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In vitro induction and growth of friable callus of *Jatropha curcas L.* (non-toxic ecotype)

Inducción y crecimiento in vitro de callo friable de *Jatropha curcas L.* (ecotipo no tóxico)

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RESUMEN

Se desarrolló un protocolo para la inducción de callos en dos accesiones no tóxicas de *J. curcas*. Para ello, se realizaron dos experimentos. En el primer experimento se determinó el método de desinfección de semillas para la obtención de explantes asépticos. Para ello se evaluó el efecto de cuatro concentraciones de hipoclorito de sodio (0%, 10%, 15% y 30%) y tres tiempos de exposición (15, 20 y 30 minutos) en dos accesiones no toxicas de *J. curcas* (I-27 y I-34) en un experimento

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factorial por duplicado, utilizando 10 repeticiones. En un segundo experimento se evaluó el efecto de cuatro concentraciones de 2,4-D (0.00, 2.2, 3.3 y 4.5 µM) sobre la inducción de callo. En los tratamientos de desinfección, los mejores resultados se encontraron en la accesión I-34 expuesta al 30% de hipoclorito de sodio durante 15 minutos debido a que se logró obtener un 75% de embriones axénicos. El mejor tratamiento para inducir callos viables se observó cuando los hipocótilos de la accesión I-34 se expusieron a 4,5 μM de 2,4-D. Nuestros resultados mostraron una respuesta diferencial en la formación de callos entre las accesiones evaluadas. I-34 fue la accesión con mayor tasa de formación de callos. Los resultados de la prueba GLM mostraron la existencia de significancia estadística en la interacción de 3 niveles. Se encontró que el uso de 4,5 M de 2,4-D en hipocótilos de adhesión I-34 fue el tratamiento más eficaz para la inducción de callo. La concentración de 2,4-D fue el factor más influyente para la inducción de callos, ya que fue el único factor principal significativo en las interacciones de 2 niveles. Por lo que se recomienda utilizar estos resultados en los programas de fitomejoramiento que se están desarrollando en este importante cultivo.

Palabras clave: Ecotipo no tóxico, *Jatropha curcas* L., embriones axénicos, inducción de callo.

ABSTRACT

A protocol for callus induction was developed in two non-toxic accessions of *J. curcas*. It was performed two experiments. The first one was established to determine the best disinfection seed method to obtaining aseptic explants. To do this a factorial experiment was conducted by duplicate, using 10 replicates, to evaluate the effect of four concentrations of sodium hypochlorite (0%, 10%, 15% and 30%) and three exposure times (15, 20 and 30 minutes) on the in vitro contamination levels of two accessions (I-27 and I-34). A second experiment was performed with the objective to evaluate the effect of four 2,4-D concentrations (0.00, 2.2, 3.3 and 4.5 µM) on callus induction using a factorial experimental design with 12 treatments. It was found significant differences among the disinfection treatments. The accession I-34 exposed to 30% of sodium hypochlorite for 15 minutes was the best treatment because produced 75% axenic embryos. It was also observed significate differences among the 2, 4 treatments. The best treatment to induce viable callus was observed when hypocotyls of the I-34 accession were exposed to 4.5 μM of 2,4-D. Our results showed a differential response in the callus formation between accessions evaluated. I-34 was the accession with the highest rate of callus formation. The results of the GLM test showed the existence of statistical significance in the 3-level interaction. It was found that the use of 4.5 M of 2, 4-D in hypocotyls of I-34 accession was the most effective treatment to callus induction. The 2,4-D concentration was the most influential factor for callus induction since it was the only significant main factor in the 2-level interaction s. Therefore, it is recommended to use these results in the plant breeding programs which are being developed in this important crop.

Keywords: Non-toxic ecotype, *Jatropha curcas* L., axenic embryos, callus induction.

