Australian Journal of Crop Science AJCS
AJCS 7(10):1511-1519 (2013) ISSN:1835-2707

Morphogenesis and regeneration of adventitious shoots in *Jatropha curcas* L.

Claudinéia Ferreira Nunes[1*], Dalilhia Nazaré dos Santos[1], Moacir Pasqual[1], Thaís Cainã Teixeira Valente[2], Ana Catarina Lima de Oliveira[1], Eduardo Alves[3], Tesfahun Alemu Setotaw[4]

[1]Federal University of Lavras (UFLA) - Department of Agriculture, Plant Tissue Culture Laboratory - Post Office Box 3037, 37200-000, Lavras, MG, Brazil
[2]Federal University of Lavras (UFLA) – Department of Biology - Post Office Box 3037, 37200-000, Lavras, MG, Brazil
[3]Federal University of Lavras (UFLA) - Department of Plant Pathology - Post Office Box 3037, 37200-000, Lavras, MG, Brazil
[4]Agricultural Research Agency of Minas Gerais (EPAMIG), CEP 37780-000, Caldas, MG, Brazil

***Corresponding author: nunescfr@yahoo.com.br**

Abstract

The successful regeneration of *Jatropha curcas* requires easily reproducible methods. Therefore, this study was undertaken to evaluate the effects of sources of explants, growth regulators, coconut water, nitrogen and flask sealing on the quality and quantity of shoots derived from leaf segments of *Jatropha curcas* after *in vitro* manipulation. The highest morphogenetic responses, 4.8 and 4.56 (numbers of adventitious shoots), were obtained from leaf cotyledon plantlets cultured in half-strength MS medium in the presence of BAP separately or combined with IAA (at a concentration of 4.4 µM of BAP + 2.8 µM of IAA). Enhanced shoot multiplication rates of 2.07 and 1.84 were observed in MS medium without coconut water and with the flask sealed with plastic film or a polypropylene lid with a filter, respectively. This result demonstrated the positive effects of minimum air circulation between the internal and external environments of the culture medium during regeneration. Concentrations of KNO_3 and NH_4NO_3 between 50 to 100 % in MS medium favored the induction of callus and shoot formation at the 60th day of culture. Observations using a scanning electron microscope permitted visualization of dedifferentiated cells, and the reinitiation of meristematic activities resulted in the formation of a unipolar structure called a vegetative primordium. From these results, it is possible to conclude that regeneration efficiency (quality and quantity of material regenerated) in *Jatropha curcas* can be increased with the use of balanced medium constituents, growth regulators and modifications of culture conditions that include the absence of light.

Keywords: Age; Coconut water; Embryo culture; Flask sealing; Growth regulator; *Jatropha curcas*.
Abbreviations: BAP_6-benzylaminopurine, CIV_cotyledonary *in vitro*, CGH_cotyledonary in greenhouse, IAA_indole-3-acetic acid, IBA_indole-3-butyric acid, KNO_3_potassium nitrate, MS_Murashige and Skoog, MGH_mature leaves of plantlets in greenhouse, NH_4NO_3_ammonium nitrate, PF_ plastic film, PLF_polypropylene lid with filter membrane permeable to gases, PL_ polypropylene lid without filter, TDZ_Thidiazuron.

Introduction

The species *Jatropha curcas*, commonly known as the physic nut, is a member of the family Euphorbiaceae and has various desirable characteristics that make it economically important in the agro-energy market due to its high oil content. Physic nuts possess many useful attributes and multiple potential uses, such as preventing soil erosion and being suitable for hedges on farmlands. From its seeds, products are extracted that are useful for the production of insecticides (Silva et al., 2012) soap, candles and pesticides. In addition, pharmacological effects present in its leaves, seeds, and seed coat have been used in preparing homemade medicines (Staubmann et al., 1999). Because of its importance as an energy source and its other attributes, the species has been given special attention by various fields of science, from molecular biology to experimental field work, with the objective of improving its genetic potential and developing appropriate conditions for its commercial propagation. To achieve this objective, tissue culture techniques have provided improved possibilities for micropropagation. The use of explants derived from embryo cultures provides a promising method for micropropagation because propagules taken from the field demonstrate a low regeneration response, in addition to issues from their probable tissue contamination. Mass production of this species using *in vitro* manipulation requires the regeneration of explants capable of responding to stimuli proceeding from a hormonal balance between growth regulators and culture conditions. Some of the advances in establishing an efficient and reproducible system of *in vitro* regeneration in *J. curcas* have been discussed by Sujatha and Mukta (1996), Wei et al. (2004), Kumar and Reddy (2010), Kumar et al. (2010), Khemkladngoen et al. (2011) and Li et al. (2012). These authors developed methodologies for differentiating buds with or without intervention from callus tissue derived from different explants of *J. curcas*. From the

