Article

Development of micropropagated plants of *Agave americana* var. Oaxacensis during greenhouse acclimatization

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Abstract

American agave var. Oaxacensis Gentry is a long-lived species that takes approximately 20 years to reach its reproductive stage. In Oaxaca, Mexico, this species is used as raw material to make distilled beverage mezcal. To increase the cultivation area with this species, it is proposed to propagate it *in vitro*, in addition to conventional propagation methods. Micropropagated plants must go through an acclimatization period, but environmental adaptation conditions, such as the substrate and nutrient supply, which affect the survival and magnitude of plant growth are unknown. The objective of the research was to evaluate the morphological characteristics of micropropagated plants of *A. americana* var. Oaxacensis that were tested for different substrate mixtures: peat-sand: 33.3% + 66.6% v/v, 66.6% + 33.3% v/v, 75% + 25% v/v; as well as, the supply of nutrients by fertigation with dilutions 0, 50 and 100% of the Steiner formulation, during 290 days of its acclimatization for which a completely randomized design with factorial arrangement was used, at the end of this period, of a total of 135 plants, 100% of these survived. The plants that reached a larger size had the following characteristics: 5.5-5.6 leaves, the greater leaf of 17.7 to 18.2 cm in length, 2.4 to 2.5 cm in width, the stem of 2.4 to 2.5 cm in diameter, and corresponded to those established in substrate with 66.6% peat + 33.3% sand v/v or 75% peat + 25% sand v/v, and which were also fertigated. The fertirrigated plants with 100% nutritive solution achieved larger size than the plants irrigated with only water.

Keywords: chlorophyll, fertirrigation, stomata

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Introduction

Most of the agave species that are established in commercial plantations, *A. tequilana*. *A. sisalana*, *A. fourcroydes* and *A. angustifolia*, are conventionally propagated by asexual methods, for example: rhizome baby grandparents or those that originate from rosette leaves (intrafoliary children) that develop when they are separated from the mother plant or it dies (Nobel, 1998; García-Mendoza, 2007), in addition there are bulbils that originate from vegetative buds in the inflorescence, their development is induced when the abscission or cut of the flower buds occurs (Arizaga and Ezcurra, 1995).

Since the early 1980s in various countries and particularly in Mexico, research has been carried out for the *in vitro* propagation of agaves (Arizaga and Ezcurra, 1995), so it has been possible to describe the driving conditions suggested at each stage of the micropropagation process (Domínguez *et al*., 2008; Enríquez-del Valle, 2008), as a complementary alternative and not exclusive of conventional methods. Currently there are reports on the in vitro propagation of *A. tequilana* (Santacruz-Ruvalcaba *et al*., 2008), *A. angustifolia* (Enríquez-del Valle, 2008; Arzate-Fernández *et al*., 2016), *A. inaequidens* Koch (Aureoles-Rodríguez *et al*., 2008), *Agave fourcroydes* Lem (Garriga *et al*., 2010), *Agave cocui* (González *et al*., 2012), *Agave grijalvensis* (Santiz *et al*., 2012), *Agave sisalana* (Carneiro-dos Santos *et al*., 2014). However, there are other agaves with biological-social value that have been little studied, such as the *Agave americana* var. Oaxacensis Gentry, which is an endemic species of Mexico, which in Oaxaca is used to obtain hard fibers (Palma-Cruz, 2000) and for the elaboration of the distilled beverage called mezcal (García-Mendoza, 2011). The production of this beverage using the afore mentioned species as raw material is limited because there are few specimens which take up to 20 years to reach the stage of maturity for harvest.

So, farmers have preferred to propagate other species of shorter reproductive cycle, such as *Agave angustifolia*, which is harvested between seven and nine years of cultivation (CONABIO, 2006). In view of the above, to respond to the interest in establishing plantations of this species, it is suggested to apply the technique of plant tissue culture or *in vitro* culture for its large-scale propagation, but also for conservation purposes, and sustainable use.

