

MICROPROPAGATION OF AGAVE (*Agave potatorum* Zucc.) THROUGH DIRECT ORGANOGENESIS

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ABSTRACT

Populations of *Agave potatorum* Zucc. have been overexploited from their habitat for the commercial production of mezcal, a traditional Mexican liquor. Since micropropagation is the only method for cloning selected genotypes of this species, this study aimed to establish an efficient protocol for the *in vitro* propagation of *A. potatorum* using individual shoots. During the propagation stage, we evaluated the interaction between different concentrations of the cytokinin benzylaminopurine (BAP: 0, 1.5, and 3.0 mg L⁻¹) and the auxin indole acetic acid (IAA: 0, 1.5, and 3.0 mg L⁻¹). Additionally, we evaluated the interaction between different concentrations of the auxins naphthaleneacetic acid (NAA: 0, 1.5, and 3.0 mg L⁻¹) and IAA (0, 1.5, and 3.0 mg L⁻¹) during the *in vitro* rooting stage. In the propagation stage, the highest number of shoots was obtained with the combinations of 3.0 mg L⁻¹ BAP + 3.0 mg L⁻¹ IAA, 1.5 mg L⁻¹ BAP + 3.0 mg L⁻¹ IAA, and 3.0 mg L⁻¹ BAP + 1.5 mg L⁻¹ IAA, which yielded 9.87, 9.73, and 9.73 shoots per explant, respectively. In the rooting stage, the best shoot development was observed in the control treatment and when only 3.0 mg L⁻¹ IAA was supplemented. Finally, after the rooting stage, plantlets were acclimatized and grown in the field, yielding a 98-100 % survival rate. In conclusion, the propagation protocol proposed in this study results in propagules suitable for establishment in the field.

Keywords: mezcal agave, interaction between growth regulators, propagules, *in vitro* propagation.

INTRODUCTION

In Mexico, most species of the genus *Agave* are of ecological, economic, and cultural importance. The Mexican territory is home to 150 of the 200 *Agave* species worldwide (Delgado-Lemus *et al.*, 2014a; Rangel-Landa *et al.*, 2015). Unfortunately, the intensive extraction of wild agave plants for tequila, mezcal, and pulque production has negatively impacted wild populations (Rangel-Landa *et al.*, 2015; Torres *et al.*, 2015).

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Agave potatorum Zucc., locally known as “maguey”, is a species endemic to the Tehuacán Valley, a region stretching across the states of Oaxaca and Puebla in central Mexico. This species is traditionally used to produce mezcal, a distilled beverage produced under Designation of Origin in seven Mexican states (Colunga-García *et al.*, 2007; Delgado-Lemus *et al.*, 2014b). Wild *A. potatorum* plants are harvested from forests just before sexual reproduction, causing severe ecological damage to their population (Félix-Valdez *et al.*, 2015; Enríquez-del Valle *et al.*, 2016a). Although not currently listed in any risk category by the Official Mexican Standard NOM-059-ECOL-2010 (SEMARNAT, 2010), *A. potatorum* may be classified as a species subjected to special protection in the future, given the intensive extraction to which it is currently subjected, its long flowering time to produce seeds (10-30 years) (Blancas *et al.*, 2010; Rangel-Landa *et al.*, 2015), and its nil asexual reproduction.

The main alternative for improving agave productivity is the selection, *in vitro* micropropagation, and the subsequent plantation establishment of elite individuals (Luna-Luna *et al.*, 2017; Aguilar and Rodríguez, 2018; Monja-Mio *et al.*, 2019). However, this approach requires developing efficient species-specific micropropagation protocols. Studies on the micropropagation of *A. potatorum* for the supply of propagules are scarce (Domínguez-Rosales *et al.*, 2008; Enríquez-del Valle *et al.*, 2016b; Luna-Luna *et al.*, 2017; Correa-Hernandez *et al.*, 2022), and some of the available protocols are not entirely efficient in semisolid medium. Therefore, the objective of this study was to establish an protocol for the efficient micropropagation of *A. potatorum*, with the ultimate goal of achieving sustainable management and avoiding further deterioration of wild populations.