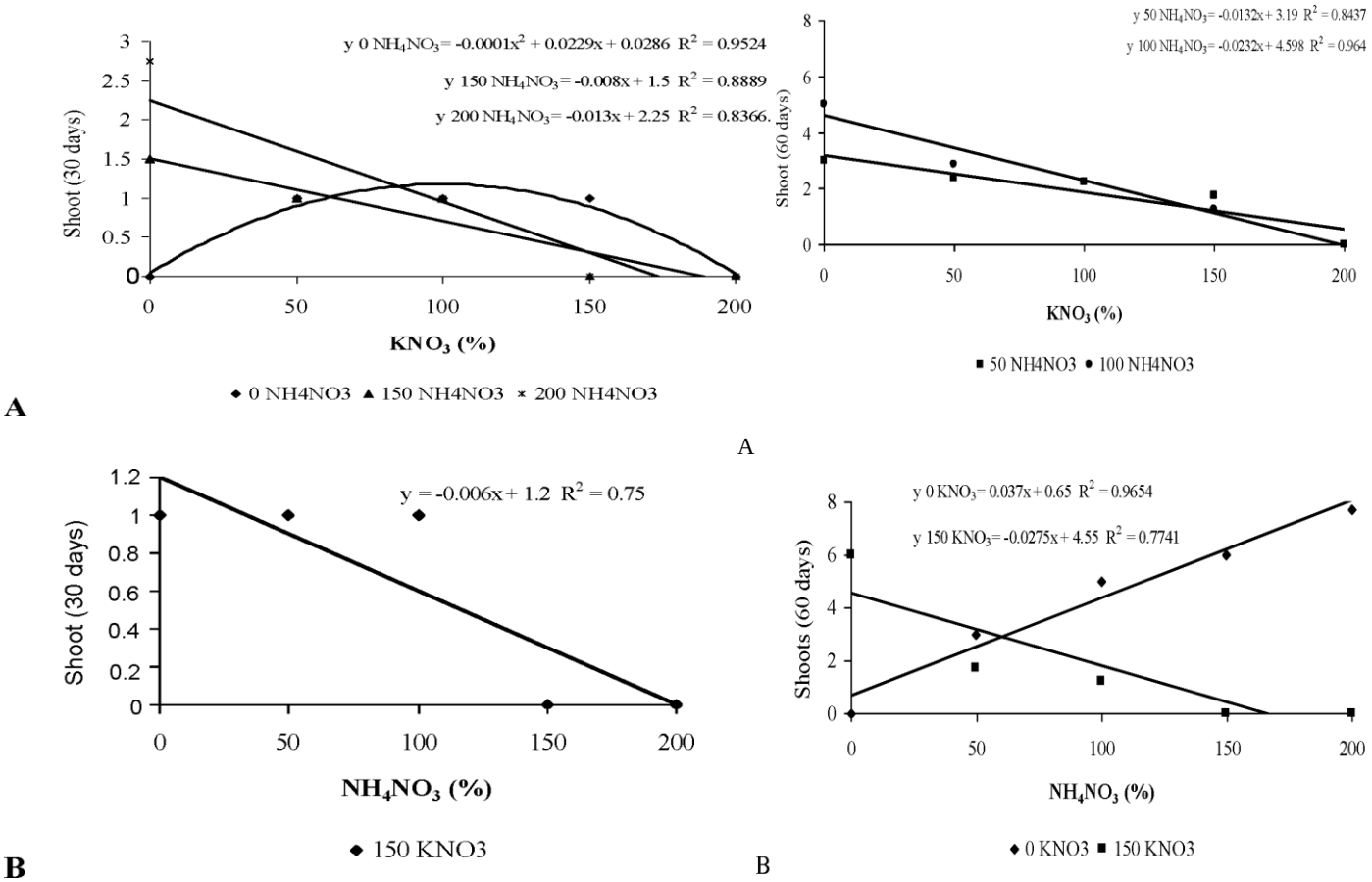

Fig 1. Effect of nitrogen concentrations in MS medium on the formation of shoots in explants of cotyledon leaves of physic nuts (*Jatropha curcas*) cultured *in vitro* for 30 days after inoculation. (A) Interactions between concentrations of NH_4NO_3 with KNO_3 on the number of shoots. (B) The effect of KNO_3 combined with different concentrations of NH_4NO_3 on the number of shoots.

