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Micropropagation of *Jatropha curcas* superior genotypes and evaluation of clonal fidelity by target region amplification polymorphism (TRAP) molecular marker and flow cytometry

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The major limitation in large-scale cultivation of *Jatropha curcas* for use as energy crop is the inconsistent and unstable seed yield due to the heterozygous nature of the plant. A reliable *in vitro* regeneration system is necessary for continuous supply of quality planting material at large-scale. In this study, the interaction between the season collection of explants and capacity of *in vitro* regeneration shoots from foliar explants was investigated. Three genotypes selected in our breeding program were evaluated. We achieved an average of 39.8, 25.5 and 10.9 shoots per explant for G1, G2 and G3 genotypes, respectively. All genotypes showed higher regeneration capacity when the foliar explants were collected in September/2012 season. Excellent results were obtained with the use of micrografting technique for the *in vitro* rooting, with a plant recovery rate of 85%. In order to confirm the genetic stability of micropropagated shoots, two analyses were performed: ploidy estimation using flow cytometry and DNA polymorphism analysis using TRAP molecular markers, which has been here reported for the first time for *J. curcas*. For G1 genotype, it was found that 4% of the plants were tetraploid and 5% of plants had polymorphic bands. No DNA polymorphisms were found in plants of other genotypes. Thus, the low or no somaclonal variation indicates that the protocol established preserves the clonal fidelity of micropropagated plants.

Key words: Organogenesis, *in vitro* micrografting, foliar explants.

INTRODUCTION

Jatropha curcas L. (Euphorbiaceae) is a perennial deciduous and monoecious shrub (3 to 10 m), native to México and Central America (Achten et al., 2008). Recently, the species has attracted the attention of the

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Abbreviations: BAP, 6-Benzylaminopurine; **GA_3,** gibberellic acid; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog medium; **TDZ,** Thidiazuron; **TRAP,** target region amplification polymorphism.

international research community due its potential for production of exceptional quality biodiesel from the oil contained in its seeds, more so in view of the potential for avoiding the dilemma of “food x fuel” (Ghosh et al., 2007; Carels, 2011). *J. curcas* oil yield per hectare is around 2000 L which can be increasing significantly by selective breeding. Its seed contain 25 to 40% oil with predominance of oleic fatty acid in triglycerides which can collaborate with oxidative stability to biodiesel (Argollo Marques et al., 2013). Moreover, the plant is considered as drought tolerant, low seed cost, has wide adaptation to different soil and climate conditions and ability of biodiesel to persist stable upon storage (Balat, 2011; Gomes et al., 2010). Despite its potentiality, the oilseed is a non-domesticated plant and still there are none cultivar for commercial plantations. The specie is xenogamic and highly heterozygous for most agronomic traits suggesting a high degree of segregation (Argollo Marques et al., 2013). Consequently, breeding programs require several years to obtain a stable cultivar.

Biotechnological tools such as *in vitro* cloning of superior genotypes may contribute to speed up the breeding process meeting the growing demand for stable and improved genetic material in the near future (Argollo Marques et al., 2013). Numerous studies have been reported on *J. curcas* organogenesis (Sujatha and Mukta, 1996; Lu et al., 2003; Wei et al., 2004; Sujatha et al., 2005; Sharma et al., 2006; 2011; Rajore and Batra, 2007; Deodore and Johnson, 2008; Kumar and Reddy, 2010; 2012; Kumar et al., 2010a; b; 2011a; Singh et al., 2010; Khurana-Kaul et al., 2010) and somatic embryogenesis (Sardana et al., 2000; Jha et al., 2007; Cai et al., 2011). Though several reports on regeneration from various explants in *J. curcas* exist, none report on the influence of season collection of explants and its interaction with different genotypes are available. Besides the genotype, the physiological, nutritional and sanitary condition of the mother plant strongly influences the *in vitro* regeneration process. Also, important is the choice of suitable culture medium and growth regulators combinations for shoots induction/elongation and *in vitro* rooting, the most critical phase. Although, previous studies have reported relative success on *in vitro* rooting of micropropagated plants, the rooting rates have been low and not exceeded 51.1% (Kumar and Reddy, 2010). Alternatively, this difficulty can be overcome using *in vitro* micrografting techniques (Silva et al., 2005). Other challenge is the generating planting material with genetic stability in order to maintain interest traits of superior genotypes (Rahman and Rajora, 2001). The somaclonal variations are genetic and epigenetic changes that occur uncontrollable, spontaneous and randomly during the *in vitro* process. It is a phenomenon that is highly undesirable of obtaining genetically uniform clones. The level of somaclonal variation may be influenced by choice of the regeneration method, genotypes, explants origin, kind and concentration of growth regulation and number and duration of the subcultives (Ahuja, 1987; Rani and Raina, 2000; Bairu et al, 2011).

