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ARTICLE

In Vitro-Propagation of *Agave tequilana* Weber cv. azul in a Temporary Immersion System

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ABSTRACT

In Mexico, there is a need to produce large quantities of plantlets for the establishment and replanting of blue (cv. azul) agave production areas. Most of these plots are within the origin denomination area (DOT, Spanish acronym) of the distilled product of this plant, known as tequila. The objective of this study was to develop an *in vitro*-propagation protocol for *Agave tequilana* Weber cv. azul using segmented stems in both: solid and liquid media. A disinfection and *in vitro* technique were developed to obtain shoots, through plantlets collected in commercial plots, which attained 100% surface-disinfection and budding rate. At the multiplication stage, the effects of 6-Benzylaminopurine (BA) (0.0, 4.4 and 13.2 μM) and kinetin (0.0, 9.4, 18.8 and 37.6 μM) were evaluated on lateral-shoot production of segmented sagittal stems. These were cultivated on Murashige & Skoog (MS) medium, with the addition of 3.0% sucrose and 8 g L^{-1} agar. It was observed that BA and kinetin increased the number of shoots per explant, obtaining up to 18 and 26, respectively. Furthermore, it was found that just the sagittal segmentation of explants increased axillary budding. On the other hand, segmented-stem bases were grown in MS liquid medium with 3.0% sucrose, inside a RITA® system, programmed by a 5 min immersion step with a frequency of every 4 h. The effect of Indole–3-Acetic acid (IAA) (0.57, 2.9, 5.7 μM) was evaluated, while maintaining a concentration of BA (13.2 μM). It was observed that the greatest concentration of IAA led to the formation of more than 20 buds per explant. These results offer a new methodology to increase the efficiency of A. *tequilana* Weber cv. azul-*in vitro* multiplication by sagittal segmentation of stems and the addition of BA and/or IAA.

KEYWORDS

Plant tissue culture; stem-disk culture; tequila; *in vitro* culture

1 Introduction

The cultivation zone for *Agave tequilana* Weber cv. azul in Mexico requires increased areas, due mostly to the greater national and international demand for Tequila, which is the distillate from that plant. If this is not achieved, there will be a shortage of agave plant heads. The growth of this crop in the origin denomination area (DOT, Spanish acronym) has generated the need to develop more efficient ways for

multiplying the *Agave tequilana* plantlets, which must comply with the conditions of agro-industrial production. The *in vitro*-culture technique is an alternative that allows the annual production of large numbers of plants in small laboratory spaces, maintaining their genetic stability [1–3]. There are several reports on micropropagation of various *Agave* species, through distinctive systems of plant regeneration [4]. Plants were obtained by direct organogenesis from callus tissue [3,5–10]. Multiple shoots were induced through basal meristems; and embryogenesis and indirect organogenesis were also used [11–17]. Furthermore, plants were regenerated by direct organogenesis from stem tissues [18]. Somatic embryogenesis was also induced, and several plants were obtained [19–22]. In addition to micropropagation, there are reports on the production of secondary metabolites by cultures of callus tissue [16,23,24].

There are challenges in the agave-*in vitro* culture that have influenced the micropropagation efficiency for different species, which have required optimization. For example, through the establishment stage, the need to transplant selected plants from the field to greenhouse conditions has been mentioned. Fungicides and bactericides were applied during preconditioning or during surface disinfection to reduce *in vitro* contamination [9,11,16]. In the multiplication stage, the proliferation procedures were developed in a complex way, and, in most cases, they were initiated from calli derived from leaves, stems or apex tissues. Once these calli were produced, their adventitious shoots were induced to generate different multiplication rates. This induction depended on the combined use of 2, 4-D and cytokinins at different levels [6,7,9,11,21], cytokinins alone [12,17], and cytokinins and auxins [10,16].

On the other hand, there are indications that the protocols for *Agave* micropropagation are based on high costs. This justifies the modification of methods through organogenesis, to achieve an efficient and cost-effective multiplication protocol at a large scale [1]. In other plant species, it was demonstrated that these costs can be reduced through automation processes, such as the application of temporary immersion bioreactors [4,25–28]. Among these systems, the RITA® (Automated Temporary Immersion System), due to its applications and benefits [29], was used in different optimization processes for micropropagation [30–33]. This system consists of a single container that has two compartments separated by a tray with a net, where the explants are kept; then, through a pneumatic mechanism, the explants are intermittently exposed to a liquid-air interface. Its principle and efficiency were compared with other systems [34–37].

