Towards the micropropagation of *Euphorbia cyathophora* Murray: a wild plant species with medicinal and ornamental potential

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ABSTRACT: *Euphorbia cyathophora Murray is a plant species with medicinal and ornamental potential but whose in vitro propagation is unknown. Two experiments were performed to know 1) the effect of light (WL) at 25±2 °C and 20* **μ***mol m⁻² s⁻¹ luminous intensity, or darkness at 20±2 °C, for the first 20 days and subsequent placement in light (DKL) as previously described, and 2) the best combination of plant growth regulators: benzyl amino purine (BAP), kynetin (KIN), 2-isopentenyl adenine (2iP) and thidiazuron (TDZ) in combination with naphthaleneacetic acid (NAA) and the effect of both light conditions. The DKL treatment did not promote the growth of leaves, cotyledons, or plantlet height; in contrast, WL produced a more developed root system with early appearance of tertiary roots. In regards to the interaction of plant regulators and light effect, only the treatment with 1 mg BAP L⁻¹ and 0.1 mg NAA L⁻¹ generated shoots in both light conditions (DKL and WL). Darkness (DKL) generated more indirect greatest morphogenic responses (callus formation).*
Key words: *Euphorbiaceae, in vitro culture, phytohormones, light conditions.*

Micropropagação de *Euphorbia cyathophora* Murray: uma especie de planta selvagem com potencial medicinal e ornamental

RESUMO: *Euphorbia cyathophora Murray é uma espécie vegetal com potencial medicinal e ornamental, mas cuja propagação in vitro é desconhecida. Dois experimentos foram realizados para conhecer 1) o efeito da luz (WL) a 25±2 °C e 20 μmol m⁻² s⁻¹ intensidade luminosa, ou escuridão a 20±2 °C, nos primeiros 20 dias e posterior colocação em luz (DKL) como descrito anteriormente, e 2) a melhor combinação de reguladores de crescimento vegetal: benzil amina purina (BAP), kynetina (KIN), 2-isopentenil adenina (2iP) e thidiazuron (TDZ) em combinação com ácido naftalenoacético (ANA) e o efeito de ambas as condições de luz. O tratamento com DKL não promoveu o crescimento de folhas, cotilédones ou altura das plântulas. Em contraste, a WL produziu um sistema radicular mais desenvolvido com o aparecimento precoce de raízes terciárias. Com relação à interação dos reguladores vegetais e efeito luminoso, apenas o tratamento com 1 mg de BAP L⁻¹ e 0,1 mg de NAA L⁻¹ gerou brotações em ambas as condições de luz (DKL e WL). Escuridão (DKL) gerou mais respostas morfogênicas mais diretas (formação de calo).*
Palavras-chave: *Euphorbiaceae, cultura in vitro, fitohormônios, condições de luz.*

INTRODUCTION

The Euphorbiaceae is one of the most diverse and cosmopolitan botanical family, represented by around 8,000 plant species (WEBSTER, 2014). Taxonomically, this family includes up of five subfamilies and 320 genera; in Mexico, there are reports of 250 species of the *Euphorbia* genus, out of the 2,160 reported worldwide (VILLASEÑOR, 2016).

One species with medicinal and ornamental potential that is naturally distributed in Mexico is *E. cyathophora* Murray, which presents colorful bracts and leaf dimorphism (oval, lanceolate, or sublinear, whole or sawed, smooth or with scarce follicles), and black, cylindrical or ovoid seeds (MARTÍNEZ et al. 2002). This

species has priority for its preservation by the Euphorbia network of the National System of Phytogenetic Resources-Mexico (COLINAS et al., 2014). However, there is a scarce of knowledge about the propagation, light conditions and effect of phytohormones in the development of explants of this plant species.

MATERIALS AND METHODS

Plant material

Plants and seeds of *Euphorbia cyathophora* were collected from the Ejido of Libertad Misión Sabinos Unidos, Municipality of Victoria, Tamaulipas (Mexico) for testing their germination and propagation under laboratory

conditions at the Center of Plant Tissue Culture, Colegio de Postgraduados Campus Montecillo.

