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***In Vitro* Propagation of *Agave guiengola* Gentry Using Semisolid Medium and Temporary Immersion Bioreactors**

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ABSTRACT

Agave guiengola Gentry is an endemic plant from a very small locality in Oaxaca, Mexico. Its conservation status is fragile and can rapidly worsen. Because of its scarcity, this agave has been used solely for ornamental purposes, but it could have other uses if more plants were available. *In vitro* propagation by enhanced axillary sprouting from stem segments was attained using Murashige and Skoog Basal Medium (MS) as well as basal medium supplemented with cytokinins 6-Benzylaminopurine (BA) or 6-(γ,γ -Dimethylallylamino)purine (2iP). The best treatment for shoot induction in semisolid medium consisted in MS supplemented with 2 mg l⁻¹ BA, obtaining a mean of 3.7 shoots per explant. Other interesting responses were observed, such as nodular callus induction using combinations of BA and 2,4-Dichlorophenoxyacetic acid (2,4-D); root induction without Plant Growth Regulators (PGR); and generation of shoot clusters. These clusters constituted an excellent explant for micropropagation in temporary immersion bioreactors, obtaining a propagation rate of 43 shoots per explant with 1 min immersion and 6 h immersion frequencies. All new plants rooted and survived the transfer to soil. This study developed an *in vitro* propagation scheme to produce individuals that can be used either for reforestation, economical purposes, or to carry out studies in this species to assess its full potential, avoiding exploitation from wild plants.

KEYWORDS

Agavaceae; temporary immersion systems; callus induction; micropropagation

1 Introduction

The genus *Agave* is native to the American continent. It comprises 200 species, of which 150 of them are endemic to Mexico, which is its center of origin and diversification [1]. These plants are adapted to arid and semiarid environments [1], and therefore to hostile conditions, such as low water availability, intense solar radiation, and extreme temperatures. Agaves are an outstanding part of arid ecosystems, and have important relations with other organisms, especially pollinators such as bats, bees, wasps, ants, among others [1]; conferring them an undisputable ecological importance. On the other hand, agaves were used by man in many ways since ancient times, and they are currently a source of food, alcoholic



beverages like tequila and mezcal, fibers and other raw materials, and have great potential in the production of sweeteners and biofuels [1,2].

A. guiengola is micro endemic to the Guiengola limestone formation at the Tehuantepec Isthmus in Oaxaca [3]; this location is not easily accessible, nevertheless, is such a small area that any disturbance could rapidly result in a devastating effect on natural populations. Currently, *A. guiengola* is used just for ornamental purposes and is considered threatened according to Mexican law [4]. Its potential for economical applications has never been fully assessed, possibly due to its inaccessibility and small population; thence is necessary to carry out studies to have a better knowledge of *A. guiengola*, since is crucial to avoid overexploitation of wild individuals. The development of efficient systems for its massive propagation is an indispensable requirement for the conservation and rational use of this species.

In vitro plant tissue culture may be part of comprehensive management strategies to preserve threatened and endangered species [5]. Micropropagation allows obtaining individuals that can be used for reforestation; and makes it possible to obtain whole plants, organs, or tissues for economical exploitation, avoiding the need to predate on natural populations. Micropropagation in the *Agave* genus was studied since the late 1980s within several species. Robert et al. [6] reported *A. fourcroydes in vitro* regeneration from callus explants using 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BA) [6]. *A. tequilana* was also regenerated from callus or stem segments using 2,4-D and BA [7,8] or Indole-3-Butyric Acid (IBA) and Kinetin [9]. *In vitro* regeneration from basal stem segments was reported in several species of the genus, examples of which are *A. cantala* [10], *A. cupreata*, *A. difformis*, *A. karwinskii*, *A. obscura*, *A. potatorum* [11]; and *A. tequilana*, *A. salmiana* sub. *crassispina*, *A. durangensis*, *A. obscura*, *A. pigmaea*, and *A. victoriae-raeinae* [8]. Other types of explants used were rizome in *A. sisalana* [12] and complete seedlings in *A. salmiana* [13]. Also, efficient systems for *in vitro* mass propagation were reported for *A. tequilana* [9,14], *A. grijalvensis* [15], and *A. angustifolia* [16]. These works used different cytokinins for *Agave* propagation, but only Domínguez et al. [11] reported the use of 6-(γ,γ -Dimethylallylamino)purine (2iP).

