

In Vitro Propagation of *Agave americana* by Indirect Organogenesis

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Abstract. Factors such as slow growth, low rates of sexual and asexual reproduction, and viability of seeds among others limit the massive propagation of *Agave americana* L. by conventional methods. In this study, callus induction and shoot proliferation was determined in *A. americana* using Murashige and Skoog (MS) medium supplemented with dichlorophenoxyacetic acid (2,4-D) and 6-benzyl adenine (BA). Meristematic tissue was used as the explants, and were placed on MS medium supplemented with 30.0 g·L⁻¹ sucrose with 0.11, 0.18, or 0.45 μM 2,4-D and 11.0, 22.0, 38.2, 44.0, 58.7, or 73.3 μM BA. Treatments were implemented according to factorial experimental design 3 × 6. After 1 month, the number of explants with callus was determined, whereas the numbers of shoots per explant were monitored after 4, 16, 20, and 36 weeks. The maximum percent of explants with callus was obtained with 0.11 μM 2,4-D and 58.7 and 73.3 μM BA, whereas the maximum numbers of shoots per explant (71) were obtained with 0.11 μM 2,4-D and 73.3 μM BA. The effect of different concentrations of indolebutyric acid (IBA) in the rooting of shoots was evaluated. There were no significant effects of IBA on the number of roots, root length, and axillary roots. Plantlets were acclimatized in the glasshouse and they did not show any phenotypic alteration. This is a highly efficient protocol for the in vitro propagation of *A. americana* via indirect organogenesis.

The *Agave* is an important species native to semiarid and arid regions, the origin center being Mexico. These plant populations spread from the southwestern United States through Central America, the Caribbean, and into northern South America (Good-Avila et al., 2006). Species of the genus *Agave* are very important owing to the wide variety of uses of these plants, as they produce food and natural fibers (Rivera et al., 2010). All species of the family Agavaceae are native to the American continent and are classified into eight genera. It is assumed that 75% of all species belonging to the Agavaceae family are found in Mexico with 55% being endemic (García et al., 2010). The genus *Agave*, belonging to the family Agavaceae, arose

≈15 million years ago (Rocha et al., 2005). *Agave americana* is an important species with different uses, native to the southern United States and Mexico, widely cultivated throughout the world. Although *Agave americana* L. is not yet a “threatened” species in Mexico, its distribution has been reduced as its natural habitat is decreasing as a result of exploitation, especially in southern Mexico. An alternative to solve this problem is to generate technological packages for the artificial reproduction of the species to ensure the production of highly productive plants and the quality of them through control of growing conditions which will allow recovery of populations that have been diminished by the looting and destruction of habitat. *Agave americana* is of economic importance because it is used for the production of an alcoholic beverage called “comiteco.” All available plants for the production of this liqueur come from vegetative propagation by suckers. To ensure the availability of adult plants required for the production of liquor, it is necessary to establish measures to promote