MATERIALS AND METHODS

Plant material and *in vitro* establishment

We used 1-year-old agave plants (*Agave potatorum* Zucc.) obtained from the community of San Diego La Mesa in Puebla, Mexico. Plants were stripped of leaves, and the apices were collected. These were washed with soap and water and transported to the laboratory. Then, apices were immersed in a solution of 100 mL of commercial soap plus two drops of Tween 20® for 5 minutes, followed by five rinses with distilled water and immersion in 70 % ethanol (v/v) for 3 minutes. Afterwards, explants were immersed in a commercial 15 % (v/v) sodium hypochlorite (NaClO) solution (a.i. 6 %) for 15 minutes. Finally, explants were washed twice with sterile distilled water. Apical buds (0.50 cm length) were excised with a scalpel and cultured in test tubes (22 × 220 mm) containing 15 mL of MS medium (Murashige and Skoog, 1962) supplemented with 3 % (w/v) sucrose, 5.0 mg L⁻¹ of 6-benzylaminopurine (BAP, Sigma-Aldrich Chemical Company, MO, USA) (Hernández-Castellano *et al.*, 2020), 100.0 mg L⁻¹ of ascorbic acid (Sigma-Aldrich Chemical Company, MO, USA), and 50.0 mg L⁻¹ of cysteine (Sigma-Aldrich Chemical Company, MO, USA). After adjusting the pH to 5.8 with 0.1 N sodium hydroxide (NaOH), 0.55 % (w/v) agar was incorporated into the medium (Phytotechnology Laboratories, KS, USA). Thirty-five milliliters of culture

medium were transferred to 500 mL glass flasks and autoclaved at 120 °C and 115 kPa for 15 min. Two apical buds per flask were incubated at 24 ± 2 °C with 8 h darkness and 16 h light photoperiods by using photon flux ($40\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$ from fluorescent lamps).

Shoot multiplication

After six months of cultivation (three subcultures of two months each), shoots measuring 2 cm in length with three or four leaves were used as explants and cultured in MS medium supplemented with 3 % (w/v) sucrose and supplemented with increasing concentrations of the cytokinin 6-benzylaminopurine (BAP: 0, 1.5, and 3.0 mg L⁻¹) and the auxin indole acetic acid (IAA: 0, 1.5, and 3.0 mg L⁻¹) (Sigma-Aldrich Chemical Company, MO, USA). The same culture medium, sterilization method, and incubation conditions described above were used to evaluate the shoot multiplication stage. Thirty-five mL of culture medium were transferred into 500 mL glass flasks. After 60 days of culture, we assessed the number of shoots per explant, shoot length, number of roots, and root length.

In vitro rooting

Shoots of 2 cm in length with no roots were used to evaluate the interaction between different concentrations of naphthaleneacetic acid (NAA: 0, 1.5, and 3.0 mg L⁻¹) (Sigma-Aldrich Chemical Company, MO, USA), and indole acetic acid (IAA: 0, 1.5, and 3.0 mg L⁻¹). The same culture medium, sterilization method, and incubation conditions described above were used. Thirty-five mL of culture medium were transferred into 500 mL glass flasks. After 60 days of culture, we assessed shoot length, number of leaves, number of roots, and root length.