It has been well established that the use of bioreactors presents several advantages over conventional *in vitro* cultures on semisolid media; such as larger nutrient and Plant Growth Regulators (PGR) availability, increased oxygenation rate, and decreased apical dominance, among others; all of which often results in a higher growth or propagation rate [17]. For differentiated plant organ culture is recommended to use bioreactors with low shear stress and an efficient mass transfer [18]. Both qualities are present in temporary immersion bioreactors (TIBs) [19]. The use of TIBs was reported in *A. angustifolia* [20]; plantlets cultured in temporary immersion BioMINT™ bioreactors exhibited a better development of stomata and wax deposition compared to plantlets cultured in semi-solid media or permanent immersion.

The aim of this work was to develop efficient *in vitro* propagation systems for *A. guiengola*, which is one of the scarcest and most threatened species in the *Agave* genus. This propagation was achieved both on semisolid culture media and in TIBs, although, the greatest efficiency was obtained by combining both culture systems. As far as we know, there are no previously reported scientific papers about *in vitro* culture and propagation of this species. Despite the fact that TIBs technology has an increasing relevance in mass propagation of several plant species, there are very few reports using this technology within the *Agave* genus.

2 Materials and Methods

2.1 Plant Material

Capsules with viable seeds were collected in April 2016 in Oaxaca, Mexico. These seeds were disinfected and *in vitro* germinated to obtain axenic seedlings. For this, seeds were washed five times with 1% antiseptic soap with 0.1% benzalkonium chloride (Dermocleen®) in water, then disinfected for 1 min in 70% ethanol, 15 min in 1% sodium hypochlorite in water, and rinsed four times under aseptic

conditions with sterile distilled water. Seeds were germinated in culture vessels containing Murashige and Skoog Basal Medium (MS) kept at $25 \pm 2^\circ\text{C}$ under white light with a 16/8-h photoperiod (led lamps, $55 \mu\text{mol m}^{-2} \text{s}^{-1}$).

2.2 Culture Media

Basal medium consisted of MS basal media prepared with analytical grade reagents, supplemented with 30 g l^{-1} sucrose. When treatments required PGR, these were added at this point; then pH was adjusted to 5.7 ± 0.2 with NaOH or HCl 1 M. For semisolid medium, 9 g l^{-1} agar (Sigma-Aldrich, St. Louis, MO, USA) were added. Then the media were distributed in glass culture vessels or twin tank bioreactors and autoclaved at 120°C for 20 min.

2.3 Preliminary Multiplication

This step was carried out to obtain enough material for subsequent experiments and to assess the best treatment with cytokinin for shoot multiplication on semisolid media. Explants were taken from the *in vitro* germinated seedlings by excising leaves and roots, leaving only the stem that contains the apical and lateral meristems. Four treatments were used, consisting of basal MS media supplemented with 1 and 2 mg l^{-1} BA, and 1 and 2 mg l^{-1} (2iP), plus a control without PGR. Ten explants were placed in each treatment and maintained for 60–65 days at a constant temperature of $25 \pm 2^\circ\text{C}$ under white light with a 16/8-h photoperiod (led lamps, $55 \mu\text{mol m}^{-2} \text{s}^{-1}$). The experiment was conducted three independent times, with a completely random experimental design. Data were analyzed with the Minitab[®] software, v. 19.2020.1; ANOVA analyses were done, and means were compared using a Tukey's test.

2.4 Effect of Auxin and Cytokinin Combinations

Treatments consisted of semisolid basal media plus BA (0, 1, 2 and 3 mg l^{-1}) and 2,4-D (0, 0.1, 0.5 and 1.0 mg l^{-1}) in a 4×4 factorial design. We used ten explants per treatment, with three repetitions. The explants were taken from the shoots generated in the preliminary multiplication step, eliminating the leaves, and leaving only the stem. After 60 d of culture, the number of explants that produced shoots, roots, regular callus, and nodular callus were calculated, as well as the number of shoots per explant, and oxidized or necrosed explants. Culture conditions were the same as described above. Data were analyzed with the Minitab[®] software, v. 19.2020.1; ANOVA analyses were done, and means were compared using a Tukey's test. Additionally, the data generated in this experiment was used to perform a response surface analysis with a full quadratic model.