Morphological, physiological/biochemistry, cytogenetic and molecular techniques can be used to detect somaclonal variations (Kwon et al., 2010; Song et al., 2012; Suman et al., 2012). Flow cytometry (Kaewpoo and Te-Chato, 2010) and RAPD and AFLP molecular markers (Sharma et al., 2011; Leela et al., 2011) were recently used to assess the chromosomal and sequence changes, respectively and assess the genetic fidelity of micropropagated plants of *J. curcas*. Target region amplification polymorphism (TRAP) is a kind of developed molecular marker system with the advantages of simplicity, high throughput, numerous dominant makers and highly reproducibility. Another advantage of TRAP in relation to other markers would be use as parts of known sequences of genes of interest as a fixed primer and a random primer that amplifies the adjacent regions of this gene (Hu and Vick, 2003). TRAP molecular marker may be useful in genotyping of germplasm banks and tagging genes of agronomic traits of interest in the cultures, the large amount of information that is generated (Hu and Vick, 2003). This technique can also be applied in order to assess changes at the DNA level in micropropagated plants to confirm the genetic stability and clonal fidelity. In accordance with Sharma et al. (2011), this kind of study should be carried at the first stages of culture in order to eliminate the possible variants and avoid its micropropagation.

This paper reports efficient micropropagation protocols via organogenesis using foliar explants for the cloning of three *J. curcas* genotypes selected in our breeding program due to its superior oil yield and quality. We investigated the interaction between the genotypes studies and the season collection of explants. The micropropagated plants were successfully rooting by use of micrografting technique. Moreover we have established for the first time, a methodology to assess the somaclonal variation in regenerated plants using polymorphic TRAP molecular markers. Additionally, we also used flow cytometry to detect changes at the level of chromosomes.

MATERIALS AND METHODS

Plant material

Matrix plants used for this study were cultivated at the experimental field, (not irrigated) situated in Campinas/São Paulo, Brazil (latitude 22°53' S; longitude 47°5' W and altitude 664 m). Three genotypes of *J. curcas* (G1, G2 and G3) were selected by our breeding program based on superior agronomic characteristics such as yield and oil content and quality.

Foliar explants organogenesis

Young apical fully developed leaves were collected from G1, G2 and G3 plants and were stored in the dark at room temperature for

Table 1. Description of the growth regulator treatments used for *in vitro* organogenesis of physic nut explants.

S/N	Description
1	Control - no growth regulators
2	1.5 mg L^{-1} TDZ
3	3.0 mg L^{-1} TDZ
4	0.2 mg L^{-1} IBA/0.5 mg L^{-1} TDZ
5	0.2 mg L^{-1} IBA/1.0 mg L^{-1} TDZ
6	0.2 mg L^{-1} IBA/2.0 mg L^{-1} TDZ
7	0.5 mg L^{-1} BAP/0.1 mg L^{-1} IBA/0.5 mg L^{-1} TDZ
8	0.5 mg L^{-1} BAP/0.1 mg L^{-1} IBA/1.0 mg L^{-1} TDZ
9	0.5 mg L^{-1} BAP/0.1 mg L^{-1} IBA/2.0 mg L^{-1} TDZ

20 h. The sterilization process involved immersion in sodium hypochlorite solution (2.5% v/v) for 15 min, followed by rinsing three times in sterile distilled water. The leaves were cut into 0.7 x 0.7 cm pieces and cultured in MS (Murashige and Skoog, 1962) medium, supplemented with 100 mg L^{-1} inositol, 10 mg L^{-1} cysteine, 25 mg L^{-1} reduced glutathione, 30 g L^{-1} sucrose, 500 mg L^{-1} hydrolyzed casein, 6 mg L^{-1} copper sulfate and different combinations and concentrations of plant growth regulators (Table 1). The culture medium was solidified using 2.4 g L^{-1} phytagel, and the pH was adjusted to 5.8 ± 0.1 prior to autoclaving at 120°C and 1.2 atm for 20 min. The explants were maintained in culture medium under a 16 h photoperiod at 25 ± 1°C, with subculture every 21 days. Each treatment comprised five repetitions, and one repetition was represented by the average of three explants. The experiments were repeated during three different periods: May 2011, November 2011 and September 2012. The number of shoots per explant was recorded after six weeks of culture.