The plant tissue culture technique consists of intensive production of plants in large quantities, genetically homogeneous, in controlled laboratory conditions and in small spaces where it is possible to manipulate the plant material throughout the year (George and Debegh, 2008). The process is carried out in sequence of several stages: 1) selection of germplasm or plant material; 2) establishment of aseptic cultures; 3) propagation multiplication; 4) rooted outbreaks in preparation for soil transplantation; and 5) plant acclimatization (George and Debegh, 2008; Enríquez-del Valle, 2008).

Stage four is considered of great importance because it is when the seedlings are transferred from in vitro conditions to containers with substrates and greenhouse conditions, to promote gradual changes in their morphology and metabolism, within 40 to 90 days, in preparation for its subsequent transfer to nursery and field. In the case of agaves, micropropagation methodologies have been developed; however, although the general procedures for plant tissue culture have been established, it is necessary to determine the specific requirements for in vitro

propagation in each species. In addition, to evaluate the performance of micropropagated plants in the later stages of acclimatization, greenhouse and field cultivation (Enríquez-del Valle *et al*., 2016).

Some studies on the acclimatization of micropropagated agave plants have been demonstrated in *A. tequilana* (Crespo-González *et al*., 2013) and *A. potatorum* Zucc. (Enríquez-del Valle *et al*., 2016) that achieve greater growth when they receive adequate supply of nutrients, either through nutritive solutions or by incorporating mineral fertilizers to the substrates, compared to plants that do not receive fertilization. However, for A. *americana* var. Oaxacensis there are no antecedents in this regard, so the objective of the study was to evaluate the growth and development of micropropagated *Agave americana*. var. Oaxacensis Gentry plants in response to different substrate conditions and doses of fertigation during greenhouse acclimatization.

Materials and methods

Obtaining plants

The research is a continuation of the work of Cruz-García *et al*. (2017), which was carried out in the plant tissue culture laboratory and greenhouses of the Technological Institute of the Valley of Oaxaca, located in Nazareno, Xoxocotlan, Oaxaca. The plants of *A. americana*. var. Oaxacensis that were used, were obtained by the formation of outbreaks from stem tissue and its subsequent rooting *in vitro*; they had an average of 6.75 leaves, 5 roots and their longest leaf was 9.78 cm long and 0.64 cm wide. They were transplanted into polyethylene pots with a capacity of 171 cm^3, which contained different substrates.

These consisted of mixtures of peat and sand in different proportions (33.3-66.6%, 66.6-33.3%, 75-25%) in relation to their volume. Its acclimatization was carried out in a chapel-type shadow house, 5.5 m wide, 12.1 m long, 3.5 m high, with metal structure, translucent corrugated sheet roof and translucent polyethylene side roofs, with solar radiation reduced to 40% , temperature from 14 to 28 °C, high relative humidity 80-90%. The plants were maintained for 45 days under these conditions, receiving once a week 10 mL of the Steiner universal solution (1984), diluted to 10% concentration. Subsequently, from day 46, the plants were moved to a second greenhouse where they were exposed to greater solar radiation, temperature between 15 and 38 °C, and relative humidity between 30 and 50%, under these conditions they were until day 270, during this time the contribution of the nutrient solution was applied at the substrate level three times per week, in accordance with established treatments.

Experimental design and data analysis

The experiment was established according to a completely randomized design with 3 x 3 factorial arrangement, where: 1) the substrate factor was established at three levels: a) peat + sand mixed 33.3 + 66.6% v/v, peat - 66.6 + 33.3% v/v mixed sand, peat + 75-25% v/v mixed sand and, 2) the type of irrigation factor was also established at three levels (water and nutrient solution (SN) Steiner (1984), in dilution at 50% and SN at 100%). So, there were nine treatments. The SN (1984) at 100% contains in mg L^{-1}: 166.01 N, 31.35 P, 277.38 K, 182.06 Ca, 49.08 Mg, 110.898 S, 1.33 Fe, 0.201 Mn, 0.077 B, 0.019 Mo, 0.0375 Zn and 0.00065 Cu.

The experimental unit was a plant and there were 15 repetitions per treatment. The experiment concluded after 270 days of acclimatization and at that time six plants were selected for each treatment to which morphological characteristics were determined as: number of leaves (NH); length (LHM) and width (AH) of the major leaf, fresh leaf weight (PFF) and dry leaf weight (PSF), volume (VF) and leaf area (AF), leaf succulence (SF), stem diameter (DT), number of main roots (NRP), root length (LR), root volume (VR), fresh root weight (PFR), dry root weight (PSR), stomatic index and chlorophyll content. Foliar succulence (SF) was determined using the formula proposed by Mantovani (1999), SF= (maximum fresh weight-dry weight (g))/surface area (cm^2).

