Article

Cell changes in avocado leaves with calcium deficiencies

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Abstract

Calcium deficiency causes susceptibility to physiological diseases in fruits and reduced yield, in addition to generating curling, chlorosis and decreased growth of young leaves. In the present work, it was carried out in 2017, where structural and ultrastructural cellular measurements of avocado leaves were obtained, using a scanning and transmission electron microscope respectively and were correlated with the foliar nutritional concentration. It was observed that the thickness of the leaf, the length of the spongy parenchyma plus epidermis and the length and width of parenchyma in palisade did not have significant involvement with the calcium concentration alone, but with the nutritional relationships: BxCa, KxCa, ZnxCa, Mn/Ca and Ca/Mn. The width of the cell wall was affected by the relations with magnesium: Mn/Ca and Ca/Mn.

Keywords: cellular and ultracellular, electron microscopy, nutritional relationships.

Reception date: November 2019
Acceptance date: February 2020

Introduction

Calcium as a divalent cation (Ca^{2+}) is required for various structural roles in cell walls and membranes, it is a counter cation for organic and inorganic anions in the vacuole, and the concentration of cytosolic Ca^{2+} functions as an intracellular messenger coordinating responses to numerous signs of development and environmental changes according to White and Broadley (2003) and serves as a metabolic factor or cofactor in various cellular activities (Ge *et al*., 2007).

According to Ge *et al*. (2007), calcium also participates in the regulation of sexual reproduction in flowers, in the germination and elongation of pollen tubes as well as in cell expansion during the fruit ripening process (Hocking *et al*., 2016). It has been reported that physical and chemical changes in cell walls are associated with pectin modification.

On the other hand, Hocking *et al*. (2016) indicate that chemical changes include depolymerization of pectins and degradation of xyloglycan, which are related to the mechanism of fruit ripening. These physical changes are associated with the continuous modification and solubilization of pectins, determined by the calcium-pectin bonds. In addition, in the absence of the cellulose-xyloglycan network, pectate bonds have a greater participation in cell exchange (Peaucelle *et al*., 2012).

According to Montanaro *et al*. (2006) in post-harvest, apoplastic calcium is important due to its participation in the cell walls of the fruits, which represents between 60-75% of the total calcium of the fruit and that contributes to its post-harvest life (Montanaro *et al*., 2014) . It has been reported that calcium deficiency is related to the required and adequate supply in the growth stage (Álvarez *et al*., 2008). It is also known that applications of post-harvest $CaCl_2$ increases calcium concentration in fruits and delays ripening (Rivera *et al*., 2017).

According to White *et al*. (2013) calcium deficiency in young leaves, storage tissues and fruits can be caused by low perspiration and distribution of calcium transported by mass flow between the tissues, since between 85-93% of the total calcium supply to the plant, it goes directly to the mature leaves and very little calcium to the fruit and other tissues (Álvarez *et al*., 2008; Montanaro *et al*., 2014).

In general, according to White *et al*. (2006) deficiency disorders are attributed to the insufficient mobilization of apoplastic calcium in old tissues and its low redistribution via phloem, in tissues where calcium supply is more via phloem than by xylem. In addition, low perspiration due to inadequate humidity and poor root growth reflects insufficient calcium absorption (Bouzo and Cortez, 2012).

Materials and methods

Morphometric analysis of avocado leaf

Samples of leaves were collected from trees in a Hass avocado orchard in the Tetela municipality of the Volcano, Morelos state, considering as deficiency patterns the degree of curling, the presence of chlorosis and the decrease in leaf growth. Leaf samples were collected with different degrees of

calcium deficiency, determined based on the visual diagnosis a critical deficiency (CaC), leaves with medium calcium deficiency (CaM) and healthy leaves (CaS). The variables thickness of the leaf, spongy parenchyma + epidermis, parenchyma length in palisade and the parenchyma width of palisade were evaluated, for which transmission electron microscopy was used. For its analysis, the Hayat methodology (2000) was followed.

