INDIRECT ORGANOGENESIS AND ESTIMATION OF NUCLEAR DNA CONTENT IN REGENERATED CLONES OF A NON-TOXIC VARIETY OF *Jatropha curcas*†

[ORGANOGÉNESIS INDIRECTA Y ESTIMACION DEL CONTENIDO DE ADN NUCLEAR EN CLONES REGENERADOS DE UNA VARIEDAD NO TOXICA DE *Jatropha curcas*]

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SUMMARY

Jatropha curcas L. is a second-generation energy crop, which produces approximately 40% oil in its seeds, which can be transformed into biodiesel. *In vitro* culture is a valuable tool for the multiplication and conservation of elite plant varieties. In *J. curcas*, there are several reports on the micropropagation of the species, but with low reproducibility. The objective of this study was to obtain the *in vitro* organogenesis of *J. curcas* and estimate the nuclear DNA content by flow cytometry during eight subcultures *in vitro*. The organogenesis of adventitious shoots was obtained in 3.3 g/l of MS, 110.25 µM of 6–(γ, γ-Dimethylallylamino) purine (2ip), 1.27 µM of indoleacetic acid (IAA) and 369.21 µM of adenine sulfate (AdS), obtaining up to 18.50 ± 0.7 shoots per explant. The development of the shoots was 1.67 ± 0.76 cm in MS medium, 4.44 µM of benzylaminopurine (BAP), 1.0 µM of IAA and 543 µM of AdS. The rooting was 13.6 ± 2.51 roots in ½ MS and 14.7 µM of indole butyric acid (IBA). The nuclear DNA content was from 0.80 ± 0.12 to 1.07 ± 0.23 pg of nuclear DNA during the eight subcultures.
Keywords: *In vitro* culture; 6–(γ, γ-Dimethylallylamino) purine; benzylaminopurine; adenine sulfate; flow cytometry; somaclonal variation; micropropagation; indol acetic acid; adventitious organogenesis.

RESUMEN

Jatropha curcas L. es un cultivo energético de segunda generación, que produce aproximadamente el 40% de aceite en sus semillas, que puede transformarse en biodiesel. El cultivo *in vitro* es una herramienta valiosa para la multiplicación y conservación de variedades de plantas de élite. En *J. curcas*, hay varios reportes sobre la micropropagación de la especie, pero con baja reproducibilidad. El objetivo de este estudio fue obtener la organogénesis *in vitro* de *J. curcas* y estimar el contenido de ADN nuclear por citometría de flujo durante ocho subcultivos *in vitro*. La organogénesis de brotes adventicios se obtuvo en 3.3 g/l de MS, 110.25 µM de 6–(γ, γ-Dimethylalilamino) purina (2ip), 1.27 µM de ácido indolacético (AIA) y 369.21 µM de sulfato de adenina (SAd), obteniendo hasta 18.50 ± 0.7 brotes por explante. El desarrollo de los brotes fue de 1,67 ± 0,76 cm en medio MS, 4.44 µM de bencilaminopurina (BAP), 1.0 µM de AIA y 543 µM de SAd. El enraizamiento fue de 13.6 ± 2.51 raíces en ½ MS y 14.7 µM de ácido indolbutirico (AIB). El contenido de ADN nuclear fue desde 0.80 ± 0.12 a 1.07 ± 0.23 pg de ADN nuclear durante los ocho subcultivos.
Palabras clave: Cultivo *in vitro*; 6- (γ, γ-Dimetilalilamino) purina; bencilaminopurina; sulfato de adenina; citometría de flujo; variación somaclonal; micropropagación; ácido indolacético; organogénesis adventicia.

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INTRODUCTION

The species *Jatropha curcas* L. belongs to the family Euphorbiaceae and is believed to have originated in Mexico and Central America (Contran *et al*., 2013). The importance of this crop resides mainly in the fact that its seeds contain approximately 40% of oil with a chemical composition appropriate for transformation into high quality biodiesel by transesterification (Li *et al*., 2016; Soares *et al*., 2016). This species also presents alternative applications, such as its use in the recovery of areas degraded by mineral exploitation and by deforestation of devastated areas, which gives added value to its industrial use (Carels, 2013; Sabandar *et al*., 2013; Dias *et al*., 2012). In addition, the latex has been attributed with medicinal properties, in particular in the treatment of skin lesions (Sabandar *et al*., 2013). The biofuel obtained from this species is ecological and biodegradable, which has attracted worldwide attention as an alternative source of sustainable energy for the future (Baran Jha *et al*., 2007). Given the importance of the species in Mexico, the varieties Gran Victoria, Doña Aurelia, Don Rafael (Zamarripa and Solís, 2013) and the ALJC01 (Góngora-Canul and Martínez-Sebastian, 2016) have been registered in the National Service of Seed Inspection and Certification (SNICS - for its Spanish acronym), the latter being of particular agronomic importance given that it is a low, monoecious shrub with an open canopy and profuse branching that presents an average of 90 bunches of fruit per plant, an oil content of approximately 40%, a yield of 2.5 tons at a planting density of 2000 plants per ha and presents a phorbol esters concentration of 0.044 ± 0.003 mg/g (Sacramento-Rivero *et al*., unpublished data), according to Makkar *et al*. (1997) is considered potentially toxic when the concentration of phorbol esters is > 0.11 mg/g. So far only a non-toxic variety has been reported from Papantla region of the state of Veracruz in Mexico, suitable for human consumption (Makkar *et al*., 1997).