the spread and protection of populations. Slow growth, low rate of asexual reproduction, and sexual reproduction limited by problems of pollination and seed viability are factors that hinder the massive multiplication of agaves by conventional methods. Besides, these factors limit the potential for improvement of cultivated species (Chen et al., 2014; Portillo et al., 2007; Robert et al., 1992). The in vitro *A. americana* reproduction is an alternative that can overcome the low amount of raw material to date and lead to the generation of protocols for the micropropagation of the species. In vitro methods can be used to propagate and preserve plant species when seed-based methods are inadequate (Pence, 2011). Successful propagation depends on several steps, including obtaining cultures free of pathogenic bacteria and fungi, establishment of shoot producing or embryogenic cultures, rooting of shoots or the outgrowth of embryos into plants, and the acclimatization of those plants to ex vitro conditions (Pospíšilová et al., 1999). Callus culture might be a useful technique to cultivate *A. americana*, offering the potential to produce plants of the desired clone and might also serve as a starting point for future plant improvement through molecular biotechnology. Several studies have been reported on the micropropagation of *Agave* species such as *Agave fourcroydes* (Robert et al., 1987), *Agave cantala* (Binh et al., 1990), *Agave parrasana* (Santacruz-Ruvalcaba et al., 1999), *Agave victoriae-reginae* (Martínez-Palacios et al., 2003; Rodríguez-Garay et al., 1996), *Agave sisalana* (Hazra et al., 2002; Nikam, 1997; Nikam et al., 2003), and *Agave tequilana* (Castro-Concha et al., 1990; Robert et al., 1992). Garriga et al. (2010) reported that the combination of 6-N-Bencilaminopurina (BAP) at a concentration of 0.75 mg·L⁻¹ and 1 mg·L⁻¹ IBA had a significant effect on survival of the explant in establishing medium for shoot proliferation. However, combining 0.5 or 0.75 mg·L⁻¹ Thidiazuron with 1.0 mg·L⁻¹ BAP and 1.0 mg·L⁻¹ IBA in the MS basal medium increased shoot multiplication percentage. Somatic embryogenesis has been reported by Portillo et al. (2007) in *A. tequilana* Weber blue cultivar using leaf as the source of explant and MS medium supplemented with 9.0 μM of 2,4-D and 1.3 μM BA. The obtaining embryogenic lines using callus induced on MS medium (Santacruz-Ruvalcaba and Portillo, 2009). Shoot proliferation was reported in *A. parrasana* Berger using MS medium supplemented with L₂ vitamins and 13.3 μM BA (Santacruz-Ruvalcaba et al., 1999). Tejavathi et al. (2007) induced somatic embryos from callus culture of *Agave vera-cruz* Mill. in MS medium supplemented with L₂ vitamins, 4.52 μM 2,4-D and 5.37 μM 1-naphthalene-acetic acid (NAA). The MS medium was supplemented with 5.37 μM NAA, 0.91 μM Zeatin (ZEA) and 40 g·L⁻¹ sucrose for embryo maturation and re-differentiation. Indirect somatic embryogenesis has been reported in *A. angustifolia* Haw from callus

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induced from zygotic embryos cultivated in quarter-strength MS medium supplemented with 60 g·L⁻¹ sucrose, 3.0 mg·L⁻¹ 2,4-D, and 1.0 mg·L⁻¹ BA (Arzate-Fernández and Mejía-Franco, 2011). Shoots were obtained in *Agave grijalvensis* B. Ullrich in MS medium plus 8.2 μM BA (Sánchez-Urbina et al., 2008). For somatic embryo induction, MS medium supplemented with 22.0 μM BA and 2.6 μM NAA was used (Santíz et al., 2012). Recently, Chen et al. (2014) reported a protocol for shoot regeneration (18.0 shoots/explant) using MS medium supplemented with 13.32 μM BA using meristems as explants. In this study, the effect of different concentrations of 2,4-D and BA on the induction of *A. americana* callus from meristem and on the number of shoots per callus were investigated. In addition, the effect of IBA on root induction was studied to obtain complete plantlets. The process of cell dedifferentiation can be seen by histological studies and determine regeneration pathways, and also confirms the presence of meristematic structures, which when divided by mitosis give rise to new structures.

Materials and Methods

Explant source and disinfection. The *A. americana* plants used in this study were obtained from a collection at the experimental site of Tuleaito, located in Comitán (NL 16°15'04', WL 92°08'03'), at an altitude of 1622 m.a.s.l. (Chiapas, México) during the period June–August. Leaves, stems, and roots were removed from 8-week-old plants, leaving only the main body which contained the meristem. The main bodies were washed and disinfected with soap (Axion®) and water for 5 min. The dissection of the explants to obtain segments of ≈1.5 cm was performed in a laminar flow hood. Explants were submerged in 0.5% agrimycin 500 (Arista Life Science) solution mixed with 0.5% captan (Pfizer) for 20 min, and rinsed three times with sterile distilled water for 5 min. They were then immersed in 70% ethanol for 5 min, and then in a solution of commercial sodium hypochlorite at 30% for 20 min, and rinsed again three times (1 min) in sterile distilled water (Vázquez-Molina et al., 2005). They were later submerged in a 0.1% HgCl₂ solution for 10 min, and rinsed three times with sterile distilled water. As the last step, the explants were submerged in a 3% (w/v) CaCl₂O₂ solution for 20 min and then washed three times with sterile distilled water for 5 min (Vázquez-Molina et al., 2005).