The two most important benefits offered by blue agave *in vitro* culture are related to the multiplication efficiency and the guarantee of phytosanitary conditions. Firstly, the *Agave* spp. micropropagation allows the production of clonal lines and several thousand plants from a single mother agave plant within 12 months *vs.* a few tens of plants that the same plant will produce in the 6th year of its life cycle. The *Agave in vitro* culture results in high-quality, high-yielding materials that produce a significant increase in yield at the plantation stage [14]. Secondly, the sterile conditions of nutritive media and the aseptic techniques for establishment and multiplication, allow to keep axenic-*in vitro* tissue cultures. There are also techniques that facilitate to remove diseases produced by endogenous microorganisms, including viruses [38]. For *Agave tequilana* Weber cv. azul the phytosanitary condition is especially important since the clonal condition of plantations favors the disease-spreading, which can have profound effects on the availability of material for plantations and on the crop productivity in the field [15].

Since almost 35 years ago, Robert et al. [14] defined a protocol reported as an efficient method to micropropagate agaves and a strategy for the management of large-scale production that has been successfully applied to *A. tequilana* Weber cv. azul and several other *Agave* species. This scheme has formed the basis for the development of other methodologies to improve that efficiency, mainly aimed at exceeding multiplication rates. This is the main source of information for companies dedicated to the production of agave plantlets. Although none of the research reports mention the cost per micropropagated plantlet (Tab. 1), the efforts in such studies suggest an optimization in the cost of inputs

and in the process technique. Our study did not estimate the degree of cost reduction in the developed methodology, but, based on the comparison with the materials and methodologies reported, it proposes a level of multiplication efficiency higher than them. This suggests possible cost savings for *A. tequilana* Weber cv. azul up to the stage of micropropagation that is addressed in this study. On the other hand, the information that the companies have shared with the public reflects a level of technology transfer derived from the referred investigations. For example, in Mexico there is a company that reports having achieved the massive propagation of more than 22 million *in vitro* blue agave plants. It also offers to guarantee the supply of "elite" material for tequila industry, and mentions a quantity of losses after transplantation in a range of less than 2%. In addition, this company reports having reached harvest in five years, with an average content of reducing sugars between 20–24 percent in the harvestable product [39]. This shows that the potential of the micropropagation technique can meet the needs that intense industrial activity requires.

Table 1: Reported methods to improve the *Agave* species multiplication rate by direct bud and shoot formation

Agave species	Plant growth regulators/type of explant	Multiplication efficiency	Reference
A. fourcroydes	2,4-D (0.11 μM) and BA (44 μM)/rhizome segments	1–6 shoots per explant/12 weeks	[6]
A. cantala Roxb.	NAA (0.41 μM), IBA (0.49 μM) and Kinetin (2.35 μM)/Shoots from rhizome	4.86 shoots per bud/40 days	[11]
Agave sisalana	60 days Induction BA (22.2 and 44.0/μM); 1st. 60 days subculture on BA (22.2 μM); 2nd. 60 days subculture on BA (2.2 or 4.4. μM)/rhizome segments	8–12 buds and shoots/120 days	[12]
A. sisalana Perrine	Kinetin (2.35 μM)/rhizome segments	5.7 shoots (time not reported)	[40]
A. parrasana Berger	BA (53.2 μM)/Shoots from *in vitro* germinated seeds	48.6 shoots per explant/60 days	[13]
A. victoriae-reginae	BA (2.2–8.8 μM)/Stem bases from *in vitro* germinated seeds	1.9–2.2 shoots per explant/12 weeks	[41]
Agave salmiana Gentry	IAA/BA (1.43/8.8 μM)/2–3 mm-thick discs sectioned from the stem core.	Propagation via regeneration of 20 shoots per explant/45 days	[16]
Agave spp.	*A. oscura* IBA/BA (2.46/4.44 μM). *A. tequilana* Pulses with 2,4-D (6.8 mM-3 d); subculture on 2,4-D/BA (0.113 μM; + 4.44 μM BA) or BA/NAA *A. duranguensis* IBA/BA (0.049/4.44 μM) *A. pigmaea* IBA/BA (0.049/13.31 μM) *A. victoria-reginae.* IBA/BA (2.46/2.22 μM) *A. salmiana* subspecies *crassispina* IBA/BA (0.49/4.44 μM) In all species, the explants were complete stem cores established from young plants	12.8 shoots/60 days 12.0 shoots/30–60 days after a pulse of 1–6 days 5.9 shoots/60 days 5.6 shoots/60 days 5.5 shoots/60 days 3.0 shoots/60 days	[9]
Agave spp.	*A. cupreata* and *A. karwinskii* (BA 6.6 and 4.4 μM). *A. difformis* and *A. obscura* (TDZ 0.9 μM). *A. potatorum* (kinetin 14.1 μM). In all species, the explants were shoot bases from *in vitro* germinated seeds.	10.5–6.1 shoots per explant/12 weeks 8.5–11 shoots per explant/12 weeks 6.9 buds per explant/12 weeks	[17]

