Short note [Nota corta]

In vitro GYNOGENESIS OF *Jatropha curcas* L. var ALJC01 †

[GINOGÉNESIS *in vitro* DE *Jatropha curcas* L. var ALJC01]

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SUMMARY

Background. *Jatropha curcas* L. is a crop whose oil can be converted to biodiesel. However, there is lack of varieties with high yields that limiting commercial production of the crop. An alternative is to obtain *in vitro* plants from unpollinated female gametophytes is known as gynogenesis which can provide an alternative to produce varieties with increased seed production. **Objective**. The objective was to establish a protocol for obtaining *in vitro* plants from unfertilized ovules of *Jatropha curcas* L. ovules. **Methodology**. The ovules were extracted from unfertilized inflorescences pretreated for 0, 1, 3 and 7 days at 2 to 5 ° C. Three combinations of plant growth regulator treatments were applied to culture media for induction of gynogenic calli. Gynogenic calli were cultured on MS basal medium with BAP and IBA for development of green, friable. The gynogenic calli from the development treatment were transferred to experiments to determine a regeneration treatment. The gynogenic plantlets were transferred to different treatments for root development. **Results**. The results show that pretreatment of inflorescences at 2 to 5°C had no significant effect on the number of ovules that formed friable white calli. Induction of gynogenic friable white calli occurred in the dark conditions, on half-strength Murashige and Skoog basal medium (½ MS) supplemented with two combinations of growth regulators: (i) 5.37 µM naphthalene acetic acid (NAA) combined with 6.65 µM 6-benzylaminopurine (BAP) and, (ii) 8.05 µM NAA with 22.09 µM BAP. Development of green, friable, gynogenic calli under light–dark conditions was possible under treatment with complete Murashige and Skoog (1962) (MS) basal medium with 6.66 µM BAP and 4.9 µM indole-butyric acid (IBA). Friable green callus formed gynogenic embryos on MS basal medium supplemented with 22.09 µM BAP and 3.40 µM paclobutrazol (PBZ), embryos regeneration occurred in photoperiod conditions. Embryos were able to develop and convert to plantlets in the treatment on MS basal medium containing 2.22 µM BAP and 0.28 µM of indole-3-acetic acid (IAA). Root development of plantlets occurred in ½ MS basal medium with 18.65 µM IBA. **Implications**. A protocol of *in vitro* gynogenesis in *Jatropha curcas* would contribute to the improvement of its cultivation, reducing the time required for the generation of pure lines that would allow us to obtain varieties with increased seed production. **Conclusions.** The work presented here describes a reproducible protocol to produce plants *in vitro* from unfertilized ovules of *Jatropha curcas* L. This methodology will facilitate to obtain homozygotic lines with significant reduction in the time required by conventional methods.

Keywords: Genetic improvement; paclobutrazol; plant growth regulator.

† *Submitted June 15, 2020 – Accepted September 3, 2020.*
ISSN: 1870-0462.

RESUMEN

Antecedentes. *Jatropha curcas* L. es un cultivo cuyo aceite se puede convertir en biodiésel. Sin embargo, existe una escasez de variedades con rendimientos altos que limitan la producción comercial del cultivo. Una alternativa es obtener plantas *in vitro* a partir de gametofitos femeninos no polinizados, lo que se conoce como ginogénesis, que puede proporcionar una alternativa para producir variedades con mayor producción de semillas. **Objetivo.** El objetivo fue establecer un protocolo para la obtención de plantas *in vitro* a partir de óvulos no fertilizados de óvulos de *Jatropha curcas* L. **Metodología.** Los óvulos se extrajeron de inflorescencias no fertilizadas pretratadas durante 0, 1, 3 y 7 días a 2 a 5 º C. Se aplicaron tratamientos con tres combinaciones de reguladores del crecimiento de plantas a los medios de cultivo para la inducción de callos ginogénicos. Se cultivaron los callos ginogénicos en medio basal MS con BAP e AIB para el desarrollo de callos verdes friables. Los callos ginogénicos del tratamiento de desarrollo se transfirieron a experimentos para determinar un tratamiento de regeneración. Las plántulas ginogénicas fueron transferidas a diferentes tratamientos para el desarrollo radicular. **Resultados**. Los resultados muestran que el pretratamiento de las inflorescencias a una temperatura de 2 a 5 ° C no tuvo un efecto significativo sobre el número de óvulos que formaron callos blancos friables. La inducción de callos blancos friables ginogénicos ocurrió en la oscuridad en el medio basal Murashige y Skoog de concentración media (½ MS) suplementado con dos combinaciones de reguladores del crecimiento: (i) ácido naftalenoacético (ANA) 5. 37 µM combinado con 6-bencilaminopurina 6.65 µM (BAP) y (ii) ANA 8.05 µM con BAP 22.09 µM. El desarrollo de callos verdes, friables y ginogénicos en condiciones de luz-oscuridad fue posible con el tratamiento con medio basal completo de Murashige y Skoog (1962) (MS) suplementado con BAP 6.66 µM y ácido indol-butírico (AIB) 4.9 µM. El callo verde friable formó embriones ginogénicos en el medio basal MS suplementado con 22.09 µM de BAP y 3.40 µM de paclobutrazol (PBZ), la regeneración de embriones se produjo en condiciones de fotoperíodo. Los embriones pudieron desarrollarse y convertirse en plántulas en el tratamiento en medio basal MS que contenía 2.22 µM de BAP y 0.28 µM de ácido indol-3-acético (AIA). El desarrollo de raíces en las plántulas se produjo en el medio basal ½ MS con 18.65 µM de AIB. **Implicaciones**. Un protocolo de ginogénesis *in vitro* en *Jatropha curcas* L. contribuiría a la mejora de su cultivo, reduciendo el tiempo necesario para la generación de líneas puras que nos permitirían obtener variedades con mayor producción de semilla. **Conclusiones**. El trabajo aquí presentado describe un protocolo reproducible para producir plantas *in vitro* a partir de óvulos no fertilizados de *Jatropha curcas* L. Esta metodología facilitará la obtención de líneas homocigóticas con una reducción significativa en el tiempo requerido por los métodos convencionales.