Establishment, conditions, treatments, and variables for experiment 1

A first experiment was set consisting on moisturizing the seeds for 24 h in a GA_3 solution (200 mg L^{-1}) plus Captan® (0.5 g L^{-1}) commercial fungicide. Then, the seed coat was cut off with a scalpel under a stereoscopic microscope, and the embryo and endosperm were detached. Embryos were placed in beakers with MS medium (MURASHIGE & SKOOG, 1962) without growth regulators. Seven embryos were placed under light conditions (WL) at 25±2 °C and 20 μmol $m^{-2} s^{-1}$ light intensity. Other seven embryos were placed in darkness at 20± 2°C during 20 days; and subsequently, under light conditions (DKL) as described for WL treatments.

Besides determining the germination percentage in WL and DKL, plant height, root growth, root length, cotyledon length and width, and number, length, and width of leaves were evaluated in nine sampling times (3, 5, 8, 12, 15, 19, 26, 41, and 48 days) after *in vitro* sowing.

Establishment, conditions, treatments, and variables for experiment 2

A second experiment was done using the explants obtained from the previous assay. This experiment included a control treatment, and 12 treatments consisted of three concentrations of benzyl amino purine (BAP) (0.05, 0.1, and 1.0 mg L^{-1}), three concentrations of kinetin (KIN) (0.05, 0.1, and 1.0 mg L^{-1}), three concentrations of 2-isopentenyl adenine (2iP) (0.05, 0.1, and 1.0 mg L^{-1}), three concentrations of thidiazuron (TDZ) (0.05, 0.1, and 1.0 mg L^{-1}), and one concentration of naphthaleneacetic acid (NAA) (0.1 mg L^{-1}).

The 12 treatments contained MS at 100% + 0.04 mg L^{-1} thiamine-HCl, 0.5 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} pyridoxine HCl, 2.0 mg L^{-1} glycine, 100 mg L^{-1} inositol, 30,000 mg L^{-1} sucrose, 0.1 mg L^{-1} NAA, and 5,000 mg L^{-1} agar gel-rite; the pH was adjusted to 5.7±0.1. The control treatment consisted of 100% MS added with 0.1 mg L^{-1} NAA.

Each treatment consisted in six beakers, each one with six stem segments (explants), approximately 0.5 cm length. After explants were planted, three beakers were placed under DKL conditions, and the remaining three bakers were exposed to WL, as previously described.

The evaluated parameters consisted on describing the morphological expression type, number of shoots, shoot length, and root number and length at 10, 20, 40, and 70 days. For subjective evaluation of the morphological expression type, five criteria were defined: 0 = no response, 1 = callus presence, 2 = root production, 3 = shoot generation, 4 = callus and root production, and 5 = root, callus, and shoot presence (HA et al., 2016).

Experimental design and statistical analysis

For the first experiment a completely randomized design was used. In the case of the second experiment, individual analyses were performed for each sampling time by using a divided plot design in which the large plots corresponded to the light conditions, and the small plots were assigned for the treatments. In addition, combined analyses were simultaneously done by considering all sampling times, light conditions, and treatments. All data were analyzed with the general lineal model (GLM) procedure by using SAS (SAS INSTITUTE, 2015).

RESULTS

Germination percentage under light conditions

In the experiment 1, the application of 200 mg GA_3 L^{-1} and the elimination of the seed coat resulted in increased germination under WL conditions (85%) when compared to DKL condition in which a 72% germination was achieved.

Plant growth under light (WL) and darkness (DKL) conditions

The embryos at DKL conditions indicated less width cotyledons than those at WL conditions; however, differences were only observed at 41 and 48 days. Length of cotyledons showed no differences between DKL and WL conditions (Table 1). Under WL conditions, explants had greater number of leaves than under DKL conditions in all sampling times; nevertheless, leaves emerged fifteen days sooner at WL conditions. Either width or length of leaves was in overall lower (0.1 until 0.6 cm) at DKL conditions than those leaves developed at WL conditions. Height of explants at WL conditions was numerically higher than those explants grown at DKL conditions in all sampling times, but only at 8 days, differences were obtained between WL and DKL conditions (Table 1).

Explants grown in WL conditions presented higher number of roots than those explants grown at DKL conditions, but significant differences were observed only at the third day. Root length showed no significant differences during 26 days; however, at 41 and 48 days, roots of explants grown at WL conditions were statistically lengthier than those from explants at DKL conditions (Table 1).

Table 1 - Morphological parameters of *Euphorbia cyathophora* with light (WL) and darkness-light conditions (DKL).