(Continued)

Table 1 (continued)

Agave species	Plant growth regulators/type of explant	Multiplication efficiency	Reference
A. tequilana	IBA (0.246 μM) y Kinetin (46.46 μM)/Shoot apex from young plants.	3.67 shoots/4 weeks	[10]
A. tequilana, A. angustifolia, A. applanata, A. salmiana, A. colimana, A. victoria-reginae, A. inaequidens, A. maximiliana	Authors offer a proven protocol for these species. BA (0–52.8 μM) and 2,4-D (0.04–0.11 μM)/ Rescued zygotic embryos, apex (5 mm) and bulbils.	Authors ensure 3–6 shoots using composed explants (2–3 shoots)/4 weeks; according to the species and/or variety.	[3]

Based on the aforementioned facts, the objective of this research was to develop a more efficient system for the *in vitro* propagation of *Agave tequilana* Weber cv. azul, through the cultivation of segmented stems. The results obtained demonstrate a significant increase in multiplication efficiency on a semi-solid medium and in a RITA® system. Up to our knowledge, both results have not been reported for this species and variety of *Agave* using segmented stems.

2 Materials and Methods

2.1 Establishment of Aseptic Cultures

The technique of establishing *Agave tequilana* Weber cv. azul *in vitro* cultures was implemented according to the procedure for garlic micropropagation by cultivating segmented-stem discs, defined by Ayabe et al. [42]. The principles applied for the multiplication of bulbous species by traditional methods such as scooping, and scoring were as described by Hartman et al. [43] and Miller et al. [44].

Agave plants, of approximately 2–3-year-old, were obtained from plantations located in the DOT, and these plants had on average stem base size of 10.0 ± 2.0 cm diameter. The bulbous bases of the plants were reduced in size by sagittal segmentation, until internal basal stem segments of 4.0 ± 1.0 cm long and 2.0 ± 1.0 cm wide were obtained. These basal segments were rinsed for one minute with tap water, adding 0.2% of Extran® MA 02 detergent (Merck, Mexico). Then, they were rinsed to remove the detergent, and the excess water was dried using sterile paper towels. Under aseptic conditions, the basal segments were reduced in size, until obtaining central portions of the stem, with a diameter of 1.0–1.5 cm. Subsequently, these segments were surface disinfected by stirring in ethanol (80% v/v) for 1 min, and then, by gently stirring in a commercial solution of Cloralex® bleach (NaOCl) (20% v/v) for 15 min, adding 0.02% Tween 80® (Phytotechnology Laboratories, KS. USA). Later, they were rinsed three times, 30 min each, with sterile distilled water to remove chlorine and detergent. Finally, the agave cylinders were cut, using an Olympus SZ60 stereoscopic binocular microscope (USA) at 15X magnification, to obtain 3.0–5.0 mm diameter stem discs, so each final explant had 1.0 mm from the base of the stem and 1.0 mm for the cataphyle-bases. Each stem disc was sagittally segmented into four parts, so that the cut passed through the center of the disc. Each fragment was then placed vertically on Murashige and Skoog basal medium (MS) containing 3.0% sucrose and 8.9 g L^{-1} agar (Phyto Technology Laboratories, KS, USA). To prepare this medium, the pH value was adjusted to 5.8 (pH/ORP/ISE, HI98191 Hanna meter, Mexico) before adding the agar. The medium was sterilized at 121°C for 15 min. The addition in the medium of BA (0.0, 4.4, 8.8, 13.2, 17.6 μM) and zeatin (0.0, 4.6, 9.2, 13.8, 18, 4 μM) was evaluated in conjunction. The explants were incubated at 25 ± 1.0°C under a 32 μmol m^{-2} s^{-1} illumination and a 16 h photoperiod of light. Each treatment consisted of six repetitions, where each one was represented by an explant (stem disk) divided

into four segments in each culture container. The evaluation was carried out after 12 weeks of cultivation. The variables measured corresponded to the percentage of contamination and the number of shoots per explant.