Acclimatization

After the rooting stage, rooted shoots were transferred to 72-cell trays containing a sterile substrate mixture of soil, peat moss (Premier, Rivière-du-Loup, CAN), and agrolite (Agrolita, Tlalnepantla de Baz, MX) (1:1:1 v/v). Plantlets were cultivated under greenhouse conditions (50 % shade, 30 ± 5 °C, 60 ± 5 % RH, relative humidity), watered twice a week, and received foliar fertilization (Grogreen® Campbell Co. De Rochelle, Ill, USA) (1 mg L⁻¹) weekly. After 30 days of acclimatization, the survival rate (plant survival percentage) was computed. Subsequently, plantlets were transferred to 38-cell trays containing unsterilized substrate mixture and under natural light with a photon flux of $130 \mu\text{mol m}^{-2} \text{s}^{-1}$. Finally, plants were transferred to field conditions in a plot at Izúcar de Matamoros, Puebla, Mexico, under a drip irrigation system.

Experimental Design and Statistical Analysis

A completely randomized experimental design was used, with a two-factor arrangement for the propagation (BAP × IAA) and rooting (NAA × IAA) stages. For the *in vitro* establishment, two apical buds were placed by flask; a total of 50 explants

were used. During the shoot multiplication stage, 25 explants per treatment, with five explants per culture flask, were used. For the acclimatization process, 144 shoots were used (two trays of 72-cell trays). An analysis of variance (ANOVA) was performed, with means compared with Tukey's test ($p \leq 0.05$). All experiments were performed in duplicate. Data analysis was carried out in SPSS (Version 22 for Windows).

RESULTS AND DISCUSSION

In vitro establishment and shoot multiplication

After 60 days of culture, a 100 % of shoot regeneration response was observed in the *in vitro* establishment process (direct formation of adventitious shoots). No contamination was observed during *in vitro* establishment. For shoot multiplication, significant differences were observed between the different BAP $\times$ IAA treatments. Additionally, an interaction was observed in the two-factor analysis for the number of shoots per explant, number of roots, and root length. On the other hand, no interaction was observed for shoot length (Table 1). The highest number of adventitious shoots was obtained by direct regeneration with 3.0 mg L⁻¹ BAP + 3.0 mg L⁻¹ IAA, 3.0 mg L⁻¹ BAP + 1.5 mg L⁻¹ IAA, and 1.5 mg L⁻¹ BAP + 1.5 mg L⁻¹ IAA, which yielded 9.83, 9.73, and 8.33 shoots per explant, respectively. In contrast, the lowest number of shoots was produced when only IAA was supplemented to the culture medium, resulting in less than two shoots per explant (Figure 1A-C). Regarding shoot length, the highest values were obtained with the control treatment and when only IAA was supplemented. In contrast, the shortest shoots occurred with 1.5 mg L⁻¹ BAP and 1.5 mg L⁻¹ + 1.5 mg L⁻¹ IAA. The highest number of roots was observed with 1.5 mg L⁻¹ BAP + 1.5 mg L⁻¹ IAA

Table 1. Shoot multiplication of *Agave potatorum* after 60 days of culture in different concentrations of BAP and IAA.

BAP (mg L ⁻¹)	IAA (mg L ⁻¹)	Response (%)	Number of shoots per explant	Shoot length (cm)	Number of roots per explant	Root length (cm)
0.00	0.00	100±0.00 a	1.60±0.19 d*	5.51±0.11 a	2.87±0.19 b	4.80±0.15 a
0.00	1.50	100±0.00 a	1.87±0.19 d	5.43±0.10 a	3.0±0.17 b	4.94±0.19 a
0.00	3.00	100±0.00 a	1.93±0.23 d	5.55±0.13 a	2.93±0.18 b	4.91±0.18 a
1.50	0.00	100±0.00 a	6.80±0.31 c	4.73±0.12 b	1.40±0.21 c	2.14±0.30 c
1.50	1.50	100±0.00 a	8.33±0.32 b	4.67±0.14 b	4.27±0.28 a	3.51±0.10 b
1.50	3.00	100±0.00 a	9.73±0.33 a	5.20±0.14 ab	4.33±0.29 a	3.57±0.09 b
3.00	0.00	100±0.00 a	7.73±0.30 bc	4.95±0.15 ab	0.93±0.21 c	0.55±0.23 d
3.00	1.50	100±0.00 a	9.73±0.37 a	4.95±0.15 ab	0.80±0.20 c	0.46±0.13 d
3.00	3.00	100±0.00 a	9.87±0.38 a	4.95±0.18 ab	1.07±0.23 c	0.73±0.15 d
Factors				<i>p</i> -value		
BAP			0.00	0.00	0.00	0.00
IAA			0.00	0.16	0.00	0.00
BAP $\times$ IAA			0.00	0.23	0.00	0.00