The selected tissues were cut 1 mm^2 parts which were fixed with 6% glutaraldehyde in 0.2M sodium cacodylate buffer pH 7.4 for 24 h. Subsequently, they were washed three times with the 0.05M sodium cacodylate buffer pH 7.4 and postfixed with 2% osmium tetraoxide for 24 h. Then, the samples were washed again with the 0.05M sodium cacodylate buffer pH 7.4 to begin a dehydration sequence with increasing series of anhydrous ethyl alcohol. Then an infiltration with toluene was made and finally, the tissues were included with araldite resin for 24 h at room temperature. Sections of the sections were made with an ultramicrotome at 20 nm at 3 mm s^{-1} with diamond knife, said cuts were contrasted with uranyl acetate and lead citrate. The observation was performed in a transmission electron microscope (MET) of the FEI brand model TECNAI 1 at 120 kV.

Soil sampling. In the orchard, 25 sampling sites were randomly selected, located in the drip areas of the tree. At each sampling site it was extracted with a stainless steel auger, a subsample of 50 g of soil at a depth of 0-30 cm. The collected sample was 1.25 kg of soil, with which a spherical figure was formed that was divided into four parts, discarding two opposite parts, until obtaining 300 g of sample for analysis.

Soil fertility was determined by analysis of the pH 1:2 soil-water ratio with potentiometer, CIC (interchangeable cations extracted with 1 N ammonium acetate pH 7). Nitrogen by the Kjeldahl method, phosphorus by the Bray method, potassium by flame emission spectrophotometry, calcium and magnesium by volumetry (EDTA 0.01 N). Iron, copper, zinc and manganese were extracted and read in an atomic absorption spectrophotometer (Etchevers, 2001), while B was determined by the azometin-H method.

Foliar sampling In 25 randomly selected trees, 4 leaves per tree were collected, located in the fifth position from the apex, healthy, without physical, chemical or biological damage, from well-lit branches, located in the four cardinal points and at an average height of 2 m; from the soil surface, making a total of 100 leaves per sample.

The leaves were placed in paper bags and kept in a cooler until they entered the laboratory, where they were washed, dried at 70 °C until constant weight, ground in a 40 mesh stainless steel mill. 0.5 g of the dry tissue were taken and ground; they were subsequently placed in a Kjeldahl flask with 4 ml of diacid mixture (4:1 sulfuric acid and perchloric acid), plus 2 ml of hydrogen peroxide (30% hydrogen peroxide) to accelerate the reaction and then the flask was placed in a Lindenberg SB digestion oven at 260 °C, until a clear and crystalline extract was obtained which was added to 50 mL with distilled water.

The extract was analyzed by determining the nutrients by the following methods: nitrogen by the Khejdahl method, phosphorus by vanadate-molybdate (yellow) photocolorimetry read on Spectronic 20 spectrophotometer, potassium by flamometry read on Corning flamometer model 410, calcium, magnesium, copper, Iron, zinc and manganese were determined on an atomic absorption spectrophotometer and boron was determined by the method of azometin-H.

Kenworthy balance indices

The results of the foliar analyzes were interpreted using the Kenworthy technique using standards proposed by Benton *et al*. (1991) and the coefficients of variation established by Maldonado (1999). The diagnosis for each treatment established as deficient values from 17 to 50, below normal with 50 to 83, normal from 83 to 117, above normal from 117 to 150 and excess from 150 to 183 (Kenworthy, 1967).

Diagnostics using the DRIS technique

For the diagnosis using the integrated diagnostic and recommendation system (DRIS), the reference values proposed by Beverly *et al*. (1984).

Nutritional relationships and their relationship with leaf morphological variables

With the data of the soil and leaf analyzes, DRIS and Kenworthy, the nutritional relationships between all the elements were obtained and subsequently related to the morphological variables leaf thickness, parenchyma in spongy+epidermis, parenchyma length in palisade and the parenchyma width in palisade.