Palabras clave: Mejoramiento genético; paclobutrazol; regulador de crecimiento de plantas.

INTRODUCTION

Jatropha curcas is a promising energy crop whose oil is suitable for biodiesel production (Reale *et al*., 2012). It is native to Mexico and Central America and has been cultivated in several African and Southeast Asian countries (Sun *et al*., 2008). Its seeds contain oil, the composition of which is adequate to produce biodiesel and jet fuel. *Jatropha curcas* is a species also considered to have industrial and environmental benefits (Bueso *et al*., 2016). Biodiesel from *Jatropha curcas* is biodegradable and more environmentally friendly than diesel derived from fossil fuels. Oil content of *Jatropha curcas* seeds can vary from 30 to 50% of total seed weight (Pramanik, 2003; Kumar *et al*., 2009). Fatty acids found in *Jatropha curcas* oil include lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidonic acid (C20:0), and gadoleic acid (C20:1). Of these, 21.1% are saturated fats and 78.9% unsaturated fats (Jain and Sharma, 2010).

The main limitation for large-scale cultivation of *Jatropha curcas* is seed production, as it has variability caused by the allogamic nature of the species (Franco *et al*., 2014). Studies have been conducted with the aim of improving the seed yield of *Jatropha curcas* to satisfy the needs of the biodiesel industry (Pan *et al*., 2016). *Jatropha curcas* has low levels of genetic diversity (Sanou *et al*., 2015), so there is an urgent need to enhance this germplasm resource with novel accessions, particularly from the center of origin of the species (Rosado *et al*., 2010). One of the strategies used to obtain varieties rapidly involves the generation of pure lines, which can be obtained through self-fertilization of a plant throughout several generations. This requires a time investment of several years, as well as extensive cultivation and economic resources, which increases the end price of the seed. Another method is the use of biotechnology, in which pure lines are generated through androgenesis by pollen cultivation or gynogenesis through ovule culture (Ibrahim *et al*., 2015; Murove and Bohanec, 2012; Chen *et al*., 2011; Germanà, 2011; Yang and Zhou, 1982).

The process of obtaining *in vitro* plants from unpollinated female gametophytes is known as gynogenesis (Germanà 2011), which can provide an alternative to produce haploid plants in certain species. The production of plants through gynogenesis was described for the first time in barley (*Hordeum vulgare* L.) and it has been used to produce *in vitro* plants in many species (Chen *et al*., 2011). Vasudevan and Ramachandran (2014) reported a method and the composition of a suitable medium for somatic

embryogenesis of *Jatropha curcas* from ovules of unopened flower buds. Tantasawat *et al*. (2015) listed several factors that affected the gynogenetic potential of the ovules, including the genotype, exposure to low temperatures (e.g., during pretreatment), growth regulators, and components of the culture medium. A protocol of *in vitro* gynogenesis in *Jatropha curcas* would contribute to the improvement of its cultivation, reducing the time required for the generation of pure lines that would allow us to obtain varieties with increased seed production, given that the current lack of varieties with high yields is limiting commercial production of the crop. This is the first report of gynogenesis in *Jatropha curcas* L.