*Values represent mean $\pm$ SE (Standard Error). Means with different letters per column are statistically different (Tukey, $p \leq 0.05$).

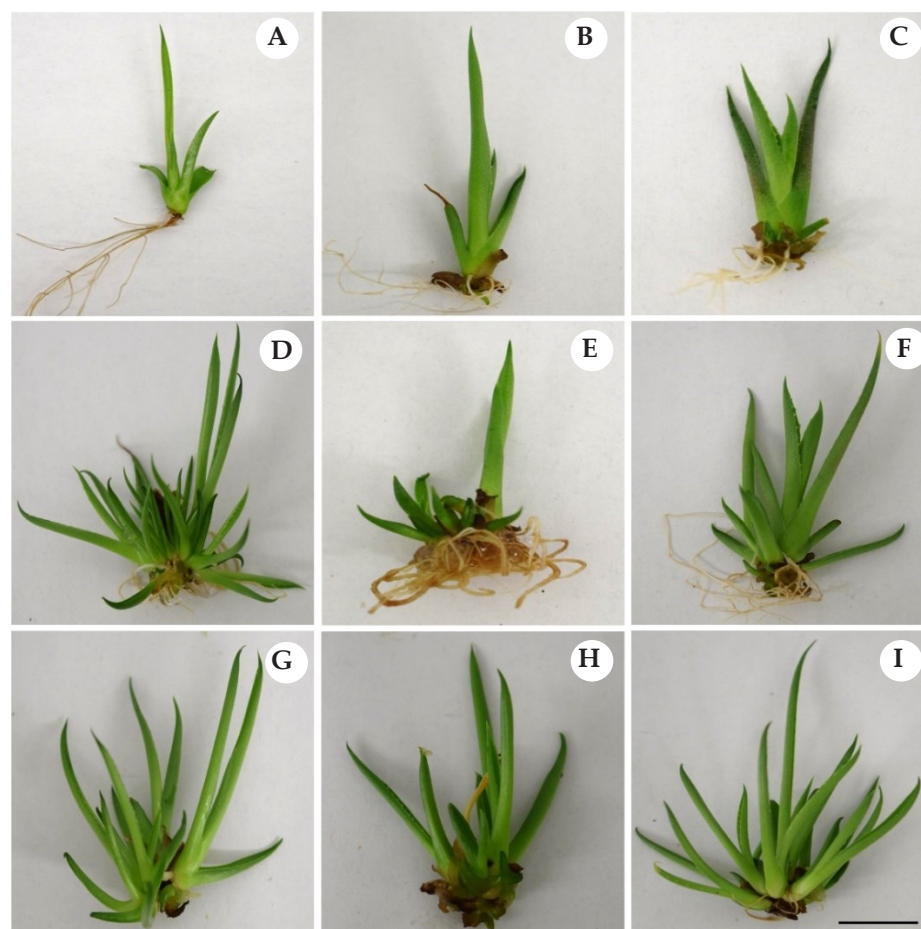


Figure 1. Effect of BAP and IAA concentration on *in vitro* propagation of *Agave potatorum*. A: 0 mg L⁻¹ BAP + 0 mg L⁻¹ IAA, B: 0 mg L⁻¹ BAP + 1.5 mg L⁻¹ IAA, C: 0 mg L⁻¹ BAP + 3 mg L⁻¹ IAA, D: 1.5 mg L⁻¹ BAP + 0 mg L⁻¹ IAA, E: 1.5 mg L⁻¹ BAP + 1.5 mg L⁻¹ IAA, F: 1.5 mg L⁻¹ BAP + 3 mg L⁻¹ IAA, G: 3 mg L⁻¹ BAP + 0 mg L⁻¹ IAA, H: 3 mg L⁻¹ BAP + 1.5 mg L⁻¹ IAA and I: 3 mg L⁻¹ BAP + 3 mg L⁻¹ IAA. Bar= 1 cm.