INTRODUCTION

Jatropha curcas L. (Euphorbiaceae) of non-toxic is quite different as compared to the toxic one due to its contents of forbol esters, being null in the non-toxic one. This shrub is distributed along Central America and México. This specie is economically relevant because of its use to produce biodiesel as well as for its various uses, such as pharmaceutical and food (Martínez-Herrera et al., 2006; Jongschaap et al., 2007). In Mexico, especially in Veracruz state, the ethno-botanical characteristics of non-toxic cultivar, commonly known as "piñón manso", was known since ancient times by the Totonac indigenous group (Achten et al., 2010; Valdes-Rodriguez et al., 2011; Martínez-Herrera et al., 2012). This edible cultivar can supply additionally environmental services like soil restructuration since it resists long period of drought (Kheira and Atta, 2009). Based on its agronomic characteristics, the non-toxic *J. curcas* germplasm has been little studied, and then more studies are needed, besides its high diversity (Pamidimarri et al., 2009; Valdes-Rodriguez et al., 2011). Moreover, there is controversy about the potential of this species in terms of yield and soil restoring in land that have been overexploited, (Abhilash et al., 2011). Hence, the non-toxic cultivar has raised worldwide interest as a promising alternative for sustainable use by rural communities not only for biofuel production but as a food (Makkar et al., 1998; Openshaw, 2000; Ouwens et al., 2007). Semi-domesticated species, there is still a need for obtaining a commercial variety with stable agronomic characteristics as a viable option for commercial use in the biofuel production chain. Therefore, the development of techniques and protocols is required to facilitate the production of elite material (Trabucco et al., 2010; Edrisi et al., 2015).

Conventional genetic improvement methods are an alternative to obtain potentially useful genotypes. However, these methods are very time-consuming and need intensive labour (Mroginski and Roca, 2009; Attaya et al., 2012). Furthermore, conventional commercial agriculture depends basically on seeds and cuttings for its propagation. These methods exhibit problems for producing plants with desirable characteristics since seed productions depends on cross-pollination and plants obtained from cuttings have weak root system (Qing et al., 2007; Valdes-Rodriguez et al., 2011). In vitro protocols can be used to produce plants with desirable characteristics much faster as compared to produce friable callus with morphogenetic capacity (i. e., high multiplication rate and reduced oxidation) (Lu et al., 2003; He et al., 2009; Deore et al., 2008; Addae-Frimpomaah et al., 2014; Moniruzzaman et al., 2016). It is well known that for a successful induction of desirable callus, the concentration of the growth regulator, the source of the explant and the genotype (accession) are important (George et al., 2008; Kumar et al., 2011; Mohebodini et al., 2011). Around of *J. curcas,* there are many studies on micropropagation technics and genetic transformation via callus. Therefore, there is still a question non answered yet, as what are the effect of the interaction among accession, source of explant and growth regulator. One

standard protocol for the production and maintenance of callus must include study aims to establish a protocol to achieve optimal in-vitro conditions for the rapid and favourable production of friable callus of non-toxic *J. curcas* those factors, but this has not been obtained yet (Attaya et al., 2012). Hence, the present study aims to establish a protocol to achieve optimal in-vitro conditions for the rapid and favourable production of friable callus of non-toxic *J. curcas.*

MATERIALS AND METHODS

Plant material. This investigation was conducted in the plant tissue culture laboratory at the Institute of Biotechnology and Applied Ecology (INBIOTECA), Universidad Veracruzana.

Seeds used came from two promising accessions (I-27 and I-34) of the non-toxic cultivar of *J. curcas*, obtained from the Germoplasm bank at the Colegio de Postgraduados (COLPOS-Campus Veracruz). These accessions have suitable characteristics regards to the following agronomic traits: floral buds, number of flower clusters, number of fruit and seed weight, so that they possess a greater potential and vigour to be included in a genetic improvement programmed (García-Alonso, 2015).