MATERIALS AND METHODS

Plant Material, Pre-treatment, and Sterilization of the Inflorescences

Unfertilized female inflorescences at the F9 stage of development (Hui *et al*. 2017) were collected from 30 cloned *Jatropha curcas* (var. ALJC01) plants in the experimental field of the Alternative Agroindustry Company located in Tizimin Yucatan, Mexico (21°08′34″N, 88°09′04″W, 21 m asl). *Jatropha curcas* plant is a low shrub with an open canopy, abundant branching, and male and female flowers. All the inflorescences were pretreated with exposure to cold treatment temperatures at 2 to 5°C for 0, 1, 3 and 7 d, after which the inflorescences were washed with water and 10 mL L^{-1} Extran (a liquid detergent concentrate), followed by a spray application of benzalkonium chloride (a biocidal cationic surfactant) at 0.5% (v/v) for 30 min to disinfect the surfaces. The inflorescences were then sprayed with 70% alcohol (v/v) in a laminar flow hood, after which a solution of 5% (v/v) sodium hypochlorite was applied for 12 min, followed by three rinses with sterile distilled water.

Induction of Gynogenic Calli

Ovules were excised from closed and disinfected female floral buds using a Nikon stereoscopic microscope (4× magnification, observation at 640 line-pairs mm^{-1} with extra low dispersion apochromatic plan 2× widefield at maximum zoom) (Nikon, China). All the callus induction treatments were established in ½ MS basal medium with the addition of 3% (w/v) sucrose and 2.2 g L^{-1} of Gelrite (Sigma-Aldrich, St Louis, MO). The pH was adjusted to 5.8 with potassium hydroxide (1 M) or hydrochloric acid (1 M). Sterilization was performed at 121°C for 15 min. For callus tissue induction, the excised ovules were planted in a 4 × 3 factorial design. The factors were the exposure time of the inflorescences to 2 to 5 °C for 0, 1, 3 and 7 d and three combinations of plant growth regulators (PGR) treatments that were applied to culture media: (i) 5.37 µM NAA combined with 6.65 µM BAP, (ii) 8.05 µM NAA with 11.09 µM BAP, and (iii) 8.05 µM NAA with 22.09 µM BAP. The experiments were conducted in triplicate and the response variable was expressed as the percentage of ovules that formed calli. All the ovules planted were incubated for 4 months in a growth chamber in the dark at 23 ± 2°C.

Development of Gynogenic Calli

The calli induced in the dark on the gynogenic callus medium [½ MS supplemented with 5.37 µM of NAA combined with 6.65 µM BAP, 3% (w/v) sucrose and 2.2 g L^{-1} of Gel rite] were selected because this medium was the one that produced the most friable white callus, then callus was transferred to light–dark conditions and a design was proposed to determine a treatment for to evaluate of callus development. Calluses were cultured on MS basal medium with BAP and IBA. The experiment for this stage was set up with a 4 × 4 factorial design with two factors: a cytokinin (BAP) and an auxin (IBA). Both factors were evaluated at four levels, BAP at 2.22, 4.44, 6.66, and 8.88 µM and IBA at 2.45, 4.9, 7.35, and 9.9 µM, resulting in a total of 16 treatments (Table 1, DT1-DT16). All media used contained MS supplemented with 3% (w/v) sucrose and 2.2 g L^{-1} of Gelrite. The pH of the culture media was adjusted to 5.8 before autoclaving at 121°C for 15 min. The treatments were performed in triplicate and the cultures were incubated for 1 month in a growth chamber under light–dark conditions. The response variable evaluated was the number of calli that remained viable (green in color, without phenolization).

Regeneration of Gynogenic Embryos

The gynogenic calli from the development treatment (DT10) were transferred to experiments to determine a regeneration treatment. Two experimental designs were prepared. The first had a 8 × 5 factorial design, where the first factor was the type of basal medium: [1) Gamborg B-5 mixture; 2) White's; 3) Schenk and Hildebrandt; 4) MS basal medium; 5) Gamborg B-5 minimal organics; 6) McCown woody plant; 7) Hoagland, and 8) Chu's N6], and the second factor consisted of five combinations of BAP and PBZ, with a total of 40 treatments (Table 2, RT1-RT40). The second experiment had a 3 × 3 factorial design: the auxin IAA at 0.05, 5.00, and 8.56 µM and the cytokinin BAP at 2.22, 6.66, and 8.88 µM were evaluated, resulting in nine treatments. In the treatments of the second design, MS was used as basal medium. The calli were incubated for 2 months in a growth chamber.