In *J. curcas*, in addition to the establishment of genetic improvement programs through the use of agrobiotechnological methods aimed at increasing seed and oil yields, it is also important to satisfy the demand and conservation for plant material from elite varieties, in this respect, no much work has been done on germplasm conservation (Kumar and Sharma, 2008); this creates the need to develop techniques of massive multiplication and conservation of the species (Panghal *et al*., 2012) to get the demand on a large scale and ensure easy supply of this elite material (Kumar *et al*., 2011a; kumar *et al*., 2015). Within this context, micropropagation protocols in *J. curcas* have been reported in toxic varieties, however, regeneration efficiency was observed in toxic cultivars compared to non-toxic cultivars (Kumar 2011, Kumar *et al*., 2011a, 2011b), this agrees with what was reported by Sharma *et al*. (2011) who have reported that non-toxic varieties of *J. curcas* are less sensitive to *in vitro* organ formation. Another factor that affects *in vitro* regeneration is the genotype / cultivar (Feyissa *et al*., 2005; Landi and Mezzetti, 2006;) in *J. curcas*, several authors report the high dependence of the genotype on *in vitro* regeneration (da Camara Machado *et al*., 1997; Kumar 2008; Kumar and Reddy 2010; Kumar *et al*., 2010) this behavior may be related to mechanisms and the endogenous content of the metabolism of plant growth regulators (sharma *et al*., 2011) which include morphogenesis using different concentrations of growth hormones, followed by optimization of the conditions for rapid regeneration (Gangwar *et al*., 2016), the induction of adventitious shoots from the petiole (Liu *et al*., 2015), the use of different types of explants and plant growth regulators for direct and indirect morphogenesis (Verma and Verma, 2015; Ali *et al*., 2015), and the induction of adventitious shoots and the development of plantlets from petiole explants (Liu *et al*., 2016), There are reports of non-toxic varieties in *Jatropha curcas* in which, regeneration was obtained by indirect organogenesis from leaf (Sujatha *et al*., 2005) and petiole (Kumar *et al*., 2010, 2011a).

Plant tissue culture is a group of techniques applied *in vitro*, which includes the culture of plant tissue in an artificial medium under controlled environmental conditions (Levitus *et al*., 2010) with the aim to generate micropropagation protocols that can genetically improve of the plants and the production of metabolites. Their applications can be studies of physiology, genetics, biochemistry; procurement of pathogen-free plants, method for the conservation and exchange of germplasm as well as *in vitro* morphogenesis (Organogenesis or somatic embryogenesis) (Roca and Mroginski, 1991).

In vitro organogenesis in plants is a morphogenetic event which occurs due to structural changes and modifications in cellular organization, resulting in a unipolar organism; in *in vitro* tissue culture it can be direct (formation from an explant) or indirect (previous induction of the callus is required) (Sharry *et al*., 2015). In general, this process can be induced through the application of plant growth regulators (PGRs), the capacity of the tissue to respond to these factors during the culture, the type of culture medium and the organic additives (Sugiyama, 1999).

During the process of *in vitro* culture, the plants regenerated from non-meristematic cells or tissues often go through a phase of callus formation, usually exhibiting phenotypic and genetic variation (Ramulu and Dijkhuis, 1986; Kaeppler *et al*., 2000). These variations can affect the quality and quantity of the clones, as well as their application in the regeneration of genetically transformed tissues (Alatar *et al*., 2017). A frequent phenomenon found in *in vitro* culture is the spontaneous appearance of

somaclonal variation (Larking and Scowcroft, 1981).

There are a number of reports in which the use of flow cytometry has enabled the analysis of changes in the stability of the clones; for example, in the species *Vitis vinifera*, Leal *et al*. (2006) reported the analysis of the ploidy level and Prado *et al*. (2010) detected somaclonal variants; moreover, in *J. curcas*, Franco *et al*. (2014) analyzed the clonal fidelity among genotypes, Rathore *et al*. (2014) genetic homogeneity and de Oliveira *et al*. (2013) performed the analysis of polyploidization. The aim of the present study was to develop a protocol of *in vitro* indirect organogenesis for *J. curcas* variety ALJC01, a high seed yield variety, in order to propagate plant material for future research and agronomic applications. Also, this is first report of *in vitro* culture of *J. curcas* in which the genetic stability of clones from one to eight subcultures was analyzed by flow cytometry.

MATERIALS AND METHODS

Establishment of *Jatropha curcas* plant material

Seeds of *J. curcas* variety ALJC01 were collected randomly from a plantation in Sucila, Yucatan, Mexico. The seeds were surface sterilized with benzalkonium chloride at 0.5% and were then placed in Extran® at 1% for 15 min and left to dry on absorbent paper for 30 min. Subsequently, the seed coat was removed and the embryos obtained were disinfected in a laminar flow cabinet by immersion in a solution of Extran® at 5 % for 5 min. Afterwards they were transferred to ethanol at 70% for 1 min, followed by agitation in sodium hypochlorite at 30% for 15 min and by three final rinses in sterile distilled water. The disinfected embryos were transferred to magenta boxes with 40 mL of MS culture medium. All the embryos were incubated at a temperature of 23±2°C and relative humidity (RH) of 60% in a darkness growth chamber for 15 days, after which they were cultivated in a photoperiod of 16/8 hours (light/darkness); the light source was provided by LED lamps with a light intensity of 60 $\mu mol^{-2}s^{-1}$.

Induction of adventitious shoots

A 3^3 factorial design was constructed in which 27 treatments with 3 repetitions were obtained. The first factor was 6–(γ, γ-Dimethylallylamino) purine (2ip) at 96.00, 110.70 and 125.40 µM, the second factor was IAA at 1.10, 1.27 and 1.44 µM and the third factor was adenine sulfate (AdS) at 282.34, 325.78 and 369.21 µM; all the treatments were prepared with the 3/4 MS medium (Table 1). For the evaluation, the initial weight of the explant and the weight at 45 days were registered, and these values were used to calculate the average weight obtained during the growth period. The number of shoots formed at 45 days was also evaluated.

