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MANAGEMENT OF AUXIN-CYTOKININ INTERACTIONS TO IMPROVE MICROPROPAGATION PROTOCOL OF HENEQUEN (*Agave fourcroydes* Lem.)

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ABSTRACT

Henequen (*Agave fourcroydes* Lem.) is a high value plant species both for agricultural and ecological uses. The species is very well known worldwide by the quality of its fiber that is widely used for industrial purposes. This species is propagated at a large scale by asexual methods but current propagation technologies are not satisfying the grower's demands. *A. fourcroydes* has been cultivated *in vitro* but it has shown some recalcitrant behaviour during the multiplication and rooting stages. The combination of 6-benzylamynopurine (BAP) (0.75 mg L^{-1}) and indolebutyric acid (IBA) (1.0 mg L^{-1}) instead of 2,4-dichlorophenoxyacetic acid (2,4-D) significantly improved explant survival and shooting during the establishment of *in vitro* young shoots. Combining thidiazuron (TDZ) (0.5 or 0.75 mg L^{-1}) with BAP (1.0 mg L^{-1}) and IBA (1.0 mg L^{-1}) in the basal medium increased the multiplication rate of henequen and significantly speeded out bud dormancy breaking. To improve rooting of the micropropagated shoots, the addition of IBA and naphthylacetic acid (NAA) was tested. The best rooting efficiency was obtained when the basal medium was supplemented with 0.5 or 0.75 mg L^{-1} of NAA, giving 100% of rooted explants and an average of 9.40 and 11.55 roots per explants, respectively. Over 94% of micropropagated plants survived the *ex vitro* weaning step and no morphological disorders were observed in any of the plants. Modification of plant growth regulators composition in the medium was a key factor to improve the efficiency of the micropropagation technology of henequen.

Key words: *Agave fourcroydes*, propagation, thidiazuron, 6-benzylamynopurine, *in vitro* culture.

INTRODUCTION

Henequen (*Agave fourcroydes* Lem.) is a cultivated species known worldwide because it produces a high quality fiber that is widely used for industrial purposes. This species was first domesticated from the wild species *Agave angustifolia* Haw. by the pre-hispanic Mayan culture (Colunga, 1998). The fiber produced by henequen is highly valued because of its resistance and size. The species is also highly resistant to poor and drought soils. For this reason henequen is considered as a good alternative for low income farmers inhabiting in vulnerable areas (Colunga, 1998). Saponin production in this species has been also recognized as a potential source of natural products for industrial uses as steroids and detergents (Robert *et al*., 1992). Its fiber can also be a good source of cellulose (Cazaurang *et al*., 1990) but also some other compounds with a potential use in the pharmaceutical and agricultural industries have been identified and it could have an unexploited potential for the production of beverages. For these reasons the species can be a suitable alternative for planting in poor and marginal soils (Eastmond *et al*., 2000). As other representatives of the Agave genus, henequen is a relatively infertile pentaploid species (n = 30) (Piven *et al*., 2001). Sexual recruitment in natural conditions or plantations is constrained by the agronomic practice of cutting the inflorescence to guarantee the quality of the fiber that could be affected during flowering (Peña *et al*., 1997).

Propagation of henequen is mainly asexual and it has been traditionally performed through basal shoots, rhizome multiplication or bulbils produced by the inflorescence. Although *A. fourcroydes* has not been widely studied by biotechnologists, successful *in vitro* micropropagation has been obtained in this species (Robert *et al*., 1992; Peña *et al*., 1997; González *et al*., 2003). However, the documented protocols have some

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limitations considering the relatively low efficiency of explant establishment to the *in vitro* conditions, the high concentrations of cytokinins during the micropropagation step that strongly induce the formation of hyperhydric shoots and the inhibition of rooting. Besides that, the above mentioned protocols included the auxin 2,4-D which has been recognize as strong inducer of variability in tissue culture, even at low concentrations (Salisbury and Ross, 1994).