Development and Conversion of Gynogenic Embryos

Different treatments were used to evaluate for development and conversion of the embryos. The 3 × 2 experimental design had two factors and three dosage levels; the factors were BAP at 2.22, 6.66, and 22.20 µM and IAA at 0.28, 8.56, and 17.13 µM. All culture media were supplemented with MS basal medium, 3% (w/v) sucrose and 2.2 g L^{-1} Gelrite (Sigma-Aldrich). The response variable was embryos converted. Embryos were incubated from 1 to 2 months in a growth chamber.

Root Development

At the cotyledon stage, the gynogenic embryos were transferred to different treatments for root development. All of the treatments were formulated with ½ MS basal medium, 2 % (w/v) sucrose and 7.5 g L^{-1} agar. A 2 × 3 factorial design was used to evaluate the effect of the basal medium at two concentrations (50 and 100% of its ionic strength) and the auxin IBA at three concentrations: 0.5, 7.3, and 18.65 µM, giving a total of six treatments. The cultures were maintained in a growth chamber *in vitro* for 1 month.

Growth Chamber Conditions

The conditions in the growth chamber were 23 ± 2°C, relative humidity of 55 to 60%, and a light–dark cycle of 16/8 h; the light was provided by LED lamps emitting 60 $\mu mol^{-2}s^{-1}$ (InfiniLEDs, Jiangmen City, China).

Data Analysis

The data from the different treatments were analyzed by ANOVA with Statgraphics Centurion version XVI (Statgraphics Technologies, Inc.). The means were compared according to the Tukey´s Test at $P = 0.05$.

RESULTS AND DISCUSSION

For induction of gynogenic calli no significant differences were observed in the percentage of ovules that formed calli (Figure 1a) when inflorescences of *Jatropha curcas* were pretreated using temperature (2-5°C) at different exposure times. Therefore, it was not necessary to apply cold pretreatment, this differs from previous reports, which indicated that the use of low temperatures as a pretreatment favored callus induction in the ovules. Shalaby (2007) obtained gynogenic ovules at a rate of 22% with treatment of 2 to 5°C for 4 d in *Curcubita pepo* L. Doi *et al*. (2013) induced the formation of embryogenic structures in infertile ovules of *Gentiana* sp. via a cold treatment for 7 and 14 d. After observing that it is not necessary to apply the pretreatment at 2-5 ° C to *Jatropha curcas* inflorescences, the subsequent induction calli was carried out without pre-treating the inflorescences.

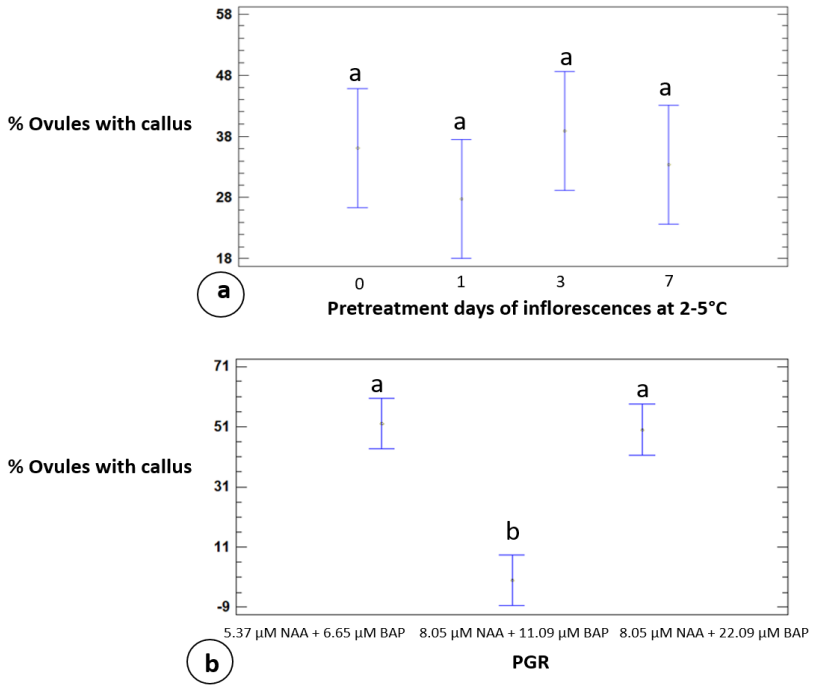

Figure 1. Means graph of percentage of callus formation in ovules of *Jatropha curcas* L. under (a) Influence of pretreatment temperature (2–5°C) at different exposure times; (b) Effect of the PGR combination. Different letters denote significant differences at P <0.05 between treatments. ½ MS basal medium was used in all treatments.