The chlorophyll content was determined in adult and young leaves through the method proposed by Moran (1982), using a Minolta SPAD-502 meter. The SPAD values were converted to µg cm^{-2} using the formula proposed by Coste *et al*. (2010). For the determination of stomata, leaves of the middle part of the rosette were used, three sections were chosen: basal, middle and apical, both of the adaxial and abaxial part, the samples obtained were observed in a Carl Zeiss brand compound microscope, at 40x, the total number of cells and stomata were subsequently counted.

With the previous data, the stomatic index (IE) was calculated using the formula proposed by Wilkinson (1979). Where: IE= number of stomata per unit area/(number of stomata per unit area + number of epidermal cells per unit area) × 100. The data obtained from each variable were subjected to analysis of variance and the Tukey test for the multiple comparison of means with a level of significance $\alpha = 0.05$. For the analysis routine, the SAS® statistical package (the SAS System for Windows 9.0) was used, the corresponding variance analysis and multiple means comparison were performed to establish which treatments caused significant effects on the variables studied.

Results and discussion

Characteristics of micropropagated plants of *Agave americana* var. Oaxacensis at the end of its acclimatization

The analysis of variance of the effect of the substrate and the fertilization on the indicators of the growth and development of the plants (Tables 1 and 2), show significant differences in the effect of the treatments. While the multiple comparison of means found that the differences ($p< 0.01$), caused by the levels of the substrate factor are manifested on most of the variables, except for foliar succulence. Irrigation type factor levels had significant different effects ($p< 0.01$) on the variable number of leaves (NH), leaf width (AH), fresh leaf weight (PFF), leaf volume (VF), leaf area (AF), stem diameter (DT) and number of main roots (NRP). The substrate-type irrigation interaction showed significant effects ($p< 0.01$) on NH, AHM, PFF, VF, AF and NRP. In an earlier study Cruz-García *et al*. (2017), reported that plants of the same species had thin leaves of herbaceous consistency at the beginning of their acclimatization and that during 240 days at this stage; gradual senescence of the leaves from the *in vitro* culture occurred and that these leaves were replaced by new leaves that were larger, 11.6 to 18.2 cm long, 1.7 to 2.5 cm wide, thickened, and herbaceous in consistency but more rigid.

They also point out that at the end of that stage 100% of the plants survived and that although they had fewer leaves than at the beginning of acclimatization, they were longer and wider, although their size was in relation to the amount of nutrients they received.

In this case, when nine months elapsed, the smallest plants were those that settled in the substrate 33.3% peat v/v + 66.6% sand v/v, irrigated with only water or with the SN-50%. As the amount of peat in the substrate increased to 66.6% and 75%, the plants reached larger sizes. Also, fertirrigated plants up to SN-100% tended to be larger than plants irrigated with only water.

So the plants that were established in the substrates with 66% peat and 33.3% sand or 75% peat + 25% sand and that were fertilized with the nutrient solution at 100% nutrient concentration, developed 5.6 and 5.5 leaves, the major leaf was 17.7 and 18.2 cm in length, as well as 2.5 and 2.4 cm in width, 34.9 and 31.8 g of fresh leaf weight, 36.1 and 34.5 cm^3 of leaf volume, 122.7 and 112 cm^2 of leaf area, 4.06 and 3.61 g of dry leaf weight; the stem of 2.5 and 2.4 cm in diameter, 6.5 and 7.3 roots, 39.8 and 32.6 cm in length; the root of 12.5 and 10.8 cm^3 of volume; 10.7 and 9.9 g of fresh root weight, 1.7 and 1.4 g of dry root weight, magnitudes that were significantly (Tukey, $p< 0.05$) greater than the values recorded for the same variables in the plants that were established in substrate with the higher proportion of sand (33.3% peat + 66.6% sand) and that were not fertirrigated (Tables 1 and 2).

