Acclimation of *Agave potatorum* Zucc. micropropagated plants

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ABSTRACT

Objective: *Agave potatorum* Zucc. is an intensively exploited wild plant species. We intend to evaluate how the concentration of mineral salts (MS), indole-butyric acid (IBA), and the incubation conditions affect *in vitro*-rooted *A. potatorum* during acclimation.

Design/Methodology/Approach: We conducted 18 treatments that resulted from combining three factors: 1) MS concentrations (50%, 75%, and 100%); 2) IBA concentrations (without auxin, 0.5 mg L^{-1}, and 1 mg L^{-1}); and 3) incubation environments (fluorescent lighting in a laboratory or exposure to solar radiation in a greenhouse). Thirteen plants from each treatment were transplanted into individual pots containing a 1:1 mixture of peat moss and perlite. These were placed in the acclimation greenhouse for 150 days, exposed to changes in solar radiation, starting from 600 μmol m^{-2} s^{-1} and conditions of high relative humidity (80-90%), and reaching outdoor conditions with full solar radiation,1400±200 μmol m^{-2} s^{-1}. All *in vitro* culture plants, both in greenhouse and laboratory, underwent the same environmental and management conditions.

Results: After 150 days of acclimation, plants micropropagated in a culture medium with 0.5 to 1 mg L^{-1} of IBA, 100% MS, and incubated in a greenhouse showed better growth.

Study limitations/Implications: The *in vitro* culture protocol was (successfully) modified by providing solar radiation in a greenhouse during the rooting of *A. potatorum* sprouts.

Findings/Conclusions: Solar radiation during *in vitro* incubation of *A. potatorum* favors its acclimation.

Keywords: Solar radiation, Adaptation, *In vitro* culture, Agave.

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INTRODUCTION

Agave potatorum Zucc. is a monocotyledonous wild species that grows in the Central Valleys, the Sierra Sur, and the Mixteca regions in Oaxaca, Mexico. Its extraction is intensive and lacks any management, which reduces its population, all the more since it is the raw material to produce tobalá mezcal —a representative distilled beverage of the state (Pérez and Casas, 2007; Enríquez-del-Valle *et al.*, 2016a). Seeds are the only form

of natural reproduction for this species. This reproduction mechanism is not enough to compensate for the number of extracted plants, which is why some producers in the Sierra Sur and the Mixteca regions have established small plantations since the late 1990s (Enríquez-del-Valle *et al.*, 2008; Enríquez-del-Valle *et al.*, 2016a). However, the demand exceeds the availability of plants (Enríquez-del-Valle *et al.*, 2021; Correa-Hernández *et al.*, 2022). Micropropagation can be a strategy to address the situation and increase plant production in the short term (Enríquez-del-Valle *et al.*, 2016b).

The micropropagation technique has proved successful in rescuing and preserving threatened species. It is also an effective technique to obtain large clonal populations from selected plants and large-scale multiplication of superior genotypes. Micropropagation consists of the aseptic asexual propagation from plant cells, tissues, or organs that enables the generation of somatic tissues, cell division, and morphogenesis, as in the production of sprouts, roots, and somatic embryos (Enríquez-del-Valle *et al.*, 2016a, 2021). Acclimation is the final stage of all micropropagation schemes, and crop survival depends on it. Micropropagation has already been used in *Agave fourcroydes* (Madrigal *et al.*, 1990), *A. tequilana* (Valenzuela-Sánchez *et al.*, 2006), *A. karwinskii* and *A. potatorum* (Domínguez *et al.*, 2008; Bautista-Castellanos *et al.*, 2020; Correa-Hernández *et al.*, 2022), *A. cocui* Trelease (Salazar *et al.*, 2009), and *A. angustifolia* (Enríquez-del-Valle *et al.*, 2005; Ríos-Ramírez *et al.*, 2017, 2018). Artificial lighting was used in all incubation stages of the cited *in vitro* cultures. During acclimation in the greenhouse, micropropagated plants of *Agave Americana* var. Oaxacensis adapted using sand or perlite as a substrate, and their size related positively to the applied fertigation dose (Enríquez-del-Valle *et al.*, 2013). Modifications can be implemented in each stage of the *in vitro* propagation scheme to increase production efficiency and plant quality. Thus, it is possible to evaluate variations in the concentration of components in the culture media, particularly mineral salts and growth regulators, such as indole-3-acetic acid (AIA), naphthaleneacetic acid (NAA), and indole-butyric acid (IBA), which induce adventitious root formation and sprout growth by cell extension in the sprouts of different species (Gilroy and Trewavas, 2001; Soto *et al.*, 2006). *Agave potatorum* Zucc. is an intensively exploited wild species. We intend to evaluate how the concentration of mineral salts (MS), indole-butyric acid (IBA), and the incubation conditions affect *in vitro*-rooted *A. potatorum* during acclimation.