Table 1. Effect of plant growth regulator (PGR) on the viability of gynogenic calli during development under light–dark conditions.

Treatment Key	PGR: BAP, IBA	Average number of viable calli	Callus color
	µM	%	
DT1	2.22, 2.45	0.00a	Brown
DT2	2.22, 4.90	0.00a	Brown
DT3	2.22, 7.35	0.00a	Brown
DT4	2.22, 9.90	0.00a	Brown
DT5	4.44, 2.45	0.00a	Brown
DT6	4.44, 4.90	0.00a	Brown
DT7	4.44, 7.35	0.00a	Brown
DT8	4.44, 9.90	0.00a	Brown
DT9	6.66, 2.45	90.00 ± 8.16f	Green
DT10	6.66, 4.90	100.00 ± 0.00g	Green
DT11	6.66, 7.35	80.00 ± 8.16e	Green
DT12	6.66, 9.90	60.00 ± 816d	Green
DT13	8.88, 2.45	20.00 ± 8.16c	Brown
DT14	8.88, 4.90	0.00a	Brown
DT15	8.88, 7.35	0.00a	Brown
DT16	8.88, 9.90	10.00 ± 0.00b	Brown

Treatments with the same letter are statistically equal according to Tukey's test ($P \leq 0.05$). DT1-DT16: Treatments in dark conditions. BAP: 6-benzylaminopurine; IBA: Indole-butyric acid. MS basal medium was used in all treatments.

Auxins are widely used for induction of gynogenesis, and their optimum concentrations have been reported to vary considerably from species to species (Chen *et al*., 2011), we decided to use NAA due to that have been reported 2,4-dichlorophenoxyacetic acid (2,4-D) to generate mutations (Neelakandan and Wang 2012; Niazian *et al.* 2017), and suppress somatic embryo development, which leads to a decline in regeneration capacity over time. (Phillips and Garda., 2019). Exogenous application of auxin and cytokinin induces callus (Ikeuchi *et al*., 2013), therefore the combined use of an auxin with a cytokinin was evaluated. The results obtained in *Jatropha curcas*, indicating that PGR have statistically significant effect on the percentage of ovules forming calli, with a confidence level of 95%. The PGR combinations that had a greater effect were 5.37 µM NAA plus 6.65 µM BAP and 8.05 µM NAA plus 22.09 µM BAP, which resulted in 50 to 52% of ovules forming white, friable, gynogenic calli (Figure 1b). Formation of the calli occurred at 1 month of culture; however, because of the slow growth of the calli, it was necessary to maintain the calli in the same induction medium over a period of 4 months for adequate growth.

In this report, the white, friable calli formed in the dark did not survive under the same induction treatment of ½ MS basal medium with 5.37 µM NAA plus 6.65 µM BAP, or ½ MS basal medium with 8.05 µM NAA plus 22.09 µM BAP when they were transferred to light–dark conditions. Therefore, experiments were conducted to establish a treatment that would permit them to develop. The PGR combination had a significant effect on the percentage of viable calli under light–dark conditions: the calli were green in color and had a friable consistency under the combination of 6.66 µM BAP and 4.9 µM IBA (DT10), producing 100% viable calli (Table 1).

The treatment established in this stage allowed the gynogenic calli to grow, and viability was estimated; however, gynogenic calli failed in regenerating. Therefore, the developed gynogenic calli were transferred to different treatments for the induction of regeneration. It was observed that 7% of the gynogenic calli cultured in the Gamborg B-5 minimal organics medium, supplemented with 3.4 µM PBZ and 22 µM BAP (RT22, Table 2) formed embryos. Similarly, Bhat and Murthy (2008) achieved regeneration of plants in ovules of *Guizothia abyssinica* (L.f.) Cass. with Gamborg B5 medium. In *Jatropha curcas* var. ALJC01, we can observe that MS basal medium with three of the PGR combinations allowed the regeneration of gynogenic embryos: 0.34 µM PBZ plus 6.65 µM BAP (RT16, Table 2), 3.4 µM PBZ with 22 µM BAP (RT17, Table 2), and 1.7 µM PBZ with 11.09 µM BAP (RT20, Table 2). Gynogenesis did not occur in any of the treatments with the other basal media Gamborg B-5 (mixture), McCown woody plant, Hoagland's, Chu's N6, White's, and, Schenk and Hildebrandt, which produced necrosis of the callus tissue. The basal medium MS had the best response, this is similar at reported in the most the species that have been cultured *in vitro* (Phillips and Garda., 2019). In this report, the effect of PBZ was necessary for regeneration of gynogenic calli of *Jatropha curcas*, this could be due to PBZ inhibits ethylene production (Grossmann *et al*., 1989) facilitating regeneration. Given that PBZ influences in production of endogenous hormones (Kucharska and Orlikowska, 2008; Junxian *et al*., 2017), it could influence in the result obtained in *Jatropha curcas*. Although PBZ has been widely used in plants (Šimko 1993, Wu *et al*., 2019), this is the first report in which PBZ has been reported as essential for regenerating gynogenic callus. The experiments of second factorial design did not induce regeneration.