Table 1. Effect of the substrate and fertilization on the growth and development indicators of the leaves of micropropagated plants of *A. americana* that were acclimatized in the greenhouse for 270 days.

Sust	Fert (%)	NH	LHM (cm)	AH (cm)	PFF (g)	VF (cm^3)	AF (cm^2)	PSF(g)	SF
33.3%T: 66.6%A	0	3.3 e	11.8 c	1.7 c	10.5 b	11.5 b	38.6 d	1.15 c	0.3 a
	50	3.5 ed	12.7 bc	1.7 c	11.9 b	13.1 b	48.5 cd	1.54 c	0.26 a
	100	3.8 cde	11.6 c	1.7 c	12.8 b	14.1 b	50.5 cd	1.56 c	0.28 a
66.6%T: 33.3%A	0	4.8 abc	16.6 ab	2.4 ab	22.1 ab	23.3 ab	84 abc	2.2 bc	0.28 a
	50	4.1 cde	15.3 abc	2 bc	22.3 ab	22.6 ab	73.2 bcd	1.94 c	0.3 a
	100	5.6 a	17.7 a	2.5 a	34.9 a	36.1 a	122.7 a	4.06 a	0.3 a
75%T: 25%A	0	4.5 bcd	15 abc	1.9 bc	17 b	18 b	63.6 cd	1.93 c	0.28 a
	50	4.5 bcd	15.1 abc	1.9 bc	17 b	18.1 b	62.9 cd	2.07 bc	0.29 a
	100	5.5 ab	18.2 a	2.4 a	31.8 a	34.5 a	112 ab	3.61 ab	0.31 a
Sust		**	**	**	**	**	**	**	ns
Fert		**	ns	**	**	**	**	**	ns
Sust∗Fert		**	ns	**	*	*	**	ns	ns

Sus= substratum; Fert= fertilization; 33.3%T:66.6%A= a portion of peat combined with two of sand; 66.6%T= 33.3%A= two portions of peat combined with one of sand; 75%T:25%A= three portions of peat combined with one of sand; NH= number of leaves; LHM= leaf length greater; AH= leaf width; PFF= fresh leaf weight; VF= leaf volume; AF= leaf area; PSF= dry leaf weight; SF= leaf succulence; **= significant F value ($p\leq 0.01$); *= significant F value ($p\leq 0.05$); ns= F value, not significant ($p\geq 0.05$).

Table 2. Effect of the substrate and fertilization on the growth and development indicators of the stems and roots of micropropagated *A. americana* plants that were acclimatized in the greenhouse for 270 days.

Sust	Fert (%)	DT (cm)	NRP	LR (cm)	VR (cm^3)	PFR (g)	PSR (g)
33.3%T: 66.6%A	0	1.7 c	2.8 e	18 c	3 d	2.3 c	0.5 c
	50	1.7 c	3.1 de	19.1 c	3.6 d	3.1 bc	0.6 c
	100	1.8 c	3.1 de	16.5 c	4.1 cd	3.4 bc	0.6 c
66.6%T: 33.3%A	0	1.9 bc	5.1 bcd	32.5 ab	10.8 ab	9.4 a	1.3 ab
	50	1.9 bc	5.3 abc	31.6 ab	9.1 abc	8.1 ab	1.1 abc
	100	2.5 a	6.5 ab	39.8 a	12.5 a	10.7 a	1.7 a
75%T: 25%A	0	1.9 bc	4.1 cde	27.5 bc	7 bcd	6.4 abc	0.9 bc
	50	1.9 bc	4.6 cde	25.5 bc	6.6 bcd	6.1 abc	0.8 bc
	100	2.4 ab	7.3 a	32.6 ab	10.8 ab	9.9 a	1.4 ab
Sust		**	**	**	**	**	**
Fert		**	**	ns	ns	ns	**
Sust∗Fert		ns	**	ns	ns	ns	ns

Sus= substratum; Fert= fertilization; 33.3%T: 66.6%A= a portion of peat combined with two of sand; 66.6%T: 33.3%A= two portions of peat combined with one of sand; 75%T: 25%A= three portions of peat combined with one of sand. DT= stem diameter; NRP= number of main roots; LR= main root length; VR= root volume; PFR= fresh root weight; PSR= root dry weight; **= significant F value ($p \leq 0.01$); *= significant F value ($p \leq 0.05$); ns= F value, not significant ($p \geq 0.05$).