and 1.5 mg L⁻¹ BAP + 3.0 mg L⁻¹ IAA, with 4.27 and 4.33 roots per explant, respectively (Figure 1D-F). The lowest number of roots was observed with 1.5 mg L⁻¹ and 3.0 mg L⁻¹ BAP, either alone or in combination with IAA, with less than 1.4 roots per explant. As for root length, the highest value was recorded in the control treatment and with IAA alone. The lowest root length was observed under supplementation with only 1.5 mg L⁻¹ and with the combinations of 3.0 mg L⁻¹ + 1.5 mg L⁻¹ and 3.0 mg L⁻¹ + 1.5 mg L⁻¹ IAA, yielding roots measuring less than 1 cm in length (Figure 1G-I).

Our study established an efficient protocol for the micropropagation of *A. potatorum*. However, there is insufficient information about the *in vitro* propagation of this species. Domínguez-Rosales *et al.* (2008) obtained 6.9 shoots per explant by supplementing the

culture medium with 3 mg L⁻¹ of Kinetin (KIN). Pérez-Molphe-Balch et al. (2012) obtained 7.8 shoots per explant using 3.0 mg L⁻¹ of KIN, which differs from the 9.87 shoots per explant obtained in this study with the 3.0 mg L⁻¹ BAP + 3.0 mg L⁻¹ IAA. The cytokine-auxin interaction is commonly used for the micropropagation of agave species (Domínguez-Rosales et al., 2008; Us-Camas et al., 2017; Aguilar and Rodríguez, 2018; Rodríguez-Garay and Rodríguez-Domínguez, 2018; Sara et al., 2020). The interaction between plant growth regulators has a synergistic or antagonistic effect on in vitro morphogenesis and meristem cell development (Su et al., 2011; Faisal et al., 2018; Gupta et al., 2020). Our results indicate synergism, evidenced by the higher number of shoots per explant, number of roots, and root length. Enríquez-del Valle et al. (2016b) reported that 1.0 mg L⁻¹ of BAP fosters the in vitro propagation of *A. potatorum*. However, this report did not mention the number of shoots per explant produced.

In vitro rooting

Significant differences were observed between the different combinations and concentrations of NAA and IAA. In addition, an interaction was observed in the two-factor analysis for shoot length. On the other hand, no interaction was observed for number of leaves, number of roots, and root length (Table 2). The greatest shoot length was observed in the control treatment and when only 1.5 mg L⁻¹ or 3.0 mg L⁻¹ of IAA were supplemented, resulting in shoots of 5.04, 5.31, and 5.77 cm in length (Figure 2A-C); the smallest length was obtained by adding 3.0 mg L⁻¹ NAA alone and 3.0 mg L⁻¹ NAA + 1.5 or 3.0 mg L⁻¹ IAA, which produced shoots 2.1 cm long. As for the number

Table 2. *In vitro* rooting and survival during acclimatization of *Agave potatorum* after 60 days of culture in different concentrations of NAA and IAA.