2.2 Multiplication on Solid Medium

The multiplication technique was implemented considering the use of MS medium, and the type and range of effective concentrations of single cytokinins or combined with auxins, according to Binh et al. [11]; Ramírez-Malagón et al. [9] and Silos-Espino et al. [16]. The differences of this study in comparison to those published consisted in the cultivation of sagittal segmented-stem bases as explants, and the avoidance of using 2,4-D as auxin.

For this stage of multiplication on semisolid medium, *in vitro* established plants were selected, and individual shoots (6–8 cm long and 1.0 ± 0.2 cm in diameter) were utilized. The leaves were removed, and four segments were obtained from the cylindrical portions of the stem bases. These were placed in a horizontal position, with the segmented section in contact with the MS medium, which contained 3.0% sucrose and 8.0 L^{-1} agar. The pH was adjusted to 5.8 before adding the agar. The BA (0.0, 4.4 and 13.2 μM) and kinetin (0.0, 9.4, 18.8 and 37.6 μM) were added independently to the medium. Explants were incubated at 25 ± 1.0°C under an illumination of 32 μmol m^{-2} s^{-1} and a photoperiod of 16 h light. Each treatment consisted of four and three repetitions for BA and kinetin, respectively. Each repetition was represented by an explant (stem disk) divided into four segments, inside a culture flask and containing 20 ml of culture medium. After six weeks of culture, observations were carried out. The variable measured was the number of shoots per explant.

2.3 Multiplication in a Temporary Immersion System

For the *A. tequilana* Weber cv. azul multiplication in the RITA® temporary immersion system, the MS medium was used, and the immersion cycle and culture conditions were based on Ramos-Castellá et al. [45]. The shoots (10–15 cm in length) established on solid medium were used. From these, lateral basal portions measuring 1.0 cm length and 1.0 cm diameter were taken from the stem. The explants were sagittal sectioned, obtaining four segments per explant, and placed into an automatic temporary immersion system (RITA®). All treatments including BA (13.2 μM) and three IAA concentrations (0.57, 2.9, 5.7 μM) were evaluated. To each RITA® container, 300 ml of liquid MS medium with 3% sucrose were added. The immersion time was programmed at 5 min with a frequency of every 4 h. The explants were incubated at 25 ± 1.0°C, under an illumination of 32 μmol m^{-2} s^{-1} and a photoperiod of 16 h light. Each RITA® represented one treatment, and each treatment had six explants sectioned into four segments.

A normality analysis was carried out for the data according to the Shapiro-Wilk method in all experiments. For the establishment stage's evaluation, given the similarities among the cytokinins (μM) concentrations, an analysis of variance was conducted for a factorial design with a completely random arrangement, evaluating two factors: Plant Growth Regulators and their levels. For the other experiments, a simple analysis of variance was conducted according to a completely random arrangement. The means separation in all experiments was performed with the Tukey test ($P \leq 0.05$).

3 Results and Discussion

3.1 Establishment of Aseptic Cultures

After 12 weeks, the segmented-stem explants generated multiple shoots, greater than 3 mm tall in each treatment, with and without cytokinins. BA and zeatin had differences in the induction of shoot number per explant ($P = 0.0000$). Likewise, it was observed that the increase in the concentration of cytokinin produced a greater shoot number ($P = 0.0000$), in the range of the evaluated concentrations (Tab. 2).

Table 2: Effect of BA and zeatin on shoot production in segmented-stem explants of *A. tequilana* Weber cv. azul after 12 weeks

BA-Zeatin (μM)	Shoots per explant*
0.0–0.0	3.00 ± 0.47 a
4.4–4.6	6.66 ± 0.68 b
8.8–9.2	7.00 ± 0.83 b
13.2–13.8	9.66 ± 1.25 bc
17.6–18.4	12.41 ± 1.50 c

*Note: Average values ± standard error, with distinctive letters within the column to the right are significantly different (Tukey; α = 0.05).