Fig 2. Effect of nitrogen concentrations in MS medium on the formation of shoots in explants of cotyledon leaves of physic nuts (*Jatropha curcas*) cultured *in vitro* for 60 days after inoculation. (A) Interaction between concentrations of NH_4NO_3 with KNO_3 on the number of shoots. (B) Interaction between concentrations of KNO_3 with NH_4NO_3 on the number of shoots.

results presented by the authors, it has been observed that independent of the regeneration method and the source of the explants used, it is possible to achieve the multiplication of *J. curcas in vitro.*The successful regeneration of *J. curcas* has been obtained via organogenesis from different explants, such as from mature leaves (Deore and Johnson, 2008), hypocotyls (Soomro and Memon, 2007; Sharma et al., 2011), epicotyls (Wei et al., 2004), auxiliary nodes (Shrivastava and Banerjee, 2008; Staubmann et al., 1999), petioles (Kumar et al., 2011) and young cotyledons (Khemkladngoen et al., 2011). This body of work has tended to demonstrate that for the successful regeneration of *J. curcas*, the source of the explants is extremely important. According to Camara Machado et al. (1997), the *in vitro* regeneration of *J. curcas* is highly genotype dependent. This characteristic can explain the fact that the methodologies developed by Sujatha and Mukta (1996), Wei et al. (2004), Kumar et al. (2010) and Kumar and Reddy (2010) were not repeatable for the *J. curcas* accessions maintained by the germplasm bank in Brazil. Therefore, more attention should be given to genetic factors. Given these facts, a methodology applicable to the *in vitro* regeneration of *J. curcas* accessions in Brazil is necessary and the present study is the first report of bud induction through the intervention of the callus. The application of these results will accompany the necessary breeding programs for *J. curcas*. As emphasized by Andrade (2003), *in vitro* regeneration will be essential for mass multiplication and genetic transformation. Our projections have indicated that *in vitro* regeneration will soon take its place in breeding programs as a routine process for the propagation of this species, due to its ease of adoption. For the aforementioned reasons, and to explore the regeneration potential of *J. curcas*, it is important to study various factors that are current obstacles to *in vitro* culturing and manipulation. This study was performed with the objective of evaluating the effects of different explants, growth regulators, coconut water, nitrogenous compounds and the sealing of flasks in promoting the regeneration of adventitious shoots in *Jatropha curcas* (L.).

Results

Effects of explant sources on shoot induction

Leaf explants placed in MS medium (Murashige and Skoog, 1962) and supplemented with different proportions of auxins/cytokinins exhibited distinct morphological changes. The explants exhibiting beige, light beige, and dark beige coloration after nine weeks of culture displayed an expansion of leaf margins, callus formation and vegetative primordia, indicating signs of dedifferentiation. Maximum light beige coloration was observed on the leaf cotyledon explants of

Table 1. Combinations of growth regulators applied to different types of explants. Coloration of callus (coloration), presence of callus that generates adventitious shoots (PAS) and numbers of adventitious shoots (NAS) originating from cotyledon leaves of plantlets *in vitro* (CIV), cotyledon leaves (CGH) and mature leaves originating from greenhouse plants (MGH) of *Jatropha curcas* cultured in MS medium with different concentrations of growth regulators. Note reference to coloration of callus: (0) dark beige (1) beige and (2) light beige*.