Callus induction and shoot regeneration. Explants were directly placed in MS (Murashige and Skoog, 1962) medium supplemented with different combinations of 0.11 μM 2,4-D (Fluka®), and 58.7 and 73.3 μM BA (Sigma®). The hormone-free MS medium was a control. A culture vessel containing 20 mL of medium with four explants was considered as an experimental unit and the experiment was repeated five times. The culture medium contained 30 g·L⁻¹ sucrose and 2.5 g·L⁻¹ phytigel (Sigma®). The pH was

adjusted to 5.7 before autoclaving at 121 °C and 1.2 kg·cm⁻² pressure for 15 min. Callus formation was evaluated every month and subculturing was done every 2 months. Callus cultures were transferred to MS hormone-free medium after the third subculture to induce shoot regeneration after 2 months. Cultures were maintained at 25 °C under continuous illumination (35 mmol·m⁻²·s⁻¹) with fluorescent lights. Regenerated shoots were separated from the callus and transferred to test tubes (25 × 130 mm), each containing 10 ml half-strength MS medium containing 30 g·L⁻¹ sucrose and 2.5 g·L⁻¹

phytagel. Cultures were maintained at 25 °C under continuous illumination (35 mmol·m⁻²·s⁻¹).

Rooting and acclimatization of plantlets. Plantlets were rooting and acclimatization according to Reyes-Zambrano et al. (2016). Two hundred and fifty plantlets with a well-developed root system were washed in running water to remove phytigel and transferred to unicef pots (12 × 10 cm) (one plantlet per unicef pot) containing peatmoss and agrolite mixture (1:1) for acclimatization. Plantlets were grown in the greenhouse with temperatures that ranged

Table 1. Effect of 2,4-D and BA on callus induction and shoot regeneration in *Agave americana* L.

Treatment	2,4-D (μM)	BA	Explant with callus (%)	No. of shoots per callus			
				4	16	20	36
1	0.11	58.7	80 a	14.0 a	39.0 a	33.0 a	65 a
2	0.11	73.3	80 a	10.0 a	40.0 a	31.0 a	71 a
3	0	0	0 b	0 b	0 b	0 b	0 b
LSD (0.05) [†]			5.7	2.8	4.4	8.8	7.3

2,4-D = dichlorophenoxyacetic acid; BA = 6-benzyl adenine.

[†]LSD = least significant difference ($P < 0.05$). Values with the same letter are not significantly different between the treatments.