The results obtained using agave segmented stems as explants are similar to those reported in other species by cultivating bulbous and rosette stems, generated from the apex or axile of rhizomes. Xue et al. [46] described the *in vitro* formation of embryogenic garlic calli from the basal plate in bulbs that conserved the apex and in aerial bulbs. In this same species, Ayabe et al. [42] demonstrated that the *in vitro* culture of segmented stem discs could increase the multiplication efficiency, compared to other methods. They also observed that the proliferation response was independent of the exogenous addition of BA and/or NAA. In the *Agave* genus, the regenerative capacity of basal tissues for *in vitro* establishment has been demonstrated. For instance, central stem tissue has been cultured by sagittal segmentation in *A. cantala* Roxb., *A. fourcroydes* Lem. and A. *sisalana* Perrine [11]; however, this was achieved by indirect regeneration of adventitia buds. Lateral sprouting has also induced through transversal segmentation of stems in *A. salmiana* Gentry [16]. In most reports, budding and shooting were induced by growing whole stems containing the apex and the stem's inner base. In our research, according to the high percentage of surface disinfection and sprouting, 100 percent of the responses observed were uniform in both: the aseptic establishment and the lateral sprouting capacity during this stage.

The direct axillary shooting in segmented stems of *A. tequilana* Weber cv. azul, compiles the postulations of the apical dominance phenomenon that derives from the presence of auxins at the apex, which by elimination, such as decapitation, can be reversed [47,48]. Due to the addition of exogenous cytokinins, lateral budding is promoted [49]. Regarding the addition of auxins for the different *Agave* species, most of the micropropagation reports are based on the whole apex culture in the presence of 2,4-D, generally with the addition of BA. This has increased indirect budding of the adventitia and, subsequently, lateral shoot proliferation [6,7,9–12,16,17,21]. This effect confuses the distinction of the origin of the organogenic response. Furthermore, only limited research was reported to determine the genetic status of sprouts [50–52]. In addition, it was observed that in different cell explants used to induce somatic embryogenesis, 2,4-D increased the appearance of cytogenetic abnormalities, related to different levels of somaclonal variation [53].

3.2 Multiplication in Solid Medium

After six weeks of culture, the number of shoots per explant was evaluated. It was observed that the addition of BA ($P = 0.0018$) and kinetin ($P = 0.0001$) increased the number of lateral shoots. While the lack of BA induced three shoots per explant, the addition of this cytokinin (8.8–13.2 μM) induced the formation of up to 18 shoots (Tab. 3). The control treatment for kinetin induced the formation of four shoots per explant, and the addition of this cytokinin (9.4–37.6 μM) induced the formation of up to 26 shoots. These responses demonstrated that the sagittal cut and segmentation of the explant itself promoted lateral dominance and multiple shooting (Fig. 1). This axillary shooting response, given this type of explant and the effects of phytohormones, up to our knowledge, has not been previously reported

in *Agave* [3,4,10,11,16]. Nonetheless, Silos-Espino et al. [16] reported that stem discs obtained by transversal segmentation of *A. salmiana* Gentry explants, produced less than two shoots per explant when placed on MS medium containing IAA 0.25 mg L^{-1}. In our results, the addition of BA and kinetin significantly increased the proliferation of lateral shoots (Tab. 3).

Table 3: Effect of two cytokinins (BA and kinetin) on axillary proliferation in *A. tequilana* Weber cv. azul segmented stems, after 6 weeks of *in vitro* culture

BA (μM)	Shoots per explant*
0.0	3.0 ± 0.81 a
4.4	11.7 ± 1.60 b
13.2	18.2 ± 3.06 b
Kinetin (μM)	**Shoots per explant***
0.0	4.3 ± 0.88 a
4.7	19.7 ± 2.02 b
9.4	22.7 ± 2.33 b
37.6	26.7 ± 1.76 b

Note: *Shapiro-Wilk Test: BA, $P = 0.11$; kinetin $P = 0.50$. Average values ± standard error with distinctive letters within the column to the right, are significatively different (Tukey; $\alpha = 0.05$).

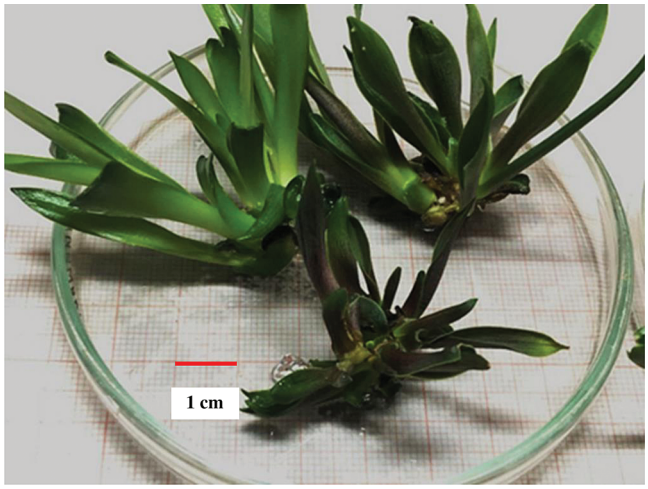

Figure 1: Axillary shoots induced by the addition of BA (13.2 μM) in segmented stems of *A. tequilana* cv. azul, after 6 weeks of culture in a solid MS medium