Elongation of regenerated shoots

Regenerated shoots of the G1 and G2 genotypes (5 mm in length) were incubated in MS medium supplemented with 10 mg L^{-1} cysteine, 25 mg L^{-1} reduced glutathione, 500 mg L^{-1} hydrolyzed casein, 6 mg L^{-1} copper sulfate, 50 mg L^{-1} adenine sulfate, 30 g L^{-1} sucrose, 7 g L^{-1} agar and different plant growth regulators. Different concentrations of gibberellic acid (GA_3) (1.5, 3.0 mg L^{-1} and 4.5 mg L^{-1}) were tested in the first experiment, and three combinations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) (0.15 /0.05, 0.3 /0.1 and 0.5 /0.2 mg L^{-1}) were tested in a second experiment. Each treatment was included ten repetitions, with each replicate consisting of three shoots on average. The explants and shoots were maintained in culture medium under a 16 h photoperiod at 25 ± 1°C, with subculture every 21 days. The number of leaves and length of the shoots were recorded after six weeks of culture.

In vitro micrografting and acclimatization

Shoot apices of 5 mm in length were isolated from the regenerated plants and used for *in vitro* micrografting onto *J. curcas* L. seedlings. Seeds without tegument were surface sterilized in sodium hypochlorite (2.5% v/v) for 20 min, rinsed three times in sterile distilled water and inoculated in solid MS medium containing 30 g L^{-1} sucrose and maintained under a 16 h photoperiod at 25 ±1°C for 40 days to obtain *in vitro* rootstocks. *In vitro* micrografted plants were incubated in MS medium supplemented with 30 g L^{-1} sucrose, 0.15 mg L^{-1} BAP and 0.05 mg L^{-1} IBA. The number of developed graft plants, the number of leaves and the leaf lengths were recorded at 30 days after inoculation. The experiment was repeated twice with ten replicates each and one plant per tube. All micrografted plants developed *in vitro* were transferred to flasks containing sterile vermiculite for acclimatization. The plants were maintained under high humidity for 40 days, followed by the gradual reduction of the humidity. The plants were transferred to pots containing a mixture of soil and commercial substratum (Plantimax ™/Eucatex) in a 1:1 ratio and maintained under greenhouse conditions.

Statistical analysis

All the experiments were set up in factorial completely randomized design. Statistical analyzes were performed using the SANEST program (Machado and Zonta, 1995). Data from the *in vitro* organogenesis and shoot elongation experiments were subjected to analysis of variance (ANOVA) and Tukey's test ($p < 0.05$) to determine the significance of the differences among the means. The organogenesis data were transformed by $\sqrt{x+0.5}$ before analysis.

Characterization of *in vitro*-regenerated shoots

Two groups of *in vitro*-regenerated shoots that had been subcultured in MS medium supplemented with 0.3 mg L^{-1} BAP /0.1 mg L^{-1} IBA were used for genetic stability analyses using flow cytometry and TRAP molecular markers.

Flow cytometry

The first group was analyzed on 2º subculture (20 shoots of each genotype: G1 and G2) and 12º subculture (30 shoots of genotype G1), and the second group was analyzed on 7º subculture (10 shoots of each genotype: G1, G2 and G3) using flow cytometry. The ploidy level of the regenerated shoots was determined by flow cytometry using the CyFlow Ploidy Analyzer (Partec Gmbh.) equipped with an UV-LED lamp. Nuclear suspensions were isolated from pieces of shoot leaves with area of 0.25 cm^2. The cell nuclei were exposed using a steel scalpel blade and stained using the CyStain UV ploidy solution kit (Partec Gmbh.), which uses 4-6-diamidino-2-phenylindole (DAPI) as a fluorochrome. The nuclei solution was filtered through a 30 µm filter and immediately analyzed. One thousand intact nuclei were evaluated in each sample. The histograms were analyzed using CyView software (Partec Gmbh.). The samples with coefficients of variation (CV) greater than 10% were discarded. The ploidy level of each sample was compared with that of a leaf from the donor plant.

Molecular marker analysis

Genomic DNA from the shoots of G1 (20 shoots), G2 (10 shoots) and G3 (10 shoots) genotypes was extracted from the leaves using the method of Doyle and Doyle (1990). The DNA polymorphisms in the samples were assessed using TRAP molecular markers. The PCR reactions were performed in final volume of 13 µL containing 1X PCR buffer, 2.7 mM $MgCl_2$, 115 µM of each dNTP, 0.6 µM of fixed and arbitrary primers, 0.6 U Taq DNA polymerase and 100 ng of genomic DNA. The PCR reaction was initiated at 94°C for 2 min, followed by 5 cycles of 94°C for 45 s, 35°C for 45 s and 72°C for 1 min; 35 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 1 min; and a final cycle of 72°C for 7 min. Twelve different combinations of primer pairs were used. The fixed *primers* were designed according to Cristofani-Yali et al. (2007) for the citrus genes, and the arbitrary

Table 2. Description of the fixed and arbitrary *primers* used in the TRAPs molecular marker technique.