MATERIALS AND METHODS

The experiment was conducted in the plant-tissue culture laboratory, the acclimation greenhouse, and the nursery of the Instituto Tecnológico del Valle de Oaxaca, located in the municipality of Santa Cruz Xoxocotlán, Oaxaca. The institute is located at 96° 44' W, 17° 02' N, at an altitude of 1530 masl.

The plant material was obtained from *in vitro* cultures of *Agave potatorum* Zucc. in its rooting stage. Said material came from 165 cm^3 flasks containing 25 mL of one of nine variants of culture media to induce the formation of adventitious roots. The composition of the culture media combined the following components: 1) 0.4 mg L^{-1} thiamine HCL; 50 mg L^{-1} myo-inositol; 30 g L^{-1} sucrose; 2) inorganic salts (MS) in different concentrations (50%, 75%, and 100%); and 3) auxin of indole-butyric acid (IBA) in different concentrations

(0, 0.5, and 1 mg L^{-1}), with a pH of 5.8 and 5.6 g L^{-1} of agar. In addition, we used different types of incubation: 1) in laboratory conditions, with fluorescent light at 35 μmol m^{-2} s^{-1}, for a photoperiod of 16 h to 8 h of darkness, with a temperature in the range of 18 °C to 29 °C; 2) in greenhouse conditions, where plant material was exposed to solar radiation, 400 μmol m^{-2} s^{-1}, under a polyethylene cover, using the natural photoperiod, and a wide range of daily temperatures (12 °C-29 °C).

Thirteen plants in the rooting stage were transplanted per treatment, each into a 250 cm^3 pot with a 1:1 mixture of peat moss and perlite. The potted plants were then placed for 63 days in the acclimation greenhouse, exposed to solar radiation, which reached 600 μmol m^{-2} s^{-1} at midday, under conditions of high relative humidity (80%-90%) produced for a period of 10 seconds every 12 minutes by an intermittent nebulization system from 11 a.m. to 3 p.m. Every day, after mist irrigation, plants were fertigated at the substrate level with 10 mL $plant^{-1}$ of Steiner (1984) nutrient solution diluted to 20%.

From day 64 to day 90, the plants were placed in the nursery under shade mesh, where they were exposed to solar radiation, which reached 900 μmol m^{-2} s^{-1} at midday, under low relative humidity (60%-70%) outdoors. From day 91 to day 150, the plants were exposed to full solar radiation, which reached 1400±200 μmol m^{-2} s^{-1} at midday. In the nursery, the plants were irrigated with water at the substrate level. Twice a week, fertigation was conducted with the same nutrient solution formulation, except the nutrient concentration was increased to 100%. Throughout acclimation, all plants from the 18 *in vitro* culture treatments underwent the same environmental and management conditions in the greenhouse and nursery. Our experiment used a completely randomized design with a 3×3×2 factorial arrangement. The 18 treatments consisted of variations in the culture media and *in vitro* culture incubation conditions in which plants were obtained. The experimental unit was a potted plant, and 13 replications per treatment were conducted. After 150 days of ex vitro growth, 10 plants from each treatment were randomly selected to assess the following variables: number of leaves, stem diameter, foliar volume, root volume, foliar area, and total dry weight. The data were used to conduct analyses of variance and means comparisons (Tukey, 0.05).

RESULTS AND DISCUSSION

Acclimation of micropropagated agave plants

After 150 days of acclimation in the greenhouse/nursery, all the plants obtained from the various *in vitro* culture conditions adapted and grew. However, although all plants were managed similarly in the greenhouse and nursery, differences in size persisted due to their distinctive *in vitro* culture environments. The *in vitro* culture conditions affected the characteristics of the plants and their performance during the acclimation stage. The analyses of variance (Table 1) show that MS concentration in the culture medium had a highly significant effect ($P \leq 0.01$) on foliar volume, root volume, foliar area, and total dry weight. The concentration of IBA in the culture medium had a significant effect ($P \leq 0.05$) on root volume and a highly significant effect ($P \leq 0.01$) on foliar area, foliar volume, and total dry weight. The *in vitro* incubation environment had a significant effect ($P \leq 0.01$) on the number of leaves, foliar volume, foliar area, and total dry weight.