Regarding the multiplication efficiency, our results are different from those of other authors (Tab. 1). For example, Nikam [40] observed in *A. sisalana* Perrine that the addition of kinetin 0.5 mg L^{-1}, in the range of 0.2 to 2.0 mg L^{-1}, induced the formation of 5.7 shoots per rhizome-explant. Santacruz-Ruvalcaba et al. [13] observed that in *A. parrasana* Berger, the presence of BA in the medium, in the range of 13.3 to 53.2 μM, induced the formation from 22 to 48.6 shoots per explant in six weeks. These explants came from shoots obtained by *in vitro* germinated seeds. The shoots were kept for weeks in a medium containing 2,4-D and BA, similarly to what was reported by Robert et al. [6] for *A. fourcroydes*. In this study, the medium without BA did not induce the formation of lateral shoots. Similarly, Martínez-Palacios et al. [41] observed in *A. victoriae-reginae*, that the addition of BA in a range of 2.2 to 8.8 μM induced the

formation of 1.9 to 2.2 shoots per explant; these explants consisted of a complete stem base, which was *in vitro* generated through seeds. The culture medium without the addition of BA induced the formation of 1.1 shoots per explant and the observations were made during 12 weeks. Then again, Rosales et al. [17] evaluated the effect of different cytokinins: BA (1–3 mg L^{-1}), 2iP (1.0–3.0 mg L^{-1}), kinetin (1.0–3.0 mg L^{-1}), thidiazuron (TDZ 0.1–0.4 mg L^{-1}) and Meta-hydroxybenzyl Adenine (MT 0.5–2.0 mg L^{-1}) on proliferation of lateral shoots in *A. cupreata* Trel and Berger, *A. difformis* Berger, *A. karwinskii* Zucc., *A. obscura* Schiede and *A. potatorum* Zucc., using stem bases as explants after 12 weeks of culture. *A. cupreata* and *A. karwinskii* (BA 1.5 and 1.0 mg L^{-1}) developed 10.5 and 6.1 shoots per explant, respectively. *A. difformis* and *A. obscura* (TDZ 0.2 mg L^{-1}) provided 8.5 and 11 shoots per explant, correspondingly. *A. potatorum* (kinetin 3.0 mg L^{-1}) produced 6.9 buds per explant. However, this study did not evaluate shoot production in a growth medium without cytokinins. Furthermore, the origin of the shoots used as explants came from seeds.

According to our results, it can be suggested that the exogenous presence of cytokinin is important in the induction of lateral dominance at the base of complete stems of *Agave tequilana* Weber cv. azul. Likewise, in this *Agave* species, the sagittal segmentation of stem base inhibits apical dominance. Also, efficiency is increased in both: shoot number and reduction of time to develop each shoot. In summary, the results obtained in this study on *Agave tequilana* Weber cv. azul are different from results previously published by other researchers (Tab. 1).

3.3 Multiplication in a Temporary Immersion System (TIS)

The *A. tequilana* Weber cv. azul explants, after 12 weeks of incubation in TIS, produced different number of shoots per explant, which were evaluated. The IAA addition (0.57–5.7 μM) enhanced the number of lateral shoots, higher IAA concentrations induced the formation of more than 20 shoots per explant (Tab. 4). The shoots depicted a normal differentiation appearance, noticing a shiny cuticle without callus formation at the bases, and without any qualitative malformation appearance or hyperhidrosis (Fig. 2). The lack of hyperhidrosis in the agave shoots is one of the main advantages of our multiplication system since this anomalous growth is a very common phenomenon when agave explants are grown in liquid medium (Fig. 2B. It was observed, by visual inspection, that all multiple shoots grew from the axile between the bases of the leaves). The shoots emerged in an orderly and concentric way, from the tissue located between the bases of the cataphylls. Likewise, the individual shoots depicted the morphology of phytomers, which consist of one or two leaves that surround the apex, a node with the ability to form new lateral buds, and a stem or basal internode that supports the entire shoot (Fig. 2C). These observations support the proposition that the shoots differentiated directly from stem disc tissue and did not occur through a process of adventitious differentiation (Fig. 2D). This study offered a novel, original report [27] of culture in liquid medium for micropropagation of *A. tequilana* Weber cv. azul, using a RITA® temporary immersion system. The increase in axillary shooting induced by IAA concentration is an effect that had not been previously evaluated. Although IAA was used in a single concentration, it was added to cytokinin treatments [7,11,16] to increase lateral proliferation. In addition, NAA was used in different concentrations with BA to induce adventitious regeneration of *A. americana*, utilizing basal leaf segments [54].