NAA (mg L ⁻¹)	IAA (mg L ⁻¹)	Response (%)	Shoot length (cm)	Number of leaves	Number of roots	Root length (cm)	Survival (%)
0.00	0.00	100±0.00 a	5.04±0.29 a*	3.87±0.19 a	3.87±0.83 a	7.03±0.35 a	99.33±0.66 a
0.00	1.50	100±0.00 a	5.31±0.34 a	3.87±0.19 a	3.93±0.70 a	6.82±0.43 a	99.33±0.66 a
0.00	3.00	100±0.00 a	5.77±0.35 a	3.73±0.21 a	4.20±0.77 a	6.91±0.43 a	99.33±0.66 a
1.50	0.00	0.00 b	3.85±0.18 b	2.80±0.20 b	0.00 b	0.00 b	98.33±1.66 a
1.50	1.50	0.00 b	3.07±0.18 bc	2.73±0.18 b	0.00 b	0.00 b	98.33±1.66 a
1.50	3.00	0.00 b	3.0±0.15 bc	2.80±0.20 b	0.00 b	0.00 b	98.33±1.66 a
3.00	0.00	0.00 b	2.19±0.13 c	1.60±0.13 c	0.00 b	0.00 b	98.33±1.66 a
3.00	1.50	0.00 b	2.15±0.16 c	1.53±0.13 c	0.00 b	0.00 b	98.33±1.66 a
3.00	3.00	0.00 b	2.19±0.12 c	1.47±0.13 c	0.00 b	0.00 b	98.33±1.66 a
Factors			<i>p</i> -value				
IAA		0.000	0.574	0.828	0.460	0.937	1.000
NAA		1.000	0.000	0.000	0.000	0.000	0.615
IAA × NAA		1.000	0.015	0.984	0.539	0.992	1.000

*Values represent mean ± SE (Standard Error). Means with different letters per column are different (Tukey, *p* ≤ 0.05).