The aim of this study was improve *in vitro* micropropagation of henequen by modifying the composition of plant growth regulators (PGRs) in the basal medium during the different stages of the culture. Improvement of multiplication rates, shoot quality and rooting efficiency were the main criteria used to select the best micropropagation conditions.

MATERIALS AND METHODS

Plant material and culture conditions

Actively growing rhizome shoots, 10 cm long, from field plants were collected from a plantation near Matanzas (23º07´21.8´´ N; 81º20´14.2´´ W), Cuba. These shoots were used as explant source and they were disinfected by a long dip in a 5% iodine solution during 20 h. After iodine treatment the explants were rinsed with distilled sterile water for 5 min. Then, a second round of disinfection for 20 min with a 4% NaOCl supplemented with two drops of Tween 80, was developed. Explants were treated once again with a NaOCl solution but at 2% for 10 min. After chlorine disinfection, the explants were washed gently with distilled sterile water for 10 min and explants were dried on a sterile filter paper.

After surface disinfection, shoots tips were removed and cultivated in modified (Robert *et al*., 1992) Murashige and Skoog basal medium (MS) (Murashige and Skoog, 1962) supplemented with 0.1 mg L^{-1} thiamine, 0.5 mg L^{-1} pyridoxine, 0.5 mg L^{-1} nicotinic acid, 2.0 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol and 30 g L^{-1} sucrose. All media were solidified with 10 g L^{-1} Technical Agar #3 (Biocen, La Habana, Cuba) and pH was adjusted to 5.7, before autoclaving at 121 °C and 1.2 kg cm^{-2} of pressure. Glass flasks (12 cm height and 8 cm diameter) containing 30 mL of medium were capped with polypropylene closures. All the explants were cultivated in growth chambers at 26 ± 2 ºC and a 16:8 h photoperiod (37.5 μmol m^{-2} s^{-1}).

In vitro establishment and shoot formation from shoot tips

To enhance the morphogenic responses from shoot tips, four concentrations (0.25, 0.50, 0.75 and 1.0 mg L^{-1}) of 6-benzylaminopurine (BAP) combined with 1.0 mg L^{-1} of indolebutyric acid (IBA) were tested. All plant growth regulators were added into the above mentioned basal medium before pH adjustment and autoclaving. The effect of cytokinin treatments on shooting, shoot length and fresh mass of shoots obtained was evaluated after 45 d of initial culture. To compare the efficiency of the assayed plant growth regulators (PGRs) interactions a control treatment consisting in a basal MS medium supplemented with 0.025 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BAP was included, considering previously reported protocols for the species (Robert *et al*., 1992).

Evaluation of thidiazuron to improve shoot regeneration during the multiplication stage

Sprouted shoots (2.5 cm height as minimum) were excised from the initial explant and cultivated on the basal medium supplemented with IBA (1.0 mg L^{-1}) and BAP (0.75 mg L^{-1}) during the first multiplication cycle. To enhance the multiplication rate the *in vitro* regenerated plantlets were transferred to the basal medium supplemented with IBA (1.0 mg L^{-1}), BAP (0.75 mg L^{-1}) and thidiazuron (TDZ) at 0.0, 0.25, 0.50 and 0.75 mg L^{-1}. The effect of PGRs interactions on adventitious shoot formation, shoot length and dry mass of shoots were evaluated to choose the best micropropagation medium. A basal medium supplemented with 0.025 mg L^{-1}, 2,4-D and 10.0 mg L^{-1} BAP previously suggested for this species (Robert *et al*., 1992) was included as a control and the experiment was evaluated 45 d after planting.

