxalate crystals in leaves of *Vitis*. *Plant J.*, 7: 633-648.
17. Franceschi, V. R. (1989). Calcium oxalate formation is a rapid and reversible process in *Lemna minor* L. *Protoplasma*, 148:130-37.
18. Sticher, L., Penel, C. & Greppin, H. (1981). Calcium requirement for the secretion of peroxidases by plant cell suspensions. *J. Cell Sci.*, 48, 345- 353.
19. Franceschi, V. R. & Nakata, P. A. (2005). Calcium Oxalate in Plants: Formation and Function. *Annu. Rev. Plant Biol.*, 56:41-71.
20. Horner, H. T., Jr. & Wagner, B. L. (1995). Calcium oxalate formation in higher plants (Ed. Khan S. R.) Calcium Oxalate in Biological Systems. CRC Press, Boca Raton, p. 53-72.

[Received: 12.10.12; Accepted: 22.11.2012]