2.5 Experiments in Temporary Immersion System

The bioreactors used were of the twin-container design, as described by Monja-Mio et al. [20], which consisted in two 1000 ml flasks for each bioreactor. The volume of culture medium in the reservoir flask was 100 ml. Immersion frequency was 1 min every 12 h. To assess the best type of explant and optimal cytokinin level to be used in the temporary immersion system, one individual shoot or one shoot cluster were placed in each container of the bioreactor, and liquid MS supplemented with 1 mg l^{-1} BA or 2iP was employed. The multiplication rate was determined after 60 days of culture.

The previous experiment allowed to determine the best PGR. To determine the optimal immersion frequency, one shoot cluster was placed in each container and 1 mg l^{-1} BA was added to the liquid medium. The compared immersion frequencies were 6, 12 and 24 h. The control treatment consisted of explants cultured on semisolid medium supplemented with 1 mg l^{-1} BA. The multiplication rate was determined after 90 days; shoot length was measured and hyperhydrated shoots were counted. Data were analyzed with a Kruskal–Wallis test and a Dunn's multiple comparison test using the GraphPrism[®] software.

2.6 Rooting and Hardening

After micropropagation in the temporary immersion system, shoots were excised from the explants and transferred to semisolid medium without PGR for rooting. Plants propagated on semisolid medium were used as control. After two months, the plants developed roots, and once they reached at least 2 cm in length, it was possible to initiate the hardening process.

First, the protocol described by Domínguez et al. [11] for other agave species was used with small modifications; it consisted of gradually opening the lid of the vessel throughout one week to allow the plants to adapt to the exterior environment. Plants were extracted from culture vessels, the remaining medium was gently washed away from the roots, and the plants were placed in 200 cm³ pots, filled with pot soil and sand 1:1 v/v. The survival rate was assessed after two weeks and revised again after 8 months.

A second hardening experiment was conducted. Rooted plants were immediately extracted from the culture vessels; the roots were gently washed with tap water, and the plants were placed in 50 cavities plastic trays and covered with a plastic bag. After two days, plants were transferred to soil; two treatments were tested, one consisted in Sphagnum peat-moss (Premier[®]) and the second on a mixture of Sphagnum peat-moss (Premier[®]) and vermiculite 1:1 v/v. Substrate was placed into a double-layer seedling tray; the upper tray was divided into 50 cavities, 90 cm³ each, and provided with a draining hole. The lower tray was not divided and had no draining holes, and it was kept half filled with tap water. The trays were placed in a transparent plastic bag; after one week the bag was lightly perforated, and the bag was completely retired after two months. Then, the survival rate and other measurements were registered.

3 Results and Discussion

The germination rate was 84%, this 60 d after disinfection and *in vitro* inoculation of the seeds. The generated axenic seedlings, 4 to 6 cm high, were used as a source of explants for the first shoot multiplication experiments.

For shoot multiplication, treatments with BA and 2iP presented less-differentiated shoot generation in comparison to the control (Tab. 1). All explants on medium without PGR developed roots (Fig. 1b), as well as 73.7 % of the explants treated with 1 or 2 mg l⁻¹ 2iP. Callus formation was observed in some BA-treated explants, and nearly 80% on 2 mg l⁻¹ 2iP-treated explants. Interestingly, cytokinins, particularly BA, induced the formation of shoot clusters. These were formed by compact groups of approximately 50–60 poorly differentiated buds (Fig. 1c).

