Short note [Nota corta]

THE EFFECT OF INOSITOL, PYRIDOXINE AND THIAMINE ON SOMATIC EMBRYOGENESIS OF *Agave angustifolia* †

[EL EFECTO DEL INOSITOL, PIRIDOXINA Y TIAMINA EN LA EMBRIOGÉNESIS SOMÁTICA DE *Agave angustifolia*]

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SUMMARY

Background: Somatic embryogenesis in *Agave angustifolia* is an option for massive *in vitro* propagation and genetic improvement of this species, this process involves the induction of embryogenic callus, the development and maturation of embryos and their germination to form a complete plant. In this sense, an optimized selection of the compounds of the culture medium is required. **Objective:** This study evaluated the effect of vitamin components (inositol, pyridoxine and thiamine) on callus formation and induction of somatic embryos in *A. angustifolia*. **Methodology:** Two consecutive assays were conducted, assay I consisted of eight treatments to determine the isolated effect and in combination of three vitamin compounds (250.0 mg of L^{-1} inositol, 0.5 mg of L^{-1} pyridoxine and 2.0 mg of L^{-1} thiamine). In assay II the effect of six concentrations of thiamine (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}) was determined. In both assays, a completely randomized experimental design was used and each treatment had 20 repetitions. **Results:** The results of assay I show that the formulation of the vitamin complex of the culture medium plays a fundamental role in this process, suggesting that thiamine is an essential compound for the induction of somatic embryos (SE) of *A. angustifolia* because calluses obtained in thiamine supplemented media (alone or in combination) had a better embryogenic response than those not supplemented with this compound; In addition, in assay II with the increase in the concentration of thiamine to 2.5 and 3.0 mg L^{-1} in the culture medium, it is possible to induce a higher number of SE per explant (50.5 and 55.9, respectively), compared to 35.8 SE induced with the original concentration of thiamine (2.0 mg L^{-1}). **Implications:** The results of this study contribute to a better understanding of the importance of the formulation of the vitamin complex and the effect of its addition in the culture medium to induce a greater number of SE in *A. angustifolia*. This can help in the beginning of programs of genetic improvement and *ex situ* conservation of this species. **Conclusion:** The results of this research suggest that thiamine is an essential compound for the acquisition of embryogenic potential in the somatic cells of *A. angustifolia*, since by increasing the concentration of this component in the culture medium it is possible to obtain a greater number of somatic embryos with high *ex vitro* survival rates.

Key words: *In vitro* culture; vitamins; inositol; pyridoxine; thiamine.

RESUMEN

Antecedentes: La embriogénesis somática en *Agave angustifolia* es una opción para la propagación masiva *in vitro* y la mejora genética de esta especie, este proceso implica la inducción de callos embriogénicos, el desarrollo y la maduración de embriones y su germinación para formar una planta completa. En este sentido, se requiere una selección optimizada de los compuestos del medio de cultivo. **Objetivo:** Este estudio se evaluó el efecto de componentes vitamínicos (inositol, piridoxina y tiamina) sobre la formación de callos y la inducción de embriones somáticos en *A. angustifolia.* **Metodología:** Se realizaron dos ensayos consecutivos, el ensayo I consistió en ocho tratamientos para determinar el efecto aislado y en combinación de tres compuestos vitamínicos (250.0 mg de L^{-1} inositol, 0.5 mg de L^{-}