Plant parameters	Light conditions	Days						
		8	12	15	19	26	41	48
Width of cotyledons (cm)	WL	0.00	0.10 a	0.24 a	0.26 a	0.39 a	0.50 a	0.51 a
	DKL	0.00	0.05 a	0.17 a	0.21 a	0.21 a	0.21 b	0.21 b
	HSD$_{(\alpha=0.05)}$	0	0.176	0.210	0.244	0.280	0.267	0.265
Leaf number	WL	0.00	0.00	1.00 a	1.00 a	2.00 a	4.00 a	5.71 a
	DKL	0.00	0.00	0.00 b	0.43 a	1.14 a	1.86 a	2.57 a
	HSD$_{(\alpha=0.05)}$	0	0	0.824	0.934	1.607	2.623	3.544
Leaf width (cm)	WL	0.00	0.00	0.11 a	0.16 a	0.27 a	0.41 a	0.64 a
	DKL	0.00	0.00	0.00 b	0.11 a	0.20 a	0.24 a	0.27 b
	HSD$_{(\alpha=0.05)}$	0	0	0.100	0.173	0.255	0.283	0.354
Leaf length (cm)	WL	0.00	0.00	0.11 a	0.63 a	0.91 a	1.09 a	1.29 a
	DKL	0.00	0.00	0.00 b	0.13 a	0.39 a	0.44 a	0.54 b
	HSD$_{(\alpha=0.05)}$	0	0	0.100	0.510	0.676	0.701	0.737
Plant height (cm)	WL	2.71 a	3.64a	4.39 a	5.73a	7.86 a	10.71 a	13.00 a
	DKL	0.59 b	1.33 a	1.83 a	2.64 a	3.41 a	4.86 a	5.64 a
	HSD$_{(\alpha=0.05)}$	1.99	2.65	3.15	3.82	4.75	6.81	7.73
Number of roots	WL	0.85 a	0.85 a	1.42 a	2.28 a	2.42 a	2.42 a	2.57 a
	DKL	0.57 a	0.57 a	1.28 a	1.28 a	1.28 a	1.42 a	1.85 a
	HSD$_{(\alpha=0.05)}$	0.53	0.53	1.01	1.29	1.21	1.23	1.44
Root length (cm)	WL	1.75 a	2.85 a	4.24 a	7.02 a	11.85 a	14.28 a	15.28 a
	DKL	1.28 a	1.71 a	2.4 a	3.54 a	4.29 a	5.92 b	6.78 b
	HSD$_{(\alpha=0.05)}$	1.53	2.10	2.93	4.65	7.35	7.77	8.46

HSD = Honest Significant Difference. Different letters in the same parameters and columns represent significant differences (Tukey, α = 0.05) between WL and DKL conditions. n = 7.

Experiment 2. In vitro growth of explants

From all treatments evaluated in this experiment, only the treatment with 1.0 mg BAP L^{-1} + 0.1 mg NAA L^{-1} generated shoots in both light conditions (Figure 1a) (Figure 3). Shoots started appearing at day 10 under WL conditions while at DKL conditions shoots appeared at day 40. Furthermore, shoot length was statistically higher at WL conditions than DKL (Figure 1b).

