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EFFECT OF AGAVE SAP ON OPUNTIA SPP. PLANT PROPAGATION

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To find a possible *Agave* honey biotechnological alternative, it was used as a supplement in a MS medium and the effect was analyzed under *in vitro* and *ex vitro* conditions on *Opuntia* genotypes. More interestingly, the treatment at 25% (MS) concentration supplemented with 15 g/L *Agave* honey was responsible for higher root induction (6.70 cm) in *Chicle* genotype, while 50% of MS medium with 20 g/L induced red pigmentation in tissues of *Xoconostle blanco* genotype, which were analyzed by Raman spectroscopy, showed a signal for β-carotene (α-carotene, 2.18 and β-carotene, 31.58 µg/100 g, respectively for the best treatment). After in the *in situ* conditions it was found that the substrate complemented with *Agave* honey and pH 6.5 presented a similar effect (plant height, 16.30 cm in a period of 180 days) in *Opuntia* plant growth when a commercial fertilizer was used (N-P-K 17% and pH 7.5). Additionally, the substrate complemented with 30 g/l of *Agave* honey and pH 7.5 resulted in higher content of protein (11.7 ± 0.03%) in tissue. The results obtained indicate that this byproduct can be used to lead the synthesis of compounds of nutritional interest in *Opuntia* genotypes under controlled conditions.
Keywords: *Agave* honey, *Opuntia*, culture *in vitro, ex vitro, Xoconostle blanco*.

INTRODUCTION

One of the variants within the *in vitro* culture, is the addition of organic compounds to the basal culture medium (Hicks, 2009), for example banana pulp and coconut water are some of the most used in micropropagation of orchids due to high content of sugars, amino acids, antioxidants, minerals, organic acids and growth promoting agents, such as cytokinins, zeatins, kinetins and purines (Arditti, 1993; Yong *et al.*, 2009). *Agave* sap is better known as *Agave* honey (sugared water; honey-water), is obtained from *Agave* spp plants (*A. salmiana, A. mapisaga, A. atrovirens*). It contains more amount of micronutrients such as iron (2.15 mg/100 g), zinc (1.41 mg/100 g), iodine, vitamin A, vitamin C, niacin, riboflavin, vitamin B-6 and vitamin E (Makola *et al.*, 2003; Silos-Espino *et al.*, 2007), carbohydrates (sucrose, glucose, fructose, inulin) and proteins (Martinez, 1999; Cruz *et al.*, 2006; Estrada-Luna, 2008; Ortiz-Basurto *et al.*, 2008). If *Agave* sap exposed in a constant firing and stirring process to concentrate about 10 times, the content produces a viscous solution known as *Agave* sap, which could have a high potential and be an alternative for biotechnological application. Additionally, the *Opuntia* plants contain different components of high nutritional value, which require their synthesis in sustainable production systems; therefore, tissue culture can be a viable alternative. One of these components are the betalains, which can be used in food industry and besides it has been attributed antioxidant bioactivity and cancer prevention (Tanaka *et al.*, 2008; Gandía-Herrera and García-Carmona, 2013). Betalains are pigments derived from betalamic acid, betaxanthins (yellow) and betacyanins (red) will be present depending on the united components in this structure. Betalains stability is affected by temperature, pH, oxygen, light, and aqueous activity (Reynoso *et al.*, 1997; Dehbi *et al.*, 2014). Some *Opuntia* genotypes have important pharmaceutical properties such as antioxidant, anti-cancer, anti-lipidemic and antimicrobial activity like *O. joconostle* (Pimienta-Barrios *et al.*, 2008) and *O. dillenii* (Shirazinia *et al.*, 2019). Considering *Agave* sap; an important source of nutrients, a study was conducted to evaluate its effect on propagation under *in vitro* and *ex vitro* conditions and to analyze some nutritionally important value components of *Opuntia* spp. The main objective of the present study was to determine an application for *Agave* honey as a complement in the synthesis of pigments in *Opuntia* genotypes.

MATERIALS AND METHODS

***Agave* sap:** To determine the use of *Agave* sap as a supplement in plant propagation under *in vitro* conditions, a liquid chromatography of the content of carbohydrates was performed (AOAC, 2005).