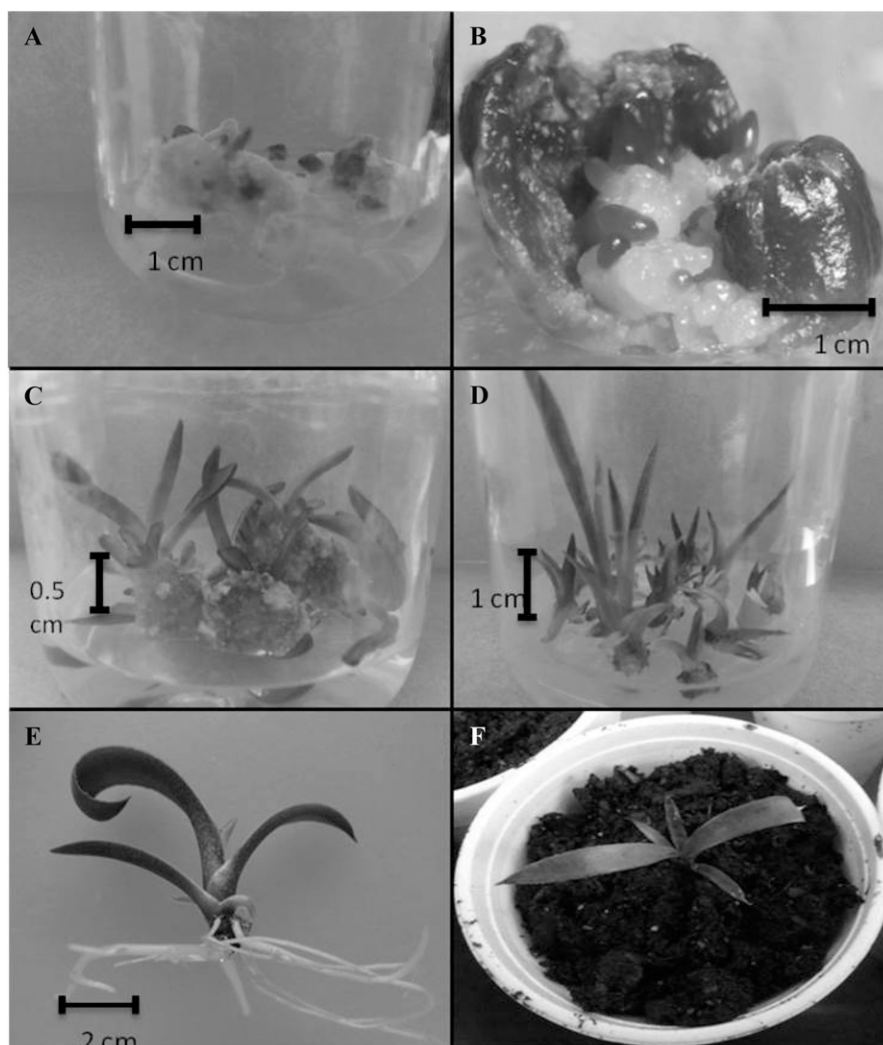


Fig. 1. In vitro regeneration of *Agave americana* L. plantlets. (A) Callus induced from meristematic tissue, (B) adventitious bud formation from callus, (C) shoots regenerated from callus, (D) plantlets derived from callus, (E) plantlets with roots, and (F) plants growing in the soil.

from 18 ± 2 °C for 4 weeks to $20\text{--}26$ °C for 8 weeks. They were then transferred to near-commercial greenhouse conditions where temperatures fluctuated between $30\text{--}35$ °C in daytime and $16\text{--}24$ °C at night. The plantlets were irrigated every third day.

Histological analysis. For detailed anatomical studies, the samples were fixed in formalin/acetic acid/ethanol/water (10:5:50:35, by volume) for 48 h, kept at 4 °C, rinsed with distilled water, and dehydrated in a graded ethanol series from 30%, 50%, 70%, 85%, 96%, and absolute ethyl alcohol (v/v). The samples were infiltrated and embedded in plastic resin JB-4 Embedding Kit® (glycol methacrylate; Polysciences, Los Angeles, CA), according to the instructions of the manufacturer. Longitudinal sections that were 3 µm thick were cut using a microtome (Microm HM 325; Thermo Scientific, Walldorf, Germany). Sections were acidified for 30 min with periodic acid (1% w/v), rinsed with distilled water repeatedly, and stained for 20 min with Schiff reagent as described by McManus (1961). Finally, the samples were stained for 5 min with 7% (w/v) Naphthol blue black mounted in Poly-mount (Polysciences, Inc.), ase spe and photographed using a microscope Scope.A1; Carl Zeiss, AxioCam Icc 5.

Data analysis. SAS statistical software (SAS Institute, 1990) was used to analyze data using a confidence limit of 5%. The linear and quadratic values of all factors and the interactions between them were tested.

Results

Callus induction. Callus formation was obtained in all treatments. Explant percentage with callus was not affected by the different combinations of 2,4-D and BA, and varied from 0% in treatment 3 to 80% in the 1 and 2 treatments ($P < 0.05$) (Table 1).

Induction of shoots and plant regeneration. The number of shoots was 71 with 0.11 µM 2,4-D plus 73.3 µM BA (Table 1). Callus were obtained after 4 weeks (Fig. 1A and B). Adventitious bud were observed and shoots were produced after 12 weeks callus induction (Fig. 1C). Plantlets derived from callus were observed after 16 weeks (Fig. 1D).

Rooting and acclimatization of plantlets. Rooting was recorded after 4 weeks. Axillary roots were observed after 11 weeks (Fig. 1E). The plantlets were acclimatized successfully (Fig. 1F) with a survival rate of 100%. The plantlets did not show any phenotypic variation.

Histological analysis. The histological analysis done with callus derived of treatment with 73.3 µM BA and 0.11 µM 2,4-D confirm adventitious shoot formation in *A. americana* (Fig. 2A). Meristematic zones were observed (Fig. 2B) with high mitotic activity and vascular bundles (Fig. 2C). Cells from organogenic centers were similar to meristematic cells of axillary buds (Fig. 2D).