Several authors agree that better acclimatization of plants obtained *in vitro* is achieved when they are established on substrates that are a mixture of organic matter and inert materials that improve porosity for aeration and drainage, in some cases they also indicate the addition of nutrients by other means such as nutritive solutions or foliar fertilizations that make available the minerals in ionic forms that the plant can take advantage of in the short term. For example, Vilchez *et al*. (2007) found that aloe vera plants (*Aloe vera* L.) showed increases in number of leaves and height when they were established in the earthworm humus substrate, in addition the plant growth was faster and more vigorous, compared to plants that they settled in river sand.

Florio and Mogollon (2011), reported that groups of *Musa* sp. cv. Roatan that settled on a substrate composed of 85% peat v/v and 15% vermiculite v/v, 90% of plants survived after 30 days of acclimatization, and were 12.24 cm high, 13.87 mm in diameter of the bud, 6.38 leaves, 13.65 cm of maximum root length, 1.7 and 0.64 g of fresh biomass; 0.27 and 0.07 g of dry biomass from outbreaks and roots, higher magnitudes compared to plants that were in other substrates: 1) coconut sawdust (100%); 2) sand, coconut sawdust and rice husk (1:2:1 v/v); 3) sand, coconut sawdust and rice husk and vermicompost (1:5:2:2 v/v). The above substrates, whether they contain organic matter or only inert materials, must have adequate porosity for aeration and drainage. Also, the supply of nutrients is beneficial for plants to achieve greater growth and yield. Abreu *et al*. (2007), when micropropagar *A. fourcroydes* transferred the plants to 47 x 69 cm polyfoam containers with 247 alveoli each with 30 cm^3 volume, with fermented henequen pulp substrate and zeolite in different proportions.

The plants were established in a greenhouse that allowed the passage of 30% of the light (≈ 558.74 and 686.55 μmol m^{-2} s^{-1}), these conditions were generated by using layers of black saran mesh. After 30 days, the plants that were established in the substrate composed of 4.46 kg of henequen pulp and 5.54 kg of zeolite, 92% of the plants survived and had an average of 7.64 cm^2 of leaf area and 59.43 mg of total dry weight.

On the other hand, Crespo-González *et al*. (2013) worked with *in vitro* micropropagated plants of *Agave tequilana* Weber var. blue with a height of 15-22 cm and 16 months of age, where they evaluated the response of plants to established treatments based on formulations of organic substrates (coconut powder (PC), peat (T) and compost of Agave bagasse (C), mixed in a volume ratio (v/v), considering the following: 1) control: 80% PC + 10% T + 10% C; 2) 100% PC; 3) 100% T; 4) 70% PC + 30% C; 5) 50% PC + 50% C; 6) 30% PC + 70% C; and 7) 100% C. In addition, the plants of all treatments for nine months received between 7 and 14 fertigation per month with iron and manganese chelates, magnesium sulfate, calcium nitrate, and NPK formulas 18-18-18 (September-December period) and 15-30-15 (January-May period).

The plants that were in substrates with 30 and 50% compost developed the pineapple with diameters of 4.7 and 5.07 cm and the stem of 3.5 and 3.75 cm, were 15 and 15.3 leaves, the major leaf was 55.4 and 51.3 cm in length, as well as 3.6 and 4 cm wide, respectively, magnitudes that were greater than 3.63 cm in diameter of pineapples, 2.10 cm in diameter of stem, 9.8 leaves, with the leaf greater than 36.9 cm in length and 3 cm in width, which they had the plants in the 100% peat substrate. The authors conclude that larger plants are obtained with substrates that contain more organic matter mixed with another material that provides good aeration and filtration.

Stomatic index in adult and young leaves of micropropagated plants of *Agave americana* var. Oaxacensis at the end of its acclimatization

Micropropagated plants that are transferred to the ex vitro condition form vegetative structures during their greenhouse acclimatization, where the plant develops leaves that show different characteristics with respect to the leaves formed in in vitro condition: cuticle, palisade-parenchyma and mesophilic. Among the changes observed, it is mentioned that the new leaves formed under greenhouse conditions have different amounts of stomata (Pospíšilova *et al*., 1999).