Opuntia genotypes established under in vitro conditions: For *in vitro* growth analysis of genotypes like *Copena,*

Vaquera, Amarilla Zacatecas, Xoconostle blanco, Cardón, Rojo pelón, Pico chulo and *Chicle*. Murashige and Skoog (1962) was used as a base culture medium in concentrations of 25, 50 and 100%, as a source of carbon 30 g/l of sucrose and *Agave* sap was used in quantities of 5,10,15 and 20 g/l. No growth regulator was added. The pH of the medium was adjusted to 6.5 and then 7 g/l (Sigma R) of agar was added, placed in glass flasks with 20 ml per flask and sterilized in an autoclave at 121°C for 20 min. The *Opuntia* stems cultivated *in vitro* were cut in small segments (approx. 1.0 cm) without apex in order to determine a better response in the growing of new shoot, four explants were established per flask with 5 replicates per treatment, then placed at incubation room with temperature of 25 °C, photoperiod 16 hours light and average photon flux density of 54 $\mu molm^{-2}s^{-1}$.In general recent research show an increased interest for cactus propagation and establishment however a universal protocol is not available yet because most plant responses to tissue culture are highly dependent on the genotype (Estrada-Luna *et al.*, 2008).

Synthesis of pigments under in vitro conditions: *Agave* honey was used in a proportion of 10, 15, 20, 25 and 30 g/l, agar (6 g/l) and a pH of 6.5 (defined previously).

Extraction and quantification of betalains: *Opuntia* tissues (*Xoconostle blanco, Cristalina, Pico chulo* and *Cardón* genotypes) with presence of red pigments were selected and analyzed following Castellanos and Yahia (2008) : 5 g of fresh pulp was weighed and milled and 40 ml of 80% (v/v) methanol was added and filtered through filter cloth, collecting the filtrate in a 50 ml volumetric flask (previously covered with aluminum foil). After, 80% of methanol was taken up, 10 ml were taken, centrifuged at 3000 rpm for 10 min, the supernatant was recovered and filtered through Whatman number 4 paper, in the samples the absorption spectrum was measured in a spectrophotometer (SPECTRONIC 20D from Milton Roy Company) in a range of 400 to 600 nm. The content of betacyanins and betaxanthins was determined by measuring the absorbance at 538 and 483 nm respectively, as the control 80% methanol solution was used. The relevant dilutions were made to obtain the absorbance value of fewer than 0.6 units.

***Pigments determination*:** The content of beta cyanins and betaxanthins was quantified as described by Castellanos and Yahia (2008) the absorbance of betalain extracts at 538 and 483 nm in a spectrophotometer Spectron 20D from Milton Roy Company. For the conversion of the absorption units to the concentration units, the expression: $B\,(mg/g) = (A.FD.PM.V)\,/\,(\varepsilon.P.L)$ was used, where A is absorbance at 538 nm for betacyanins and 483 nm for betaxanthine, FD is dilution factor at the time of reading in the spectrophotometer, PM is molecular weight (betanin = 550 g/mol or indicaxanthine 308 g/mol), V is extracted volume, εx is molar extinction coefficient (60,000 L / mol.cm for betanin, 4.8000 L / mol.cm For betaxanthines), L is cell length (1 cm) and P is sample weight.