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[1] piridoxina y 2.0 mg de L^{-1} tiamina). En el ensayo II se determinó el efecto de seis concentraciones de tiamina (1.5, 2.0, 2.5, 3.0, 3.5 y 4.0 mg L^{-1}). En ambos ensayos, se utilizó un diseño experimental completamente al azar y cada tratamiento tenía 20 repeticiones. **Resultados:** Los resultados del ensayo I muestran que la formulación del complejo vitamínico del medio de cultivo desempeña un papel fundamental en este proceso, sugiriendo que la tiamina es un compuesto esencial para la inducción de embriones somáticos de *A. angustifolia* (ES) debido a que los callos obtenidos en los medios suplementados con tiamina (sola o en combinación) tuvieron una mejor respuesta embriogénica que los que no se suplementaron con este compuesto; Además, en el ensayo II con el aumento de la concentración de tiamina hasta 2.5 y 3.0 mg L^{-1} en el medio de cultivo, es posible inducir un mayor número de ES por explante (50.5 y 55.9, respectivamente), en comparación con los 35.8 SE inducidos con la concentración original de tiamina (2.0 mg L^{-1}). **Implicaciones:** Los resultados de este estudio contribuyen a una mejor comprensión sobre la importancia de la formulación del complejo vitamínico y el efecto de su adición en el medio de cultivo para inducir un mayor número de ES en *A. angustifolia*. Esto puede ayudar en el inicio de programas de mejora genética y de conservación *ex situ* de esta especie. **Conclusión:** Los resultados de esta investigación sugieren que la tiamina es un compuesto esencial para la adquisición del potencial embriogénico en las células somáticas de *A. angustifolia*, ya que al aumentar la concentración de este componente en el medio de cultivo es posible obtener un mayor número de embriones somáticos con altas tasas de supervivencia *ex vitro*.
Palabras clave: Cultivo *in vitro*; vitaminas; inositol; piridoxina; tiamina.

INTRODUCTION

Mexico is the center of origin of the genus *Agave*, which includes many economically important species that are sources of food, fibers, pharmaceutical, ornamentals and beverages, among others (Portillo *et al.,* 2007).

One of these species is *Agave angustifolia* Haw., used in different states in Mexico to make mescal. In recent years, the increased production of this alcoholic beverage has caused over-exploitation of the species. Both gathering plants and seeds and destruction of its natural surroundings have significantly reduced wild populations (Nikam *et al*., 2003).

A. angustifolia can reproduce vegetatively through bulbils or rhizome shoots. In optimal environmental conditions, the flowering of this species occurs only once during its lifetime (8 -15 years old), but every reproductive cycle fewer viable seeds are obtained because of endogamic depression (Tejavathi *et al.*, 2007). For this reason, the urgent need to obtain a large number of uniform individuals of *elite* genotypes has led to development of *in vitro* culture techniques for massive propagation of *A. angustifolia* via somatic embryogenesis (Arzate-Fernández and Mejía-Franco, 2011; Reyes-Díaz *et al*., 2017).

In vitro somatic embryogenesis is the result of cell reprogramming toward embryogenesis. This process forms the basis of cell totipotential in higher plants and can be induced by different biochemical or molecular signals caused by exogenous factors, such as growth regulators, macro and micro nutrients, amino acids, sugars and vitamins, added to the culture medium (Fehér *et al.*, 2003). Reyes-Díaz *et al.* (2017) suggested that the vitamin complex formulation used in the phase of callus induction is correlated with acquisition of embryogenic competence in somatic cells of *A. angustifolia*. They concluded that, with the components of the L2 vitamin complex (inositol, pyridoxine and thiamine) (Phillips and Collins, 1979), a larger number of somatic embryos can be obtained than with other vitamin formulations.

Vitamins are nitrogenous compounds that act as enzymatic cofactors in diverse metabolic processes. Inositol (vitamin B8) is used by plants as a sucrose supplement, supplying considerable energy during the stage of cell reprogramming. Also, there is evidence that pyridoxine (vitamin B6) participates in reactions of transamination and decarboxylation of amino acids (protein biosynthesis) and thiamine (vitamin B1) participates in oxidative decarboxylation reactions, intervenes in carbohydrate metabolism, and is essential in the Krebs cycle for energy production and nitrogen assimilation (Fehér *et al.*, 2003; Murray, 2010).

This study evaluates the effect of the components of the L2 vitamin complex (VCL2: inositol, pyridoxine and thiamine) on callus formation and somatic embryo (SE) induction in *Agave angustifolia*.

MATERIALS AND METHODS

The experiment was realizated in the Laboratory of Plant Molecular Biology of the Center for Research and Advanced Studies in Plant Breeding of the Universidad Autónoma del Estado de México, located on the University Campus "El Cerrillo", Toluca, State of Mexico.

Plant material

The initial explants were mature *A. angustifolia* zygotic embryonic axes (Figure 1A) obtained from wild seeds collected in the municipality of Zumpahuacán, State of Mexico.