In the present investigation it was observed that, at the beginning of its acclimatization, the leaves of the plants of *A. americana* var. Oaxacensis micropropagated had a stomatic index with an average of 4.55% for the basal section, 7% for the middle section and 7.93% for the distal section, however on the underside of the leaves an average of 4.16, 7.26 and 9.32% was obtained for the basal, middle and distal section respectively. At the end of this stage, the stomatic index had increased in the different sections of the leaf. In the adaxial surface (beam) in its basal, middle and distal section, an average of 4.82, 9.06 and 10.67% of stomatic index were obtained, respectively, while for the abaxial surface (underside) averages of 5.93, 9.24 and 10.86 % respectively were obtained.

Table 3. Effect of the substrate and fertilization on the stomatic index of the beam and underside of adult leaves of *A. americana* var. Oaxacensis after 270 days of adaptation.

Sust	Fert (%)	IEHB (%)	IEHM (%)	IEHD (%)	IEEB (%)	IEEM (%)	IEED (%)
33.3%T: 66.6%A	0	5.3 a	9.5 ab	12.6 a	6 a	9 a	11.6 ab
	50	5.2 a	8.9 ab	10.9 abc	6.7 a	9.8 a	11.1 ab
	100	4.5 a	9.5 ab	10.7 abc	6.6 a	9.6 a	9.7 b
66.6%T: 33.3%A	0	5 a	7.5 b	10.2 abc	5.3 a	8.3 a	9.6 b
	50	4.6 a	9.5 ab	9.4 bc	6 a	10.7 a	12.2 a
	100	4.3 a	7.6 b	8.9 c	5.2 a	8.4 a	10.4 ab
75%T:25%A	0	4.6 a	9.5 ab	10.2 abc	5.3 a	9.2 a	11.6 ab
	50	4.9 a	10.1 a	11.8 ab	6 a	9.8 a	11.3 ab
	100	4.6 a	9 ab	10.9 abc	5.8 a	7.9 a	9.8 b
Sustrato		ns	**	**	ns	ns	ns
Fertilización		ns	ns	ns	ns	**	**
Sust∗Fert		ns	ns	ns	ns	ns	**

Sust= substratum; Fert= fertilization; 33.3%T: 66.6%A= a portion of peat combined with two of sand; 66.6%T: 33.3%A= two portions of peat combined with one of sand; 75%T: 25%A= three portions of peat combined with one of sand. Sust= substratum; Fert= fertilization; IEHB= stomatic index beam base section; IEHM= stomatic index beam middle section; IEHD= stomatic index beam distal section; IEEB= stomatic index underside base section; IEEM= stomatic index underside middle section; IEED= stomatic index underside distal section; **= significant F value ($p \leq 0.01$); *= significant F value ($p \leq 0.05$); ns= F value, not significant ($p \geq 0.05$).

According to the analysis of variance, the levels of the substrate factor had significant different effects ($p < 0.01$) on the variable stomatic index of the beam in the middle and distal section of the larger-sized leaf of *Agave americana* var. Oaxacensis (Table 3). The levels of the type of irrigation factor showed significant effects ($p < 0.01$) different only for the stomatic index variables in the middle and distal section. The substrate-type irrigation interaction had a significant effect, only for the stomatic index variable in the distal leaf section.

Regarding the stomatal index, determined in the new leaves formed *ex vitro*, it was observed that the distribution of stomata is more homogeneous than *in vitro* conditions since the middle and apical section of both the bundle and the underside have index values very similar stomatal (Table 4). The leaves that the plants of *A. americana* var. Oaxacensis formed *in vitro* had a lower stomatic index, compared to the new leaves that the plant developed during its adaptation.

This characteristic could be due to an increase in the size of the epidermal cells (Figure 1); therefore, in the field of observation of the microscope, fewer of them were observed, which affected the denominator of the formula to calculate the stomatic index. Stranburger *et al*. (1986), mention that the number of large epidermal cells that reduces the denominator of the IE formula per unit of analyzed area is possibly related to the optimization of water storage capacity.