Table 1: Effect of 6-Benzylaminopurine (BA) and 6-(γ,γ -Dimethylallylamino)purine (2iP) on shoot generation in *A. guinegola*

Treatment	Shoots per explant (Mean + SE)	Explants with shoot clusters (%)	Explants with callus (%)	Explants with roots (%)
Control	3.4 + 0.54 a	10 c	0 b	100 a
1 mg l ⁻¹ BA	0.5 + 0.17 b	80 a	5 b	20 b
2 mg l ⁻¹ BA	0.7 + 0.21 b	95 a	30 b	5 b
1 mg l ⁻¹ 2iP*	1.6 + 0.42 b	35 bc	31.6 b	70 a
2 mg l ⁻¹ 2iP	1.2 + 0.31 b	60 ab	78.9 a	70 a

Note: N = 20 for all treatments, except * (n = 19). Equal letters, within columns, indicated there was no statistical differences with the Tukey test at $P \leq 0.05$

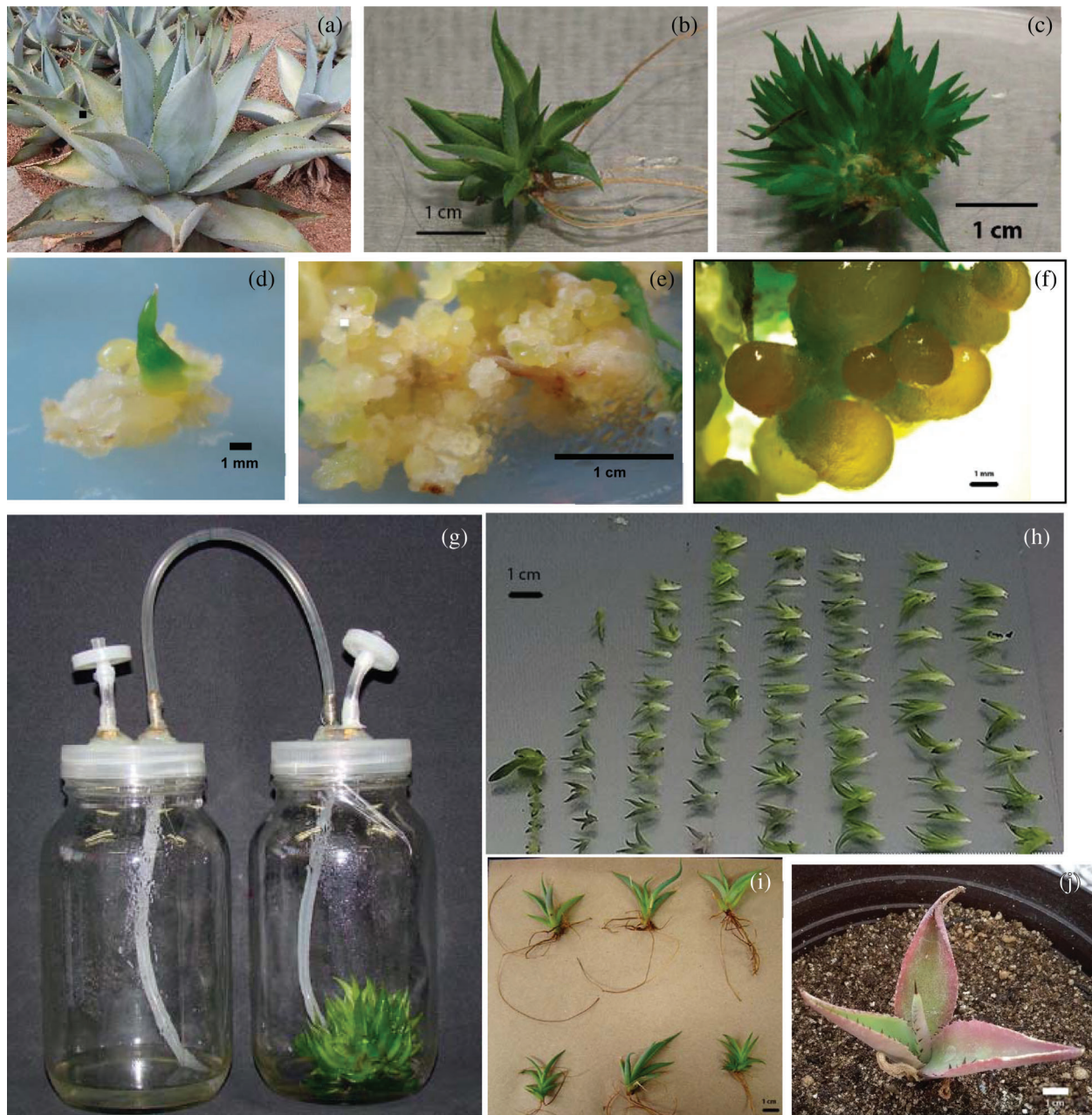


Figure 1: Different characteristics of a *A. guiengola*-*in vitro* propagation system. (a) Adult plants; (b) differentiated shoot with well-developed roots from control treatment; (c) shoot conglomerate generated on semisolid medium with 1 mg l^{-1} BA; (d) compact callus generated on semisolid MS with 1 mg l^{-1} 2,4-D; (e) nodular callus observed on semisolid medium supplemented with 1 mg l^{-1} BA and 1 mg l^{-1} 2,4-D and (f) globular structures observed on stereo microscope; (g) employed temporary immersion system; (h) shoots generated by a single explant inoculated in a TIBs, with an immersion frequency of 12 h; (i) shoots generated in TIB and rooted on semisolid medium without PGR; (j) shoot generated in TIB, after 8 months of being transferred to soil

Regarding the response to the cytokinin/auxin combination, several authors reported that cytokinins combined with small concentrations of auxins resulted in more vigorous shoots in some *Agave* species [6,7,8,10,13,21,22]. However, in *A. guiengola*, combinations of BA/2,4-D and 2,4-D alone always resulted in callus tissue induction (Tab. 2, Figs. 2d and 2e); the combination of 2 mg l⁻¹ BA and 0.1 mg l⁻¹ 2,4-D generated the highest amount of callus. Some treatments, particularly 1 mg l⁻¹ BA combined with 0.1, 0.5 and 1.0 mg l⁻¹ 2,4-D, produced