Identification	Gene	Type	5' → 3'
01F	*ACC synthase*	Fixed	TCCCCGAGGCACAGCATC
02F	*Caffeic acid O-methyltransferase*	Fixed	ACAGGGCCAAAGGTAAAC
05F	*NADP-dependent glyceraldehyde 3-phosphate dehydrogenase*	Fixed	ACGCGTCCGCCACTCTCA
06F	*Chlorophyll a/b- binding protein*	Fixed	TGGCAGCATCGTCAACT
07F	*SRG1*	Fixed	GGCACCGCACTCACCATC
08F	*Miraculin-like protein 2*	Fixed	GTGGCGAATTTTGACTGT
10F	*Ein3-like protein*	Fixed	CAGTTTCTTGTTGCTACG
02R	*Caffeic acid O-methyltransferase*	Fixed	AGCGCGTCCTGGTGATGC
03R	*Sucrose synthase*	Fixed	ATATACCCCAGCCAATGT
06R	*Chlorophyll a/b- binding protein*	Fixed	GGAGACGGCGGGCTTAGA
07R	*SRG1*	Fixed	TGCTCTGGTTTCGGACAA
09R	*DNAJ*	Fixed	CGCATCCTCGCCGTATTG
P2	-	Arbitrary	GACTGCGTACGAATTTGC
P4	-	Arbitrary	GACTGCGTACGAATTTGA

primers were designed according to Li and Quiros (2001) (Table 2). Formamide was added at an equal volume to the amplified PCR products for the posterior denaturation step, involving heating at 94°C for 3 min. Electrophoresis was performed using a 5% denaturing polyacrylamide gel containing 7 M urea and TBE buffer under the following conditions: fixed power (75 W) for 2 h, followed by gel staining using silver nitrate (0.2%), according to Creste et al. (2001). DNA polymorphisms were identified by comparing the profiles of the PCR-amplified fragments of the regenerated shoots with those of the respective donor plants.

RESULTS AND DISCUSSION

Foliar explants organogenesis

The season collection of foliar explants into matrix plants had a great influence in the shoot regeneration of the three studied genotypes. All genotypes had better responses when the leaf explants were collected in September 2012 (Figure 1). The G1 genotype showed great oscillations in the regeneration capacity with averages of 10.2, 1.4 and 24.8 shoots per explant in May 2011, November 2011 and September 2012, respectively. The other two genotypes had smaller oscillations during the periods analyzed (Figure 1). September represents the beginning of the spring season in the southern hemisphere, a time with warmer days and the beginning of the rainy season. This climate induces the growth of new branches and leaves by physic nut plants, a deciduous species. Despite all explants were collected from fully developed leaves, those that were collected during this period (September/2012) were likely younger than those that were collected during May and November, 2011. Therefore, it is expected that the climate and explant age positively influenced the induction of *J. curcas* organogenesis during the experiment realized in September 2012.

In two season collections (May/2011 and September/2012) the results demonstrated the occurrence of genotype dependence in the *J. curcas* organogenesis using foliar explants. Similar results were found by other authors who reported different responses in the direct organogenesis induction of various *J. curcas* genotypes using cotyledonary explants (Kumar et al., 2010b) or petiole explants (Kumar and Reddy, 2010). These studies considered that the different responses of genotypes to organogenesis may be related to different levels of endogenous hormones (especially cytokinin) found in each genotype. The effect of different culture media in each genotype was only performed in experiment realized during September 2012, in which regeneration rate was superior. In this season collection of explants were achieved an average of 39.8, 28.9 and 10.9 shoots per explants for the genotypes G1, G2 and G3, respectively (Figure 2). These averages were higher than those reported by other authors (3.5 to 10 shoots per explants) (Shukla et al., 2013; Zhang et al., 2013). For all genotypes, the control treatments without growth regulators (treatment 1) did not regenerated none adventitious shoot. For G1 and G3 genotypes, the combinations of thidiazuron (TDZ) and IBA (treatments 4 to 9) were more efficient than the treatments containing TDZ alone (treatments 2 and 3). Different results were observed for the G2 genotype, for which treatments with TDZ alone also effectively induced the regeneration of shoots.