On day 10, the treatment with 0.1 mg TDZ L^{-1} at DKL produced the highest number of roots. Contrastingly, on days 20, 40, and 70, the treatment
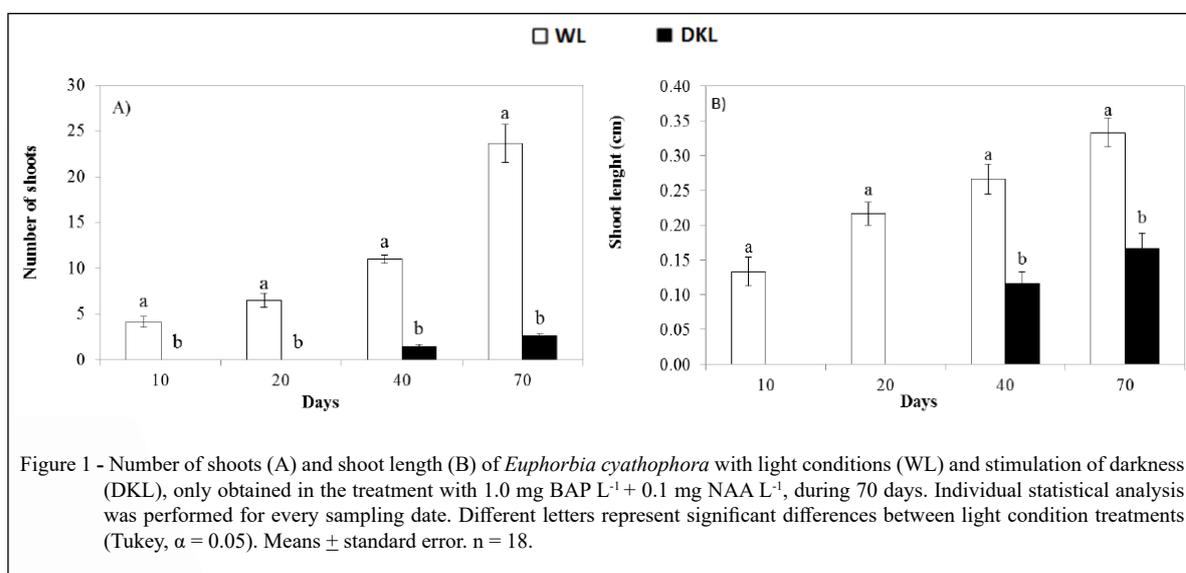
Figure 1 - Number of shoots (A) and shoot length (B) of *Euphorbia cyathophora* with light conditions (WL) and stimulation of darkness (DKL), only obtained in the treatment with 1.0 mg BAP L^{-1} + 0.1 mg NAA L^{-1}, during 70 days. Individual statistical analysis was performed for every sampling date. Different letters represent significant differences between light condition treatments (Tukey, α = 0.05). Means $\pm$ standard error. n = 18.

with 1.0 mg BAP L^{-1} at DKL conditions the explants had a significantly higher number of roots than the remaining treatments (Table 2).

Treatment with 0.05 mg TDZ L^{-1} at DKL conditions resulted in longest roots, on days 10 and 20. On day 40, treatments with 0.05 mg TDZ L^{-1} and 1.0 mg 2iP L^{-1} at DKL conditions produced longest roots, while on day 70, treatments with 0.05 mg TDZ L^{-1} at DKL, and control at WL conditions generated the longest root values (Table 2).

Types of morphological expression of the explants

Type of morphological expression of the explants as a response of treatment application in four sampling dates is presented in figure 2. On days 10 and 20, DKL conditions favored the expression of roots in treatments with 0.05 mg 2iP L^{-1} and 0.05 mg TDZ L^{-1}, but the treatment with 0.1 mg TDZ L^{-1} favored the formation of callus and roots. On days 40 and 70, DKL conditions produced the formation of callus and roots in explants for 0.05 mg KIN L^{-1}, 0.05 mg TDZ L^{-1}, 1.0 mg KIN L^{-1}, and 1.0 mg 2iP L^{-1}. Conversely, WL conditions produced callus and root at days 40 and 70, only for the control. At all sampling dates, the treatment with 1.0 mg BAP L^{-1} generated roots, calluses, and shoots explants exposed to both light conditions.

In general, DKL condition generated greater callus development in all treatments when compared to WL condition. Also, DKL condition promoted oxidized calluses with few green regions (data not shown).