Development of adventitious shoots

A second 3^3 factorial design was used for root elongation, using PGR at 4.44, 6.66 and 8.88 µM, IAA at 1.0, 1.7 and 2.4 µM and the organic additive AdS at 543, 1086 and 814.5 µM; 27 treatments with three repetitions were established (Table 2). The experimental unit was a shoot with an initial length of 0.5 cm, which was measured after 45 days to determine its final length and to obtain total growth.

Rhizogenesis of shoots obtained from *J. curcas*

Shoots 2 cm in length were used to induce rhizogenesis. The experimental design was also a 3^2 factorial arrangement, with the factors being the ionic concentration of the MS basal medium at 25, 50 and 100% ionic strength and the IBA auxin at 14.7, 17.7 and 20.7 µM; the experimental design was constructed with and without phloroglucinol (Phl) (2.4 mgL^{-1}). The variables evaluated after 30 days were the percentage of rooted shoots, length and number of developed roots.

Estimation of nuclear DNA content

For the isolation of nuclei, 100 mg of callus with leaf from *J. curcas* and 100 mg of leaf from *Zea mays* L. 'CE-777' (reference standard with 2C = 5.43 ; Lysak and Doležel 1998) were weighed and subsequently chopped with a razor blade in a Petri dish with 1000 µL of WPB solution (0.2 M Tris·HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfate, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7.5) (Loureiro *et al*., 2007). The nuclear sample was then filtered with a 40 µM nylon mesh and 50 µL of Propidium iodide (1mg/1mL) was added to stain nuclear DNA. After an incubation period of 15 min, the sample was analyzed in a BD Accuri C6® flow cytometer equipped with a blue and red laser, two light scatter detectors, and four fluorescence detectors with optical filters optimized for the detection of many popular fluorochromes. Data was obtained using BD Accuri™ C6 Plus software in the format of FCS 3.1 format; for each sub-cultured generation of *J. curcas* (until the eight subculture), three samples were analyzed in triplicate in three days.

The average fluorescence of 2C peaks of *J. curcas* ALJC01 variety and of *Zea mays* was obtained and the information was used to estimate the nuclear DNA content as follow:

$$Estimation\ of\ nuclear\ DNA\ (pg) = Reference\ DNA\ content\ (pg)\left(\frac{Mean\ position\ of\ G1\ sample\ peak}{Mean\ position\ of\ G1\ reference\ peak}\right)$$

The estimated value of the nuclear DNA was converted to base pairs, considering 1 pg of DNA correspondent to $0.978x10^9$ bp (Doležel *et al.*, 2003).

Statistical Analyses

Statistical software Statgraphics® Centurión XVI, (2009) (Statgraphics Technologies, Inc. The Plains, Virginia, URL: http://www.statgraphics.com/) was used for all calculations. Analysis of variance (ANOVA) was performed at the significance level of P≤0.05, when appropriate; means were separated by using Tukey's test (P≤0.05). All data were distributed normally. Statistic model for induction and adventitious shoots development was a factorial design 3^3, for adventitious shoots rhizogenesis a factorial design 3^2 and for nuclear DNA content a single-factorial ANOVA.

RESULTS

Induction of adventitious shoots

The best response in the induction of adventitious shoots was obtained with a MS medium at 75% of its ionic strength (3/4 of MS) with 110.25 µM of 2ip, 1.27 µM of IAA and 369.21 µM of AdS (T17), which facilitated 18.50±0.70 shoots (Table 1).

Table 1. Average of weight gain and number of adventitious shoots in *Jatropha curcas* ALJC01 explants after 45 days of culture in 27 different treatments

Treatments	PGR 2ip (µM)	PGR IAA (µM)	PGR AdS (µM)	Weight gain of explants (g)	Average of adventitious shoots
T1	096.00	1.10	282.34	3.08±0.38^{cde}	0.50±0.700^{a}
T2	110.70	1.10	282.34	2.46±1.20^{abcde}	1.50±0.700^{a}
T3	125.40	1.10	282.34	0.14±0.05^{a}	00.0±0.000^{a}
T4	096.00	1.44	282.34	2.55±0.48^{abcde}	8.00±1.410^{abc}
T5	110.70	1.44	282.34	2.42±1.81^{abcde}	15.5±0.70^{bc}
T6	125.40	1.44	282.34	0.88±0.13^{abc}	00.0±0.000^{a}
T7	096.00	1.27	282.34	1.30±0.53^{abcde}	4.00±1.410^{ab}
T8	110.70	1.27	282.34	2.75±0.90^{bcde}	0.50±0.700^{a}
T9	125.40	1.27	282.34	3.62±0.64^{e}	9.50±0.700^{abc}
T10	096.00	1.10	369.21	3.08±1.00^{cde}	9.50±0.700^{abc}
T11	110.70	1.10	369.21	0.73±0.16^{abc}	0.50±0.700^{a}
T12	125.40	1.10	369.21	1.72±0.67^{abcde}	6.50±2.120^{abc}
T13	096.00	1.44	369.21	3.40±0.26^{de}	4.50±0.70^{ab}
T14	110.70	1.44	369.21	1.42±0.11^{abcde}	0.00±0.000^{a}
T15	125.40	1.44	369.21	0.79±0.65^{abc}	3.00±1.410^{ab}
T16	096.00	1.27	369.21	2.56±1.56^{abcde}	2.50±0.70^{ab}
T17	110.70	1.27	369.21	3.64±0.39^{e}	18.5±0.70^{c}
T18	125.40	1.27	369.21	1.99±0.51^{abcde}	12.0±1.41^{abc}
T19	096.00	1.10	325.78	0.64±0.16^{abc}	1.00±0.70^{a}
T20	110.70	1.10	325.78	0.85±0.33^{abc}	7.00±1.410^{abc}
T21	125.40	1.10	325.78	0.69±0.14^{abc}	0.00±0.000^{a}
T22	096.00	1.44	325.78	1.19±1.16^{abcde}	2.00±0.000^{ab}
T23	110.70	1.44	325.78	1.50±1.14^{abcde}	1.00±0.000^{a}
T24	125.40	1.44	325.78	1.00±0.95^{abcd}	3.50±0.700^{ab}
T25	096.00	1.27	325.78	2.12±0.72^{abcde}	0.00±0.000^{a}
T26	110.70	1.27	325.78	2.24±0.85^{abcde}	1.50±0.700^{a}
T27	125.40	1.27	325.78	0.51±0.25^{ab}	0.00±0.000^{a}