Effect of NAA and IBA treatments on rooting

Shoots (3 cm height and three expanded leaves) regenerated in the basal medium supplemented with 1.0 mg L^{-1} IBA, 1.0 mg L^{-1} BAP and 0.50 mg L^{-1} TDZ were individualized and cultivated in a rooting medium containing different concentrations of IBA and NAA (naphthylacetic acid). Auxins were assayed using a bifactorial design of four concentrations for NAA and IBA (0.25, 0.50, 0.75 and 1.0 mg L^{-1}). Rooting efficiency, number of roots per explant, fresh mass of shoot and shoot length increase were used as main criteria to choose the best PGR and concentration for the rooting step. Evaluation of the experiment was developed after 30 d of shoot culture on rooting media.

For *ex vitro* cultivation, rooted plantlets were washed thoroughly with distilled unsterile water, to remove the remaining culture medium from the roots, and planted in garden pots containing zeolite, decomposed henequen pulp and compost in 0.6:0.3:0.1 (w/w) proportions and 0.7 of apparent density. Cultivation of *ex vitro* plants occurred in a greenhouse, illumination was reduced 70% during 45 d and plants were irrigated twice a day.

Statistical analysis

All the experiments were repeated twice and 20 repetitions

were used for all the treatments. Significant differences were determined by a non parametric ANOVA (Kruskal-Wallis test), employing a completely random design. To analyze the data obtained as percentage a y = arcsin x transformation was done before determining differences among treatments by the Student-Newman-Keuls´s test ($p \leq 0.05$). All the statistical analyses were performed using StatGraphics Centurion XV (StatPoint Technologies, Warrenton, Virginia, USA).

RESULTS AND DISCUSSION

In vitro establishment and plant recovery from of shoot tips

Establishment of shoot tips and shoot development from introduced shoot tips were obtained in all treatments and there were significant differences between the PGRs treatments (Table 1, Figure 1). Supplementing the basal medium with 1.0 mg L^{-1} IBA and 0.75 mg L^{-1} BAP yielded the highest percentage of shoot formation (95%, $p \leq 0.05$). Concentration of BAP had also a significant influence on plant development after *in vitro* introduction of shoot tips as demonstrated by fresh mass (264.11 ± 12 mg) and shoot length (3.07 ± 0.23 mg). Shoots growing on basal medium supplemented with 1.0 mg L^{-1} IBA and 0.75 mg L^{-1} BAP doubled the fresh mass obtained in the control treatment (0.025 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BAP). Furthermore, a significant reduction of shoot development was obtained when cytokinin concentration was increased from 0.75 to 1.0 mg L^{-1}.

Addition of IBA instead of 2,4-D, as auxin source, during *in vitro* establishment could enhance shoot elongation after bud breaking and IBA could also be less toxic than 2,4-D in this critical stage of the *in vitro* micropropagation protocol. However, it seems that shoot development for *in vitro* established henequen plants is strongly depending on cytokinin concentration in the medium, considering that significant differences were obtained when the medium was supplemented with BAP at 0.75 mg L^{-1} but shoot efficiency felt down when a higher concentration of BAP was added to the medium. This could indicate that the amount of cytokinins necessary for shooting and plant growth is relatively low for these growing tissues.

Use of *A. fourcroydes* apical shoots as explant sources to produce embryogenic *calli* has been previously reported by González *et al*. (2003). Other authors have used henequen marrow, a tissue with high meristematic activity, as explant source for establishment of *in vitro* shoot cultures (Robert *et al*., 1992; Peña *et al*., 1997) and to induce adventitious shoot formation. However, in both protocols they supplemented the basal medium with 2,4-D as auxin source instead of IBA and added higher concentrations of BAP. In contrast, we found that reducing the amount of BAP in the media not only enhanced shoot initiation, but also their further growth and development.