DISCUSSION

In the first experiment, it was observed that the application of gibberellins and the removal of the seed coat allowed a higher percentage of germination in WL and DKL conditions. In this regards, physical seed coat wear or removal may allow water and oxygen diffusion, and eliminate or decrease the effects of the seed germination inhibitors (RAVEN et al., 2013; SUN et al., 2006). In addition, gibberellins are directly implied in the promotion of seed germination (DAVIES, 2010). These phytohormones (gibberellins) are synthesized in several plant organs with active growth region like embryos or meristems (MIYASHIMA et al., 2013). The later explains in part how the GA$_3$ can break the seed dormancy of *E. cyathophora*, and may replace or compensate environmental requirements such as light or temperature.
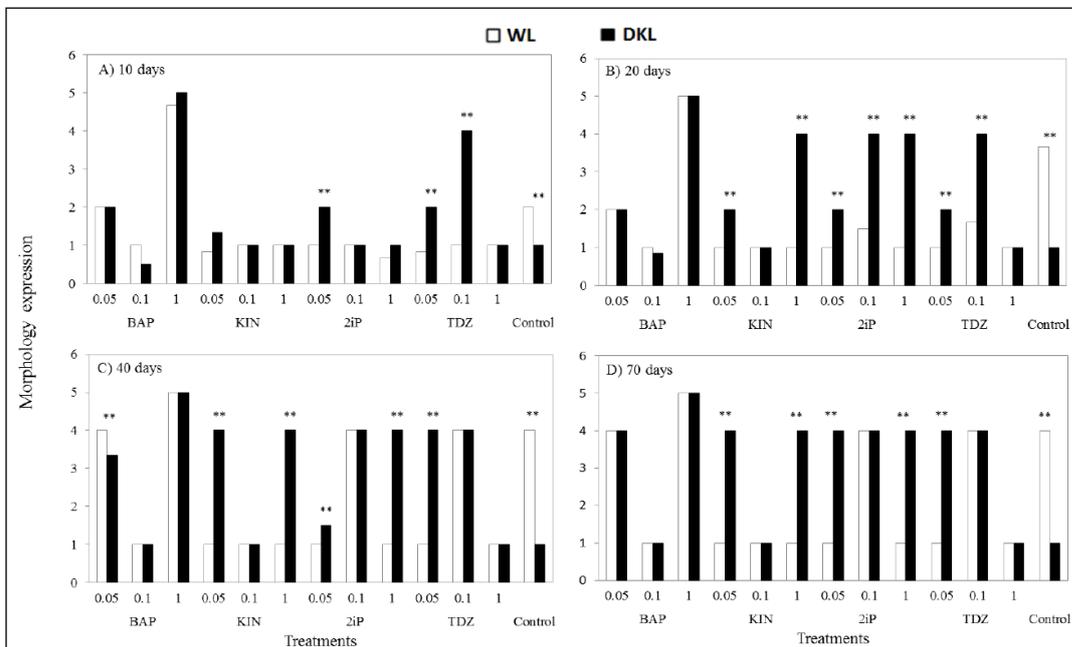
Figure 2 - Morphological expression of *Euphorbia cyathophora* explants as an effect of the application of growth regulators, light conditions (WL) and darkness-light conditions (DKL), at 10 (A), 20 (B), 40 (C), 70 days (D) 70. BAP = benzyl amino purine, KIN = kinetin, 2iP = 2-isopentenyl adenine and TDZ = thidiazuron. Individual statistical analysis was performed for each sampling date, and asterisks denote significant differences between light condition treatments (Tukey, α = 0.05), n = 18.

Table 2 - Number of roots and root length of *Euphorbia cyathophora* with light (WL) and darkness-light conditions (DKL).

Parameter		----------Number of roots----------						
Days		------10------		------20------		------40------		------70------	
Light conditions		WL	DKL	WL	DKL	WL	DKL	WL	DKL
BAP (mg L⁻¹)	0.05	1.16cd	1.16cd	1.66de	1.50de	2.00de	1.66e	2.00de	1.66e
	0.1	0.00d	0.00d	0.00e	0.00e	0.00f	0.00f	0.00f	0.00f
	1.0	1.50bc	0.00d	1.50de	9.00a	1.50ef	9.00a	1.50ef	9.00a
KIN (mg L⁻¹)	0.05	0.00d	1.00cd	0.00e	2.16cd	0.00f	2.50cde	0.00f	2.50cde
	0.1	0.00d	0.00d	0.00e	0.00e	0.00f	0.00f	0.00f	0.00f
	1.0	0.00d	0.00d	0.00e	2.33cd	0.00f	2.33de	0.00f	2.66cde
2iP (mg L⁻¹)	0.05	0.00d	2.66ab	0.00e	2.66cd	0.00f	2.66cde	0.00f	3.00cde
	0.1	0.00d	0.00d	1.83d	3.00cd	1.83de	3.33cd	1.83e	3.50bcd
	1.0	0.00d	0.00d	0.00e	2.66cd	0.00f	3.33cd	0.00f	3.50bcd
TDZ (mg L⁻¹)	0.05	0.00d	3.00a	0.00e	3.83be	0.00f	4.00bc	0.00f	4.00bc
	0.1	0.00d	4.00a	1.50de	5.00a	1.50ef	5.00b	1.50ef	5.00b
	1.0	0.00d	0.00d	0.00e	0.00e	0.00f	0.00f	0.00f	0.00f
Control		1.33bcd	0.00d	2.00d	0.00e	2.00de	0.00f	2.00de	0.00f
HSD(α= 0.05)		------1.47------		------1.68------		------1.63------		------1.58------	
Parameter		----------Root length----------						
Days		------10------		------20------		------40------		------70------	
Light conditions		WL	DKL	WL	DKL	WL	DKL	WL	DKL
BAP (mg L⁻¹)	0.05	0.20cd	0.40b	0.63b	0.45bcd	0.75bc	0.66c	0.75bcd	1.16cd
	0.1	0.00e	0.00e	0.00g	0.00g	0.00g	0.00g	0.00h	0.00h
	1.0	0.20cd	0.00e	0.20ef	0.28def	0.20fg	0.28ef	0.20fgh	0.28defgh
KIN (mg L⁻¹)	0.05	0.00e	0.10de	0.00g	0.45bcd	0.00g	0.50cde	0.00h	0.55cdefg
	0.1	0.00e	0.00e	0.00g	0.00g	0.00g	0.00g	0.00h	0.00h
	1.0	0.00e	0.00e	0.00g	0.28def	0.00g	0.61cd	0.00h	0.98abc
2iP (mg L⁻¹)	0.05	0.00e	0.33bc	0.00g	0.50bc	0.00g	0.58cd	0.00h	0.71bcde
	0.1	0.00e	0.00e	0.13fg	0.23ef	0.16fg	0.23efg	0.16gh	0.23efgh
	1.0	0.00e	0.00e	0.00g	0.50bc	0.00g	0.98ab	0.00h	1.13ab
TDZ (mg L⁻¹)	0.05	0.00e	0.82a	0.00g	0.91a	0.00g	1.08a	0.00h	1.33a
	0.1	0.00e	0.23cd	0.20ef	0.36cde	0.20fg	0.36def	0.20fgh	0.71bcde
	1.0	0.00e	0.00e	0.00g	0.00g	0.00g	0.00g	0.00h	0.00h
Control		0.20cd	0.00e	0.23ef	0.00g	0.56cd	0.00g	1.26a	0.00h
HSD(α= 0.05)		------0.14------		------0.18------		------0.26------		------0.51------	