nodular calluses that exhibit structures with a globose aspect that may be somatic embryos (Figs. 1e and 1f). Embryogenic calluses were reported for such characteristics using 2,4-D and BA in *A. tequilana*, *A. sisalana* and *A. angustifolia* [23]. Synthetic auxins, in particular 2,4-D, induce the development of poorly differentiated tissues but it antagonize development of organized structures. For this reason, they have been used to induce callus and stimulate the early stages of somatic embryogenesis. However, this mechanism of action inhibits shoot regeneration. This has been reported for other *Agave* species [24] and was confirmed in the experiment carried out with *A. guiengola* (Figs. 2a, 2c and 2d). Interestingly, the induction of nodular callus required the simultaneous presence of 2,4-D and BA (Fig. 2d). This indicates a synergy between the two types of PGR.

Table 2: Morphogenetic responses in *A. guiengola* to cytokinin and auxin combinations

Plant growth regulator (mg l ⁻¹)	Shoots per explant (Mean + SE)	Explants with shoot conglomerates (%)	Explants with roots (%)	Explants with regular callus (%)	Explants with nodular callus (%)	Oxidized or necrosed explants (%)
BA 2,4-D						
0.0 0.0	0.30 + 0.1189 c	0 d	76.7 a	0 d	0 c	0 b
0.0 0.1	0.14 + 0.0673 c	0 d	0 c	0 d	83.3 a	6.7 ab
0.0 0.5	0.03 + 0.0333 c	0 d	0 c	80.0 a	3.3 c	0 b
0.0 1.0	0 c	0 d	0 c	60.0 ab	6.7 c	20.0 a
1.0 0.0	3.70 + 0.6798 a	80.0 a	3.3 b	16.7 cd	0 c	0 b
1.0 0.1	0.80 + 0.2971 b	6.7 d	0 c	0 d	100.0 a	0 b
1.0 0.5	0.03 + 0.0333 c	0 d	0 c	0 d	100.0 a	0 b
1.0 1.0	0.03 + 0.0333 c	0 d	0 c	0 d	100.0 a	0 b
2.0 0.0	1.53 + 0.3171 b	53.3 bc	0 c	33.3 bc	6.7 c	3.3
2.0 0.1	0 c	43.3 c	0 c	66.7 a	0 c	10.0 ab
2.0 0.5	0.03 + 0.0333 c	0 d	0 c	3.3 cd	90.0 a	0 b
2.0 1.0	0 c	0 d	0 c	26.7 cd	40.0 b	0 b
3.0 0.0	1.59 + 0.4506 b	73.3 ab	0 c	16.7 cd	0 c	13.3 ab
3.0 0.1	0.47 + 0.1571 b	3.3 d	0 c	76.7 a	20.0 bc	0 b
3.0 0.5	0.20 + 0.0884 c	0 d	0 c	3.3 cd	93.3 a	0 b
3.0 1.0	0.03 + 0.0333 c	0 d	0 c	66.7 a	33.3 b	0 b