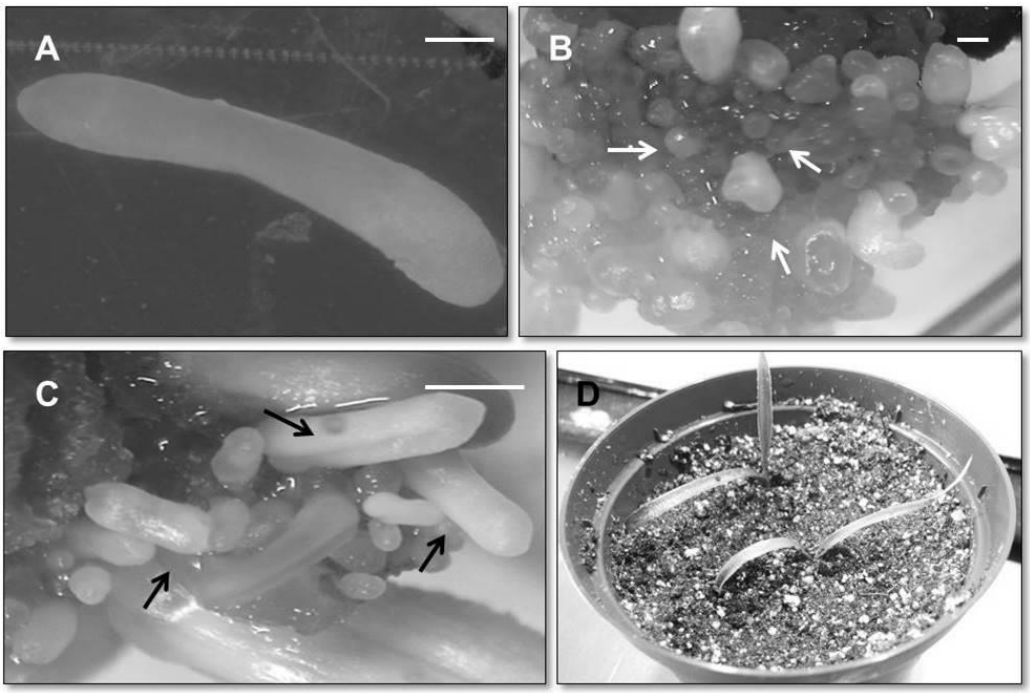

Figure 1. Somatic embryogenesis in *Agave angustifolia*. A. zygotic embryonic axis used as explant. B. Callus with somatic embryos in globular-torpedo stage (arrows) 60 d after initiating culture (daic) in treatment 1. C. Somatic embryos in cotyledonal stage (arrows) 120 daic. D. Adapted plantlets in greenhouse 200 daic. Barr = 1 mm.

The seeds were submerged in a soap solution and rinsed under running water for 15 min to eliminate dust and debris. Then, in aseptic conditions, they were submerged in 96% ethanol for 1 min and later placed in a solution of 2% sodium hypochlorite for 15 min under constant shaking. Finally, they were rinsed three times with sterile distilled water and stored at 4ºC for 48 h until use.

Callus formation and somatic embryo (SE) induction

The *A. angustifolia* zygotic embryonic axes were established in culture medium for callus formation and SE induction. This medium was reported by Arzate-Fernández and Mejía-Franco (2011).

To determine the effect of the VCL2 (alone or in combination) in the culture medium, two assays were conducted consecutively:

Assay I. The effect of 250.0 mg L^{-1} inositol, 0.5 mg L^{-1} pyridoxine and 2.0 mg L^{-1} thiamine (Phillips and Collins, 1979) in eight treatments were evaluated (Table 1).

Assay II. The effect of six concentrations of thiamine (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}), and together with better concentration of inositol and pyridoxine that had been used to yield the highest rate of SE were evaluated on embryogenic callus formation and SE induction of *A. angustifolia* (Table 1).

pH of the culture medium was adjusted to 5.7, gelled with 8.0 g L^{-1} agar and sterilized at a temperature of 121°C (1.1 kg cm^{-2} pressure) for 20 min. All the treatments were incubated in darkness for 60 days at 25 ± 2°C (Arzate-Fernández and Mejía-Franco, 2011).