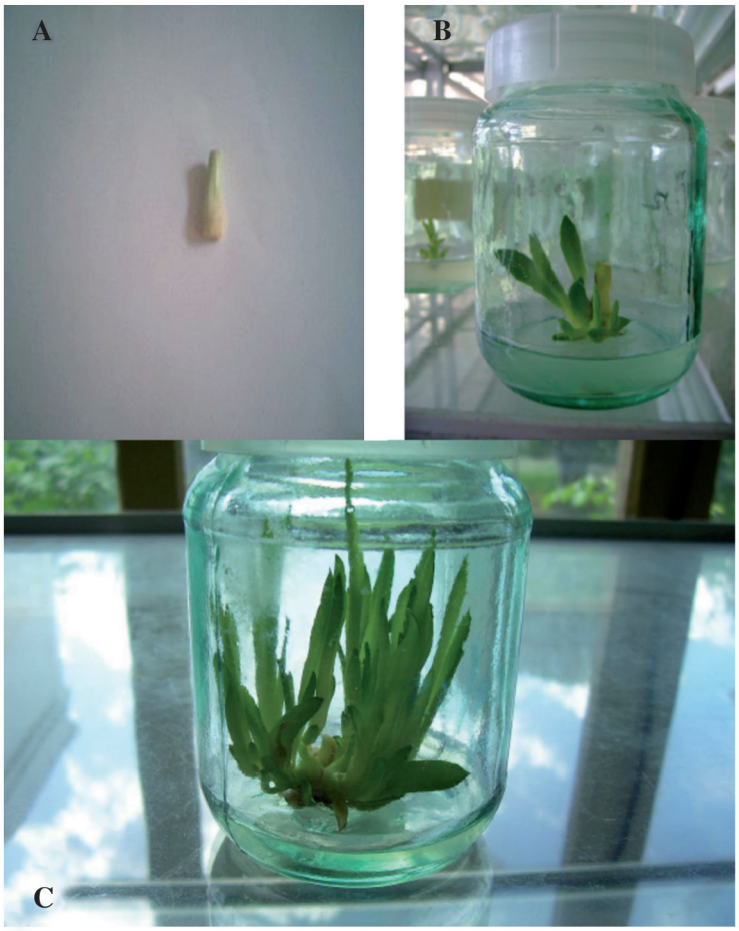
Auxin/cytokinin ratio during *in vitro* tissue culture can play a critical role to induce the morphogenic response in higher plants (García *et al*., 2008). The expression of the morphogenic potential under *in vitro* culture can also depend on certain specific interactions, as demonstrated for embryogenic and non-embryogenic calli. In *Pinus caribaea* Morelet it was found that development of certain calli and plant regeneration depended on specific auxin/cytokinin interactions: NAA/6-furfuryl aminopurine (kinetin) in the medium produced 100% friable *calli* without influence of the NAA/kinetin ratio. However, for NAA and BAP cultures it was necessary to have a 1:2 NAA/BAP ratio to obtain the same results (Akaneme and Eneobong, 2008). Peres and Kerbauy (1999) found that alteration in endogenous auxin/cytokinin ratio, favouring cytokinins, strongly influenced plant development under *in vitro* conditions.

In general, supplementing the basal medium with IBA, as auxin source, and BAP as cytokinin source, increased the efficiency of *in vitro* plant development in the establishment stage of henequen.

Table 1. Morphogenic development of henequen (*Agave fourcroydes*) under different plant growth regulator concentrations in the establishment stage 45 d after disinfection and *in vitro* planting.

Growth regulators	*In vitro* established apex shoot	Fresh mass of shoot	Shoot length
mg L^{-1}	%	mg	cm
0.025 2,4-D-10.00 BAP (Control)	50b	111.30c	1.72c
1.00 IBA-0.25 BAP	70b	137.50c	1.89c
1.00 IBA-0.50 BAP	75b	176.27b	2.17b
1.00 IBA-0.75 BAP	95a	264.11a	3.07a
1.00 IBA-1.00 BAP	60b	199.42b	2.33b

Different letters represent significant differences among treatments according to Kruskall Wallis and Student Newman Keuls ($p \leq 0.05$).
2.4-D: 2.4-dichlorophenoxyacetic acid; BAP: 6-benzylaminopurine; IBA: indolebutyric acid.