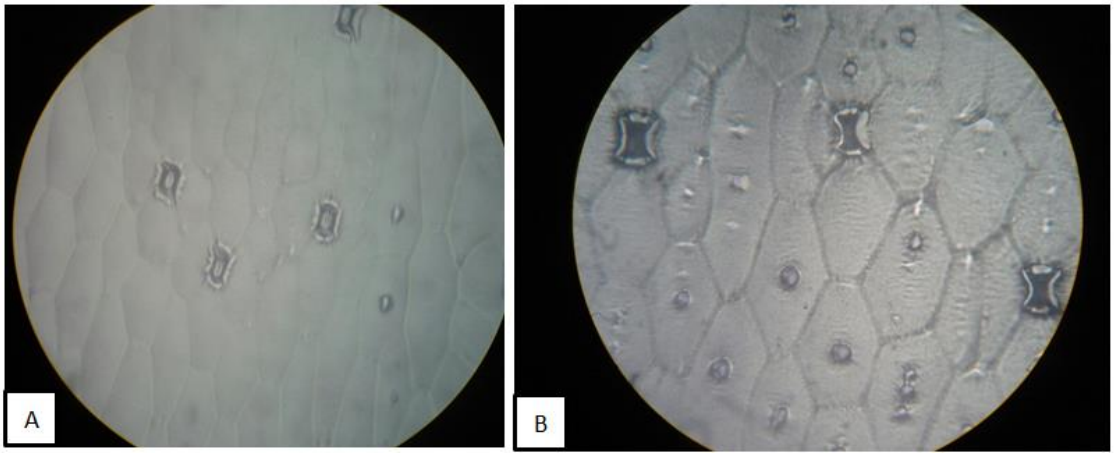

Figure 1. Stomas observed under a microscope in a 40x field. A) stomata present in *A. americana* var. Oaxacensis in *in vitro* culture; and B) stomata in plants adapted in the greenhouse.

The analysis of variance showed that the substrate factor, the type of irrigation factor and the substrate-type interaction of irrigation had significant effects on IEEM, but not on the variables IEHB, IEHM, IEHD, IEEB and IEED. The foregoing denotes that the stomatic index values determined in the different sections of the new leaves of *A. americana* var. Oaxacensis, is not influenced by the type of substrate or the supply of nutrients in the irrigation (Table 4).

Table 4. Effect of the substrate and supply of nutrients in irrigation on the stomatic index of the beam and underside of new leaves of *A. americana* var. oaxacencis after 270 days of adaptation.

Sust	Fert (%)	IEHB (%)	IEHM (%)	IEHD (%)	IEEB (%)	IEEM (%)	IEED (%)
33.3%T: 66.6%A	0	9.2 a	12.1 a	13.5 a	8.7 a	13.8 a	14.6 a
	50	8.6 a	12.3 a	12 a	9.4 a	11.9 abc	12.9 a
	100	7.5 a	11.2 a	11.6 a	8.3 a	10.5 bc	12 a
66.6%T: 33.3%A	0	6.5 a	10.4 a	11.5 a	9.1 a	10.3 bc	12 a
	50	8.2 a	12.1 a	11.7 a	8.1 a	12.3 ab	12.6 a
	100	8.7 a	10.4 a	11.4 a	9.2 a	12.1 abc	11.9 a
75%T:25%A	0	7.5 a	10.5 a	10.9 a	9.1 a	11.4 bc	12.5 a
	50	7.6 a	10.9 a	11.7 a	8.8 a	11.4 bc	13.1 a
	100	7.6 a	9.7 a	10.5 a	7.6 a	9.9 c	12.1 a
Sustrato		ns	ns	ns	ns	*	ns
Fertilización		ns	ns	ns	ns	*	ns
Sust∗Fert		ns	ns	ns	ns	**	ns

Sust= substratum; Fert= fertilization; 33.3%T: 66.6%A= a portion of peat combined with two of sand; 66.6%T: 33.3%A= two portions of peat combined with one of sand; 75%T: 25%A= three portions of peat combined with one of sand. Sust= substratum; Fert= fertilization; IEHB= stomatic index beam base section; IEHM= stomatic index beam middle section; IEHD= stomatic index beam distal section; IEEB= stomatic index underside base section; IEEM= stomatic index underside middle section; IEED= stomatic index underside distal section; **= significant F value ($p \leq 0.01$); *= significant F value ($p \leq 0.05$); ns= F value, not significant ($p \geq 0.05$).