Maturation of somatic embryos (SE)

The calli with SE at the globular stage obtained in the previous assays were transferred to a culture medium for maturation of the SE to a cotyledonal stage for 60 days more in darkness at 25 ± 2°C, which is 120 daic. The culture medium consisted of MS salts (Murashige and Skoog, 1962) reduced to 50% of their original concentration, supplemented with 0.1 mg L^{-1} 2,4-D and 30 g L^{-1} sucrose. At this stage, the number of SE per callus induced was quantified (only embryos in cotyledonal stage were counted).

Germination of somatic embryos (SE) and plantlet regeneration

Ten somatic embryos in cotyledonal stage were selected at random per treatment. Germination occurred in MS medium without plant growth regulators and in a photoperiod of 16 h light until they reached an average height of 5 cm. Regenerated plants with vigorous roots were transferred to pots containing a substrate of agrolite, compost and sand (1:1:1) and taken to a greenhouse for adaptation.

Table 1. Treatments evaluated and their effect on embryogenic response in *Agave angustifolia.*

Assay	Treatment	Vitamins (mg L^{-1})†			Embryogenic callus $^{+}$ (%)	Somatic embryos in cotyledonal state per callus $^{++,*}$	
		Inositol (B8)	Pyridoxine (B6)	Thiamine (B1)			
I	1	250.0	0.5	2.0	60.0	35.8 ± 0.3	a
	2	0.0	0.0	2.0	55.0	14.6 ± 0.6	c
	3	250.0	0.0	2.0	25.0	15.8 ± 0.0	c
	4	0.0	0.5	2.0	15.0	17.2 ± 0.9	b
	5	250.0	0.0	0.0	20.0	0.0 ± 0.0	e
	6	250.0	0.5	0.0	30.0	3.6 ± 0.7	d
	7	0.0	0.5	0.0	10.0	0.0 ± 0.0	e
	8	0.0	0.0	0.0	15.0	0.0 ± 0.0	e
II	1	250.0	0.5	1.5	60.0	23.2 ± 2.17	c
	2	250.0	0.5	2.0	60.0	36.0 ± 2.32	b
	3	250.0	0.5	2.5	80.0	50.5 ± 2.48	a
	4	250.0	0.5	3.0	70.0	55.9 ± 2.00	a
	5	250.0	0.5	3.5	60.0	24.9 ± 2.15	c
	6	250.0	0.5	4.0	40.0	16.1 ± 0.63	d

†Phillips and Collins, 1979; $^{+}$60 and $^{++}$120 days after initiating culture. *Same letters indicate no statistically significant difference at 5% ($P \leq 0.05$), according to the Tukey test of comparison of means.

Statistical analysis

In both assays, we used a completely randomized design and each treatment had 20 replications. Each replication (a Petri dish) had 20 zygotic embryonic axes, and each zygotic embryonic axis was one experimental unit. Percentage of calli obtained was calculated 60 d after initiating culture (daic).

The data on callus percentage obtained and number of SE induced per callus were subjected to an analysis of variance (ANOVA) using the software *Statgraphics* version 5.0. In those treatments where significant differences were observed, a Tukey comparison of means test was conducted with a significance level of 5%.

RESULTS AND DISCUSSION

Callus formation, induction and maturation of somatic embryos (SE)

Assay I. In this study, des-differentiation of zygotic embryonic axes was visible 5 daic, leading to callus formation. However, SE induction in the globular-torpedo state was evident as of 20 and up to 60, daic (Fig. 1B) (Table 1).

Moreover, it was observed that, although the culture medium contained plant growth regulators, the presence or absence of some vitamin compound influenced callus formation and consequently SE induction. In this sense, highly significant differences were observed among the evaluated treatments. Treatment 1, which contained the three components of VCL2 (inositol, pyridoxine and thiamine), obtained the highest average percentage of callus formation (60%) and the largest number of SE in cotyledonal state (35.8) 120 daic (Figure 1C). In contrast with treatment 8, which did not include the VCL2, obtained 15% callus formation and did not observe somatic embryogenesis (Table 1). Also, the absence of any of the vitamin components considerably affected these variables, as can be observed in treatments 3, 4, and 6.