***Opuntia genotypes established under greenhouse conditions*:** The *Opuntia* plants *Xoconostle blanco, Cristalina, Pico chulo* and *Cardón* from *in vitro* culture were established under greenhouse conditions on fine sand substrate and *Prosopis* soil (1:1 ratio) with different nutrient solutions and pH as indicated below: I; 30 g/l *Agave* sap and pH 6, II; 30 g/l *Agave* sap with pH 6.5, III; Fertilizer Triple 17 with pH 6.0, IV; Fertilizer Triple 17 with pH 6.5, V; Fertilizer Triple 17 with pH 7.0, VI; Fertilizer Triple 17 with pH 7.5 VII; 30 g/l *Agave* sap plus tap water (pH 8.3). The Nutrients and carbon sources were applied twice a week in a solution of 10 ml per plant. Fertilizer Triple 17 was used in a ratio of 3 g/l. At 150 days were determined the morphological variables and qualities of best appearance. In the contrasting genotypes, its proximal composition, minerals, and content of α and β-carotenes (two samples of *Opuntia* plants) were determined. For the above, the edible part of whole *Opuntia* plants was cut off and ground to perform the analysis. The residual moisture content, fat and inorganic matter (ash) was determined by gravimetry (ASTM-F2103-18, 2018). The total elemental nitrogen in the sample was determined by the micro-Kjeldahl method. The content of α and β-carotenes was determined by high-performance chromatography (HPLC) following the technique described by Mejia *et al.* (1988) once milled the sample was treated with tetrahydrofuran (THF), homogenized and filtered. A volume of the supernatant was measured and injected into an Agilent 1260 HPLC-PDA (λ = 460 nm) HPLC equipment using a C18 microsorb column of 4.6 mm diameter, 10 cm length and 3 μm inner layer. The mobile phase was 58% acetonitrile, 35% methanol, and 7% THF 260 °C. External standards were used for quantification. For mineral content was used a Varian model DUO AA 240 FS, with a vapor generator for hydrides and mercury model VGA 77. For the determination of Calcium (Ca) and Magnesium (Mg) were measured in the flame of acetylene with nitrous oxide. For the determination of Zinc (Zn), the diluted hydrolyzed sample was used and measured with acetylene flame. For Potassium (K) the samples were diluted in lithium Chloride (LiCl) to 0.216 N, measuring them by the flame of acetylene. The equipment was calibrated before each measurement with a standard curve for each metal.

***Opuntia tissue microscopy*:** *Xoconostle blanco* and *Cristalina* genotypes from greenhouse cultivation with presence and/or absence of pigments were cut (portions of 0.5 cm of amplitude and length by 1 mm of thickness) from tissue with a scalpel, preferably from the immediate portion to the epidermis. The Raman spectra of the samples were obtained by placing them onto an aluminum substrate and then under a DMLM microscope (Leica) integrated to the Raman system (Renishaw 1000B, approximated resolution of 2 cm^{-1}). The Raman system was calibrated with a silicon semiconductor using the Raman peak at 520 cm^{-1}. The excitation wavelength

was 830 nm, and the laser beam was focused (spot size of approximately 2 μm on the surface of the sample with a 50X objective. The laser power irradiation over the samples was approximately 10 mW. Measurements were made at several points in each selected sample, and 60 seconds integration time, although only a representative spectrum is shown.

***Data analysis*:** An experimental complete block design was used at random with 10 repetitions and 15 treatments. Analysis of variance was applied to the results of each test and separation of media according to the Tukey (0.05) test. For some data analysis of the different experiments, the average was preferably used and some tangible evidence was confirmed by microscopic observations.

RESULTS AND DISCUSSION

***Agave sap (Agave honey) composition*:** The carbohydrate composition was: fructose (11.93 g/l), glucose (15.07 g/l) and sucrose (23.55 g/l) in 100 g fresh weight.

***Morphological Opuntia response in different culture media*:** In general, all explants showed null explant growth and little response in shoot growth, however in root formation the response was significant for some treatments (Table 1 and Figure 1a), the best medium was MS 25% (1962) supplemented with 15 g/l of *Agave* sap (6.70 cm roots/explant of the *Chicle* genotype), which was superior to the 100% and 50% MS supplemented with 30 g/l of sucrose in *Copena* genotype and *Vaquera*, respectively. The medium devoid of mineral salts and supplemented with 25 g/l of *Agave* honey generated vigorous roots, which is useful for *ex vitro* establishment. In general, there was a shoot per explant. The low vigor plant found in culture media supplemented with 10 g/l of *Agave* sap (1.20 cm per explant) and the poor morphological response of the explants is attributed to the lack of growth regulators and the limited amount of minerals and *Agave* sap. In spite of the above, it is feasible to decrease the amount of MS and supplement with *Agave* sap to induce rooting. In an outstanding way in a pair of treatments, it was found that the pH adjusted to 6.5 induces the synthesis of red pigments in tissues and roots (Figure 1b), for which it was decided to perform another experiment to find the inducing possible agent. Differences in color among in red prickly pears may be due apart from the red betanin pigment concentration, to another pigments concentration as indicaxanthin. Orange-yellow pigment was also present in red prickly pears but in less amount (Butera *et al.*, 2002; Díaz *et al.*, 2006).