Statistical differences were observed in the interaction of BAP with IAA showing a direct effect on % conversion, interaction of 2.22 µM BAP with 0.28 µM IAA generated 49% conversion (Figure 2).

Table 2. Embryos formed in gynogenic calli of *Jatropha curcas* variety ALJC01.

Basal media	Treatment Key	PBZ and BAP combination µM	Average number of gynogenic embryos/ calli, per treatment
Gamborg B-5 (mixture)	RT1	0.34, 6.65	0.00a
	RT2	3.40, 22.00	0.00a
	RT3	0.34, 22.00	0.00a
	RT4	3.40, 6.65	0.00a
	RT5	1.70, 11.09	0.00a
White's	RT6	0.34, 6.65	0.00a
	RT7	3.40, 22.00	0.00a
	RT8	0.34, 22.00	0.00a
	RT9	3.40, 6.65	0.00a
	RT10	1.70, 11.09	0.00a
Schenk and Hilderbrandt	RT11	0.34, 6.65	0.00a
	RT12	3.40, 22.00	0.00a
	RT13	0.34, 22.00	0.00a
	RT14	3.40, 6.65	0.00a
	RT15	1.70, 11.09	0.00a
Murashige and Skoog	RT16	0.34, 6.65	14.50 ± 3.00c
	RT17	3.40, 22.00	30.00 ± 1.63e
	RT18	0.34, 22.00	0.00a
	RT19	3.40, 6.65	0.00a
	RT20	1.70, 11.09	22.50 ± 1.73d
Gamborg B-5 (minimal organics)	RT21	0.34, 6.65	0.00a
	RT22	3.40, 22.00	7.00 ± 1.15b
	RT23	0.34, 22.00	0.00a
	RT24	3.40, 6.65	0.00a
	RT25	1.70, 11.09	0.00a
McCown woody	RT26	0.34, 6.65	0.00a
	RT27	3.40, 22.00	0.00a
	RT28	0.34, 22.00	0.00a
	RT29	3.40, 6.65	0.00a
	RT30	1.70, 11.09	0.00a
Hoagland's	RT31	0.34, 6.65	0.00a
	RT32	3.40, 22.00	0.00a
	RT33	0.34, 22.00	0.00a
	RT34	3.40, 6.65	0.00a
	RT35	1.70, 11.09	0.00a
Chu's N6	RT36	0.34, 6.65	0.00a
	RT37	3.40, 22.00	0.00a
	RT38	0.34, 22.00	0.00a
	RT39	3.40, 6.65	0.00a
	RT40	1.70, 11.09	0.00a

Treatments with the same letter are statistically equal by Tukey ($P \leq 0.05$). PBZ, paclobutrazol; BAP, 6-benzylaminopurine.

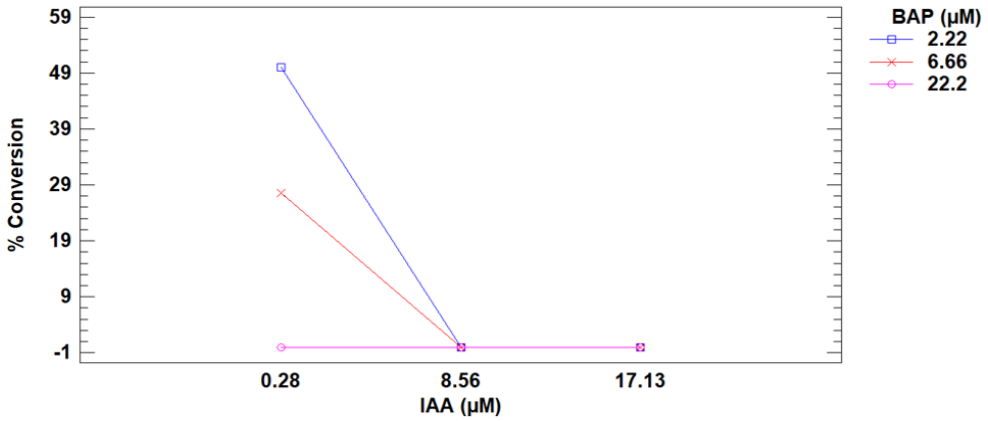

Figure 2. Effect Interaction of BAP and IAA in % conversion of *Jatropha curcas* gynogenic embryos.