Note: BA: 6-Benzylaminopurine; 2,4-D: 2,4-Dichlorophenoxyacetic acid. N = 30 for all treatments. Equal letters, within columns, indicated there was no statistical differences with the Tukey test at $P \leq 0.05$

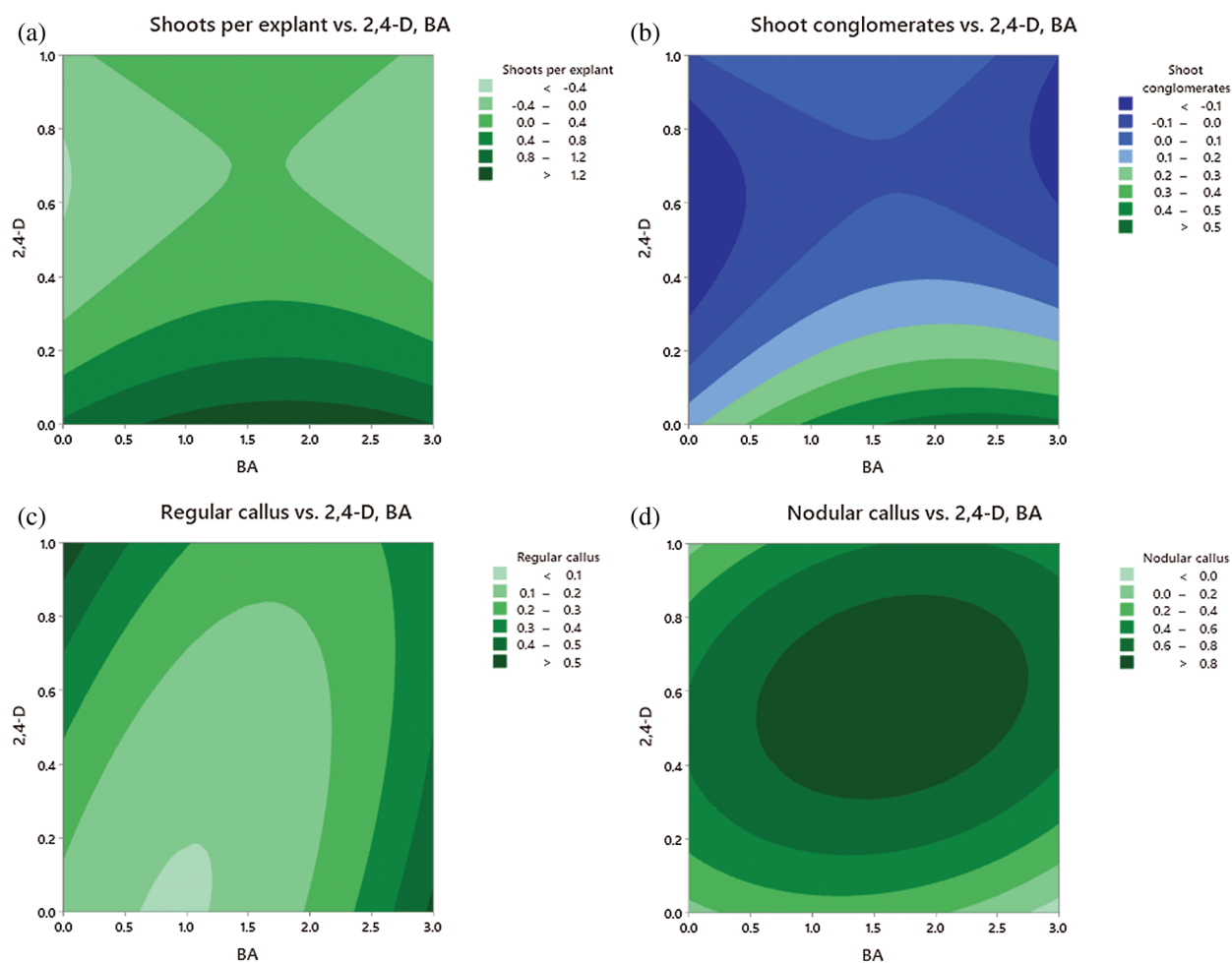


Figure 2: Contour response surface graphs of influences of 6-Benzylaminopurine (BA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) on the *in vitro* morphogenic responses of *A. guiengola*. (a) Shoots produced per explant; (b) probability of obtaining shoot conglomerates; (c) probability of obtaining regular callus, and; (d) probability of obtaining nodular callus. BA and 2,4-D concentrations in mg l^{-1}

Treatments with BA alone generated shoots in *A. guiengola* explants (Tab. 2, Fig. 2a); the best treatment was 1 mg l^{-1} BA, with a mean of 3.7 shoots per explant; this result is similar to those reported for other species, such as in *A. salmiana* sub. *crassispina*, in which Ramírez-Malagón et al. [8] reported a mean of 3 shoots per explant, and Angeles-Espino et al. [9] reported 3.67 in *A. tequilana*. However, Puente-Garza et al. [13] reported 14 shoots per explant in *A. salmiana*; Ramírez-Malagón et al. [8] reported 12 shoots per explant in *A. tequilana* and 12.8 in *A. oscura*. Nevertheless, we observed that some treatments do not produced well-differentiated individual shoots but shoot clusters (Fig. 2b). In these, the shoots were too small and poorly differentiated, so it was not possible to separate them for rooting, but it was necessary to take further steps to attain their growth and full differentiation. Since an average of 3 shoots per explant were fully developed and the rest remained too small, it is possible that the first ones inhibited the development of the rest. As Gahan et al. [25] pointed out, the first meristem formed *in vitro* may inhibit the development of further meristems.

In this experiment, oxidation and necrosis of some explants exposed to the highest concentrations of PGR were also observed (Tab. 2). This may indicate that the use of higher concentrations would be toxic to tissue.