Ultrastructural analysis in avocado leaves

In tissue samples of a cross section in avocado leaves, ultrastructural analysis was performed by X-ray spectroscopy and scanning electron microscopy. In the same way as in the samples for transmission electron microscopy, the samples followed the same processing until post-fixation with 2% osmium. The samples after post-fixation and after three washes with the 0.05 M sodium cacodylate buffer pH 7.4, were mounted in stubs for high vacuum observation in a scanning electron microscope brand FEI model Quanta 450 at a voltage of 25 kV and an emission current of 90-105 µA.

The X-ray energy dispersion spectrometry analysis was performed using an Edax smart insight detector from the Ametek company coupled to the scanning electron microscope. The voltage used was 30 kV at 10 (mm) WD.

Analysis of data

The cellular dimensions and nutritional concentrations obtained were processed by SAS and Microsoft Excel using 5% significance in the Pearson correlation tests.

Results and discussion

Morphometric analysis of avocado leaf

The uniformity in the thickness of the leaf, the boundary between one type of cell and another, density of trichomes, uniformity in the arrangement and distribution of the cells, curvature of the leaf, uniformity in the size of the same type of cells and clarity to observe the distribution and cell type, they degeneratively manifested themselves, from the healthy leaf to the leaf with critical calcium deficiency. This is attributed to the structural role that calcium has in cell walls and in the membrane, in addition to its collaboration in maintaining the stability and integrity of the cell (White *et al*., 2017). For its part, the thickness of the sheet shown in Table 1, in all treatments was less than reported by González *et al*. (2011) with Hass avocado with 221.03 µm and by Morales *et al*. (1992) with 'Fuerte' avocado leaf samples taken in the sun with 257.06 µm and in the shade with 154.31 µm.

Table 1. Mean and coefficient of variation of the morphometry analysis in the cross-section.

Parameter (µm)	Treatment	Mean (µm)	CV (%)
Leaf thickness	CaS	114.62 ±12	7.7
	CaM	89.62 ±5.48	4.36
	CaC	111.85 ±26.92	17.69
Parenchyma in spongy + epidermis	CaS	67.17 ±9.47	9.73
	CaM	49.85 ±6.97	9.44
	CaC	54.25 ±5.69	6.89
Parenchyma length in palisade	CaS	48.87 ±5.58	7.04
	CaM	42.17 ±4.22	6.4
	CaC	58.95 ±21.84	22.49
Parenchyma width in palisade	CaS	8.44 ±1.31	10.95
	CaM	8.18 ±0.44	3.05
	CaC	8.75 ±1.32	11.39

CaC= leaf with calcium deficiency; CaM= with medium calcium deficiency; CaS= healthy leaf.

The parenchyma length in palisade was presented on the leaf with calcium deficiencies, which may be due to the lack of calcium stimulates cell expansion due to an increase in cell wall fluidity (Chebli and Geitmann, 2017). The length of the spongy parenchyma and adding the length of the epidermis did not approach the reported values of 75.71 µm and the variability of the data collected was greater in the healthy leaf than the deficient one (González *et al*., 2011).

Foliar and soil analysis

In Table 2 it is observed that for an acidic soil the concentration of phosphorus and potassium that were very high in relation to the calcium and magnesium contents, thus presenting an imbalance as interchangeable bases, which is attributed to fertilizer applications considering only NPK, conventional fertilization (Horneck *et al*., 2011). This imbalance in the

exchangeable bases can be seen with the Ca, Mg and K ratios, where the Ca/Mg ratio, despite being low, remains in the ideal range, while both potassium ratios are well below the proportion of optimal cations (PPI, 1988).

Table 2. Analysis of the diagnosis of soil fertility.

Parameter (mg kg^{-1})		Nutritional classification	Exchangeable bases	
Inorganic nitrogen	28	Medium	Calcium	11.91 (%)
Phosphorus	25.38	High	Magnesium	3.5 (%)
Potassium	502.56	Excess	Potassium	4.69 (%)
Calcium	655.32	Low	Sodium	0.07 (%)
Magnesium	116.8	Medium	Ca/Mg	3.41 meq/100 g
Sodium	4.74	Deficient	Ca/K	2.54 meq/100 g
Iron	71.87	Excess	Mg/K	0.746 meq/100 g
Manganese	10.43	Low		
Zinc	1.62	Low		
Copper	3.09	Excess		
Boron	0.77	Low		
ρb	1.11 g cm^{-3}			
Organic material	4.03%			
pH	5.25			
CIC	27.5 meq/100 g			

ρb= bulk density; CIC= cation exchange capacity.