Table 1. Variance analysis of the characteristics of micropropagated plants of *Agave potatorum* after 150 days of acclimation.

Source	DF	Mean squares and significance					
		NL	SD (cm)	VS (cm^3)	RV (cm^3)	FA (cm^2)	TotDW (g)
Treat	17	2.92 **	6.09 ns	17.30 **	8.36 **	6061.47 **	1.27 **
Sal	2	3.44 ns	9.13 ns	42.44 **	16.22 **	9117.10 **	3.88 **
IBA	2	1.81 ns	6.38 ns	17.61 **	12.07 *	4919.37 **	2.20 **
Inc	1	15.02**	2.59 ns	76.05 **	3.76 ns	42331.04**	2.87 **
Sal×IBA	4	1.71 ns	6.47 ns	7.59 *	4.88 ns	3113.37**	0.41 ns
Sal×Inc	2	4.24 *	4.23 ns	18.72 **	20.24 **	5180.08**	1.54 *
IBA×Inc	2	2.04 ns	5.86 ns	4.02 ns	0.29 ns	1680.11ns	0.52 ns
Sal×IBA×Inc	4	1.21 ns	5.98 ns	5.53 ns	5.32 ns	1616.77ns	0.22 ns
Error	162	1.31	6.18	3.09	2.6	796.1	0.34
Total	179						

VS=variation source; DF=degrees of freedom; IBA=indole-butyric acid; Inc=incubation; NL=number of leaves; SD=stem diameter; FV=foliar volume; RV=root volume; FA=foliar area; TotDW=total dry weight. **=highly significant F value (P≤0.01); *=significant F value (P≤0.05); ns=not significant F value (P>0.05).

The agave plants obtained in culture media with MS concentrations at 100% and 50% had 7.8 and 7.4 leaves, a stem diameter of 1.7 and 1 cm, 7 and 5.4 cm^3 of foliar volume, 4.5 and 3.5 cm^3 of root volume, 145.6 and 122 cm^2 of foliar area, and 5.91 and 4.39 g of total dry weight respectively. In each case, the magnitudes observed were significantly different (Tukey, 0.05). Previous studies of *Agave potatorum* in its rooting stage showed that a higher concentration (100%) of indole-butyric acid (IBA) and auxins increased the number of roots and the stem diameter compared to plants grown in a culture medium with a lower MS concentration (75%) (Bautista-Castellanos *et al*., 2020). Similarly, a higher MS concentration in the rooting stage has a positive effect: it increases the size, number of leaves, foliar volume, foliar area, and total dry weight (Enríquez-del-Valle *et al*., 2021). The same results were obtained for *Agave americana* var. Oaxacensis (Miguel-Luna *et al*., 2013).

Auxin-type growth regulators have been widely studied and used to root various plant species, such as cuttings of *Caesalpinia echinata* Lam. and *Malvaviscus arboreus* Cav., rooted with indole-3-butyric acid (IBA) (Endres *et al*., 2007; Loss *et al*., 2009). Said regulators are also used in *in vitro* culture media for various species. For instance, when cultivating *A. angustifolia in vitro*, IBA is used for the multiplication of sprouts and rooting of plants since it reduces the time required for roots and adventitious sprouts to emerge (Enríquez-del-Valle *et al*., 2005). Miguel-Luna *et al*. (2013) report similar data for *in vitro* cultures of *Agave americana* var. Oaxacensis. The same happens with other species, such as *A. potatorum* (Bautista-Castellanos *et al*., 2020; Enríquez-del-Valle *et al*., 2021). The previous results concur with our research, in which plants from a culture medium with an IBA content of 1 and 0 mg L^{-1} obtained values of 7.7 and 7.5 leaves, 1.1 and 1 cm in stem diameter, 6.6 and 5.5 cm^3 in foliar volume, 4.2 and 3.4 cm^3 in root volume, 139.99 and 122.05 cm^2 in foliar area, and 5.7 and 4.6 g of total dry weight respectively (Table 2). In each case, significantly different magnitudes were observed (Tukey, 0.05).

Table 2. Characteristics of *Agave potatorum* Zucc. plants obtained under different *in vitro* culture conditions after 150 days of *ex vitro* development.