$^{a-e}$ Similar letters correspond to treatments statistically equal according to Tukey test p<0.05. All treatments were prepared with Murashige and Skoog medium (¾ MS)

Development of adventitious shoots

Shoot development was observed at 45 days of culture. In treatment T1 (4.44 µM of BAP, 1.0 µM of IAA and 543 µM of AdS), shoot elongation up to a length of 1.67±0.76 cm was achieved (Table 2). Statistically significant differences were found regarding the number of leaves (P<0.05), with an average leaf development of 18.00±2.65 leaves in treatment T1. Significant statistical differences were observed in the number of shoots (P<0.05); the treatment which presented the most significant shoot development was T2 with an average of 7.67±1.53 shoots (Table 2).

Rhizogenesis of the developed shoots

Organogenesis of roots was obtained in *J. curcas* plantlets in culture media with the addition of IBA (Table 3). The treatments which presented the best response to root organogenesis were T10 (1/4 MS + 14.7µM of IBA), T15 (1/2 MS + 20.7 µM of IBA) and T16 (MS + 14.7µM of IBA); however, the highest number of roots generated was observed in treatment T15 (1/2 MS + 20.7 µM of IBA) with an average of 13.6±2.51 roots and a length of 1.90±0.81 cm (Figure 1).

Table 2. Adventitious shoot development of *Jatropha curcas*. Information about total stem growth, number of leaves and number of adventitious shoots is also given.

Treatments	PGR			Total stem growth (cm)	Leaf numbers	Number of Adventitious shoots
	BAP (µM)	IAA (µM)	AdS (µM)			
T1	4.44	1.0	543.00	1.67±0.76^{g}	18.00±2.65^{e}	4.67±1.53^{def}
T2	6.66	1.0	543.00	0.37±0.32^{abcd}	15.33±8.14^{de}	7.67±1.53^{f}
T3	8.88	1.0	543.00	0.00±0.00^{a}	1.000±1.73^{ab}	1.00±1.73^{ab}
T4	4.44	2.4	543.00	0.90±0.79^{def}	8.000±8.00^{abcd}	1.00±1.00^{ab}
T5	6.66	2.4	543.00	0.13±0.15^{ab}	6.670±8.08^{abcd}	4.00±1.00^{bcde}
T6	8.88	2.4	543.00	0.07±0.12^{ab}	1.670±2.89^{ab}	0.67±1.15^{a}
T7	4.44	1.7	543.00	0.27±0.21^{abc}	3.330±3.51^{abc}	3.00±2.00^{abcde}
T8	6.66	1.7	543.00	0.13±0.23^{ab}	4.330±7.51^{abc}	4.33±2.52^{cde}
T9	8.88	1.7	543.00	0.20±0.17^{abc}	2.330±4.04^{abc}	4.67±3.79^{def}
T10	4.44	1.0	1086.0	0.07±0.06^{ab}	1.670±1.53^{ab}	2.67±0.58^{abcde}
T11	6.66	1.0	1086.0	0.57±0.51^{bcdef}	8.000±7.21^{abcd}	1.00±0.00^{ab}
T12	8.88	1.0	1086.0	0.00±0.00^{a}	0.000±0.00^{a}	3.33±1.53^{abcde}
T13	4.44	2.4	1086.0	0.73±0.15^{cdef}	13.67±3.06^{cde}	1.33±0.58^{ab}
T14	6.66	2.4	1086.0	0.17±0.29^{ab}	2.330±4.04^{abc}	1.00±0.00^{ab}
T15	8.88	2.4	1086.0	0.00±0.00^{a}	2.330±4.04^{abc}	1.00±0.00^{ab}
T16	4.44	1.7	1086.0	0.00±0.36^{f}	0.330±3.46^{cde}	3.67±2.52^{abcde}
T17	6.66	1.7	1086.0	0.00±0.00^{a}	0.330±0.58^{a}	3.67±1.53^{abcde}
T18	8.88	1.7	1086.0	0.23±0.21^{abc}	2.000±2.65^{ab}	2.33±1.15^{abcde}
T19	4.44	1.0	814.50	0.30±0.44^{abc}	8.000±8.54^{abcd}	3.67±3.21^{abcde}
T20	6.66	1.0	814.50	0.53±0.47^{abcde}	16.33±7.51^{de}	4.67±0.58^{def}
T21	8.88	1.0	814.50	0.50±0.36^{abcde}	15.00±1.73^{cde}	4.33±0.58^{cde}
T22	4.44	2.4	814.50	0.27±0.21^{abc}	2.330±1.15^{abc}	2.33±2.31^{abcd}
T23	6.66	2.4	814.50	0.13±0.06^{ab}	2.000±2.65^{ab}	3.67±1.53^{abcde}
T24	8.88	2.4	814.50	0.37±0.15^{abcd}	10.67±6.66^{cdef}	5.33±3.51^{ef}
T25	4.44	1.7	814.50	0.37±0.55^{abcd}	9.330±10.6^{bcde}	1.67±2.08^{abc}
T26	6.66	1.7	814.50	1.03±0.40^{df}	8.000±3.00^{abcd}	3.33±3.21^{abcde}
T27	8.88	1.7	814.50	0.03±0.06^{ab}	2.670±2.31^{abc}	3.33±0.5^{abcde}