MS: Murashige and Skoog basal medium; IBA: indolebutyric acid; BAP: benzylamynopurine; TDZ: thidiazuron.

Figure 1. Morphogenic response in *Agave fourcroydes* explants. A) Apex shoot explant (2.5 cm length) used for *in vitro* initiation of shoot cultures; B) *In vitro* shoot initiation from the shoot apex on MS basal medium supplemented with 1.0 mg L^{-1} IBA and 0.75 mg L^{-1} BAP 45 d after planted; C) *In vitro* multiplication from individualized plantlet on MS basal medium supplemented with 1.0 mg L^{-1} IBA, 1.0 mg L^{-1} BAP and 0.75 mg L^{-1} TDZ after 45 d.

Evaluation of thidiazuron to improve adventitious shoot regeneration during the multiplication stage

Addition of TDZ affected the efficiency of shoot production during the multiplication step (Table 2) and produced significant differences among the tested concentrations of this PGR. Supplementing the medium with 0.5 and 0.75 mg L^{-1} of TDZ induced the highest shoot multiplication frequency in henequen (Figure 1C). Although shoot production was not different when compared to the control treatments, these PGR concentrations did not produce hyperhydric shoots, while 10% of the shoots were hyperhydric on the controls. The same concentrations of TDZ also reduced bud breaking times compared to the control treatment and the other TDZ treatments.

Most of the shoots were regenerated from the basal part of the plantlets near the bottom of the first leaf. This morphogenic behaviour may be due lateral offshoot production or axillary branching which resulted in the formation of small clump of rootless shoots.

During the multiplication stage a strong effect of TDZ to initiate the morphogenic response of the established plantlets was found, and it was expressed as a reduction in bud break. This result can be associated to the reduction of the apical dominance when 0.5 and 0.75 mg L^{-1} of TDZ was added to the basal medium, as occurs in *Scutellaria baicalensis* Georgi, where the exogenous addition of TDZ increase the efficiency of *in vitro calli* differentiation and plant regeneration (Zhang *et al*., 2005). Thidiazuron has been used for micropropagation of a wide range of plant

Table 2. Effects of plant growth regulator synergisms at different concentrations on shoot induction and plant development from *in vitro* established plants of henequen (*Agave fourcroydes*) during the multiplication stage.

Growth regulators	Days required for bud break	N° of shoots/explant	Dry mass	Percentage of hyperhydric plants
mg L^{-1}			mg	%
0.025 2,4-D + 10.00 BAP (Control)	16.40b	3.43a	89.8	10
1.00 IBA + 1.00 BAP + 0.00 TDZ	17.90c	1.95c	84.4	0
1.00 IBA + 1.00 BAP + 0.25 TDZ	16.05b	2.50b	90.9	0
1.00 IBA + 1.00 BAP + 0.50 TDZ	15.35a	3.35a	90.3	0
1.00 IBA + 1.00 BAP + 0.75 TDZ	14.95a	3.45a	90.5	0

Different letters represent significant differences among treatments according to Kruskall Wallis and Student Newman Keuls ($p \leq 0.05$).
2.4-D: 2.4-dichlorophenoxyacetic acid; BAP: 6-benzylaminopurine; IBA: indolebutyric acid.

species because it induces higher frequencies of shoot regeneration than the commonly used cytokinins like BAP (Sunpui and Kanchanapoom, 2002).