Table 4: Axillary shoot production in *A. tequilana* cv. azul using segmented stems as explants and growing in RITA® in the presence of BA and IAA

IAA μM + (BA 13.2 μM)*	Shoot per Explant†
0.57	5.33 ± 0.98 a
2.9	12.16 ± 1.68 b
5.1	20.60 ± 1.20 c

Note: *The concentration of 13.2 μM BA was added to all treatments. † Shapiro-Wilk Test: $P = 0.1612$. Average values ± standard error with distinctive letters within the column to the right, are significantly different. (Tukey; $\alpha = 0.05$).

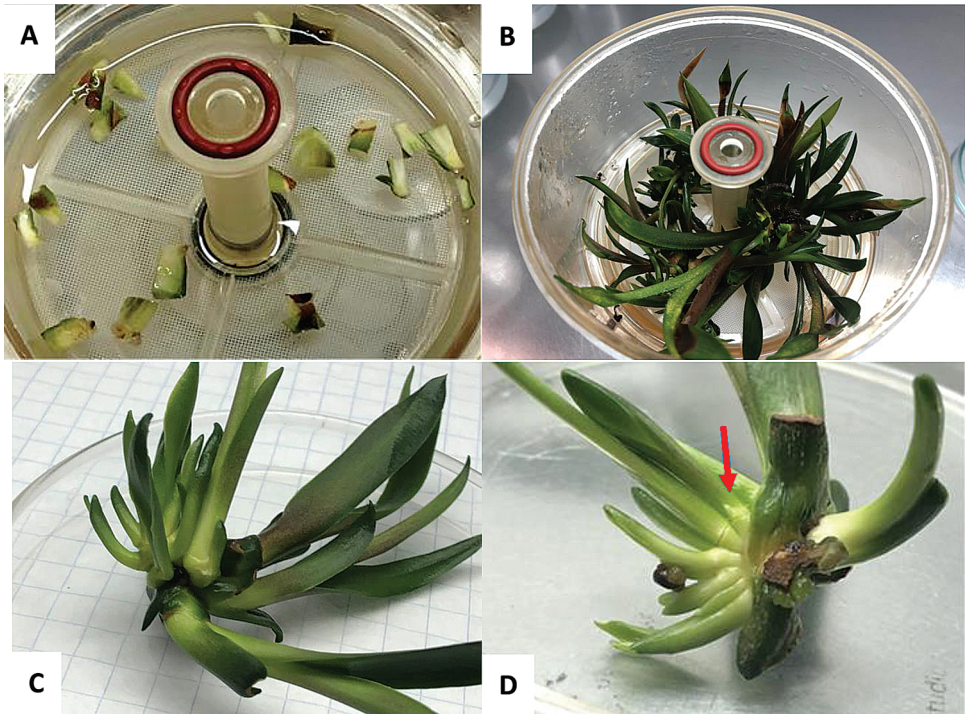

Figure 2: Culture of segmented stems, as explants, of *A. tequilana* Weber cv. azul (A). Direct formation of multiple axillary shoots within RITA® after 12 weeks in MS liquid medium (B). A segment (C) showing multiple shoots grew from the axile between the bases of the leaves (arrow) (D)

The use of IAA and BA in this study also has a remarkable difference compared to earlier reports. Previously, central stem segments were used, but intact base and apex segments were included. In this way, the explant proliferated in lateral and adventitious buds by adding 0.025 mg L^{-1} 2,4-D and 10.0 mg L^{-1} BA [6]. Likewise, the following regeneration systems were developed through the formation of calli at the apex base, applying pulses of high concentration of 2,4-D (6.8 mM) for three days. Subsequently, the basal cluster of adventitious and apex buds was maintained in a medium containing a combination of 0.113 µM 2,4-D + 4.44 µM BA [9]. It was also proposed that the multiplication scheme should be the subculture of explants composed of 2–3 shoots providing BA in the medium [0–12 mg L^{-1} (0–53.2 µM)] and 2,4-D [0.001–0.025 mg L^{-1} (0.04–0.11 µM)] [3,14].