***Opuntia explants pigmented through Agave honey*:** *Agave* honey at 30 and 25 g/l and pH of 6.5 induced a red pigmentation and a morphological response variable to the number of explants with 1.60 and 5.2 cm root in *Xoconostle blanco* and *Cristalina* genotype, respectably (Table 2). The culture medium containing 30 g/l showed vigorous explants and root formation; however, no shoots were present. In the culture medium with 25 g/l of *Agave* honey, three roots were observed with great vigor in the explants, it was also observed in the culture medium supplemented with 20 g/l *Agave* honey that showed a good rooting generated but without shoots and only with slight callosity at the base of the explant as well as the previous one. A concentration higher or lower than 25 g/l *Agave* honey causes discoloration, necrosis, and death in *Opuntia* explants. For the above mentioned we consider that red pigmentation was induced by stress condition therefore, this process is feasible of its manipulation. The genotypes *Xoconostle blanco* and *Cristalina* (Figure 1b) the red

Table 1. Morphological response of *Opuntia* genotypes on varying concentrations of MS, (1962) media and carbon sources at 60 days of *in vitro* culture

Treatment	MS (1962) (%)	Carbohydrate source (g/l)	Root		Shoots	Genotypes
			No. explant	No. Length (cm)	No. / explant	
1	100	30[a]	6.10 ± (1.9) ab	1.67 ± (0.42) abc	1.00 ± (0.01) ab	*Copena*
2	50	30[a]	6.00 ± (3.8) ab	1.70 ± (0.43) abc	1.20 ± (0.42) a	*Vaquera*
3	50	5[b]	3.20 ± (1.5) a-d	1.43 ± (0.45) a-d	0.90 ± (0.31) abc	*Amarilla Zac.*
4	50	10[b]	1.50 ± (1.4) cd	0.39 ± (0.37) f	0.60 ± (0.51) a-de	*X. blanco*
5	50	15[b]	2.20 ± (0.8) bcd	1.75 ± (0.95) ab	0.10 ± (0.31) de	*Amarilla Zac.*
6	50	20[b]	5.70 ± (4.4) abc	0.84 ± (1.90) c-f	0.70 ± (0.94) a-d	*Cardón*
7	25	5[b]	3.50 ± (2.6) a-d	0.56 ± (0.29) edf	0.00 ± (0.00) e	*Rojo pelón*
8	25	10[b]	4.10 ± (1.2) a-d	1.36 ± (0.37) a-de	0.30 ± (0.48) cde	*Pico chulo*
9	25	15[b]	6.70 ± (3.2) a	1.14 ± (0.71) b-f	0.70 ± (0.82) a-d	*Chicle*
10	25	20[b]	5.10 ± (3.0) a-d	2.06 ± (1.90) a	0.30 ± (0.48) cde	*Amarilla Zac.*
11	0	30[b]	1.50 ± (0.7) cd	0.41 ± (0.26) f	1.00 ± (0.01) ab	*X. blanco*
12	0	25[b]	4.00 ± (2.5) a-d	1.50 ± (0.45) abc	1.00 ± (0.01) ab	*Cristalina*
13	0	20[b]	3.20 ± (1.0) a-d	1.49 ± (0.51) abc	1.00 ± (0.01) ab	*Amarilla Zac.*
14	0	15[b]	3.30 ± (2.5) a-d	1.23 ± (0.53) a-f	0.50 ± (0.52) b-e	*Cardón*
15	0	10[b]	1.20 ± (0.7) d	0.53 ± (0.40) ef	0.10 ± (0.31) de	*San Juanera*

Means with the same letter in a vertical sense are statistically equal, according to the Tukey test (p = 0.05). a Sucrose and b *Agave* honey.