Experiment 1

Pre-treatment for obtaining aseptic embryos. A total of 160 seeds (80 seeds from mature fruits for each accession) were thoroughly washed with soap and water. Subsequently, seeds were placed in a 0.36% (v/v) Microdyn solution in 100 ml sterile distilled water under constant stirring for 24 h. Then, seeds were transferred to a laminar flow hood to remove the head under aseptic conditions taking care not to damage the embryo. Afterwards, seeds were pre-disinfected in 80% (v/v) ethanol solution for 60 seconds and rinsed 3 times with sterile distilled water.

Germination of aseptic embryos

Axis embryos were extracted from disinfected seeds using a scalpel. Shafts with embryonic leaves were placed in flasks with MS medium 50% (Murashige and Skoog, 1962) supplemented with 3% w/v sucrose (Jalmek®, Nuevo Leon, Mexico), 50 mg L-1 L-cysteine hydrochloride, 2.5% (w/v) Phytagel® (Sigma-Aldrich, St. Louis, MO) and 400 μM Gibberellic acid (GA3) (Sigma-Aldrich, St. Louis, MO). The pH of the culture medium was adjusted to 5.8 ± 1 before being sterilized in a vertical autoclave (Felisa® FE-398, Zapopan, Jalisco, Mexico) for 15 min at 120 ° C and 104 KPa for 15 minutes.

This research includes two experiments. The first one was about the best method for disinfection. A completely randomized design was used with 8 disinfection treatments with Sodium hypochlorite (NaOCl) and 10 replicates (Table 1). This experiment was carried out twice. These were supplemented with 2 drops of Tween® 80 (P4780 SIGMA-ALDRICH, Saint Louis, Missouri, USA). Last, seeds of the various treatments were rinsed three times with sterile distilled water.

Table 1. Treatments with sodium hypochlorite (NaOCl) for disinfection.

Accesion	NaOCl %	Immersion minutes
	0	30
I-27	10	30
	15	20
	30	15
	0	30
I-34	10	30
	15	20
	30	15

All treatments were made with 10 replicates by duplicate.

Evaluation of embryo contamination and germination

All tissues were incubated under dark conditions for 7 days followed by a photoperiod of 16/8-h light/dark photoperiod for 23 days at a 24 ± 2 ° C, under a light intensity of 40-50 µmol m-2 s-1 using white light lamps. Finally, 30 days after the onset of germination, the percentage of embryo contamination and germination was recorded for each accession and disinfection treatment.

Experiment 2

Callus induction. The experiment 2 assessed callus induction, comparing the effect of three factors. assessed, namely: 1) accessions (two levels, I-27 and I-34); 2) four concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D): 0.00 µM, 2.2 µM, 3.3 µM and 4.5 µM and, 3) source of explants (hypocotyl and cotyledon). Each experimental unit comprised 3 segments of each explant type (0.5 cm of hypocotyls or 1 cm2 of cotyledon). The basal medium for callus induction was (MS) 100% medium supplemented with the different concentrations of (2,4-D), previously mentioned, 3% (w/v) sucrose (Jalmek®, Nuevo Leon, Mexico), 50 mg l-1 L-cysteine hydrochloride and 2.5% (w/v) Phytagel® (Sigma-Aldrich, St. Louis, MO, USA).

The pH of the culture medium was adjusted to 5.8 ± 1 before sterilization in a vertical autoclave (Felisa® FE-398, Zapopan, Jalisco, Mexico) at 120° C and 104 KPa for 15 minutes. All tissues were incubated under a photoperiod of 16/8 h light/ dark photoperiod for 23 days at a 24 ± 2° C, under a light intensity of 40-50 µmol m-2 s-1 using white light lamps. After 15 days, explants were subcultivated in the same medium and under the same environmental conditions, placing individual callus in separate flask so that each experimental unit contained a single explant only.

Evaluation of callus obtained. Biomass was recorded as fresh weight (g) of the callus, from the beginning of the induction until day 45 of initiated the experiment at 7-d intervals. Calluses were weighted, followed by incubation. Fresh weight was recorded under a laminar flow hood using sterilized aluminium paper placed on a semi-analytical scale (XY100-2C, Changzhou Xingyun Electronic Equipment Co., Ltd. Jiangsu, China).