Discussion

Efficient protocols for micropropagation have been obtained through axillary shoots

(Madrigal-Lugo et al., 1989; Robert et al., 1992), organogenesis (Hazra et al., 2002; Nikam 1997; Robert et al., 1987; Valenzuela-Sánchez et al., 2006), or somatic embryogenesis (Martínez-Palacios et al., 2003; Nikam et al., 2003; Rodríguez-Garay et al., 1996). All these protocols are evidence that the in vitro regeneration of each *Agave* species is dependent on plant growth regulators, a fact that has been confirmed by the results of this study. However, the micropropagation efficiency is variable. Nikam (1997) reported 5.7 shoots per explant with 0.5 mg·L⁻¹ kinetin in *A. sisalana*. Santacruz-Ruvalcaba et al. (1999) obtained 22.0 shoots per explant with 3.0 mg·L⁻¹ BA, whereas Martínez-Palacios et al. (2003) found 2.2 shoots per explant in *A. victoriae-reginae* cultivated in MS medium plus 1.0 mg·L⁻¹ BA. Axillary meristems were used as explants, and results are evidence of the heterogeneous response in *Agave* species. Valenzuela-Sánchez et al. (2006) found 19.5 shoots per explant in *A. tequilana* with plantlet dedifferentiation through indirect organogenesis. The plant growth regulators play an important role in controlling the morphogenetic response of in vitro cultures. Endogenous growth regulator content is known to differ from one meristematic center of the callus to another (Norton, 1986), with the balance between the endogenously and exogenously supplied hormones determining the morphogenetic response. Tejavathi et al. (2007) obtained embryogenic callus in *A. vera-cruz* in MS medium supplemented with

4.52 µM 2,4-D or 5.37 µM NAA and then in MS medium supplemented with 5.37 µM NAA, 0.91 µM ZEA, and 40 g/L sucrose. Yan-Mei et al. (2013) found callus formation in new leaves used as explant and this response was attributed to auxin-cytokine exogenous concentrations, explant type, and medium culture in *Agave* hybrid N° 11648. For higher callus induction percentage, 2,4-D (1.0 or 2.0 mg·L⁻¹) was more effective than NAA in combination with BA (1.0 or 2.0 mg·L⁻¹) with immature leaves as explant. Santacruz-Ruvalcaba et al. (1999) found that 2,4-D has a great influence for shoot production; however, shoot number was reduced with 2,4-D at 0.11 and 0.18 µM, whereas lowest concentrations (0.04 µM) promoted 26.0 shoot per explant in *A. parrasana* in combination with 13.3 µM BA. Chen et al. (2014) reported the unique study in *A. americana* L. where they obtained direct organogenesis in basal and leaves segments (base, middle, and apex) as explants. These authors obtained 18.5 shoots per explant with basal stem cultured in MS medium plus 13.32 µM BA after 50 d. Basal leaf segments cultivated in MS medium supplemented with 13.32 µM BA and 2.68 µM NAA promoted 52.0% callus formation and 84.6 shoots per explant after 90 d. In our work with another explant (apical meristem) and auxin, a high percent of shoot regeneration (71 shoots/callus) was obtained after 36 weeks. This result is better in comparison with protocols for different *Agave* species micropropagation, *A. fourcroydes* Lem (Garriga et al., 2010;