	Types of explants								
Combinations of growth regulators (μM)	(CGH)	(MGH)	(CIV)	(CGH)	(MGH)	(CIV)	(CGH)	(MGH)	(CIV)
	Coloration			PAS			NAS		
1 -TDZ 2.3 + BAP 2.2 + AIB 0.5	2.00 aA	2.00 aA	1.00 bA	1.00 aA	0.00 bB	1.00 aA	0.64 cB	0.32 cB	2.92 bA
2 - TDZ 4.5	2.00 aA	1.00 aA	2.00 aA	1.00 aA	1.00 aA	1.00 aA	0.96 cB	4.12 aA	1.40 cB
3 - BAP 4.4	2.00 aA	1.00 aA	2.00 aA	1.00 aA	1.00 aA	1.00 aA	4.80 aA	2.64 bB	4.80 aA
4 - TDZ 4.5 + AIB 2.5	0.00 cB	0.00 cB	2.00 aA	0.00 bA	0.00 bA	0.00 bA	0.00 dA	0.00 cA	0.00 dA
5 - TDZ 9.0 + AIB 5.0	0.00 cA	0.00 cA	0.00 cA	0.00 bA	0.00 bA	0.00 bA	0.00 dA	0.00 cA	0.00 dA
6 - BAP 4.4 + AIB 2.5	0.00 cB	1.00 bA	1.00 bA	0.00 bA	0.00 bA	0.00 bA	0.00 dA	0.00 cA	0.00 dA
7 - BAP 8.8 + AIB 5.0	0.00 cA	0.00 bA	0.00 cA	0.00 bA	0.00 bA	0.00 bA	0.00 dA	0.00 cA	0.00 dA
8 - TDZ 4.5 + IAA 2.8	1.00 bA	2.00 aA	1.00 bA	1.00 aA	1.00 aA	1.00 aA	0.84 cB	1.60 bB	3.40 bA
9 - TDZ 9.0 + IAA 5.7	1.00 bB	2.00 aA	2.00 aA	1.00 aA	1.00 aA	1.00 aA	1.00 dC	2.00 bB	3.60 bA
10 - BAP 4.4 + IAA 2.8	2.00 aA	1.00 aB	2.00 aA	1.00 aA	1.00 aA	1.00 aA	3.60 bB	3.40 aB	4.56 aA
11 - BAP 8.8 + IAA 5.7	1.00 bB	1.00 aB	2.00 aA	1.00 aA	1.00 aA	1.00 aA	0.84 cC	2.00 bB	4.16 aA

* Means followed by different letters, small letters in the horizontal direction and capital letters in the vertical direction, within each variable evaluated represent significant differences among them at 5% probability using the Scott-Knott test.

Table 2. Influence of concentrations of coconut water associated with culture flasks. Percentage of shoots (buds) (PS), number of shoots (buds) (NS), length of shoots (buds) (LS), leaf percentages (LP) and percentages of root primordia (PRP) in explants of leaf cotyledons of physic nuts (*Jatropha curcas*) cultured in MS medium with different concentrations of coconut water and sealing: plastic film (PF), polypropylene lid without filter (PL) and polypropylene lid with membrane filter permeable to gases (PLF)*

	(PF)	(PL)	(PLF)
Coconut water (mLL^{-1})		PB (%)	
0	96.34 aA	39.53 aB	47.12 aB
50	34.21 bA	32.81 aA	41.40 aA
100	0.00 cB	25.08 aA	0.00 bB
150	0.00 cA	0.00 bA	0.00 bA
200	0.00 cA	0.00 bA	0.00 bA
		NS	
0	2.07 aA	1.05 aB	1.84 aA
50	0.60 bA	0.57 aA	0.62 bA
100	0.00 bA	0.56 aA	0.00 bA
150	0.00 bA	0.00 bA	0.00 bA
200	0.00 bA	0.00 bA	0.00 bA
		LS (cm)	
0	1.59 aA	1.29 aA	1.82 aA
50	1.35 aA	0.36 bB	0.95 bA
100	0.00 bA	0.00 bA	0.00 cA
150	0.00 bA	0.00 bA	0.00 cA
200	0.00 bA	0.00 bA	0.00 cA
		LP (%)	
0	52.81 aA	23.36 aC	45.30 aB
50	23.98 bA	0.00 bB	28.12 bA
100	0.00 cA	0.00 bA	0.00 cA
150	0.00 cA	0.00 bA	0.00 cA
200	0.00 cA	0.00 bA	0.00 cA
		PRP (%)	
0	0.00 bA	0.00 bA	0.00 bA
50	25.08 aA	0.00 bB	28.12 aA
100	27.34 aA	22.81 aB	0.00 bC
150	0.00 bA	0.00 bA	0.00 bA
200	0.00 bA	0.00 bA	0.00 bA

* Means followed by different letters, small letters in the vertical direction and capital letters in the horizontal direction, differ significantly using the Scott-Knott test at 5% probability.