Qualitative characteristics of callus: To study the qualitative characteristics the cotyledon and hypocotyls explants from two promised accessions were classified in six categories: friable-green, friable-white, compact-green, compact-white, compact-brownish, soft white (Avilés et al., 2010). We used the Munsell card (Munsell Color Company Inc. Baltimore) to determine the code of identification. Each treatment was placed on previously sterile petri dish. In axenic conditions, was compared the colour of each callus with Munsell cards.

Cell viability assay: Since green friable callus exhibited higher biomass during the 40 days process, cell viability was measured in this type of callus only (Fig. 3). Suspension cells isolated from hypocotyl green callus (50 mg) were maintained in Erlenmeyer flasks (250 ml) containing 100 ml MS medium and 1000 µl 2,4-D. Sixteen flaks were placed on a rotary shaker (Orbit 1000, Lab Net International, Inc. Edison, N.J. USA) at 110 rpm and 22 ° C. Callus was transferred into fresh medium every 4 days.

Two microbiological staining methods were used to determine cell viability: a) Evans Blue staining test and b) acetocarmine test (Durzan, 1988; Baker and Mock, 1994). Aliquots of two ml were taken from each flask and then incubated in 0.25% Evan's blue stain for 5 minutes. In the case of acetocarmine, aliquots were placed in 1% acetocarmine stain for 4 minutes. This process was repeated twice for each method. Stained and unstained cells of samples of 200 cells were observed and counted using zoom stereomicroscope, 10X and 40X objective lens (zoom ratio of 1:12.86) (Olympus SZX12, Olympus Latin America). Direct cell count was done using a Neubauer counting chamber with clips, double ruled (Blaubrand® Schoot de México, Tlalnepantla, Mexico) (Baker and Mock, 1994).

Statistical analysis. For all variables, the normality of the data was corroborated using the Shapiro-Wilks test. Added to the above the Levene test was used to verify homoscedasticity. When the variables did not meet, the assumptions were processed using percentages transformed with the angular function of Bliss [ArSen $\sqrt{}$ x + 1/100] (Zar, 1999). Later, an analysis of variance (ANOVA) and a post hoc Tukey HSD test (p = 0.05) were performed. In the case of binary data (presence or absence) were analyzed using a Generalized Linear Model (GLM), with the binomial family and the logit link function, followed by pos-hoc contrasts. Add to this, were analyzed the 3-level interaction (accession * growth regulator concentration * explant source) (Agresti and Kateri, 2011).

RESULTS

Experiment 1: Effect of NaOCl in the establishment of non-toxic *J. curcas* embryos

The results of the analysis of variance revealed significant differences between the disinfection treatments. A higher number of mature pathogen-free embryos were observed in the 30 v/v NaOCl treatment (F 3.66 = 7.93, p = 0.0001). Significant differences were observed between accessions since I-34 resulted less contaminated than I-27 (F 1.66 = 4.17, p = 0.045). Interaction accession * NaOCl concentration was not significant (F 3.66 = 0.75, p = 0.52) (Table 2).

Table 2. Effect of different NaOCl treatments (0, 10.15, 30 v/v) on non-toxic *J. curcas* seeds.

Accessions	NaOCl (%)	Contamination (%)	Germination (%)
I-27	0	81 ± (5.7)^{a}	00 ± (0.0)^{d}
	10	70 ± (11.7)^{a}	18 ± (5.7)^{bc}
	15	56 ± (10.0)^{ab}	9.9 ± (5.0)^{cd}
	30	40 ± (12.1)^{ab}	40 ± (7.4)^{ab}
I-34	0	86 ± (10.1)^{a}	3.3 ± (3.3)^{cd}
	10	52 ± (8.1)^{ab}	40 ± (10.0)^{ab}
	15	26 ± (8.3)^{bc}	63 ± (7.8)^{a}
	30	25 ± (10.4)^{bc}	75 ± (10.0)^{a}

Numbers are the mean ± standard error. Means in columns with different letters are significantly different at p ≥0.05 of the confidence limit 95% (Tukey test). NaOCl: Sodium hypochlorite.