Our results show that the combinations of TDZ and IBA were the most efficient in *J. curcas* shoot regeneration from foliar explants (Figures 2 and 3a). Notably, BAP did not affect the rate of shoot regeneration, therefore, the use of this hormone was considered unnecessary. Similar results were verified by Khurana-Kaul et al. (2010) who also showed that the combination of TDZ and

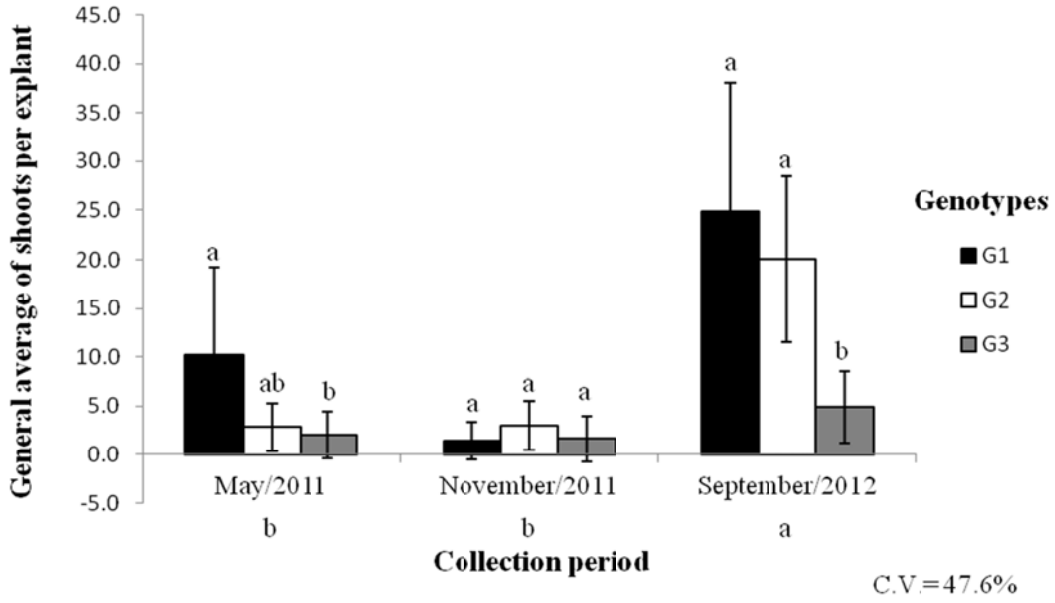

Figure 1. Mean of number of regenerated shoots per explant from somatic organogenesis of physic nut. Data are represented by the average of all treatments (medium) used for the physic nut genotypes during different season collection. The mean values with different letters between genotypes in the same period and different letters between collection period differ according to Tukey's test (p<0.05).

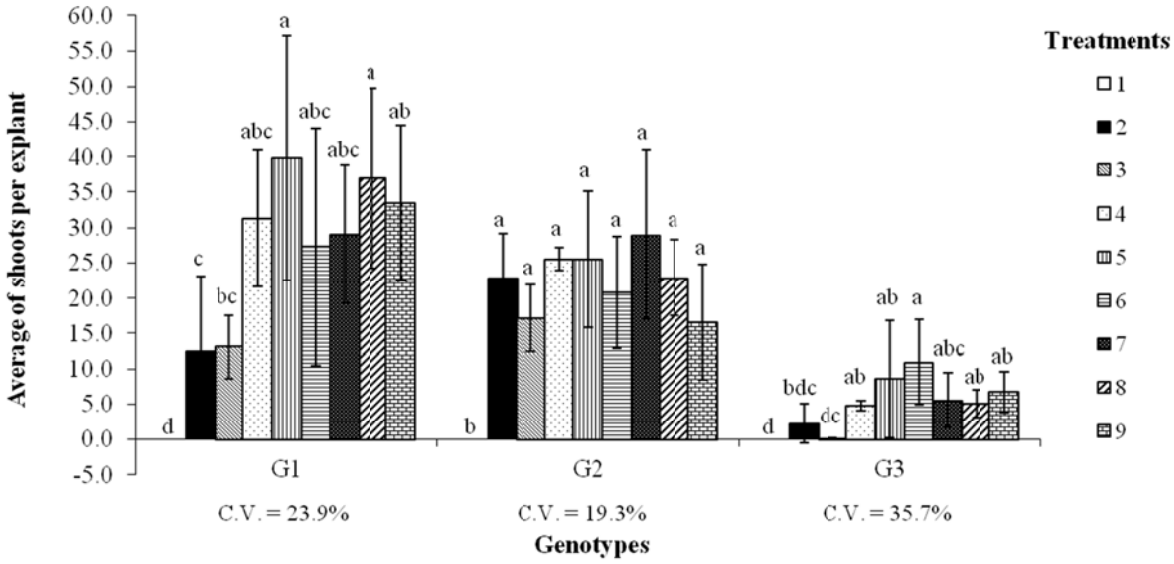

Figure 2. The mean of number of regenerated shoots per explant in nine treatments (medium) used to induce the somatic organogenesis of physic nut explants of different genotypes. Data for September 2012 experiment are shown. The mean values with different letters for the same genotype differ according to Tukey's test (p<0.05).

IBA was more effective than the combination of BAP and IBA in *J. curcas* shoot regeneration using foliar segments as explants.