In *Trifolium pratense*, both Phillps and Collins (1979) and Myers *et al.* (1989) obtained a larger number of shoots from cultured callus in a medium supplemented with the L2 vitamin complex (VCL2) than in media to which the MS vitamin complex (VCMS) was added or media without vitamins. To this respect, Shils *et al*. (2001) indicate that the vitamin complexes contain nitrogenous organic elements that are not synthesizable by the organism (with some exceptions) but are essential for plant growth and development and for maintaining adequate metabolism. When higher plants are cultured *in vitro*, the absence of vitamins is a limiting factor in growth as can be observed in treatment 8 of our study.

Vitamins fundamentally act as enzymatic cofactors in diverse metabolic processes. For example, inositol, which is an integral part of several types of membranes of some cell organelles, is used by plants to supplement sucrose, contributing considerable energy during the stage of cellular reprogramming. Likewise, pyridoxine, although it is not considered essential in tissue culture (as demonstrated in treatment 7), has been shown to participate in reactions of transamination and decarboxylation of amino acids (protein biosynthesis) (Murray, 2010).

In addition, when we analyzed the response of the zygotic embryonic axes of *A. angustifolia* to the treatments with thiamine (alone or in combination), treatments 1, 2, 3 and 4, we observed that it was possible to induce a larger number of SE, in contrast with those treatments that lacked it (treatments 5, 6, 7 and 8) (Table 1).

Assay II. As we mentioned above, largest average number of SE in cotyledonal stage (35.8) was obtained with the treatment that contained the three components of the vitamin complex L2 (Phase I). This suggests that thiamine plays a fundamental role in acquiring embryogenic competence of *A. angustifolia* somatic cells. To further test the veracity of this assumption, in Assay II of our study we evaluated the effect of six thiamine concentrations (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}) in combination with 250 mg L^{-1} inositol and 0.5 mg L^{-1} pyridoxine on embryogenic callus formation and SE induction (Table 1).

In Table 1, it can be observed that SE induction was achieved in the six treatments evaluated. However, it can also be noted that when reducing or increasing the concentration of thiamine in the culture medium (treatments 1 and 6), the number of SE decreases relative to the number induced in the original concentration (2.0 mg L^{-1}). Our results coincide with Goyer (2010), who indicate that low and high concentrations of thiamine diphosphate negatively affect the quantity of mRNA and, consequently, it also affects protein biosynthesis in the cell preproduction stage.

Nevertheless, in treatments 3 and 4 there were no significant differences, suggesting that by increasing thiamine concentration up to 2.5 and 3.0 mg L^{-1} in the culture medium it is possible to induce a larger number of SE per explant (50.5 and 55.9, respectively), compared with the 35.8 SE induced with the original concentration of thiamine (2.0 mg L^{-1}) (Assay I). A possible explanation of these results lies in that thiamine is a vitamin of the B complex with a broad spectrum of metabolic activities. That is, it is a fundamental enzymatic cofactor that participates in oxidative decarboxylation reactions and intervenes in carbohydrate metabolism (dehydrogenase pyruvate), in the citric acid cycle (α-ketoglutarate dehydrogenase), in the phosphate pentose via (transketolase) and in metabolism of isoleucine, leucine and valine (α-ketoacid dehydrogenase). In addition thiamine has recently been attributed with specific functions in regulating primordial gene expression in the stage of cell reproduction, a capacity that before was considered to be more that of macronutrients (Al-Khayri, 2001, Bunik and Fernie, 2009; Goyer, 2010; Murray, 2010).

Finally, of the 10 somatic embryos in germinated cotyledonal stage by treatment, the frequency of conversion to plantlets varied from 90 to 100%, with a survival percentage of 100% at 200 daic (Figure 1D).

The results of our study contribute to better understanding of the addition of vitamins (especially thiamine or vitamin B1) to culture medium to induce a larger number of SE on *A. angustifolia* from zygotic embryonic axes. This can aid in *ex situ* conservation of the species and in initiating genetic improvement programs. It can also set the bases for mass cloning of agave plants to guarantee sufficient plant material for growers interested in establishing commercial plantations, which will contribute to more rational exploitation of the resource.