$^{a-g}$ Similar letters correspond to treatments statistically equal according to the Tukey test $p<0.05$. All treatments were prepared with MS medium

Figure 1. Plantlet of *Jatropha curcas.*

Estimation of nuclear DNA content

The average fluorescence of 2C peaks obtained from the histograms of 1 to 8 subcultures was 3634.32±1519.02 for *J. curcas* ALJC01 variety and 22922.1±10448.1 for *Zea mays* 'CE-777' (Figure 2). The average for the nuclear DNA content estimated for *J. curcas,* according to Carvalho *et al*. (2008), is 0.85 pg ±0.006; in this report, the nuclear DNA content in *J. curcas* for each one of the subcultures was found to range between 0.80±0.12 and 1.07±0.23. Statistically significant differences were found for 3rd and 4th month of *in vitro* culture with a confidence level of 95.0% (Table 4).

Table 3. Rhizogenesis of the developed shoots in *Jatropha curcas in vitro* plants. Information about the percentage of explants with root development, number of roots and root length is provided.

Treatments	IBA µM + 2.4 mgL^{-1} Phl.	MS gL^{-1}	Root percentage	Number of roots	Root length (cm)
T1	14.7	1.32	66.6	5.00±1.70^{abc}	1.16±1.00^{abc}
T2	17.7	1.32	33.5	0.33±0.00^{a}	0.50±0.00^{ab}
T3	20.7	1.32	00.0	0.00±0.00^{a}	0.00±0.00^{a}
T4	14.7	2.2	33.3	0.33±0.00^{a}	0.50±0.00^{ab}
T5	17.7	2.2	66.6	7.30±2.30^{abc}	1.53±1.50^{abc}
T6	20.7	2.2	33.3	2.00±0.00^{ab}	0.66±0.00^{ab}
T7	14.7	4.4	33.3	0.66±0.00^{a}	0.40±0.00^{ab}
T8	17.7	4.4	00.0	0.00±0.00^{a}	0.00±0.00^{a}
T9	20.7	4.4	66.6	2.00±1.70^{ab}	1.80±2.30^{bc}
Treatments	IBA µM				
T10	14.7	1.32	66.0	11.6±2.88^{bc}	0.86±0.70^{ab}
T11	17.7	1.32	100	2.66±1.15^{ab}	0.56±0.05^{ab}
T12	20.7	1.32	66.6	6.00±0.76^{abc}	0.66±0.76^{ab}
T13	14.7	2.2	33.3	1.30±0.57^{a}	0.83±0.28^{ab}
T14	17.7	2.2	00.0	0.00±0.00^{a}	0.00±0.00^{a}
T15	20.7	2.2	100	13.6±2.51^{c}	1.90±0.81^{bc}
T16	14.7	4.4	100	5.30±1.15^{abc}	2.86±0.32^{c}
T17	17.7	4.4	33.3	3.00±1.00^{ab}	0.40±0.69^{ab}
T18	20.7	4.4	00.0	0.00±0.00^{a}	0.00±0.00^{a}

$^{a-c}$ Similar letters correspond to treatments statistically equal according to the Tukey test $p<0.05$.