The results of the major stomatic index in young leaves of *A. americana* var. Oaxacensis acclimatized coincide with Bazaldua-Muñoz *et al*. (2008), who report that micropropagated *Physalis ixocarpa* plants presented a gradual increase in stomatic density of both the beam and the underside depending on the acclimatization time.

Chlorophyll content in adult and young leaves of micropropagated plants of *A. americana* var. Oaxacensis adapted in greenhouse

According to the analysis of variance (Table 5), significant differences ($p \leq 0.001$) were found regarding the chlorophyll content in adult and young leaves of *A. americana* var. Oaxacensis in response to the substrate in which they were established and to the supply of nutrients in irrigation, that the substrate-type irrigation interaction had no significant effect. The micropropagated agave plants that were in the substrates with two and three portions of peat combined with one of sand and also received 100% nutritive solution respectively, their older leaves were 43.64 and 42.91 µg cm^{-2} of chlorophyll, magnitudes significantly (Tukey, $p < 0.05$) greater than 23.95 µg cm^{-2} that had the leaves of the plants that were established in the substrate with a portion of peat combined with two of sand and that did not receive additional nutritional solution (Table 5). The young leaves of the plants of *A. americana* var. Oaxacensis had more chlorophyll than the older leaves. The highest value of chlorophyll 75.85 µg cm^{-2} was observed in the young leaves of the plants that were in the substrate with two portions of peat combined with one of sand and that were also fertilized with the 100% Steiner solution.

Table 5. Effect of the substrate and supply of nutrients in irrigation on chlorophyll content in leaves of *A. americana* var. Oaxacensis after 270 of its adaptation in the greenhouse.

Substratum	Fertilization (%)	Chlorophyll (µg cm^{-2})	
		Adult leaf	Young leaf
33.3%T: 66.6%A	0	23.95 d	38.9 d
	50	26.78 cd	46.57 dc
	100	30.27 bcd	50.98 bcd
66.6%T: 33.3%A	0	32.92 bc	55.75 bcd
	50	35.68 abc	64.14 abc
	100	43.64 a	75.85 a
75%T:25%A	0	35.68 ab	54.67 bcd
	50	38.69 ab	61.09 abc
	100	42.91 a	67.11 ab
Sustrato		**	**
Fertilización		**	**
Sustrato∗fertilización		ns	ns

**= significant F value ($p \leq 0.01$); *= significant F value ($p < 0.05$); ns= F value, not significant ($p \geq 0.05$). Means with equal letters are not statistically different (Tukey, $p < 0.05$); 33.3%T: 66.6%A= a portion of peat combined with two of sand; 66.6%T: 33.3%A= two portions of peat combined with one of sand; 75%T: 25%A= three portions of peat combined with one of sand.

The greater growth of the plants established in these substrates could be attributed to the availability of a greater amount of nutrients in the substrate with a high proportion of peat and those provided by the nutrient solution. The above is consistent with Wu *et al*. (2008), who report that the nutritional status of plants is positively related to chlorophyll content and in turn to photosynthetic activity. While Zarco-Tejada *et al*. (2004), mention that in plants with vigorous growth they are generally expected to contain high concentrations of chlorophyll, in relation to less vigorous plants.

Conclusions

The substrate and the nutritional supply during the acclimatization of plants of *A. americana* var. Oaxacensis micropropagated determine its growth and development. All the treatments tested allowed the total of plants to survive, however, those that were established in substrates with two portions of peat combined with one of sand and that also received nutritive solution at 100% concentration, showed better growth characteristics and their adult and young leaves had the highest chlorophyll contents. The stomatic index values in adult and young leaves of the plants were not affected by the type of substrate or fertilization.

During acclimatization of *A. americana* var. Oaxacensis is recommended to use substrates that contain sufficient organic matter, with good drainage and aeration, in addition to considering liquid fertilization as a quick way to make nutrients available.

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