In many plant species, auxins are necessary for root induction [26], increasing the cost and time during the rooting stage; auspiciously, in *A. guinegola* explants, roots were observed almost exclusively in the control treatment (Fig. 1b), therefore, in this species it was not necessary to employ exogenous PGR to develop an appropriate *in vitro* root system.

For the experiments in TIBs, individual shoots and shoot clusters were used as explants, in order to attain *A. guinegola* propagation through sprouting of lateral meristems or by direct organogenesis.

In the semisolid system, 2iP induced bigger and more differentiated shoots, but BA produced shoot clusters more often. In TIBs, BA had a better response; apparently, BA added to liquid medium does not generate new shoots but induces enlargement and development of small undifferentiated ones in a shorter period (two to three months). Clearly, shoot clusters are better than individual shoots (Tab. 3) to use as explants in TIBs.

Table 3: Effect of explant type and cytokinin in a temporary immersion system for *A. guinegola*

Plant growth regulator	Explant type	n	Shoots per explant (Mean + SE)
1 mg l ⁻¹ BA	Individual shoots	6	2.8 + 0.96 b
	Shoot conglomerates	3	38.3 + 2.33 a
1 mg l ⁻¹ 2iP	Individual shoots	3	1.7 + 1.67 b
	Shoot conglomerates	3	8.0 + 4.62 b

Note: Equal letters, within columns, indicated there was no statistical differences with the Tukey test at $P \leq 0.05$.

For the development of protocols using TIBs, is crucial to assess the optimal immersion times and frequencies. Agaves are adapted to low water availability; therefore, the shortest possible immersion time, 1 min, was employed (Tab. 4). Longer immersion times would favor the intake of nutrients but would also increase the rate of hyperhydration. There were no statistical differences between immersion times of 6, 12 and 24 h and the control on semisolid medium. However, shoots obtained at a 6 h immersion frequency achieved a significant increase in length than shoots from other treatments and were morphologically best differentiated. This probably due to a higher availability of nutrients and cytokinin with respect to the other frequencies tested; only 12.2% shoots were hyperhydrated (Tab. 4). Ríos-Ramírez et al. [16] also used shoot clusters as explants in *A. americana*. They attained 45.2 shoots per explant; and they achieved their development using BA.