plantlets *in vitro* (CIV), followed by the leaf cotyledons of plants from the greenhouse (CGH). The lowest intensity of coloring was observed on explants of mature leaves taken from the greenhouse (MGH). Abundant beige coloration was observed on explants of mature leaves, similar to those of other explants. Leaves (CGH) with dark beige coloration produced the highest number of explants, in contrast with the explants of leaves (CIV) that displayed the lowest levels of dark beige color (Table 1). The absence of light and the growth regulators TDZ and BAP combined with IAA were crucial for promoting the dedifferentiation of the explants and for consequently restarting meristematic activity in the adventitious shoots (Table 1). A higher number of shoots was observed in leaf explants (CIV), followed by leaf explants (CGH) and (MGH) in the presence of isolates of 4.4 µM BAP and/or combined with 2.8 µM IAA (Table 1). The performance of explants in treatments cultured with the presence of IBA did not correspond to our expectations because these explants displayed a low frequency of shoots or the absence of organogenetic competence.

Effects of coconut water and flask sealing on shoot induction

A high percentage of shoots (96.34%) was obtained in flasks sealed with PF in the absence of coconut water. In contrast, the lowest percentage of shoots (25.08%) was observed in flasks sealed with PLF with a concentration of 100 $mL.L^{-1}$ of coconut water (Table 2). For this variable, increases in the concentration of coconut water resulted in a reduction and/or absence of shoots. The presence of coconut water most likely caused a disequilibrium of nutrients in the culture medium that hampered the induction of shoots. Similar responses were also observed for the numbers of shoots (Table 2), where the highest number of shoots was observed in flasks without coconut water that were sealed with PF (2.07/explants) and PLF (1.84/explants). A more favorable response for shoot length was obtained in the absence of coconut water using PLF (1.82 cm), followed by PF (1.59 cm) and PL (1.29 cm) (Table 2). The highest percentage of explants with numerous leaves (Table 2) was observed in flasks without coconut water that were sealed with PF (52.81%), and the lowest was observed in 50 $mL.L^{-1}$ of coconut water sealed with PF and PLF. The most favorable response for root primordia was observed using a combination of 50 $mL.L^{-1}$ of coconut water with PLF and PF (28.12 and 25.08%, respectively), while 27.34% was observed using concentrations of 100 $mL.L^{-1}$ of coconut water sealed with PF (Table 2).

Effects of ammonium/nitrate ratios on shoot induction

The concentration of nitrogen in the MS medium significantly influenced the shoot induction response at the concentrations tested. It is also possible to observe regenerative capacity in the explants after 30 days of culturing in that this competency is maximized after 60 days of culturing. The highest numbers of shoots (1.5 and 2.3) were recorded after 30 days of culturing, and these values increased to 3.0 and 5.0 after 60 days of culturing. These results confirmed the significance of variations in the nitrogen concentration (N) (Figs. 1a and 2a) and in other essential components of the MS medium during this influential period in the process of plant development. High concentrations of KNO_3 combined with low concentrations of NH_4NO_3 were not necessarily sufficient for promoting regeneration because this combination corresponded with a low number of shoots (1.2) (Fig. 1b). Quadratic behavior was also noted that in the absence of NH_4NO_3 combined with KNO_3 and estimated by a regression equation, and the maximum number of shoots (1.3) corresponded to 100% KNO_3 (Fig 1a). The increased concentration of KNO_3, up to 100%, promoted a significant increase in the number of shoots. However, concentrations of KNO_3 higher than 100% can be toxic and can reduce the number of shoots. Concentrations less than 50% were not efficient in producing high numbers of shoots in explants of *J. curcas*. In contrast, the best responses in number of shoots (7.0) were observed in the absence of KNO_3 combined with increasing percentages of NH_4NO_3 (Fig. 2b). In addition, the presence of KNO_3 at a 150% concentration in combination with decreasing percentages of NH_4NO_3 negatively affected the number of shoots (1.0).