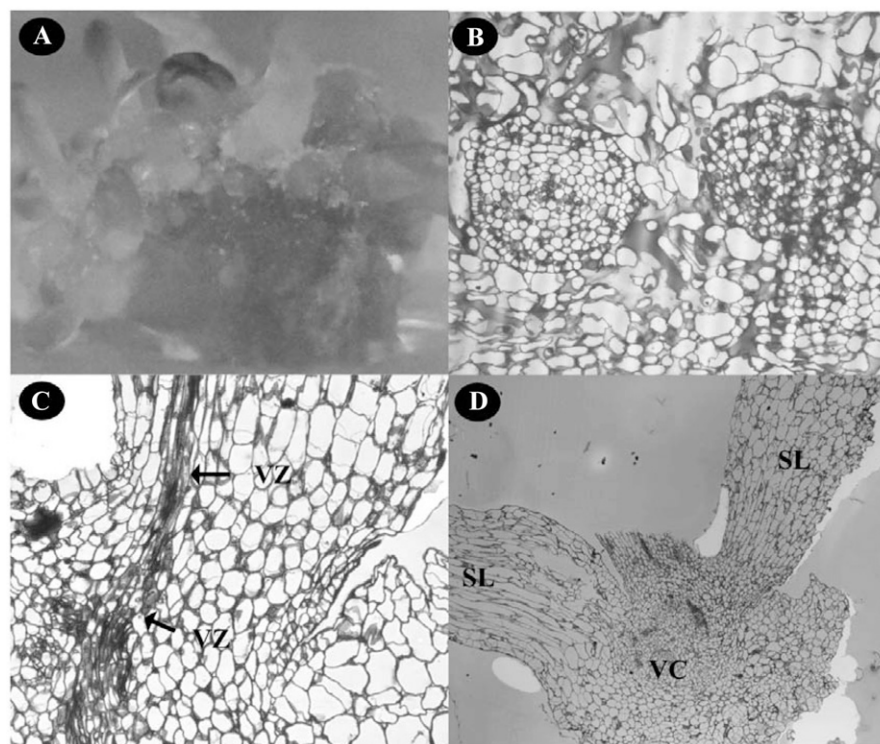


Fig. 2. Histological analysis of organogenesis indirect of *Agave americana* L. (A) Callus induced from meristematic tissue at 36 weeks of culture, (B) formation of meristematic zones, (C) vascular tissue, and (D) a development shoot derived from meristematic tissue consists of shoot apical meristem connected with vascular tissue. Sheath leaves (SL), vascular connection (VC), vascular zone (VZ).

Infante et al., 2003), *A. tequilana* (Santacruz-Ruvalcaba and Portillo, 2009; Torres-Morán et al., 2010), *A. parrasana* (Santacruz-Ruvalcaba et al., 1999), *A. vera-cruz* (Tejavathi et al., 2007), *A. angustifolia* (Arzate-Fernández and Mejía-Franco, 2011), and *A. grijalvensis* (Sánchez-Urbina et al., 2008; Santíz et al., 2012). The rooting of shoots of *A. americana* was not a significant factor. Rooting response was the same in comparison with other *Agave* species, such as *A. arizonica* (Powers and Backhaus, 1990), *A. sisalana* (Binh et al., 1990; Das, 1992), and *A. parrasana* (Santacruz-Ruvalcaba et al., 1999) in which MS medium without plant growth regulators was sufficient for root production. However, shoots of *A. fourcroydes* (Robert et al., 1987, 2006) and *Agave Sisal* (Hazra et al., 2002) required both 2,4-D and indoleacetic acid for rooting to occur. This suggests that the rooting of shoots of different species of agave is dependent on growth regulators. In conclusion, the results demonstrated the development of highly efficient protocol for plantlet regeneration. This protocol could be applied for massive plant propagation purposes for alcohol, fiber, and secondary metabolite production. Callus culture of *A. americana* could be a prerequisite for many techniques for in vitro manipulation, cellular line selection, and for genetic improvement programs.