Elongation of the shoots

High percentage of shoot oxidation was observed in both genotypes evaluated (G1 and G2) in the presence of GA_3 in the culture media. Despite oxidation process, treatment containing 4.5 mg L^{-1} GA_3 promoted a good shoots elongated rate (10.3%) and the best average length (0.97 cm) for G1 shoots. For G2 genotype the higher elongated shoots rate (16.6%) was obtained in treatment without GA_3, with an average length of 0.50 cm. The combination of BAP (0.3 mg L^{-1}) and IBA (0.1 mg L^{-1}) promoted the

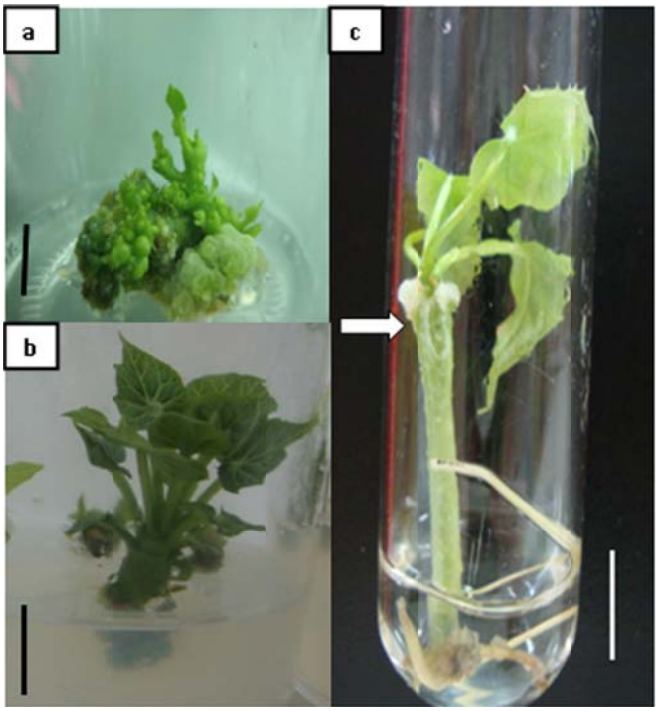

Figure 3. a) Shoot formation through somatic organogenesis from genotype G1 leaf explants. **b)** Shoot elongation from genotype G1 in culture medium containing BAP and IBA. **c)** Micrografted physic nut plant. Bar = 1 cm.

Table 3. Physic nut shoots elongation for genotypes G1 and G2 using different concentrations of BAP and IBA.

Treatment	Shoot Size (cm)		Number of Leaves	
	G1	G2	G1	G2
B1 (Control)	0.6^{a}	0.6^{a}	3.1^{a}	2.3^{ab}
B2 (0.15 mg L^{-1} BAP + 0.05 mg L^{-1} IBA)	0.9^{a}	0.7^{a}	3.8^{a}	1.6^{b}
B3 (0.3 mg L^{-1} BAP + 0.1 mg L^{-1} IBA)	1.4^{a}	1.0^{a}	4.5^{a}	3.5^{a}
B4 (0.5 mg L^{-1} BAP + 0.2 mg L^{-1} IBA)	0.6^{a}	0.7^{a}	2.5^{a}	3.6^{a}
C.V.	31.2%	31.7%	28.5%	35.1%

Average values with different letters in each column differ according to Tukey's test ($p<0.05$).

best shoot elongation (1.4 and 1.0 cm) and the largest number of leaves per shoot (4.5 and 3.5) for G1 and G2 genotypes, respectively (Table 3 and Figure 3b). The increasing of plant growth regulator concentration was directly proportional to increasing of percentage of elongated shoots for G1, ranging from 30% (control, without plant growth regulators) to 68.2% (B4 treatment: 0.5 mg L^{-1} BAP/0.2 mg L^{-1} IBA). The B4 treatment also reduced explant oxidation from 65 (control) to 9.1%. Approximately, 59.1 and 25% of the shoots cultivated in the B4 treatment formed basal calli in G1 and G2 explants, respectively. Basal callus formation is not desirable because this can hamper the posterior stage of *in vitro* rooting, the most critical of organogenesis process in *J. curcas*. Therefore, it was possible to conclude that the use of BAP and IBA in the culture medium was more efficient than GA_3 for shoot elongation.