Micromorphological aspects of callus tissue

Evaluation of callus morphology was performed using scanning electronic microscopy (SEM), which permitted a clear visualization of cellular changes (Figs. 3a-f). SEM analysis showed evidence that the presence of BAP separately or combined with IAA (at a concentration of 4.4 µM of BAP + 2.8 µM of IAA), when applied to explants that originated from the cotyledon leaves of plantlets *in vitro*, demonstrated high regenerative competency when compared with the other explants evaluated (Figs. 3c-f). It can be inferred that the cytokinin BAP more efficiently promotes the regenerative success of *J. curcas* through organogenesis and that it also influences the regulation of cell division. Cytokinin BAP stimulated and regulated the process of new shoot formation. It is also possible to observe regenerative competency in explants in the absence of or at a minimum concentration of coconut water in flasks, which permits minimal gas circulation from the external medium (Figs. 3a-b). These treatments can be considered crucial to signaling cellular dedifferentiation and consequently restarting the meristematic characteristics that favor regeneration in this species.

Discussion

In this study, the source of the explants and cultures significantly influenced the shoot induction and regeneration responses. The results of this experiment demonstrated that incubation in dark conditions could reduce the degradation of endogenous and/or exogenous growth regulators and could improve *in vitro* growth and development (Ibrahim and Debergh, 2001). The results obtained in this experiment supported the response of callus regeneration from leaf explants obtained using the same growth regulator for this species (Staubmann et al., 1999). Independently or in combination, the growth regulators can induce callus formation. However, the regenerative competence of the callus tissue can differ depending on the type of explants used because certain types of plant tissue have more favorable regeneration responses than others. Based on the results obtained in this study, it can be inferred that explants taken during the early stages of development (e.g., cotyledon leaves) showed higher totipotentiality than did explants taken during advanced stages of development, such as mature leaves. Similar results were reported by Kumar and Reddy (2010), who reported an *in vitro* explant of *J. curcas* with a high rate of regeneration and shoot number compared with *in vivo* explants. Gray (2004) offered an explanation for this type of response. For this author, this response occurred due to differences in the concentrations of endogenous growth