Table 2. Root and shoot formation on culture medium (MS 50%) containing sucrose and *Agave* honey and a pH of 6.5 at 60 days

Treat.	*Agave* honey (g/l)	Root		Explants				Genotype
		No. / explant	Length (cm)	Living	Dead	Pigmented		
						No.	Proportion (%)	
1	30	1.60±(0.84) a	0.42±(0.26) b	5	10	5	75	*X. Blanco*
2	25	5.00±(2.53) a	1.46±(0.45) a	11	0	7	63.6	*Cristalina*
3	20	2.80±(1.03) a	1.56±(0.56) a	12	5	0	0	*Amarilla Zac.*
4	15	3.60±(2.62) a	1.40±(0.58) a	3	14	0	0	*Cardón*
5	10	1.20±(0.91) a	0.60±(0.42) b	0	12	0	0	*San Juanera*

Means with the same letter in a vertical sense are statistically equal, according to the Tukey test (p = 0.05).

pigmentation is outstanding in genotypes of *Opuntia* that exclusively produce fruits of green color.

***Pigment content*:** For treatments that induced red pigmentation in explants of *Xoconostle blanco* and *Cristalina*, it was found that the tissues might contain betacyanins and betaxanthins (Table 3 and Figure 1b) in a proportion of 41.4% and 53%, respectively of *Opuntia* fruit (*Rojo pelón*) cultivated under agronomic conditions. It is convenient to mention that the amount of pigments in the *in vitro* cultures is low, for this reason, it will be needed more research to make the synthesis system more efficient. Betalains (betacyanins and betaxanthins) are natural water-soluble pigments that could potentially be used as dyes (red and yellow) and have antioxidant activity (Tesoriere *et al.,* 2005; Allegra *et al.,* 2005; Moreno *et al.*,2008; Marshall-Craig and Olcott-Marshall, 2010). They are found in *Opuntia* and *Hylocereus* genus (Stintzing *et al.,* 2005; Vaillant *et al.,* 2005) and in recent years studies on their properties have been increased in several species of the genus *Opuntia* (Sreekanth *et al.,* 2007; Osorio-Ezquivel *et al.,* 2011). Khatabi *et al.* (2016) demonstrated that differences in betalain content in the juice and pulp of prickly pear fruits could be attributed to variability in prickly pear ecotypes, physiologies, and growth conditions. In the extraction of betalains, conventional and nonconventional methods have been used (Barba *et al.,* 2017). The above demonstrates that with the use of supplements such as *Agave* honey and pH modification, it makes possible the synthesis of these important pigments under controlled conditions. In the meantime, more research will be needed to improve this process.

Table 3. Betacyanins and betaxanthines content in 5 g of *Opuntia* (mg/g) tissues

	Tissues	
	Betacyanins (mg/g)	Betaxanthines (mg/g)
Red fruit (Rojo pelón, *O. ficus indica*).	0.04106667	0.01533583
Tissue red explant (*O. joconostle, X. blanco*).	0.01769167	0.00879083
Tissue green explant (*O. joconostle, X. blanco*).	0.00229167	0.00596750