Seed germination, there was significant differences with respect to NaOCl concentration. Seeds exposed to 30 v/v NaOCl treatment germinated in higher percentages than seeds exposed to lower NaOCl concentrations (F 3.66 = 26.70, p = 0.0001). Differences between accessions were also significant. Again, seed from I-34 germinated more than seed of I-27 (F 1.66 = 29.05, p = 0.0001). With respect to the interaction (accession * NaOCl concentration) it was significant. It was found that accession I-34 showed higher percentage of germination (75%) under 30 v/v NaOCl treatment (F 3.66 = 4.83, p = 0.0042) (Table 2).

Experiment 2: Effect of 2, 4-D treatments in callus induction and friable callus biomass

The results of GLM test showed statistical significance in the 3-level interaction: accession * growth regulator concentration * explant source (X2 = 8.16, df = 3, p = 0.0427). It was found that treatments I-34 * 4.5 μM 2, 4-D * hypocotyls were the most effective (Fig. 1).

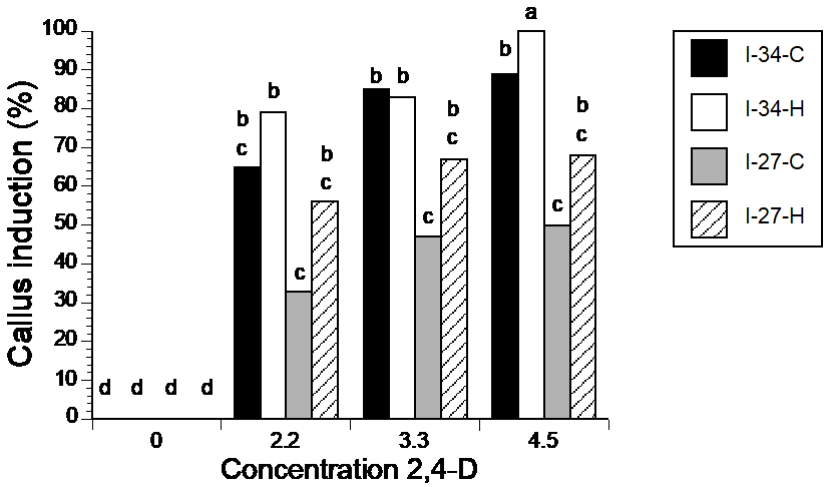

Fig 1. Percent of callus formation among treatments. *Values followed by the same letters are not significantly different according to LGM contrast test.

Concentration of 2,4-D has the most influential factor in callus induction since it was the only significant main factor and influenced the significance of 2-level interactions (Table 3).

Table 3. Main effect tests after GLM analysis for callus induction using two different accessions of *J. curcas*, two explant types and, four concentrations of 2,4-D.

Source	*df*	L-R ChiSquare	Prob>ChiSq
2,4-D concentration	3	130.10389	<0.0001*
Explant	1	3.7482494	0.0529
2,4-D*Explant	3	8.7680883	0.0325*
Accession	1	3.7956766	0.0514
2,4-D concentration* Accession	3	10.489508	0.0148*
Explant * Accession	1	2.8623662	0.0907
2,4-D concentration * Explant * Accession	3	8.1635751	0.0427*

An increase in the biomass of friable callus was recorded, regardless of the type of accession. Green friable callus started to develop after 15 days from the beginning of the induction. The highest percentage of friable callus occurred in treatment I-34 * 4.5μM of 2,4-D * hypocotyl (Table 4), which coincides with callus that displayed the highest weight gain (1.56 g) during the induction phase ($F_{2, 231} = 263.4$, $p = 0.0001$). These same treatments formed 100% friable callus at 45 days of induction.