CONCLUSIONS

The results of this research suggest that thiamine is an essential compound for the acquisition of embryogenic potential in the somatic cells of *Agave angustifolia*, since by increasing the concentration of this component in the culture medium it is possible to obtain a greater number of somatic embryos with high survival rates *ex vitro*. This information contributes to a better understanding of the factors that regulate the embryogenic process and can help in *ex situ* conservation of the specie and in the beginning of genetic improvement programs.

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Conflict of interest. The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Compliance with ethical standards. The authors confirm that this research was supervised by the Internal Bioethics Committee of the Universidad Autónoma del Estado de México under the authorization of the project 4729/2019CIS.

Data availability. Data are available with Dr. Jesús Ignacio Reyes-Díaz (jird.rd@gmail.com) upon reasonable request.

REFERENCES

Al-Khayri, J. M. 2001. Optimization of biotin and thiamine requirements for somatic embryogenesis of date palm (*Phoenix dactylifera* L.). In Vitro Cellular & Developmental Biology Plant. 37:453-456. DOI: 10.1007/s11627-001-0079-x.

Arzate-Fernández, A. M. and Mejía-Franco, R. 2011. Capacidad embriogénica de callos inducidos en ejes embrionarios cigóticos de *Agave angustifolia* Haw. Revista Fitotecnia Mexicana. 34(2):101-106. URL: https://www.revistafitotecniamexicana.org/documentos/34-2/4r.pdf

Bunik, V. I. and Fernie, A. R. 2009. Metabolic control exerted by the 2-oxoglutarate dehydrogenase reaction: a cross-kingdom comparison of the crossroad between energy production and nitrogen assimilation. Biochemistry Journal. 422: 405–421. DOI: 10.1042/BJ20090722.

Fehér, A., Pasternak, T. P. and Dudits, D. 2003. Transition to somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Culture. 74: 201-228. DOI: 10.1023/A:1024033216561.

Goyer, A. 2010. Thiamine in plants: Aspects of its metabolism and functions. Phytochemistry. 71:1615–1624. DOI: 10.1016/j.phytochem.2010.06.022.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and biossays with tabacco tissue cultures. Physiology Plant. 15: 473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x.

Murray, R. K. (editor). 2010. Harper. Bioquímica ilustrada. 28a edición. México: Mac Graw-Hill. 518-535.

Myers, J. R., Grosser, J. W., Taylor N. L. and Collins, G. B. 1989. Genotype-dependent whole plant regeneration from protoplasts of red clover (*Trifolium pratenseÿ* L.). Plant Cell, Tissue and Organ Culture. 19: 113-127. DOI: 10.1007/BF00035811.

Nikam, T. D., Bansude, G. M. A. and Aneesh-Kumar, K. C. 2003. Somatic embryogenesis in sisal (*Agave sisalana* Perr. Ex. Engelm). Plant Cell Reports. 22:188–194. DOI: 10.1007/s00299-003-0675-9

Phillips, G. C. and Collins, G. B. 1979. *In vitro* tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop Science. 19: 59–64.

Portillo, L., Santacruz-Ruvalcaba, F., Gutiérrez-Mora, A. and Rodríguez-Garay, B. 2007. Somatic embryogenesis in *Agave tequilana* Weber cultivar azul. In Vitro Cellular & Developmental Biology Plant. 43:569–575. DOI: 10.1007/s11627-007-9046-5

Reyes-Díaz, J. I., Arzate-Fernández, A. M., Piña-Escutia J. L. and Vázquez-García, L. M. 2017. Media culture factors affecting somatic embryogenesis in *Agave angustifolia* Haw. Industrial Crops and Products. 108: 81-85. DOI: /10.1016/j.indcrop.2017.06.021.

Shils, E., Olson, J. A., Shike, M. and Ross A. C. (ed). 2001. Nutrición en salud y enfermedad. Novena edición. México. McGraw-Hill, 443-541.

Tejavathi, D. H., Rajanna, M. D., Sowmya, R. and Gayathramma, K. 2007. Induction of somatic embryos from cultures of *Agave vera-cruz* Mill. In Vitro Cellular & Developmental Biology Plant. 43:423–428. DOI: 10.1007/s11627-007-9088-8.