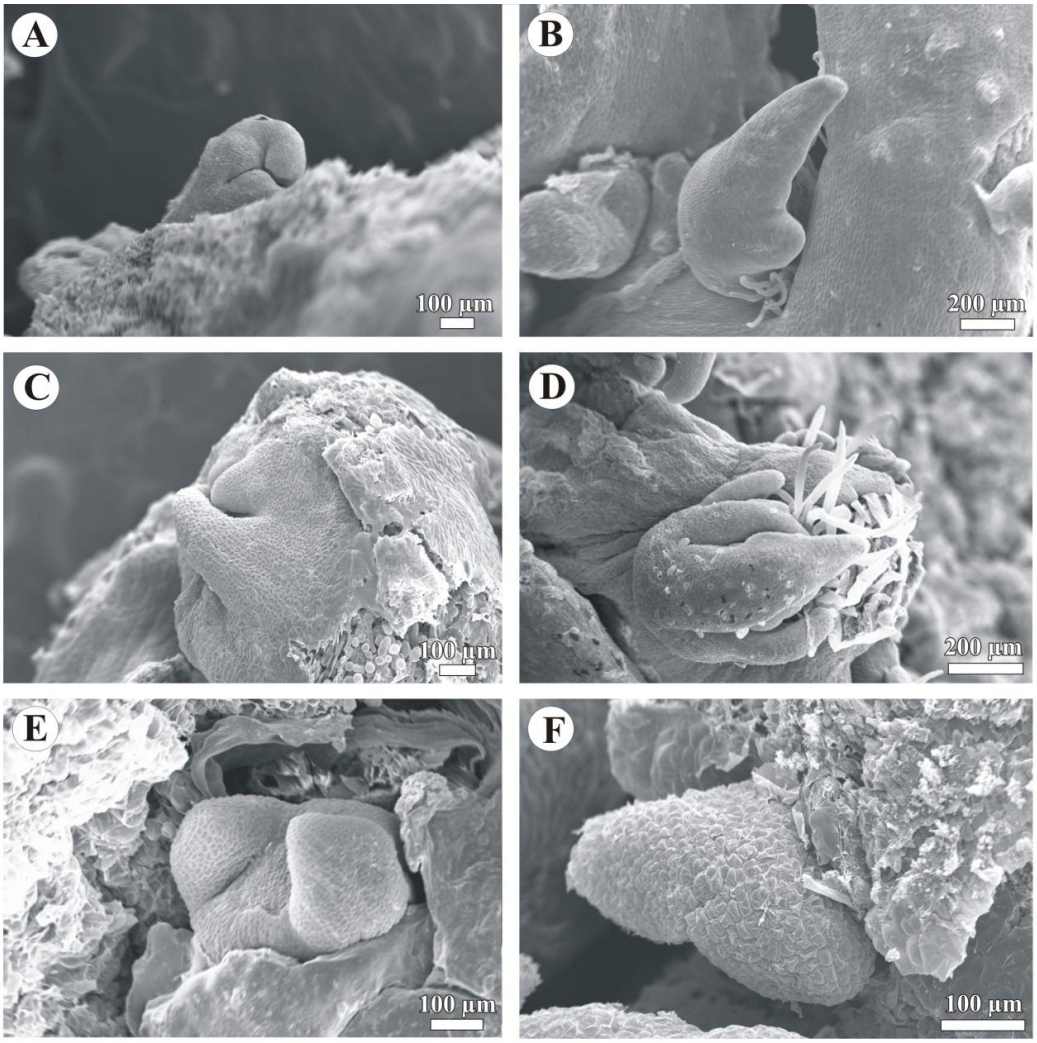

Fig 3. Dedifferentiation of the callus originated from explants of leaf cotyledons of *Jatropha curcas* incubated in the absence of light and visualized using scanning electron microscopy (a-b). Development of vegetative primordia of cotyledon leaves of plantlets *in vitro*, cultured in MS medium in the absence of coconut water and sealed with plastic film; (c-f) callus cultured in the presence of 4.4 µM of BAP + 2.8 µM of IAA.

regulators and the metabolism of the explant. The results obtained in this study regarding the use of growth regulators are consistent with the reports of Rajore and Batra (2005), who obtained high numbers of shoots from the apical segment of *J. curcas* with the addition of IAA and BAP to the culture medium. Similarly, Shrivastava and Banerjee (2008) also obtained a satisfactory number of shoots from axillary explants from *J. curcas* using 13.3 µM of BAP + 4.9 µM IBA in MS medium. These results highlighted the significance of using leaf cotyledons as a viable source for obtaining a morphogenetic response. It was also revealed that an adequate proportion of growth regulators for manipulating and optimizing the quality and number of adventitious shoots in *in vitro* cultures of *J. curcas* was important. Shoot induction also suffered from the influence of coconut water and container sealing. The present study demonstrated that the absence or presence of coconut water in minimal concentrations, in flasks sealed with plastic film (PF) and polypropylene lids with filters (PLF), positively influenced the efficiency of callus induction and shoot regeneration in leaf explants of *J. curcas.* In contrast, increasing the concentration of coconut water in flasks sealed with polypropylene lids without filters (PL) did not show a positive effect on callus induction and shoot regeneration. The minimum response was observed for most of the variables measured, except for the number of root primordia, which increased with increasing concentrations of coconut water. Therefore, it can be inferred that the absence of regeneration in the explants was due to the use of coconut water in the culture medium. Similar observations were reported for apical stems and leaf segments from *J. curcas*, which produced 1 to 3 shoots per explant (Rajore and Batra, 2005; Deore and Johnson, 2008), supporting the results obtained in this study. Studying the effects of coconut water concentration and flask sealing showed that high concentrations of coconut water are not advantageous for *in vitro* regeneration of this species because the number of shoots formed was significantly lower and morphologically inferior in treatments with high concentrations of coconut water. Our results showed that the absence of 50 $mL.L^{-1}$ of coconut water in the culture medium in flasks sealed with