The need of supplying the media with higher concentrations of cytokinins is clearly indicated by the results obtained with the treatment combining the lowest BAP concentrations in the absence of TDZ. Synergic effects between BAP and TDZ, at low concentrations in the culture media, are strong obtaining more efficient shoot formation and growing of shoots than media using high BAP concentrations and 2,4-D, as in the control treatment. The effect of auxin/cytokinin ratios can play a critical role on *in vitro* multiplication in *Agave* species (Ramírez-Malagón *et al*., 2008). This auxin/cytokinin ratio for *in vitro* micropropagation of henequen has been tested previously (Robert *et al*., 1987; 1992). Robert *et al*. (1987) found that direct shoot induction efficiency from stem explants was increased when the cytokinin/auxin ratio was 10.0 mg L^{-1} BAP/0.025 mg L^{-1} 2,4-D. But a lower cytokinin/auxin ratio (1.0 mg L^{-1} BAP/0.25 mg L^{-1} 2,4-D) increased *callus* production and reduced shoot formation. However, Das (1992) achieved the best multiplication efficient when only cytokinins were added into the micropropagation medium for *A. sisalana* Perrine, similar responses were found in *A. parrasana* A. Berger (Santacruz Ruvalcaba *et al*., 1999).

Effect of NAA and IBA treatments on rooting efficiency after multiplication

Addition of 0.5 or 0.75 mg L^{-1} NAA into the rooting medium increased the efficiency of root forming plantlets after multiplication, both of them giving 100% of root formation (Table 3). There was also a significant effect of auxin concentrations on root formation demonstrating that addition of NAA was more efficient than IBA. The highest root production per individual shoot was obtained when the medium was supplemented with 0.75 mg L^{-1} (11.55 ± 1.2) of NAA while the shoot length increase of the individualized plant was higher in the medium containing 0.5 mg L^{-1} of NAA (3.95 ± 0.35 cm).

In our *in vitro* culture conditions, shoots obtained using the micropropagation protocol previously

Table 3. *In vitro* root formation efficiency and morphological development in henequen (*Agave fourcroydes*) under different plant growth regulator during the rooting stage.

	Auxin	Rooted plants	N° of root/plant	Fresh mass shoot	Shoot length increase
	mgL^{-1}	%		mg	cm
NAA	0.25	91b	6.87c	125.00ab	3.17b
	0.50	100a	9.40b	129.00a	3.95a
	0.75	100a	11.55a	118.55b	2.43bcd
	1.00	93.75b	10.65ab	118.20b	2.16cd
IBA	0.25	70.07d	2.95d	38.21c	2.20cd
	0.50	80.9c	4.41d	38.29c	3.02bc
	0.75	71.4d	4.00d	38.40c	2.53bcd
	1.00	42.3e	3.91d	25.91d	1.77d

Different letters represent differences among treatments according to Kruskall Wallis and Student Newman Keuls ($p \leq 0.05$).
NAA: naphthylacetic acid; IBA: indolebutyric acid.

established for this species (Robert *et al*., 1992; Peña *et al*., 1997), can reduce or delay their ability for rooting after successive sub-cultivation on the suggested medium for multiplication (data not shown). This would suggest that endogenous cytokinin/auxin ratio is moved to the cytokinins and not to the auxins. Azcón-Bieto and Talón (2000) suggested that lower cytokinin/auxin ratio favour rooting of plants, hence lowering BAP concentrations in the micropropagation medium from 10.0 to 1.0 mg L^{-1} could increase rooting efficiency.

Using high concentrations of cytokinins during the multiplication stage (10.0 mg L^{-1} BAP) along with high concentrations of ammonium and nitrate ions could produce hyperhydric shoots with a low ability to growth and develop normally in *Agave* species (Castro-Concha *et al*., 1990). Although using a high concentration of agar in the medium to reduce hyperhydricity (Castro-Concha *et al*., 1990) of the shoots was employed, we still observed hyperhydric plantlets with the highest BAP concentration. This could support the idea of reducing BAP levels and including slight concentrations of TDZ in the medium to enhance cytokinin:cytokinin:auxin synergisms instead of cytokinin shocks, as a way to improve plant regeneration.