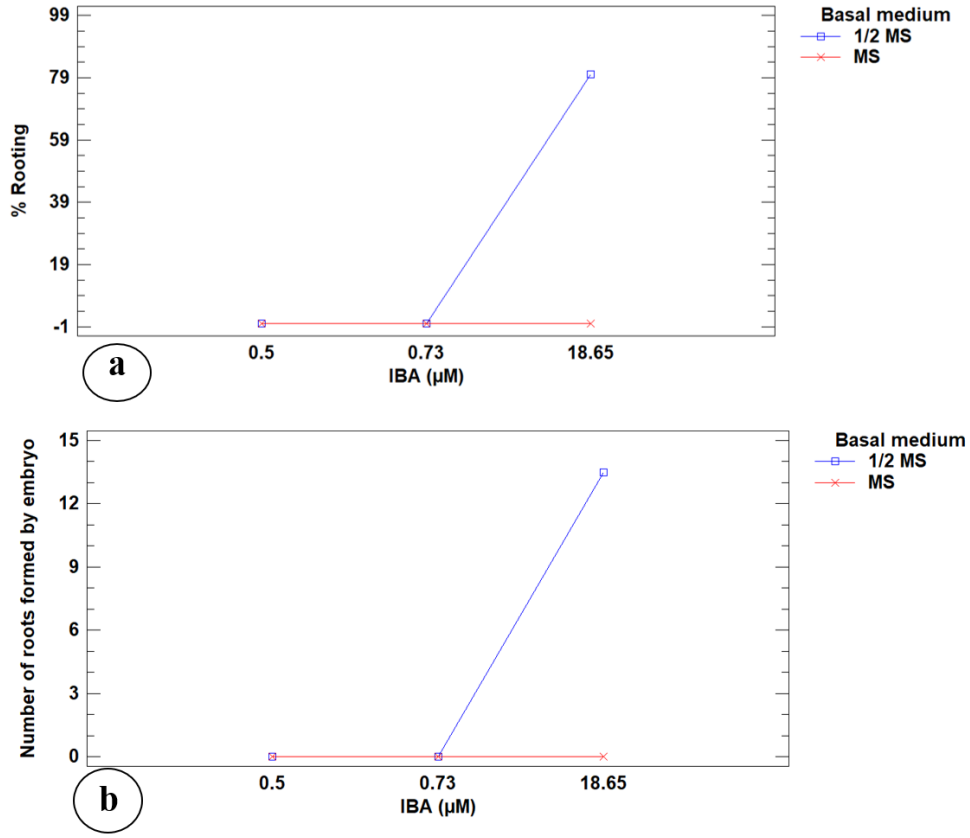

Figure 3. Effect Interaction of MS basal medium and IBA in (a) % rooting; (b) the number of roots formed per gynogenic embryo de *Jatropha curcas*.

The effect of IBA on rooting has been reported in *Jatropha curcas* [Gopale and Zunjarrao, 2013 reported a rooting efficiency of 72.9% with ½ MS supplemented with the addition of three auxins (15 µM IBA, 5.7 µM IAA, and 5.5 µM NAA); Panghal *et al.* (2012) reported 67.6% root development in ½ MS with 57 µM IAA + 0.97 µM IBA; Sharma *et al.*, 2011 reported rooting in ½ MS supplemented with 14.7 µM IBA, 5.7 µM de IAA; and Maharana *et al.* (2012) reported 5.6% rooting with ½ MS with 2 µM IBA]. In this study rooting occurred in presence of IBA without other PGR. A significant difference ($P \leq 0.05$) in the percentage of converted