Table 4. Effect of two accession with different explant source and four concentration of 2,4-D on callus induction in non-toxic *J. curcas*, at 40 days of culture.

Accession	Explant	2,4-D (μM)	N	Callus weight (g)	Callus appearance
I-34	C	2.2	20	0.70±0.00 ^{d}	Soft-Brownish
	Hyp		19	0.61±0.00 ^{e}	Friable-Green
I-27	C	2.2	21	0.56±0.01 ^{e}	Compact-Brownish
	Hyp		16	0.49±0.00 ^{f}	Compact White
I-34	C	3.3	20	0.78±0.00 ^{c}	Friable-White
	Hyp		23	0.99±0.00 ^{b}	Friable-Green
I-27	C	3.3	20	0.68±0.01 ^{d}	Compact-Brownish
	Hyp		21	0.49±0.00 ^{f}	Compact-Green
I-34	C	4.5	18	0.98±0.00 ^{b}	Friable-White
	Hyp		22	1.56±0.02 ^{a}	Friable-Green
I-27	C	4.5	22	0.79±0.00 ^{c}	Friable-White
	Hyp		22	0.67±0.00 ^{d}	Friable-Green

Numbers are the mean ± standard error. Means in columns with different letters are significantly different at p ≥0.05 of the confidence limit 95% (Tukey test); (μM): micromole; C: cotyledon; Hyp: hypocotyl; 2,4-D: 2.4 di-chlorophenoxyacetic acid.

Quality of callus. Regarding the quality of callus, two features were considered, texture and colour (Table 4; Fig. 2 A, B, C). The appearance of the callus obtained also indicated their friable condition. A total of 8 treatments were evaluated; from these, the qualitative friable condition was obtained in accession I-34 from hypocotyls treated with 2.2 μM, 3.3 μM and 4.5 μM 2,4-D (Table 4; Fig 2 E): Even, friable callus was obtained from cotyledons by increasing the concentration of 2,4-D. In the case of accession, I-27, friable callus was obtained at higher concentration of 2,4-D (Table 4). Only cotyledons explants formed friable-white callus, but this type of callus did not respond to viability test, except for I-34-C-4.5 μM (Table 4, Fig 2, G, 3).

Accession I-27 treated with 2.2 μM and 3.3 μM 2,4-D resulted in compact callus of varying colour: white, green and brown (Table 4, Fig. 2 F, H). Finally, accession I-34 treated with 2.2μM produced soft, non-friable and brown callus. It is remarkably to note that green friable callus from hypocotyls also showed a high increase in biomass.

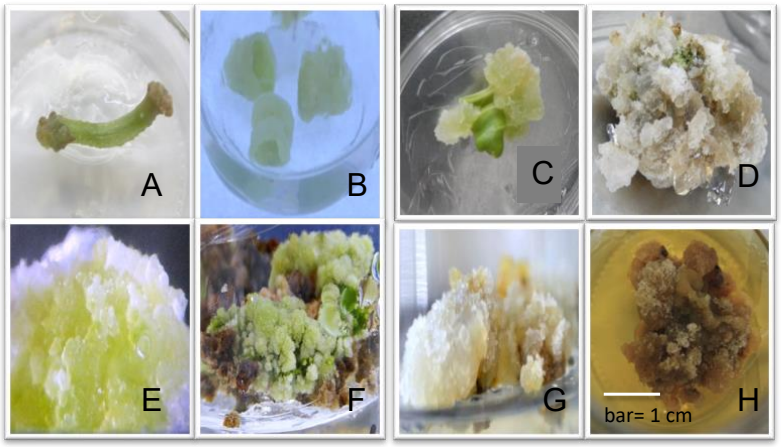

Fig 2. Effect of treatments with growth regulator 2,4-D treatments on two explant sources (hypocotyl and cotyledon) of non-toxic *J. curcas*, with 4.5 μ M 2,4-D: A) hypocotyl, 2 days; B) callus induction, hypocotyl; C) cotyledon, 10 days; D) callus induction, cotyledon; E) friable-green callus from hypocotyl, 15 days; F) compact-brownish callus from hypocotyl; G) accession I-34; (treatment I-34-C-4.5 μM); H) compact-brownish callus from cotyledon.