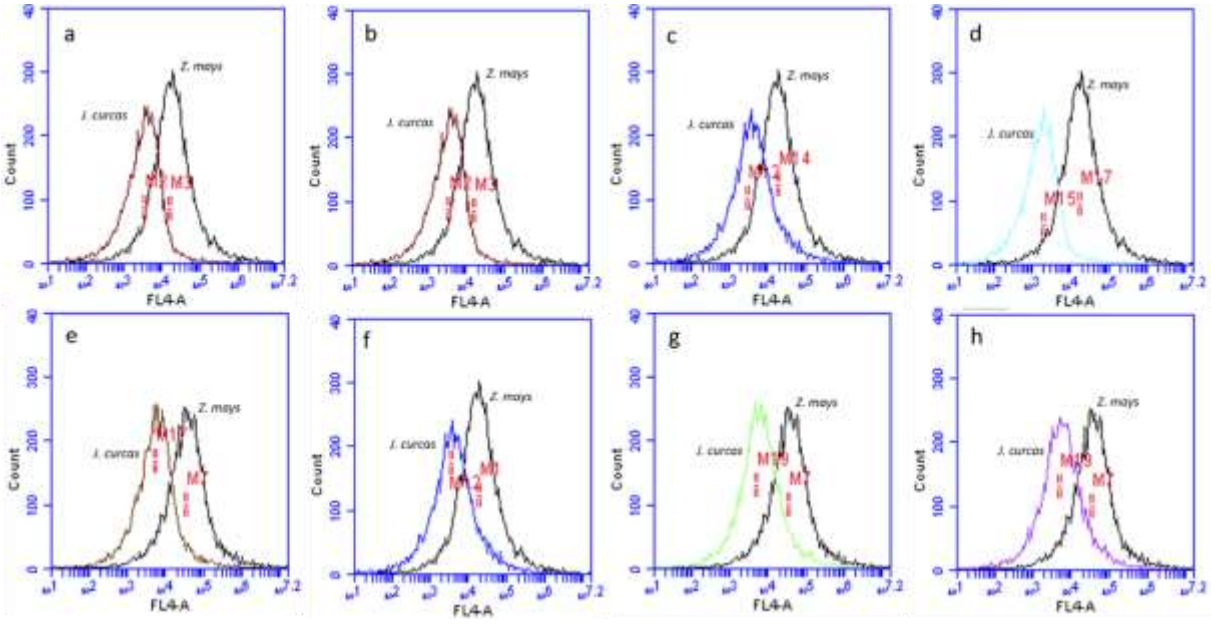

Figure 2. Histograms obtained in the flow cytometry of *Jatropha curcas* explants originating from 1 to 8 subcultures. a-h) first to eighth subculture *in vitro*.

DISCUSSION

Induction of adventitious shoots

The results obtained in this work are similar to those obtained by Gangwar *et al*. (2016) who achieved the formation of 25 shoots; it is pertinent to mention here that after 30 days it was possible to induce morphogenetic response in 70% from the cotyledonary leaf explants and at 45 days of culture the development of real leaves was observed (Figure 3). The results of the analysis of variance carried out ($P<0.05$) showed that the development of the shoots was due to the interaction of the principal effects 2ip, IAA and AdS.

In Figure 4a an average of 7 shoots can be observed with the interaction of the concentration of 125.40 µM of 2ip and 1.27 µM of IAA; in Figure 4b we can see that 125.40 µM of 2ip with 369.21 µM of AdS generated the same number of shoots, while in Figure 4c it was possible to observe the interaction of 1.27 µM of IAA and 369.21 µM of AdS which promoted the formation of 11 shoots.

George *et al*. (2008) have indicated that AdS acts as a precursor of the natural synthesis of cytokinin or improves the natural biosynthesis of AdS and thus, the compounds produced could be more efficient in causing a physiological response than the cytokinins added to the culture medium. These benefits may often be noticed when they are associated with cytokinins, which may corroborate the interactions observed in Figure 4. It has also been demonstrated that AdS can serve as a precursor of the synthesis of zeatin (McGaw *et al*., 1984), which would indicate the possible effect on the induction of adventitious shoots in *J. curcas*. These results exceeded those obtained in the patent of Sreenivasachar *et al*. (2011) who obtained an average of 3 to 4 shoots. Our results also surpass those reported in the report of Varshney and Johnson (2010) and Geetaa and Sudheer (2011) who obtained the formation of 10 shoots per explant with the processes of indirect and direct organogenesis, respectively.

Recently, several authors (Liu *et al*., 2016; Verma and Verma, 2015; Liu *et al*., 2015) have reported the formation of 6 to 25 shoots per explant by means of direct and indirect organogenesis, using a diversity of *J. curcas* explants.

Development of adventitious shoots

AdS is an organic additive which has an effect on both the induction and elongation of the *in vitro* adventitious shoots of *J. curcas*; this may be due to the fact that the interaction of AdS with the cytokinins facilitates the growth and development of the shoots in *in vitro* culture (Nwankwo and Krikorian, 1983). This has been corroborated in a report by Shrivastava and Banerjee (2008) who were also able to achieve the induction and development of *J. curcas* shoots by combining AdS with BAP and other additives such as glutamine, L-arginine and citric acid. Figure 5 shows the elongated shoots of *J. curcas* at 45 days of culture with the growth of lateral shoots and development of leaves.

Table 4. Peak averages of histograms in explants of *Zea mays* and explants of *Jatropha curcas*; DNA content estimated from one to eight subcultures *in vitro*.

Period of Subculture *in vitro* (months)	Average fluorescence of G1 peak of *J. curcas*	Average fluorescence of G1 peak of *Zea mays*[1] *L.*	Estimated DNA content for *J. curcas* (pg)	x 10^9 bp[2]
1	2796.98	12985.57	1.02±0.13[ab]	1.06
2	1951.32	12141.18	0.89±0.16[ab]	0.87
3	2445.68	12904.38	1.07±0.23[b]	1.05
4	2007.84	13224.10	0.82±0.02[a]	0.80
5	4925.31	32155.34	0.83±0.09[a]	0.81
6	5242.80	33041.56	0.86±0.05[ab]	0.84
7	4928.03	33165.54	0.80±0.12[a]	0.78
8	4991.40	33147.85	0.81±0.09[a]	0.79

[a, b] Same letters correspond to statistically similar treatments according to Tukey ($p<0.05$); pg: picograms of DNA**;** [1]*Zea mays* L. 'CE-777' reference standard with 2C= 5.43 pg of DNA (Lysák and Doležel 1998); [2]1 pg of DNA corresponds to 0.978 x10^9 bp (Doležel *et al*., 2003).

Figure 3. Adventitious shoot induction of *Jatropha curcas*: a) explant with callogenesis; b) plantlet with 30 days of induction; and c) plantlet with adventitious shoot morphogenesis at 45 culture days, leaf development can be observed on shoots.