BAP = benzyl amino purine, KIN = kinetin, 2iP = 2-isopentenyl adenine, and TDZ = thidiazuron. HSD = Honest *Significant* Difference; Different letters in the same treatment, light conditions and sampling time, represent significant differences (Tukey, α= 0.05), n = 18.

During micropropagation, the modification of the light intensity or radiation favors morphogenesis and differentiation of organs in some plant species (ARAB et al., 2014). In addition, such modification also decreases the oxidation of explants, then favoring leaf development and mesophyll differentiation, cell division, stomata development, and chlorophyll synthesis (THORPE et al., 2012; ISAH, 2015). In the present experiment, the DKL condition did not promote the early appearance of leaves in *E.*

Figure 3 - (A) Shoots of *Euphorbia cyathophora* grown in MS medium added with 1.0 mg L⁻¹ BAP; (B) Callus formation (morphological expression) in treatment with 0.1 mg L⁻¹ BAP, and (C) Root development in the treatment with 0.1 mg L⁻¹ TDZ. Explants were grown under light conditions (WL) and darkness-light conditions (DKL) during 90 days.
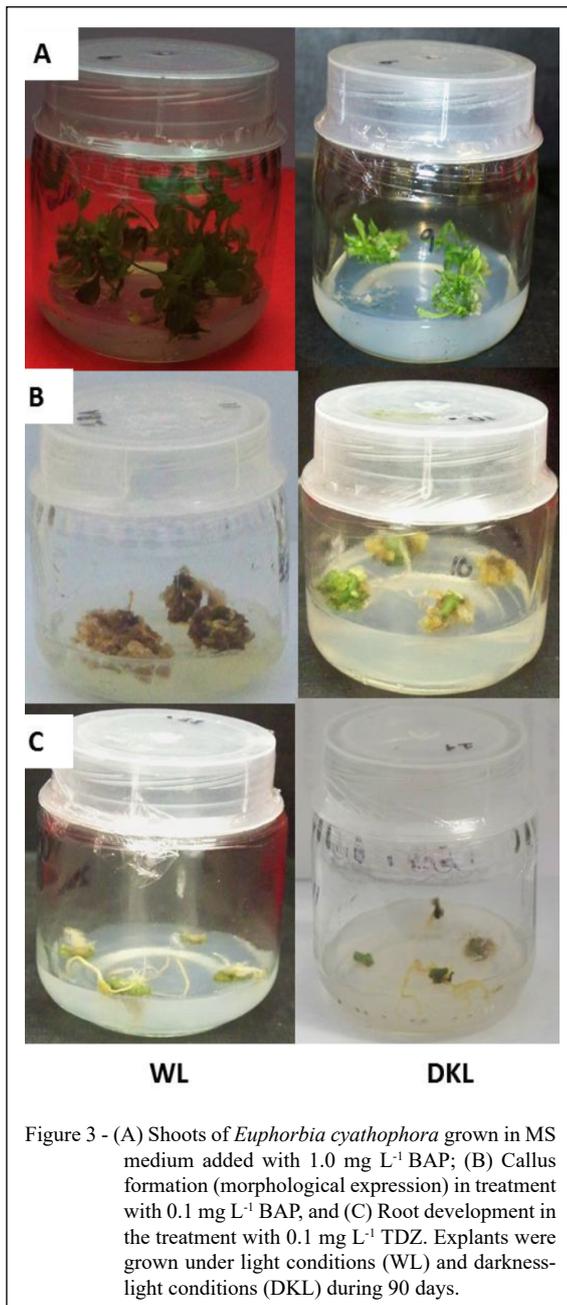
cyathophora or the growth of explants in which the cotyledons and leaves were smaller (Table 1). Similar results were reported by MARTIN et al. (2005) in *E. nivula* explants exposed to darkness conditions which resulted in the inhibition of morphogenesis.