The apparent density is within the range reported in andosols, while the content of organic matter despite being in the range reported, is moderately low to its potential (Alcalá *et al*., 2009). The cation exchange capacity is high (Cottenie, 1980).

Given the low pH value, it is considered an acidic soil, which favors the low calcium content and the excesses of iron and copper. Where excess copper is attributed, in addition to low pH, to the applications of Cu compounds as a fungicide (Maldonado *et al*., 2007; Molano, 2015).

Integrated diagnostic and recommendation system (DRIS) and Kenworthy

Through the DRIS analysis, a relationship and interpretation of the nutrient concentrations in the avocado leaves was obtained. According to the data in Table 3, in all three treatments there was the same trend in concentration and nutritional requirement, with phosphorus being the nutrient of greatest requirement followed by calcium, because both are at very poor levels in the plant, contrary as reported by Maldonado *et al*. (2007) with Kenworthy balance indices, where the concentration of calcium and phosphorus was high in the leaves. On the other hand, nitrogen, magnesium, boron and manganese were reported as normal, being that with DRIS they were found to be deficient.

Table 3. Analysis and foliar nutritional diagnosis.

Treatment		Leaf composition (%)					Leaf composition (mg kg^{-1})				
		N	P	K^{+}	Ca^{+2}	Mg^{+2}	Fe^{+2+3}	Mn^{+2}	Zn^{+2}	Cu^{+2}	B
CaS	C	0.88	0.02	0.86	0.27	0.26	131.23	61.4	38.9	58.03	50.27
	IB	-104.09	-661.12	58.06	-123.01	-329.74	2.16	-180.74	75.82		
	Nutrition requirement order										
	P> Ca> Mg> N> Mn> K> Fe> Zn										
CaM	C	0.93	0.03	0.95	0.26	0.22	130.6	54.63	47.97	74.2	40.09
	IB	-164.27	-495.75	26.04	-207.22	-246.21	22.39	-141.65	61.29		
	Nutrition requirement order										
	P> Ca> N> Mg> Mn> Fe> K> Zn										
CaC	C	1	0.03	0.91	0.26	0.24	118.23	69.7	44.8	71.5	51.03
	IB	-227.93	-513.73	-13.58	-503.33	-166.42	30.12	-87.2	28.41		
	Nutrition requirement order										
	P> Ca> N> Mg> Mn> K> Zn> Fe										

CaC= leaf with calcium deficiency; CaM= with average calcium deficiency; CaS= healthy leaf; C= concentration in the leaf; IB= DRIS index.

On the other hand, zinc and potassium were determined at an optimum level in DRIS, while what was reported in the Kenworthy indices indicated that zinc was the most deficient nutriment in avocado orchards in Michoacán (Maldonado *et al*., 2007).

This shows that, in addition to be an orchard with a severe nutritional imbalance, calcium deficiencies were not manifested in the visual symptoms of the culture during sampling. Because the symptomatology due to calcium deficiencies does not have an immediate occurrence in plant tissues (Kong *et al*., 2014), since calcium, being a structural element, manifests its absence in the new leaves that begin with its structural constitution and that they are of low perspiration, contrary to mature leaves, which have already culminated their growth stage and have a higher transpiration rate (White and Broadley, 2003; Meagy *et al*., 2013); however, the reduction in calcium concentration occurs throughout the plant at the time of presenting a deficiency but does not manifest itself uniformly in it.