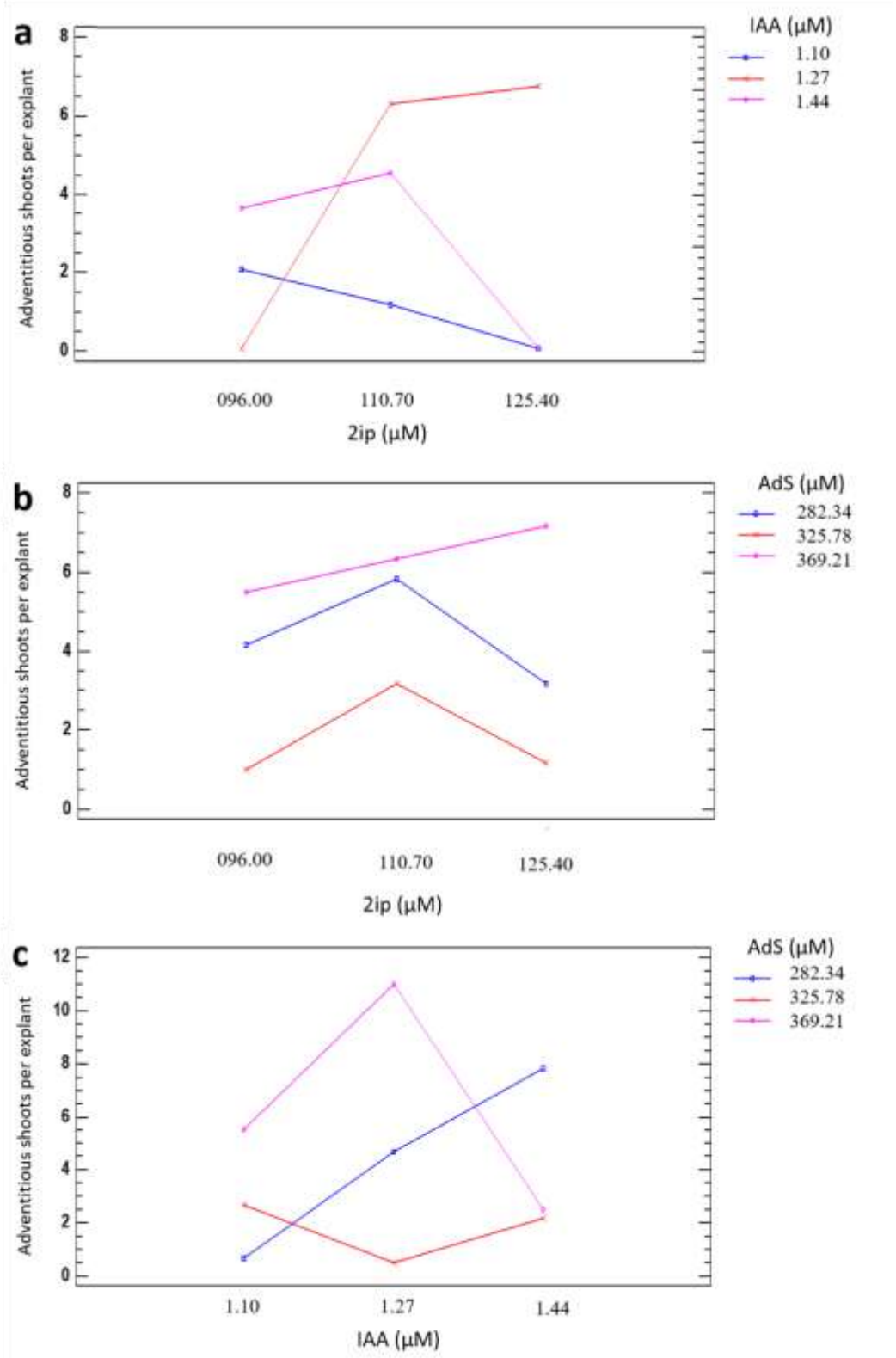

Figure 4. Principal factor interactions in adventitious shoot organogenesis of *J. curcas* a) interaction of 6–(γ, γ-Dimethylallylamino) purine (2ip) with IAA, b) interaction of 2ip with AdS and c) interaction of IAA with AdS.

Rhizogenesis of the developed shoots

Dewir *et al*. (2016) indicated that, in order to achieve a successful micropropagation system, optimal conditions for rooting and shoot development are required given that the search for a good number of healthy roots will allow the plantlets to establish in the soil and will promote normal growth and development. Although a few authors have reported that higher concentrations of IBA can induce higher levels of secondary metabolites and ethylene (Baker and Wetzstein, 1994; De Klerk, 2002), which could lead to the inhibition of the root formation process, the results obtained in this work showed that both the lowest (14.7 µM) and the highest (20.7 µM) IBA concentrations used, gave similar results. Even

though the analysis of variance ($p<0.05$) did not indicate significant statistical differences, it seems that the ionic strength of the medium has a greater effect on the organogenic response of the roots, as can be seen in the Pareto diagram of Figure 6. It was interesting to note that, although the effect of Phl on root induction has been indicated (Daud *et al*., 2013) the results obtained with the MS medium treatments at different concentrations and the addition of different concentrations of IBA + Phl (Table 3) did not give efficient results. In fact, the highest number of roots obtained with Phl was in T5 (1/2 MS + 17.7 µM of IBA + 2.54 mg·L^{-1} of Phl) which generated 7.30 ± 2.30 roots, exceeded only by T15 with 13.6 ± 2.51 roots (Table 3).

Estimation of nuclear DNA content

The variation may have been due to the method of nuclear extraction, the type of fluorochrome and the cytometer used for the analysis of the sample (Doležel *et al*., 1992). In addition, the standard used for the measurement was *Zea mays L. 'CE-777'*, which differs from the standard *Raphanus sativus* 'Saxa' used by Carvalho *et al.* (2008) in the estimation of the nuclear DNA content of *J. curcas*; Despite the fact that the propidium iodide adheres stoichiometrically to the DNA bases and can mark the nuclear DNA (Riccardi and Nicoletti, 2006), the rapid, active decondensation and condensation of the DNA (Belmont, 2003) could be another factor which may be influencing the results obtained, avoiding homogenous DNA staining in some nuclear populations.