Our study proposes an alternative to this multiplication scheme, avoiding the use of 2,4-D in *A. tequilana* Weber cv. azul micropropagation. This is because this synthetic auxin has risks of cytogenetic effects and somaclonal variation, which were observed in several *in vitro* systems [53,55–58]. In the same way, physiological changes were detected in the *Agave* plantlets when the successive subcultures were achieved in the presence of 2,4-D [51].

This study retakes the previous research strategies in *Agave* by (A) segmenting the explant [11] and favoring axillary shooting above the adventitia, and (B) exploring the effect of IAA activity at moderate BA concentrations [16]. In this sense, up to our knowledge, these results have not been previously reported for *A. tequilana* Weber cv. azul, indicating that together with the sagittal segmentation of the stem, BA activity is important for elimination of apical dominance (Tab. 2). Likewise, the IAA concentration induced the additional increase in axillary shooting. These results emphasize the importance

of adjusting exogenous auxins and cytokinins on *in vitro* morphogenic responses [47,59,60]. Furthermore, our observations appeared that the handling of the explant and the temporary immersion of *A. tequilana* Weber cv. azul increased proliferation and had considerable development in terms of efficiency. These optimization conditions would foster the development of reliable systems to evaluate multiple modifiable factors in the RITA®, or the evaluation and comparison with other available systems in this technology [25,36,37,61–64].

The biochemical mechanism for the induction of lateral shoot proliferation in *A. tequilana* Weber cv. azul stems is related to the auxin activity, inducing apical dominance according to IAA-polar transport chemiosmotic model [65], and its interaction with cytokinins that promote lateral dominance in orthotropic stems [66]. Cytokinins regulate cell division by affecting the controls that govern cell cycle progression and depend on auxins to participate in cell division by controlling the activity of cyclin-dependent protein Kinases (CDKs) [67].

The addition of BA, zeatin and kinetin in the media were carried out to evaluate their effectiveness in inducing lateral dominance, given the natural existence of endogenous auxins. Tabs. 2 and 3 clearly show that the exogenous cytokinins induced a lateral dominance effect due to a greater shoot number.

In the liquid culture medium, the exogenous addition of IAA over a relatively high concentration of BA, demonstrated this optimization effect, by increasing the number of lateral shoots, according to the auxin/cytokinin interaction mechanism [59]. Tab. 4 depicts that the IAA induces lateral shoots that can reach an optimum, given the presence of a relatively high level of BA.

Other important factors in this study are the explant type and the sagittal cut effect. Central stem blocks are used in the most common methods for agave micropropagation (Tab. 1). Robert et al. [14] induced the formation of adventitious shoots on apical cubes of 0.8 cm^3 by adding 2,4-D (0.11 μM) and BA (44.4 μM). They noted that the meristem region produced most of the shoots. They stated that what is needed for induction was the undifferentiated meristematic tissue that lies below. In all the experiments in this study, we consider that the induction of shoots in stem discs has its origin in meristems separated from the central meristem, according to the concept of Steeves et al. [68] and Wardlaw [69]. Although there were no histological studies in this study, the formation of shoots between the leaf bases, without the presence of cytokinins, shows a natural behavior of lateral dominance. This effect is enhanced by exogenous concentrations of cytokinins and is optimized with IAA.

4 Conclusions

The *in vitro* culture of segmented stem discs, as explants, proved to be a simple and efficient technique for the micropropagation of *Agave tequilana* Weber cv. azul in semi-solid and liquid media. During the establishment stage, sagittal segmentation in the explants, without the presence of plant growth regulators, induced the formation of lateral shoots. The addition of BA or zeatin (17.6–18.4 μM) in the medium increased axillary sprouting, inducing the formation of 12.41 shoots on average. In the multiplication stage, subculturing segmented stem discs, without the addition of plant growth regulators, induced the formation of up to four shoots, on average. The addition of BA (4.4–13.2 μM) and kinetin (4.7–9.4 μM) increased the proliferation efficiency to 13.0 and 20.6 shoots on average, respectively. The cultivation of segmented stems in a RITA® added with BA (13.2 μM), induced the formation of 5.33 shoots, on average. The addition and increase of IAA concentration (0.57–5.1 μM), in the presence of BA (13.2 μM), boosted the multiplication efficiency, generating the formation of up to 20.6 shoots on average. Therefore, we propose that the use of segmented stems combined with the addition of cytokinins and auxins, using solid and liquid media, will promote more efficient micropropagation systems.

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