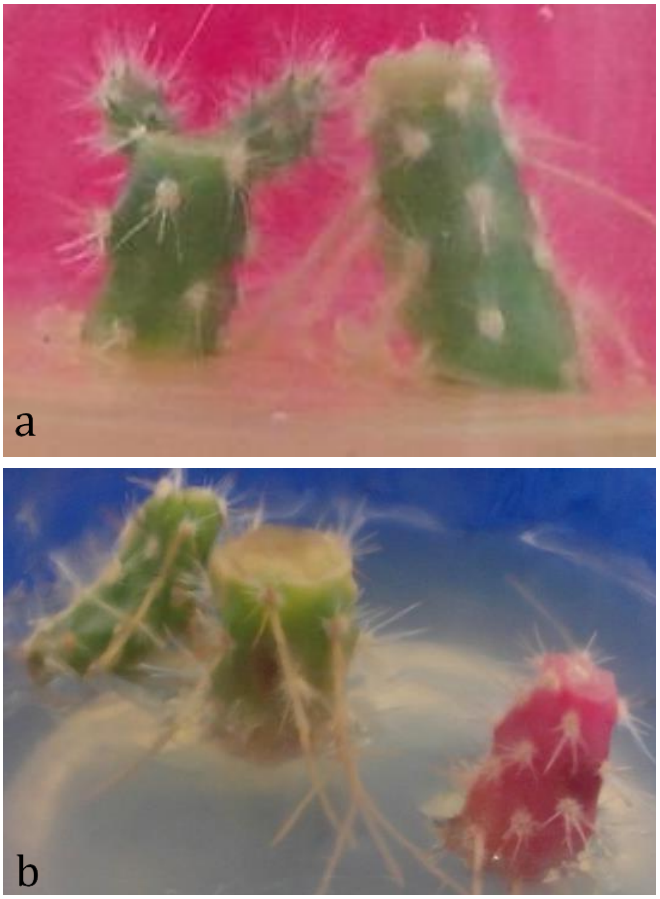

Figure 1. Behavior of *Opuntia* explants in culture media supplemented with *Agave* honey. a) Growing of shoots and roots formation in *Vaquera* genotype with 50% MS medium supplemented with 30 g of *Agave* honey and b) *Xoconostle blanco* genotype grown in 50% MS, 30 g/l of *Agave* honey and pH 6.5.

***Effect of Agave honey and minerals under greenhouse conditions*:** *Opuntia* plants established on the substrate under greenhouse conditions with different nutrient solutions showed morphological differences and color of their tissues.

Table 4. Behavior of *Opuntia* plants under nutrition at 180 days of culture with twice-weekly application of 10 ml of nutrient solution and irrigation every 4 days with running water

Components of nutritive solution	Plant height (cm)	Stem dimension (cm)	
		Thickness	Amplitude
I *Agave* honey (30 g/l) pH 6	18.7 ± (9.9)	1.46 ± (0.8)	4.64 ± (1.2)
II *Agave* honey (30 g/l) pH 6.5	16.3 ± (2.4)	0.74 ± (0.4)	4.20 ± (1.4)
III Triple 17 (3 g/l) pH 6.0	16.6 ± (2.7)	1.10 ± (0.6)	3.86 ± (0.4)
IV Triple 17 (3 g/l) pH 6.5	19.1 ± (3.6)	1.10 ± (0.3)	4.52 ± (0.7)
V Triple 17 (3 g/l) pH 7.0	19.1 ± (3.4)	1.24 ± (0.1)	4.48 ± (0.7)
VI Triple 17 (3 g/l) pH 7.5	21.1 ± (3.3)	1.22 ± (0.4)	4.90 ± (1.1)
VII *Agave* honey (30 g/l) irrigated with normal water	18.0 ± (3.8)	1.47 ± (0.5)	4.00 ± (0.5)

The substrate supplemented with Fertilizer Triple 17 at pH 7.5 induced the higher height (21.1cm), thickness (1.2 cm) and amplitude (4.9 cm) of the plants, very similar to the substrate where *Agave* honey at pH 6.5 (Table 4 and Figure 2a-d). Also, in relation to plants composition (Table 5), the same treatment showed the higher content of ash and carotenes. It is convenient to mention that plants of *Xoconostle blanco* (in the majority of the treatments) showed a green color pigmentation of greater intensity, tending to red (Figure 3a). By analyzing the pigmented plants (*Xoconostle blanco*) by means of the Raman spectroscopy, two main peaks (Figure 3b) were observed which correspond to β-carotene (DE Gelder *et al.*, 2007) at 1155 cm^{-1}, ν (C-C) stretching, and at 1522 cm^{-1} assigned to ν (C = C) in-phase stretching (Marshall-Craig and Olcoott-Marshall, 2010). The β-carotene Raman signal was found from the chloroplasts of pigmented tissue cells (Figure 3c). Finally, when comparing the nutritional quality of *Xoconostle blanco* plants with higher pigmentation and better growth rate, it was found a higher contain of β-carotene, while those that grew with *Agave* honey (30 g/l) were characterized by slightly higher protein content (Table 5).

Table 5. Proximal composition and content of carotenes in *Opuntia* cultivated with a nutritive solution.