For the multiplication of elite genotypes of *J. curcas* on a larger scale, it is important to confirm the genetic stability of the regenerated explants and to demonstrate the reliability of the regeneration systems, as demonstrated by Rathore *et al*. (2014) who evaluated *J. curcas* genotypes from tissues regenerated *in vitro*; moreover, detection of the ploidy level of DNA by flow cytometry was found to be a practical and rapid strategy for the selection of diploid, mixoploid and tetraploid plantlets induced *in vitro* from *J. curcas* meristems (de Oliveira *et al.*, 2013). Also, indirect organogenesis is associated with higher levels of genetic instability.

Soares *et al*. (2016) reported variation in the genetic stability over three generations of *J. curcas* subcultured by indirect organogenesis, which would suggest that this variation increases with each successive subculture.

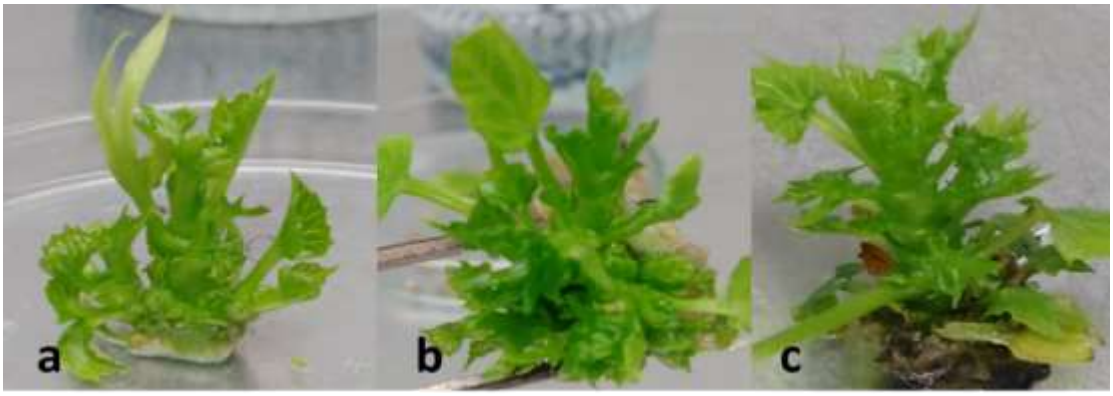

Figure 5. Adventitious shoot development of *J. curcas* during 45 culture days: a) principal shoot development with lateral shoot growth; b) lateral shoot development without apical dominance; and c) development of one shoot with apical dominance.

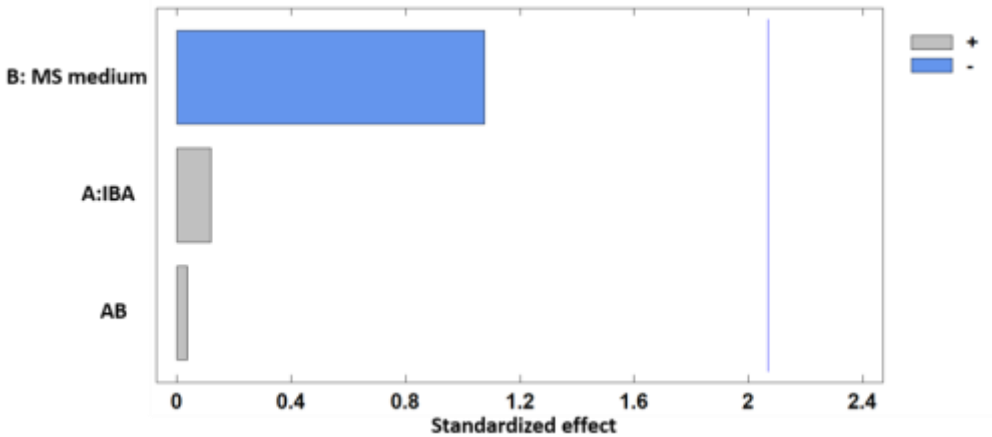

Figure 6. Standard Pareto diagram for number of formed roots.

The analysis by flow cytometer of the nuclear DNA content in *J. curcas* plantlets, from one to eight subcultures, demonstrated that the genetic stability of the clones regenerated from *J. curcas* callus remains stable, indicating that the protocol reported in the present work is suitable for the propagation of *J. curcas* by indirect organogenesis, which will ensure that the clones can be maintained over eight generations without leading to changes in the nuclear DNA content. This protocol could be used for the genetic improvement of the species in the future, given that plant tissue culture and the techniques of molecular biology are biotechnological tools which can complement conventional reproduction, accelerate genetic improvement and satisfy the demand for the availability of uniform clones in large quantities (Mukherjee *et al*., 2011).

CONCLUSION

The leaf explant cells presented totipotency, allowing the induction of adventitious shoots with which it was possible to develop a protocol of morphogenesis via indirect organogenesis in *J. curcas* L. var. ALJC01. The protocol obtained can be used as an *in vitro* propagation technique for this valuable crop and for future studies on the regeneration of genetically transformed explants, which may be developed in this species. The genetic stability of the clones regenerated by indirect organogenesis was also assured in eight generations of *J. curcas* subcultured *in vitro*.

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Author's Contributions statement

GJHC and GLP conducted experiments and wrote the manuscript. IMRB, GLP and GJHC designed and analyzed all the experiments. CCGC, GMS and EAAC provided seeds of *J. curcas* and reviewed the manuscript. JL, AUV and LGIA contributed with comments and reviewed the manuscript.

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