Colorimetric tests for evaluation of quality callus. Cells of green-friable callus reacted to both colorimetric tests. In the case of Evan's blue, an average (±s. e.) of 72.8% (±4.32) of viable cells were observed while in those exposed to acetocarmine an average of 81.83 % (±4.14) were reactive. This evidence shows that these calluses are suitable for subculture (Fig. 3).

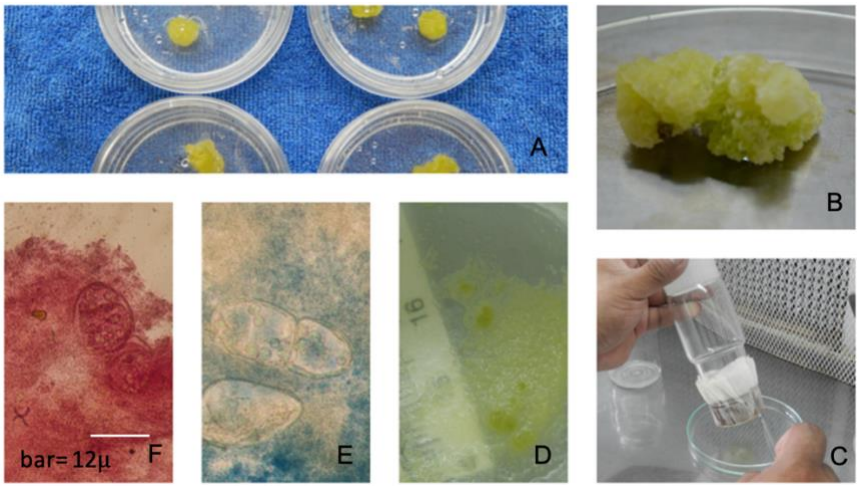

Fig 3. Viability of callus cells after 40 days of inoculation. A) hypocotyl 10 days; B) friable-green callus of hypocotyl 40 days; C) aliquot extraction by Soxhlet D) disgreged cells; E) staining cell with Evans blue; F) staining cells with acetocarmine.

DISCUSSION

An optimal combination of accessions, explants and concentrations of 2,4-D was determined in the formation of callus of non-toxic *J. curcas*. Additionally, a disinfection treatment that allows 75% of axenic seedlings was developed.

The importance of disinfestation in the phase of establishment is mostly to control pathogens because it has been observed that during the in vitro culture these may occur in subsequent subcultures and grow and affect explants (Misra et al., 2010). Thus, it is important to consider the seed source (i.e. field or greenhouse) since there are differences in the obtained axenic tissues (Shtereva et al., 2014). Our results indicate that a soaking seeds in a 30% solution of sodium hypochlorite for 15 minutes was enough for obtaining axenic material for callus induction purpose. This time and concentration differ with those found by Warakagoda and Subasinghe, (2009) who used 100% NaOCl with 30 minute immersions to get 100% germination of toxic *J. curcas* seeds. In our study both, the time of immersion and the percentage NaOCl were reduced paralleling the disinfection method used with Paulownia spp. (Shtereva et al., 2014). Some authors have noted that the use of NaOCl alone is insufficient to obtain aseptic cultures (Ramakrishna et al., 1991).

They pointed out that NaOCl is effective in eliminating pathogen species including Fusarium spp, Epicoccum purpurascens and Bacillus spp, but is ineffective on Alternaria alternata also, they noted that mercury chloride is more effective on Alternaria alternata but less effective on Fusarium spp, Epicoccum purpurascens and Bacillus spp. These authors suggest the use of different disinfectants in combination to improve the effectiveness in the establishment of aseptic cultures. However, in our study it was not necessary to convene disinfestation methods for obtaining a high percentage of axenic seeds.