Literature Cited

- Arzate-Fernández, A.M. and R. Mejía-Franco. 2011. Embryogenic capacity of induced calli on zygotic embryonic axis of *Agave angustifolia* Haw. Rev. Fitotec. Mex. 34(2): 101–106.
- Binh, L.T., L.T. Muoi, H.T.K. Oanh, T.D. Thang, and D.T. Phong. 1990. Rapid propagation of *Agave* by in vitro tissue culture. Plant Cell Tiss. Org. Cult. 23:67–70.
- Castro-Concha, L., V.M. Loyola-Vargas, J.L. Chan, and M.L. Robert. 1990. Glutamate dehydrogenase activity in normal and vitrified plants of *Agave tequilana* Weber propagated in vitro. Plant Cell Tiss. Org. Cult. 22:147–151.
- Chen, Y., X. Chen, F. Hu, H. Yang, L. Yue, R.N. Trigiano, and Z.M. Cheng. 2014. Micropropagation of *Agave americana*. HortScience 49: 320–327.
- Das, T. 1992. Micropropagation of *Agave sisalana*. Plant Cell Tiss. Org. Cult. 31:253–255.
- García, H.E., G.S. Méndez, and M.D. Talavera. 2010. El género agave spp. En México: Principales usos de importancia socioeconómica y agroecológica. Revista Salud Pública y Nutrición, Edición Especial No. 5, 112.
- Garriga, C.M., O.G. González, G.S. Alemán, C.E. Abreu, B.K. Quiroz, P.D.S. Caligari, and R. García-González. 2010. Management of auxin-cytokinin interactions to improve micropropagation protocol of henequen (*Agave fourcroydes* Lem). Chil. J. Agr. Res. 70(4):545–551.
- Good-Avila, S.V., V. Souza, B.S. Gaut, and L.E. Eguiarte. 2006. Timing and rate of speciation in *Agave* (Agavaceae). Proc. Natl. Acad. Sci. USA 103(24):9124–9129.
- Hazra, S.H., S. Das, and A.K. Das. 2002. Sisal plant regeneration via organogenesis. Plant Cell Tiss. Org. Cult. 70:235–240.
- Infante, D., G. González, L. Peraza-Echeverría, and M. Keb-Llanes. 2003. Asexual genetic variability in *Agave fourcroydes*. Plant Sci. 164(2):223–230.
- Madrigal-Lugo, R., F. Pineda-Estrada, and R. Rodríguez-De la O. 1989. *Agave*, p. 206–227. In: P.V. Ammirato, D.A. Evans, R.W. Sharp, and Y.P.S. Bajaj (eds.). Handbook of plant cell culture. Vol. 5. Ornamental Species, Chapter 9. McGraw-Hill, New York, NY.
- Martínez-Palacios, A., M.P. Ortega-Larrocea, V.M. Chávez, and B. Robert. 2003. Somatic embryogenesis and organogenesis of *Agave victoriae-reginae*: Consideration for its conservation. Plant Cell Tiss. Org. Cult. 74:135–142.
- McManus, J.F.A. 1961. Periodate oxidation techniques, p. 171–201. In: J.F. Danielli (ed.). General cytochemical methods. Vol. 2. Academic Press, New York, NY.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15(3):473–497.
- Nikam, T.D. 1997. High frequency shoot regeneration in *Agave sisalana*. Plant Cell Tiss. Org. Cult. 51:225–228.
- Nikam, T.D., G.M. Bansude, and K.C. Aneesh-Kumar. 2003. Somatic embryogenesis in sisal (*A. sisalana* Perr. Ex. Engelm). Plant Cell Rpt. 22(3):188–194.
- Norton, M.E. 1986. Explant origin as a determinant of in vitro shoot proliferation in *Prunus* and *Spiraea*. J. Hort. Sci. 61:43–48.
- Pence, V.C. 2011. Evaluating costs for the in vitro propagation and preservation of endangered plants. In Vitro Cell. Dev. Biol. Plant 47(1): 176–187.
- Portillo, L., F. Santacruz-Ruvalcaba, A. Gutiérrez-Mora, and B. Rodríguez-Garay. 2007. Somatic embryogenesis in *Agave tequilana* Weber cultivar azul. In Vitro Cell. Dev. Biol. Plant 43:569–575.
- Pospišilová, J., I. Tichá, P. Kadleček, D. Haisel, and Š. Plzáková. 1999. Acclimatization of micropropagated plants to ex vitro conditions. Biol. Plant. 42(4):481–497.
- Powers, D.E. and R.A. Backhaus. 1990. In vitro propagation of *Agave arizonica* Gentry and Weber. Plant Cell Tiss. Org. Cult. 16(1):57–60.