Rooting of plantlets, as well as plant growth development, was most efficient when the regenerated shoots were cultivated on basal MS medium supplemented with 0.5 and 0.75 mg L^{-1} of NAA (Table 3). Using NAA to promote the root formation from *in vitro* regenerated shoots of *A. fourcroydes*, differs with the results obtained by Madrigal *et al*. (1989) in *A. fourcroydes* and Santacruz-Ruvalcaba *et al*. (1999) in *A. parrasana* that achieved successful rooting in MS medium supplemented with IBA and indole-3-acetic acid (IAA), respectively.

Rooting of plantlets obtained after several subcultures under high concentrations of BAP (10.0 mg L^{-1}) reduced root induction (data not shown). Nevertheless, the use of NAA would allow successful rooting of plantlets in spite of the number of subcultures.

Substitution of the 2,4-D as auxin during the micropropagation technology of henequen can be key factor to reduce the probability of somaclonal variability since this plant growth regulator is a potential source of genetic instability (Trigiano and Gray, 1999). González *et al*. (2003) did not detect genetic variability using 2,4-D to induce somatic embryogenesis in henequen, however long culture periods could have different effects.

Ex vitro plant survival reached 94% from plantlets rooted in the medium containing 0.75 mg L^{-1} of NAA. Rooted plants from *in vitro* cultures did not show variation in morphology or growth characteristics when compared with *ex vitro* propagated plants 40 d after soil culture under greenhouse conditions.

CONCLUSIONS

Synergisms between BAP (1.0 mg L^{-1}) and TDZ (0.5-0.75 mg L^{-1}) along with IBA (1.0 mg L^{-1}) as auxin source induced high regeneration frequencies in *Agave fourcroydes* with no production of hyperhydric shoots. Supplementing the rooting medium with NAA instead of 2,4-D also induce root formation more efficiently and was more effective for plant development and growth. A total of 94% of the rooted plantlets survived and grew when transferred to the greenhouse conditions.

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RESUMEN

Manejo de la interacción auxina-citoquinina para mejorar el protocolo de micropropagación de henequén (*Agave fourcroydes* Lem.) El henequén (*Agave fourcroydes* Lem.) es una especie vegetal de gran valor agrícola y ecológico conocida mundialmente por la calidad de su fibra usada con fines industriales. Esta especie se propaga a gran escala por métodos asexuales, pero las tecnologías de propagación actuales no son capaces de satisfacer la demanda de los productores. *A. fourcroydes* ha sido propagado *in vitro* pero muestra comportamiento recalcitrante durante las fases de multiplicación y enraizamiento. En este estudio se demostró que la combinación de 6-benzilaminopurina (BAP) (0.75 mg L^{-1}) y ácido indolbutírico (IBA) (1.0 mg L^{-1}), en lugar de ácido 2,4-diclorofenoxiacético (2,4-D), mejoró significativamente la supervivencia de los explantes y la brotación durante la fase de establecimiento *in vitro* a partir de brotes jóvenes. La combinación de thidiazuron (TDZ) (0.5 ó 0.75 mg L^{-1}) con BAP (1.0 mg L^{-1}) e IBA (1.0 mg L^{-1}) en el medio basal incrementó la tasa de multiplicación del henequén y aceleró la ruptura de la dormancia de las yemas. La mayor eficiencia de enraizamiento se obtuvo cuando el medio basal fue suplementado con 0.5 o 0.75 mg L^{-1} de ácido naftalenacético (NAA), obteniéndose un 100% de plantas enraizadas y un promedio de 9.40 y 11.55 raíces por planta. El 94% de las plantas sobrevivió el proceso de aclimatización *ex vitro* y no se observaron desórdenes fisiológicos en ninguna de las plantas. La modificación de la composición de reguladores del crecimiento vegetal en el medio de cultivo fue un factor clave para mejorar la eficiencia de la tecnología de micropropagación de henequén.

Palabras clave: *Agave fourcroydes*, propagación, thidiazuron, 6-benzilaminopurina, cultivo *in vitro*.

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