Table 4: Effect of immersion frequency in *A. guinegola*. shoot conglomerates cultivated on semisolid medium were used as control

Immersion frequency	n	Shoots per explant (Mean + SE)	Shoot length (Mean + SE)	Hyperhydrated shoots (%)
Control	3	11.7 + 4.0555 a	0.83 + 0.0578 c	0
6 h	4	43.0 + 6.9160 a	1.41 + 0.0473 a	12.2
12 h	3	38.33 + 2.3330 a	1.09 + 0.0391 b	0
24 h	6	45.33 + 10.5500 a	1.27 + 0.0739 b	33.8

Note: Equal letters, within columns, indicated there was no statistical differences according to a Dunn's multiple comparisons test.

After the shoots were rooted, hardened and transferred to soil as described previously [11], the plants obtained from TIB had a survival rate of 81.3% after 14 days, while plants obtained from semisolid cultures had a 77.3% survival rate in the same period; there were no statistical differences. However, after 8 months, the survival rate dropped to 45.8% and 57.1%, respectively.

Due to low survival rates, the second hardening protocol was completed. After 100 d, survival rates of TIB-generated plants were 100% in peat-moss and 80% in peat-moss and vermiculite. Plants from semisolid cultures in both treatments had a survival rate of 80%. The natural habitat of *A. guiengola* has a warm sub-humid climate, with temperatures between 10°C and 20°C, and an annual precipitation of 600–1000 mm [27], which is more temperate and humid than the habitats of other agaves species, hence the need for a higher environmental humidity for young plants. *In vitro* culture conditions cause some morphological and physiological alterations, and plantlets exhibit juvenile leaves, impaired stomatic and photosynthetic mechanisms and poor water management, among other deficiencies [28]. Therefore, during the first 30 d after hardening 80% of the plantlets lost some leaves, and the remaining ones had a slight dehydrated appearance; in consequence their size decreased. During the next 30 d there was a slight increase in size, but more important, the emergence of new leaves of healthier appearance was observed, which is clear evidence of the successful hardening of the plantlets.

Eight-month plants obtained from TIB and semisolid medium increased their diameter 276.8% and 206.8%, respectively. Leaves were ovate-lanceolate; terminal spines were dark brown, not decurrent and rounded above and below; the leaf margins presented dark brown teeth, four per cm (Fig. 1g); all of these are consistent with characteristics reported in adult wild plants [3]. Leaves were light green, some of them with reddish margins, and not light gray or white-glaucous as reported for wild individuals [3]. Plants germinated in potted soil from the same seed batch and approximately same age exhibited the same characteristics than *in vitro* propagated plants, except reddish margins (data not shown). Nevertheless, some species acquire its characteristic coloration until maturity, and color is also highly dependent of light intensity and composition, which could be quite different between the greenhouse and the natural environment. All aspects considered, the morphology of plants micropropagated in temporary immersion bioreactors after hardening was normal.

4 Conclusions

The use of BA or 2iP on semisolid medium in *A. guiengola* did not result in a higher generation of well differentiated shoots than the control treatment. However, the use of these cytokinins on semisolid medium, particularly BA, induced the generation of shoot clusters, which are shoot aggregates too small and poorly differentiated, hence it is not possible to separate nor count them. Using BA on these shoot clusters in TIBs instead of semisolid medium, small and indistinguishable shoots were capable of growth and development at higher rates, producing in average 43 shoots per explant. The culture conditions were MS supplemented with 1 mg l⁻¹ BA, 1 min immersion time and 6 h immersion frequency. The shoots obtained in TIBs were rooted on semisolid medium without PGR, and the use of an efficient protocol for hardening resulted in a 100% survival rate of the plantlets transferred to soil; and they exhibited a normal morphology. Hence, we propose that combining several techniques in different stages leads to a more efficient propagation scheme.

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