In vitro micrografting of shoots and acclimatization

The *in vitro* micrografting of *J.curcas* shoots onto seedlings of the same species was successful (Figure 3c). We obtained 85% of developing shoots of 20 *in vitro* micrografted plants. The mean number of leaves and the mean leaf length of the micrografted plants were 2.1 and 1.1 cm, respectively, after 30 days of culture. The plants also showed numerous well-developed roots. There are many reports of lower *in vitro* rooting percentages (22 to 52%) for *J. curcas* micropropagated shoots (Kumar et al.,

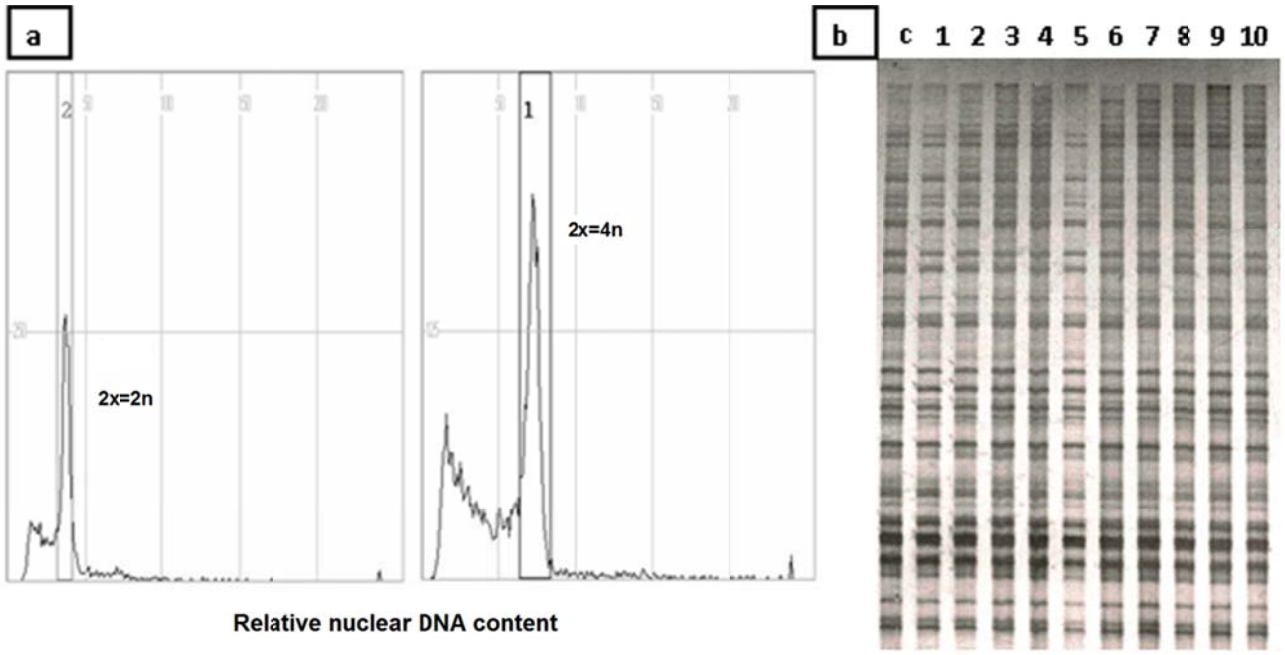

Figure 4. a) Histogram obtained from the results of the flow cytometry analysis of two *in vitro*-regenerated physic nut plants: one diploid and the other tetraploid. **b)** Electrophoretic analysis of DNA amplification through TRAPs molecular markers showing genetic similarities among donor plants **(c)** and *in vitro* shoots (1-10).

2010b, 2011b; Kumar and Reddy, 2010), with rare exceptions, such as 83% obtained by Daud et al. (2013) using woody plant medium containing IBA and phloroglucinol and 86% obtained by Li et al. (2008) using MS medium supplemented with IBA. This difficulty has also been observed in other woody plants. Silva et al. (2005) compared the efficiency between two techniques, shoot rooting and *in vitro* micrografting, for the development and recovery of *in vitro* shoots of the 'Pera' sweet orange. The results showed 58% of plants rooted when the *in vitro* rooting was used and 100% of the plants recovered after *in vitro* micrografting. The high efficiency of the plants recovery in this study showed that *in vitro* micrografting might be a viable alternative as a replacement for the induction of *in vitro* rooting in shoots micropropagated via organogenesis. This is the first report in *J. curcas* micrografting. In addition, the acclimatization of micrografted plants was also considered successful because an average of 76.5% of plants survived. These plants might be transferred to the greenhouse after 30 days of acclimatization.