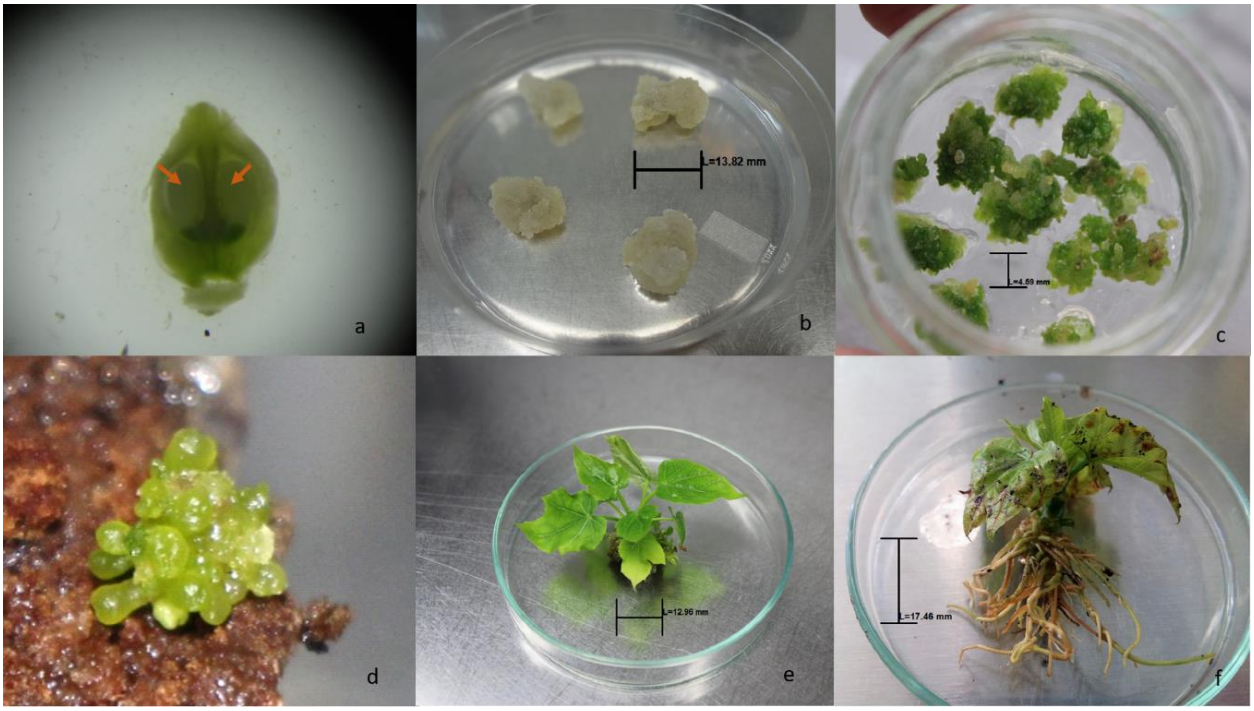

Figure 4. Gynogenesis stages to obtain *in vitro* plants of *Jatropha curcas* L. var. ALJC01. (a) Ovules (arrows); (b) Induction of gynogenic calli in the darkness; (c) Development of gynogenic calli; (d) Embryos at the globular and torpedo stages; (e) Plant at three months of age; (f) Plant with roots.

embryos that developed roots with the addition of IBA. The interaction that generated up to 79% of roots was the combination of ½ MS with 18.65 µM IBA (Figure 3a) obtaining up to 13 ± 3 roots per embryo (Figure 3b). The differences in the types and concentrations of PGR required for root development reported by Gopale and Zunjarrao (2013); Panghal *et al*. (2012); Sharma *et al*. (2011); Maharana *et al*. (2012) may be caused by different factors, including the genotype and the type of explant, given that both the genetic component and the endogenous PGR content have an influence on morphogenic processes.

The stages for producing *Jatropha curcas* ALJC01 plants from ovules is presented in Figure 4. The only antecedent, to date, is the patented EP 2 315 518 B1 method (Vasudevan and Ramachandran 2014), in which a methodology is described for the induction of somatic embryos from ovules. This is in contrast to the information reported in this study, where gynogenic embryogenesis was obtained in calli of *Jatropha curcas* ovules and is the first time that PBZ is reported as key for regeneration gynogenic calli.

CONCLUSIONS

In this study, the use of unfertilized ovules was shown to be a feasible tool for obtaining *in vitro Jatropha curcas* plants within a period of 10 to 12 months, which could be used in *in vitro* plant breeding. This will permit a significant reduction in the time required by conventional methods, which can take up to 3 or 4 yr. Moreover, this methodology will facilitate homozygotic lines, whereas the presence of only one set of chromosomes will permit the detection of mutations controlled by recessive genes.

Acknowledgments
The authors thank the Alternative Agroindustry Company and CONACYT-PEI 2014.

Author's Contributions Statement. GLP conducted the experiments, contributed analytical tools, analyzed the data, and wrote the manuscript. AUV and ARD conducted the experiments. GJHC conducted the experiments and wrote the manuscript. CC-C, GMS, and EAAC contributed analytical tools. All authors read and approved the manuscript.

Funding. The research reported here was financed by the CONACYT-PEI 2014, Fund (project No. 217871).

Conflict of Interest. All authors declare that they have not conflict of interest.

Compliance of ethical standards. This research complies with the ethical standard required for the research.

Data availability. Data are available with the first author (glopez@ciatej.mx).

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