It was found statistical difference between the accessions used for callus induction. There are several studies that highlight the importance of the origin of the genotypes, cultivar or accession for in vitro processes (Kumar et al., 2011; Grootboom et al., 2008). In addition, it is reported that the toxic in comparison to non-toxic ecotype also have different response (Kumar et al., 2016). Accession I-34 was taken from the field by having good agronomic characteristics. García-Alonso, (2015), reported that this access has increased on floral buds, number of flower clusters, number of fruit and seed weight in compared with others accession established in the experimental field. Paralleling these qualities, I-34 resulted the most effective accession since their callus exhibited the highest biomass of 1.56 g specially when obtained from hypocotyls treated with 4.5 μM 2,4-D. It is possible that their agronomic qualities match with superior properties for in vitro.

With respect to the response associated with the use of auxin 2,4-D in callus formation, it coincides with other studies of induction of callus in toxic *J. curcas* (Filippov et al., 2006; Santos et al., 2015). They have underlined the role of this regulator in the cell dedifferentiation process, however, they also pointed out that increasing the concentration of this auxin in 6 μm and 8 μM in MS medium (Murashige and Skoog, 1962), results in the production of hyper hydrated callus, which are unsuitable to achieve the regeneration of *J. curcas* plants. Apparently, the response detected here

was the most suitable one. In this study, we considered that callus weight is an important attribute associated with callus quality and the morphogenetic response (Zhong et al., 2012). It is known that the increase in size is closely related to the absorption of the auxin and this, in turn, with somaclonal variation (Sáenz et al., 2005). The best treatments were those involving genotype I-34 added with the highest concentrations of 2,4-D. In this case, friable callus had a mean weight of 1.56 g. Some studies on callus induction in *J. curcas* have also reported that auxin 2,4-D is an excellent callus inducer (Fitch and Moore, 1990). Callus can be sub-cultured and maintained for periods up to 8 to 12 months for multiple uses. A globular friable texture is typical of callus with a high growth rate (cell division) (Solís-Ramos, 2013). Our results are consistent with the findings of other authors who obtained the maximum biomass 21 days after the beginning of callus induction (Kaewpoo and T-chato, 2009). According to these authors, the growth rate of the callus declines once this period of time has passed on. Our study also recorded that those callus that acquired a brownish compact appearance ceased growing after 21 days of induction (Papadakis et al., 2001). Cell viability is defined as the number of healthy cells in a sample and a cell is considered viable when could live, grow and develop (Palta et al., 1978; Stoddart, 2011). The measurement of cell viability plays a fundamental role in all forms of cell culture but regardless of the type of assay used. These changes are similar to those reported in other studies and these changes also reduce both physiological and biochemical responses that ended in the decline of cell division rate (Krikorian et al., 1995; Nogueira et al., 2007; Semenza, 2007; He et al., 2009; Santos et al., 2015). For this reason, green callus of *J. curcas* should be sub cultured within the first 21 days after induction.

CONCLUSION

It can be concluded that NaOCl was more effective to aseptic embryos as compared to others disinfected agents generally more contaminants or toxic such as mercury, chloride. In relation to the use of 2,4-D it is concluded that exists effect in the interaction among accessions plus type of explant and growth hormones was statistically significant. To optimize callus production has relationship with the combination of the following factors: type of accession (I-34), type of explant (hypocotyl) and the growth hormone (4.5 μM 2,4-D) to obtain friable callus by also using MS media growth. These results may serve as the foundation for further work on the genetic improvement in this culture using elite non-toxic cultivar or accessions and to keep in mind the disinfection method to set up aseptic vitro culture. These non-toxic accessions may turn out to be the suitable genetic material to establish protocols for mass multiplication and regeneration of plants for genetic improvement used in commercial plantation as a raw material to produce food resources in rural areas. It is recommended to consider the interactions among the factors tested and the quality and aseptic seeds in future vitro culture studies.

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