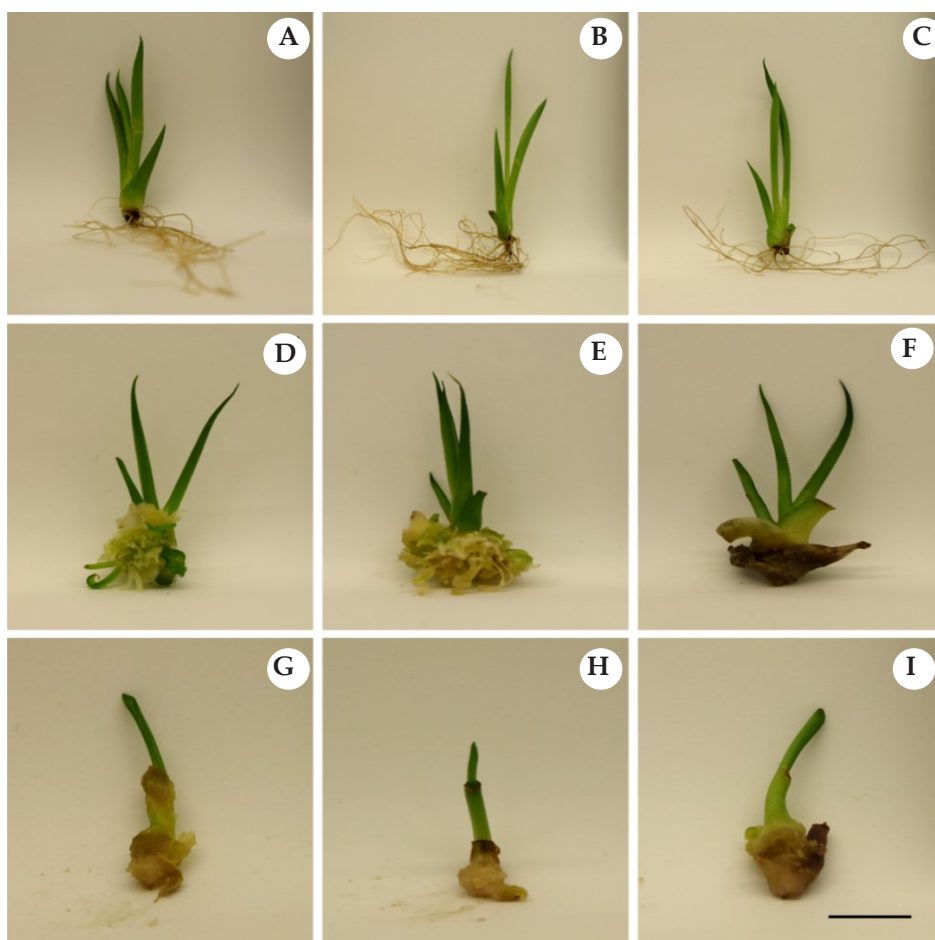


Figure 2. Effect of NAA and IAA concentrations on *in-vitro* rooting of *Agave potatorum*. A: 0 mg L⁻¹ NAA + 0 mg L⁻¹ IAA, B: 0 mg L⁻¹ NAA + 1.5 mg L⁻¹ IAA, C: 0 mg L⁻¹ NAA + 3 mg L⁻¹ IAA, D: 1.5 mg L⁻¹ NAA + 0 mg L⁻¹ IAA, E: 1.5 mg L⁻¹ NAA + 1.5 mg L⁻¹ IAA, F: 1.5 mg L⁻¹ NAA + 3 mg L⁻¹ IAA, G: 3 mg L⁻¹ NAA + 0 mg L⁻¹ IAA, H: 3 mg L⁻¹ NAA + 1.5 mg L⁻¹ IAA and I: 3 mg L⁻¹ NAA + 3 mg L⁻¹ IAA. Bar= 1 cm.

of leaves, the highest number of leaves was observed with the control treatment and with IAA alone, yielding 3.8 leaves per shoot on average. The lowest number of leaves per shoot was obtained by adding only 3.0 mg L⁻¹ NAA and with the combination of 3.0 mg L⁻¹ NAA + 1.5 or 3.0 mg L⁻¹ IAA, which produced 1.4 to 1.6 leaves per shoot. As for the number of roots, the highest number was observed in the control treatment and with IAA alone, with 3.8 to 4.2 roots per shoot; the remaining treatments produced no roots (Figure 2D-F). Regarding root length, the longest roots were resulted from the control treatment and IAA alone, with roots up to 7 cm in length; the remaining treatments produced no roots. It is worth mentioning that the combinations of 0 mg L⁻¹ IAA + 1.5 mg L⁻¹ NAA and 1.5 mg L⁻¹ IAA + 1.5 mg L⁻¹ NAA promoted the formation of callous tissue at the base of the explants (Figure 2G-I).

In this study, the auxin-auxin interactions did not show any root induction in *A. potatorum*. Shoots showed the greatest root production either with no auxin supplementation or by supplementing IAA alone. Enríquez-del Valle *et al.* (2016b) achieved 100 % *in vitro* rooted shoots of *A. potatorum* by adding 1.0 mg L⁻¹ indolebutyric acid (IBA) to the MS culture medium. However, these authors reported that all *A. potatorum* shoots formed adventitious roots, even in culture media with no auxin supplementation. On the other hand, Domínguez-Rosales *et al.* (2008) achieved 87 % rooted shoots in this same species using MS medium with no plant growth regulators (RCV).

In our study, we observed the formation of callus tissue with 1.5 mg L⁻¹ of NAA and with the combined treatment of 1.5 mg L⁻¹ of NAA + 1.5 mg L⁻¹ of IAA. The production of callus tissue in *Agave tequilana* Weber has been used to promote somatic embryogenesis (Delgado-Aceves *et al.*, 2019; Monja-Mio *et al.*, 2019). The formation of callus tissue during morphogenesis promotes somaclonal variation in the plants produced (Ramírez-Mosqueda and Iglesias-Andreu, 2015). This type of variation is undesirable when the aim is massive propagation to preserve the genetic heritage of the parent materials (Domínguez-Rosales *et al.*, 2008; Leva and Rinaldi, 2017). In the case of *A. potatorum*, one advantage is that the MS culture medium requires no auxin supplementation to promote root formation during micropropagation. Correa-Hernández *et al.* (2022), in *A. potatorum* during *in vitro* temporary immersion, found that explants had a scarce or no root system, and the shoots showed high survival percentages (95-98 %) in all treatments evaluated.