Factor	Characteristics of plants		
	NL (cm)	**SD (cm^2)**	**FV (cm^3)**
MS Salt (%)			
50	7.4±1.22[a]	1.0±0.29[a]	5.4±1.81[b]
75	7.4±1.21[a]	1.0±0.29[a]	5.7±1.7[b]
100	7.8±1.16[a]	1.7±4.26[a]	7.0±2.41[a]
IBA dosage (mg L^{-1})			
0	7.5±1.1[a]	1.0±0.3[a]	5.5±1.9[b]
0.5	7.6±1.3[a]	1.6±4.2[a]	6.1±2.0[ab]
1	7.7±1.1[a]	1.1±0.3[a]	6.6±2.1[a]
Incubation environment			
lab	7.2±1.11[b]	1.1±0.5[a]	5.4±1.77[b]
gh	7.8±1.23[a]	1.4±0.3[a]	6.7±2.22[a]
Factors	Characteristics of plants		
MS Salt (%)			
	VR (cm^3)	AF (cm^2)	PSToT(g)
50	3.5±1.7b	122±32.26b	4.39± 1.80 b
75	3.6±1.4b	127.6±29.98b	5.26±1.84ab
100	4.5±2.03[a]	145.6±41.02[a]	5.91± 2.36 a
AIB dosage (mg L^{-1})			
0	3.4±1.4b	122.05±35.1[a]	4.6±1.95 b
0.5	4.0±1.9ab	133.20±37.2[a]	5.3±2.27 ab
1	4.2±1.8[a]	139.99±33.7[a]	5.7± 2.35 a
Incubation environment *			
lab	3.7±1.53[a]	116.4±29.72b	4.88± 2.01 b
gh	4.0±1.98[a]	147.0±35.32[a]	5.60± 2.20 a

IBA=indole-butyric acid; gh=greenhouse; lab=laboratory; NL=number of leaves; SD=stem diameter; FV=foliar volume; RV=root volume; FA=foliar area; TotDW=total dry weight. Means with identical letters in the same column within each block of rows are not statistically different (Tukey 0.05). Each mean is followed by its standard deviation. *TotDW=comparison in incubation environment; means with the same letter in the same column are not significantly different (t, 0.05).

The results of our study show that after 150 days of *ex vitro* growth, the plants from *in vitro* cultures incubated in a greenhouse exceeded the values of plants obtained from *in vitro* cultures incubated in the laboratory by, on average, 7.7% in number of leaves, 21.42% in stem diameter, 19.4% in foliar volume, 7.5% in root volume, 28.8% in foliar area, and 12.85% in total dry weight (Table 2). The above values suggest that, at the end of the rooting stage, the plants obtained from *in vitro* cultures incubated in the greenhouse developed leaves with morphology and physiology closer to those of plants considered adapted to the *ex vitro* environment. Consequently, these plants suffered less stress and growth arrest when compared to plants from *in vitro* cultures incubated in the laboratory.

It is possible that exposure to solar radiation (400 μmol m^{-2} s^{-1}) during incubation in the greenhouse caused the in vitro cultures in their sprout-rooting stage to make gradual adaptations. Hence, this period serves as a pre-adaptation stage, as Teixeira-da Silva *et al.* (2005) described for the micropropagation of *Musa* and *Cymbidium*.

The characteristics of plants obtained from *in vitro* cultures incubated in the greenhouse and those obtained from *in vitro* cultures incubated in the laboratory coincide with the description of Miguel-Luna *et al.* (2013), who reported morphological variation in the in vitro rooting stage of *Agave americana* var. Oaxacensis incubated in two environments: a laboratory with fluorescent lighting and a greenhouse exposed to solar radiation. However, they did not evaluate the ex vitro performance of the micropropagated plants. In our study, we prove that the morphological characteristics of *Agave potatorum* plants resulted from the composition of the culture medium and the physical incubation environment and that said morphological characteristics affected the plants' *ex vitro* growth.

CONCLUSIONS

Agave potatorum Zucc. sprouts established in culture media with 1 mg L^{-1} of IBA produced plants with wider leaves, greater total dry weight, and larger stem diameter when compared to plants obtained in culture media without IBA. As the concentration of MS in the culture medium increased, the plants obtained were larger. The *in vitro* sprouts incubated in a nursery environment produced plants with larger stem diameters than the *in vitro* sprouts incubated in a laboratory environment. After five months of *ex vitro* development, plants from sprouts rooted in culture media with 0.5 to 1 mg L^{-1} of IBA, 100% concentration of inorganic salts (MS), and incubated in a greenhouse reached a larger size than plants from *in vitro* cultures incubated in the laboratory.

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