Relating the analysis of soil and foliar, a concordance with the deficiency of calcium in the soil and in the plant is observed; however, in the case of phosphorus, its content in the soil is reported high while in the plant it is deficient and the one with the highest requirement according to the DRIS indices, which is attributed to the phosphorus being absorbed by the plant through the radical hairs and the root tip (Fernández, 2007), which is severely affected by calcium deficiency that reduces meristematic points in the plant due to its function in cell division (Ge *et al*., 2007; Hepler and Winship, 2010; Wei *et al*., 2015). In addition, it has been observed that calcium participates as a stimulant in phosphorus absorption (Fernández, 2007).

Nutritional relationships

The significant nutritional relationships for the parenchyma length in palisade are presented in Tables 4 and 5. The important relationships were: N/B, K/B, Ca/B, Mg/B, Fe/B, Zn/B, Cu/B, B/Cu and BxCa. While for the width of the fabric they were: N/B, K/B, Ca/B, Mg/B, Fe/B, Zn/B, Cu/B,

B/Cu and BxCa, as you can see, the participation boron, calcium and zinc were the most recurrent, with boron being the main participant. Individually, boron reported a significant correlation in the parenchyma length while zinc was the significant nutrient for the width.

Table 4. Significant Pearson correlations between nutritional relationships and avocado leaf morphometry.

Parameter	Fe/Mn	N/B	Fe/B	Zn/B	Cu/B	KxFe	Fe/Mg
Length Pem (µm)	**-0.69644^{Z}**	**-0.6684**	**-0.66733**	**0.69338**	**0.70864**		
	0.0119^{Y}	0.0175	0.0177	0.0124	0.0099		
Width Pem (µm)	**-0.85483**	**-0.64281**	**-0.66063**	**-0.68916**	**0.63386**	**-0.74854**	**-0.57932**
	0.0004	0.0242	0.0194	0.0132	0.0269	0.0051	0.0484
Width P.C. (nm)	**0.59046**					**0.79368**	**0.64336**
	0.0432					0.0021	0.024

Z= Pearson's correlation coefficient; Y= Level of significance; correlations in bold= $p \leq 0.05$ Pem= palisade parenchyma; PC= cell wall; µm: microns; nm: nanometers.

For the length of the parenchyma only the Zn/B and Cu/B ratios were directly proportional, the rest of them were inverse. Similarly, in the case of parenchyma width only in a Cu/B ratio, increasing tissue width increases, the rest of the relationships were reversed to this parameter.

The width of the cell wall, only with iron, reported a significant correlation; however, jointly, KxFe, Mn/Ca, Fe/Mg, Fe/Mn, ZnxFe, Ca/Mn were identified with an acceptable level of significance. Contrary to the parenchyma width in palisade, in the width of the cell wall only the Mn/Ca ratio was inverse.

For the thickness of the sheet, the data are shown in Table 5, where the related elements: N/B, Fe/B, B/Cu and BxCa were representative, while individually they did not show significance, in addition, B/Cu and BxCa were inverse relationships.

Table 5. Significance of Pearson's correlations between nutritional relationships and avocado leaf morphometry.

Parameter	P/B	K/B	Ca/B	Mg/B	N/Ca	B/Ca	ZnxK	ZnxCa	Mn/Ca	Ca/Mn	ZnxFe
Length Pem (µm)	**-0.594^{Z}**	**-0.588**	**-0.639**	**-0.675**							
	0.0415^{Y}	0.0445	0.0254	0.0159							
Width Pem (µm)					**-0.641**	**-0.549**	**-0.68**	**-0.557**			
					0.0247	0.0648	0.015	0.0598			
Width PC (nm)									**-0.586**	**0.6634**	**0.7164**
									0.0452	0.0187	0.0088

Z= Pearson's Correlation Coefficient; Y= Level of significance; correlations in bold= $p \leq 0.05$; Pem: palisade palisade; µm: microns; nm: nanometers.

Table 5. Significance of Pearson's correlations between nutritional relationships and avocado leaf morphometry (continuation).