Although, the plant height was statistically similar in both light conditions, the number of leaves and width of cotyledons were greater at WL conditions than that observed in plants at DKL conditions. Similarly, those plants at WL conditions showed higher number of roots and root length than DKL conditions.

The WL condition favored the production of roots of the third order at 19 days; in contrast, plants at DKL condition generated roots of third order until day 48. Furthermore, plants exposed to light along the experimentation resulted in more developed root system with early appearance of tertiary roots. In this regard, a better root development favors the absorption of nutrients and water by plants, as well as the promotion and development of plant growth (FAGERIA, 2012).

Results from the second experiment exhibited that the explants subjected to 1.0 mg BAP L⁻¹ + 0.1 mg NAA L⁻¹ promoted the shoot growth, especially under WL condition (Figure 1). LUNA et al. (2014) obtained enhanced shoot growth of *Agave americana* by testing five BAP doses; however, the application of 6 mg BAP L⁻¹, resulted in smaller shoots and scarce root development.

The DKL condition in combination with 0.1 mg TDZ L⁻¹ or 1.0 mg BAP L⁻¹ concentrations favored root morphogenesis in *E. cyathophora*, whereas the root length enhanced at concentrations of 0.05 mg TDZ L⁻¹ under DKL conditions. It is important to point out that an inversely proportional effect was observed between these variables in which the higher root number, the lower root length. Commonly, IBA is used to generate roots in explants of several ornamental plants such as *Ficus carica*, *F. benjamina*, *Gerbera jamesonii*, *Yucca aloifolia*, *Dracaena sanderiana, Jatropha curcas*, *Glochidion multiloculaire*, *Euphorbia antisyphilitica*, and *E. pugniformis* (ROUT et al., 2006; ASLAM et al., 2013). Nevertheless, there are reports in where BAP, alone or combined with NAA, efficiently promotes the root generation in *G. jamesonii*, *Manihot esculenta*, and *Dieffenbachia amoena* (ELSHEIKH et al., 2013).

In regards to the morphological expression of explants, callus generation was achieved at treatments with 0.1 mg BAP L⁻¹, 0.1 mg KIN L⁻¹, and 1.0 mg TDZ L⁻¹ under both light conditions. This allows inferring that light condition may determine the callus production in *E. cyathophora* explants. Nevertheless, the *in vitro* callogenesis process is influenced by several factors like the type of explants, plant species, and hormone application in the growth medium (VERMA et al., 2016).

CONCLUSION

The darkness stimulus (DKL) in embryos did not promote the growth of cotyledons, leaves,

or plant height. In contrast, the presence of light (WL) resulted in more developed root system with early appearance of tertiary roots. Furthermore, the application of 1.0 mg BAP L^{-1} + 0.1 mg NAA L^{-1} generated the shoot formation in explants, starting on day 10 under WL, and on day 40 under DKL conditions. Darkness stimulus in explants resulted in high number and growth of roots. The interaction of DKL condition with KIN, TDZ, BAP, and 2iP generated a greater callus development. This study provides basic information of the generation of calluses, shoots, and roots during micropropagation of *E. cyathophora*.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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