- Reyes-Zambrano, S., C. Lecona-Guzmán, J. Ambrosio Calderón, M. Abud-Archila, R. Rincón-Rosales, V. Ruiz-Valdiviezo, and F. Gutiérrez-Miceli. 2016. Plant growth regulators optimization for maximize shoots number in *Agave americana* L. by indirect organogenesis. Gayana Botánica 73(1):124–131.
- Reynoso-Santos, R., J.A. García-Mendoza, W. López-Báez, and A. López-Luna. 2011. Identificación taxonómica de especies de agave utilizadas para la elaboración del licor Comité en Chiapas. Folleto Técnico N° 14, INIFAP.
- Rivera, G., V. Bocanegra-García, and A. Monge. 2010. Traditional plants as source of functional foods: A review. CYTA J. Food 8(2): 159–167.
- Robert, M.L., J.L. Herrera, E. Castillo, G. Ojeda, and M.A. Herrera-Alamillo. 2006. An efficient method for the micropropagation of *Agave* species. Methods Mol. Biol. 318:165–178.
- Robert, M.L., J.L. Herrera, F. Contreras, and K.N. Scorer. 1987. In vitro propagation of *Agave fourcroydes* Lem (Henequen). Plant Cell Tiss. Org. Cult. 8:37–48.
- Robert, M.L., J.L. Herrera, J.L. Chan, and F. Contreras. 1992. Micropropagation of *Agave* spp., p. 307–329. In: Y.P.S. Bajaj (ed.). Biotechnology in agriculture and forestry. Vol 19. High-tech and micropropagation III. Springer-Verlag, Heidelberg, Berlin, Germany.
- Rocha, M., A. Valera, and L.E. Eguiarte. 2005. Reproductive ecology of five sympatric *Agave littaea* (Agavaceae) species in central Mexico. Amer. J. Bot. 92(8):1330–1341.
- Rodríguez-Garay, B., A. Gutiérrez, and B. Acosta-Dueñas. 1996. Somatic embryogenesis of *Agave victoriae-reginae* Moore. Plant Cell Tiss. Org. Cult. 46:85–87.
- Sánchez-Urbina, A., L.M.C. Ventura-Canseco, T. Ayora-Talavera, M. Abud-Archila, M.A. Pérez-Farrera, L. Dendooven, and F.A. Gutiérrez Miceli. 2008. Seed germination and in vitro propagation of *Agave grijalvensis*, an endemic endangered Mexican species. Asian J. Plant Sci. 7(1):752–756.
- Santacruz-Ruvalcaba, F., H. Gutiérrez, and B. Rodríguez. 1999. Efficient in vitro propagation of *Agave parrasana* Berger. Plant Cell Tiss. Org. Cult. 56(3):164–167.
- Santacruz-Ruvalcaba, F. and L. Portillo. 2009. Thin cell suspension layer as a new methodology for somatic embryogenesis in *Agave tequilana* Weber cultivar azul. Ind. Crops Prod. 29(2):609–614.
- Santíz, J.A., R. Rincón-Rosales, and F.A. Gutiérrez-Miceli. 2012. In vitro propagation of *Agave grijalvensis* an endemic species from Chiapas under special protection. Gayana Botánica 69 (numero especial):23–30.
- SAS Institute. 1990. Statistic guide for personal computers, p. 1–102. Version 6.04. SAS Institute, Cary, NC.
- Tejavathi, D.H., M.D. Rajanna, R. Sowmya, and K. Gayathamma. 2007. Induction of somatic embryos from cultures of *Agave vera-cruz* Mill. In Vitro Cell. Dev. Biol. Plant 43(5): 423–428.
- Torres-Morán, M.I., M. Escoto-Delgado, S. Molina-Moret, D.M. Rivera-Rodríguez, A.P. Velasco-Ramírez, D. Infante, and L. Portillo. 2010. Assessment of genetic fidelity among *Agave tequilana* plants propagated asexually via rhizomes versus in vitro culture. Plant Cell Tiss. Org. Cult. 103(3): 403–409.
- Valenzuela-Sánchez, K.K., R.E. Juárez-Hernández, A. Cruz-Hernández, V. Olalde-Portugal, M.E. Valverde, and O. Paredes-López. 2006. Plant regeneration of *Agave tequilana* by indirect organogenesis. In Vitro Cell. Dev. Biol. Plant 42:336–340.
- Vázquez-Molina, D.E., A. De los Santos, K.A. Lecona-Guzmán, O. Súmano-Muñiz, M. Velázquez-Méndez, R. Rincón-Rosales, M.A. Oliva-Llaven, L. Dendooven, and F.A. Gutiérrez-Miceli. 2005. Sugar cane buds as an efficient explant for plantlet regeneration. Biol. Plant. 49(4):481–485.
- Yan-Mei, Z., L. Xin, C. Zhi, L. Jun-Feng, L. Jun-Ying, and Z. Wen-Zhao. 2013. Shoot organogenesis and plant regeneration in *Agave* hybrid, No. 11648. Scientia Hort. 161:30–34.