Ploidy of the regenerated shoots

Flow cytometry analysis of G1 shoots belonging to first group (second subculture) showed that 96% of the regenerated shoots were diploid and 4% were tetraploid (Figure 4a). The analysis performed in the same genotype after the 12th subculture showed the same proportion of tetraploidy plants. We can suggest that the variation in the ploidy level occurred at the beginning of *in vitro* culture and it was perpetuated during ten *in vitro* subcultures of shoots in the elongation medium. The regenerated shoots of the G2 genotype showed no changes in the ploidy level, yielding 100% diploid shoots after two subcultures. The same result was observed in the second group of plants in which 100% of the shoots evaluated after the 7th subculture (G1, G2 and G3 genotypes) were diploid, with ploidy levels identical to those of the donor plants. Kaewpoo and Te-Chato (2010) obtained similar results using epicotyl and hypocotyl *J. curcas* explants for callus and shoot induction. The callus, leaves and stem shoots were analyzed by flow cytometry, showing no variation in ploidy.

DNA polymorphisms in regenerated shoots

In accordance with established methodology in this paper, TRAP molecular markers resulted in a total of 266 bands, with an average of 22.2 bands per *primer* pair. In comparison with the donor plant, four polymorphic fragments were observed (1.5% of the total) in the molecular profile of genotype G1. All polymorphic fragments were in the same shoot sample. This polymorphic sample represented 5% of the total samples evaluated for this genotype. No polymorphism was

detected in the micropropagated shoots of the G2 and G3 genotypes (Figure 4b). The low DNA polymorphism observed in G1 genotype may be related to the high concentration of plant growth regulators used at the beginning of *in vitro* culture process, mainly in the shoot induction stage.

Sharma et al. (2011) observed similar results after evaluating the meristematic explants (axillary shoot buds) of *J. curcas* using others molecular markers (RAPD and AFLP). The authors also related few or no polymorphism among the genotypes studied. Leela et al. (2011) using RAPD analysis also recorded no somaclonal variation of regenerants. In accordance with some authors, the use of pre-existing meristems such as shoot tips and axillary buds from the hardwood shoot cuttings lower the risk of somaclonal variations (Ahuja, 1987; Ostry et al., 1994; Wang and Charles, 1991). These kinds of competent and pre-determined explants have less necessity of high growth regulator concentrations for micropropagation process when compared with organogenesis or somatic embryogenesis process using no meristematic explants. Others factors like type and concentration of plant growth regulators used in the medium, rate of multiplication, formation of adventitious shoots, increased culture period and genotype influence the rate of somaclonal variation (Bairu et al, 2011; Sharma et al., 2011). Our results show that even using non meristematic explants (leaf segments) and indirect regeneration *in vitro* (with prior callus formation) process has been possible to establish efficient and specific protocols for *in vitro* cloning of superior genotypes selected in our breeding program. Besides highly efficient on the shoots induction, these established protocols showed genetic stability of the micropropagules obtained.

Additionally, TRAP molecular markers identified polymorphism among the donor genotypes in 7.1% of the bands. 100% of DNA polymorphism was observed among genotypes with different origins. It is observed that G1 and G2 genotypes, originating from Brazil, showed identical molecular profiles, but both genotypes differed from G3, originating from Mexico. These results indicated the possibility to use these molecular markers for study of genetic diversity in *J. curcas* germplasm.

Kwon et al. (2010) evaluated the genetic diversity of *Vicia faba* L. germplasm and verified that TRAP molecular markers were able to efficiently distinguish divergent groups from different geographical origins. Creste et al. (2010) used TRAP molecular markers with primers for the genes likely involved in sucrose metabolism and the drought response to establish different clusters for 60 sugarcane genotypes. At the same time, similar studies has been realized by our time using fixed primers designed from specific ESTs involved in oil and phorbol esters metabolisms. The results (yet not published) will be helpful for the study of the genetic variability of these traits in our germplasm collection.

The results obtained in this work suggest low somaclonal variation in G1 elite genotype which was found only 4% of tetraploid plants and 1.5% of polimorfic bands. Zero somaclonal variation was founded in G2 and G3 superior genotypes. These variations were detected in beginning stage of organogenesis process. Thus, the results indicate adequate genetic stability and clonal fidelity of micropropageted superior genotypes by protocols now established. The superior traits conservation is very important for commercial production of *J. curcas* clonal variety. In addition, the *in vitro* cloning protocols established can be used to introduce genes of interest into the superior genotypes via genetic engineering as our team have done currently (data yet not published).

Conclusion

The season collection of foliar explants into matrix plants had a great influence in the shoot regeneration of the three studied genotypes. All genotypes had better responses when the leaf explants were collected in September 2012. There was genotype dependence in the organogenesis process: the G1 genotype showed a better shoot regeneration average than the other two genotypes independently of explants season collection. Micrografting proved to be a promising technique as a substitute during *in vitro* rooting of regenerated shoots, with a plant recovery rate of 85%. The evaluation of genetic homogeneity in tissue culture regenerates of *J. curcas* using Flow Cytometer and TRAP molecular marker showed low or no somaclonal variation indicating that the protocol established preserves the clonal fidelity of micropropagated plants.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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