Components	*Opuntia joconostle* Genotype (*Xoconostle blanco*)	
	Triple 17 y pH 7.5	*Agave* honey (30 g/L)
Humidity (%)	95.3±1.10	96.05±0.17
Ashes (%)	1.4±0.05	0.5±0.02
Protein (%)	9.5±0.05	11.7±0.03
Grease (%)	0.150±0.01	0.165±0.01
Total carbohydrates (%)	4.18	3.50
A-carotene ug / 100 (g)	2.189	0.330
B-carotene ug / 100 (g)	39.582	16.679
Copper (ppm)	0.0	0.1
Zinc (ppm)	1.5	1.7
Iron (ppm)	3.8	3.1
Sodium (%)	0.0038	0.0036
Potassium (%)	0.000918	0.00123
Calcium (%)	0.056	0.062

In plants, sugars represent not only energy sources and structural components but also act as physiological signals from sucrose-rich medium, thereby improving anthocyanin production (Mohan *et al.*, 2010). The above inferred that the carbohydrates of *Agave* honey could help the *Opuntia* nutrition and/or mineral conditions of the soil. This kind of culture procedure could be used to strengthen its properties and/or direct the synthesis of compounds of greater interest in a directed manner in *Opuntia* plants.

Figure 2. *Opuntia* genotype cultivated in different nutrient solution. a) *Agave* honey (30 g/l) pH 6.0, b), Triple 17 (3 g/l) pH 7.0, c) Triple 17 (3 g/l) pH 7.5 and d) irrigated with normal water.

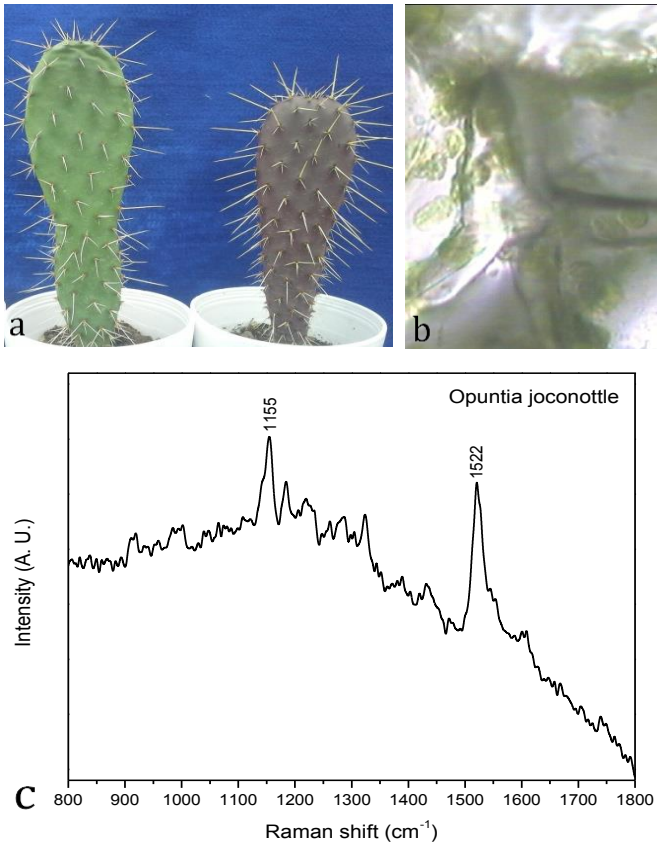

Figure 3. Presence of pigments in *Opuntia* genotypes a), *Cristalina* and *Xoconostle blanco* genotype which is slightly pigmented red, b), Chloroplasts of *Xoconostle blanco* where the presence of β-carotenes was indicated and c), representative Raman spectrum of β-carotenes found in red pigments from the chloroplasts of pigmented tissue cells.

***Conclusions*:** *Agave* honey is a source of carbon and allows the rooting and/or propagation of *Opuntia* plants under *in vitro* conditions, and it makes possible to reduce costs, and, on the other hand, induces the synthesis of pigments in some *Opuntia* tissues genotypes that do not even produce fruits of red color like the *Cristalina* genotype. In addition, nutrient solutions supplemented with fertilizer plus *Agave* honey at pH 6.5 improved growth and it is an important factor to induce the synthesis of pigments at least in the genotype *Xoconostle blanco*, which would be an important method for pigment synthesis in the near future or compounds of high nutritional value of *Opuntia* under controlled conditions in an efficient and sustainable way.

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