Parameter (µm)	N/B	B/Cu	BxCa	Fe/B	NxMg	Zn/N	KxCa	KxFe	K/B	K/N	N/Ca	B/Ca
Leaf thickness	**-0.666**[Z]	**0.57**	**0.624**	**-0.684**								
	0.018[Y]	0.053	0.03	0.014								
Length Pes + EP				**-0.595**	**0.665**	**-0.574**	**-0.609**	**-0.729**	**-0.562**	**-0.632**	**0.674**	**0.628**
				0.041	0.018	0.051	0.035	0.007	0.057	0.027	0.016	0.029

Z= Pearson's correlation coefficient; Y= level of significance; correlations in bold= $p \leq 0.05$; Pes= spongy parenchyma; EP= epidermis; µm= microns.

For the length of the sponge parenchyma + epidermis the significant relationships were: K/N, NxMg, Zn/N, KxCa, KxFe, K/B, Fe/B, N/Ca and B/Ca. Individually, boron, potassium and nitrogen also represented an important degree of significance over tissue length measurement. On the other hand, calcium, iron, zinc and magnesium were not significant individually, but when interacting with other nutrients they manifested their significance. Only the NxMg, N/Ca and B/Ca ratios were directly proportional.

Ultrastructural analysis in avocado leaves

Table 6 shows that the highest percentage of calcium abundance detected was in the leaves with medium calcium deficiency, then the healthy leaves and finally the deficient leaves, the healthy leaves being the ones with the greatest variability and those with the highest percentages. The highest calcium demand is found in meristematic areas, so that calcium deficiencies affect young leaves that have not completed their growth stage, affecting their structure, contrary to what happens in mature leaves (Hepler and Winship, 2010; Wei *et al*., 2015).

The thickest cell walls were those present in leaves with average calcium levels, then the deficient leaves and finally, the healthy leaves that had the lowest thickness.

Table 6. Dispersion analysis of cell wall width (nm) in palisade parenchyma.

Treatment	Mean (nm)	Standard deviation	Coefficient of variation (%)
CaS	132.38 ±42.05	48.64	36.77
CaM	351.92 ±192.28	141.15	40.11
CaC	297.13 ±82.4	88.95	29.94

CaC= leaf with calcium deficiency; CaM= with an average level of calcium; CaS= healthy leaf.

Changes in cell wall integrity probably result from cellulose activities, where cell cohesion is decreased due to cell mismatch and pectin degradation (Tesfay and Magwaza, 2017). The absence of calcium stimulates cell expansion due to an increase in cell wall fluidity caused by the demethyl esterification of pectins, in addition, the importance of calcium also lies in the stability of pectins since their physical properties are associated with their bond with calcium (Chebli and Geitmann, 2017).

The firmness of the cell wall affects the post-harvest quality of the avocado, since it has been observed that the softness of the fruit is due to a weak structure in the cell wall, in addition to a loss in the integrity of the membrane, cellulose hydrolysis and hemicelluloses, as well as depolymerization of pectins and starch (Tesfay and Magwaza, 2017).

Conclusions

The nutritional status of the soil was related to the foliar diagnosis of the avocado crop, since the acidic pH, the low levels of Ca, Mn, Zn and B, and the excesses of K, Fe and Cu were related to a nutritional imbalance in plant. In addition, the concentrations of N, K, Zn and B, were significant to the morphological changes of leaves, being N and B directly proportional, while K and Zn were inverse to the measures of cell morphology, but no nutritional concentration was significant over the width of the cell wall except for Fe.

The concentration of Ca did not show significant differences with the cell wall width and was not related to the symptomatology due to deficiency manifested in the leaves, since in all the treatments a poor concentration was reported and, after phosphorus, it was the second most nutrient required by the plant based on the DRIS diagnosis.

When relating Ca to N, K, Mn, Zn and B, it was significantly different in the morphology measurements of the leaf, while only Mn had significance in the thickness of the cell wall. While the nutritional relationships with significance in morphology dimensions were: N/B, B/Cu, BxCa, Fe/B, NXMg, Zn/N, KxCa, KxFe, K/B, K/N, N/Ca, B/Ca, P/B, Ca/B, Mg/B, ZnxK, ZnxCa, Mn/Ca, Ca/Mn. In the case of the cell wall the significant relationships were Mn/Ca, Ca/Mn and ZnxFe.

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