Acclimatization

In the acclimatization stage, all plantlets obtained from the rooting treatments had survival rates above 98 %. Figure 3 shows *ex vitro* plantlets transplanted to greenhouse and field conditions with a drip irrigation system.

The high survival rate (98-100 %) observed in our study contrasts with the studies of Domínguez-Rosales *et al.* (2008) and Enríquez-Del Valle *et al.* (2016a), who reported a

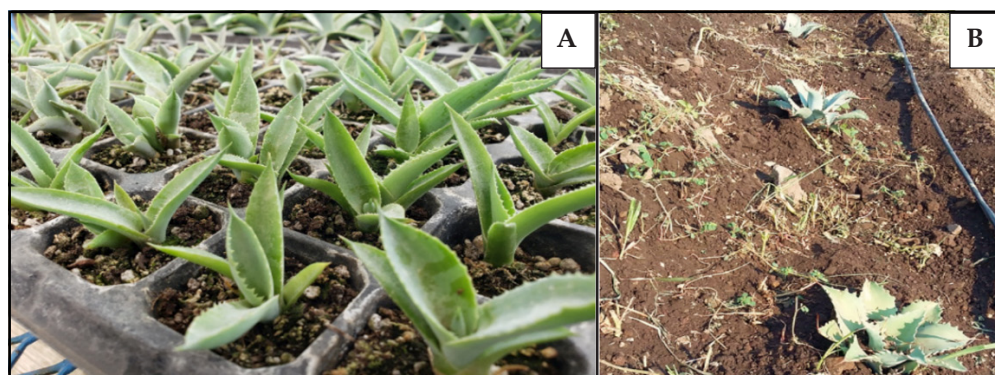


Figure 3. *Ex vitro* plants of *Agave potatorum*. A: Plants after four months of greenhouse acclimatization; B: Plants after four months in field conditions.

73 % survival for *A. potatorum* plants propagated *in vitro*. On the other hand, Enríquez-Del Valle *et al.* (2016b) did not report survival rates during acclimatization. In this study, the shoots obtained with all treatments achieved high survival rates irrespective of the presence or absence of roots. Survival during acclimatization is related to the plant vigor and a highly developed foliar system (Monja-Mio *et al.*, 2015; Martínez-Estrada *et al.*, 2019; Vieira *et al.*, 2020). Additionally, the wax content and thickness of the cuticle in this species, coupled with the type of metabolism (CAM), allowed a high survival rate during the acclimatization process. There are also external factors regulating *ex vitro* survival, such as water content (irrigation), fertilization, type of substrate, and environmental conditions (light, temperature, and relative humidity, among others) (Hoang *et al.*, 2020; Vieira *et al.*, 2020). Moreover, the anatomy and physiology of the genus *Agave*, together with appropriate greenhouse management, probably contributed to the adequate development of plantlets *ex vivo* without affecting their survival. Luna-Luna *et al.* (2017) observed that high fertigation during the acclimatization stage ensures the optimal development of *A. potatorum* plants obtained *in vitro*.

CONCLUSIONS

This study established an efficient protocol for the micropropagation of *Agave potatorum*, as evidenced by the high survival rates of regenerated plants. The interaction of 1.5 mg L⁻¹ of BAP and 3.0 mg L⁻¹ of IAA is effective for the shoot multiplication of this species, resulting in a greater number of shoots per explant. While the culture medium without NAA and IAA is suitable for promoting rooting *in vitro*. This research will contribute to the development of propagules intended to establish plantations. These plantations will prevent the intensive exploitation of wild populations, as this species will likely be subject to special protection by the official Mexican Standard NOM-059-ECOL-2010 (SEMARNAT, 2010) in the foreseeable future.

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