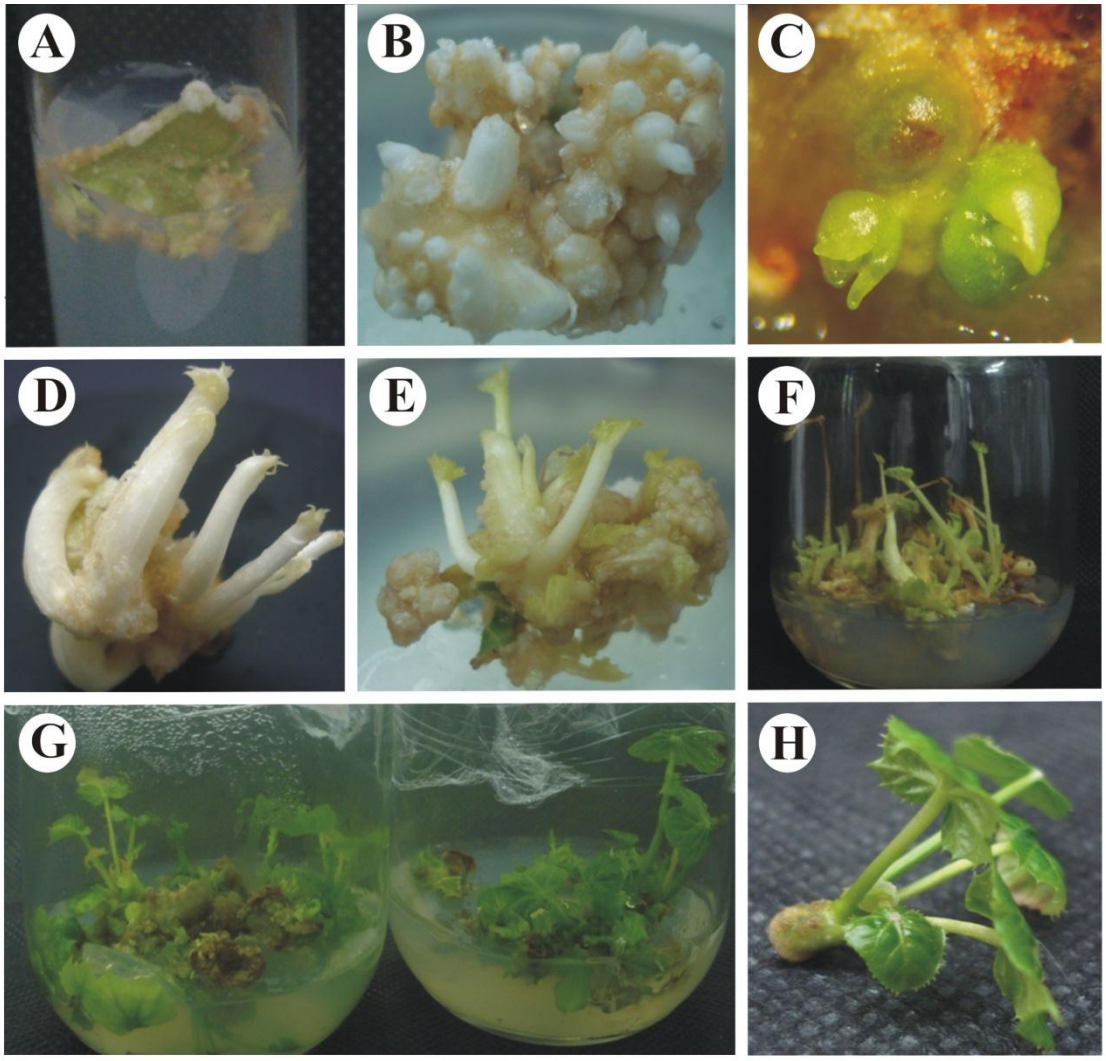

Fig 4. Callus induction and regeneration of plants from embryo cultures of *J. curcas*. (a) Induction of callus from explants of leaf cotyledons of plantlets *in vitro*; (b) morphogenesis showing induction of buds; (c) aspects of buds; (d-e) bud elongation; (f) plant regeneration from shoots; (g) multiplication and elongation of shoots and (h) regenerated shoot isolated for root induction.

plastic film or lids with polypropylene membranes is recommended for successful regeneration in *J. curcas*. The inhibiting effects of coconut water during the induction of regeneration in *J. curcas* can be explained by the presence of high proportions of glucose and mineral salts, combined with the existing constituents of MS medium, which is already rich in mineral salts and other elements necessary for the formation and development of *in vitro* cultures. In general, the presence of culture medium in flasks with adequate concentrations of water produced high internal relative humidity. The high humidity in the flask with the PL can affect the growth and morphogenesis of *in vitro* cultures and resulted in a low growth rate for explants. There is information to consider with regard to the influence of nitrogen concentrations on the culture medium. The source of the N should be considered during the application of culture medium because N given in the form of nitrate is only abundant at the initial phase of explant establishment. However, as the explants grew, the medium began to be deficient in this nutrient. When the N is provided in the form of nitrate and ammonia, N in the form of nitrate is consumed more rapidly at the beginning, whereas later, N is made available in the form of ammonia for consumption by the explant. This result can justify the necessity of combining both nitrogen sources in the *in vitro* culture. It is likely that the combination of a high dose of NH_4NO_3 with a low to zero dose of KNO_3 positively affects shoot formation because nitrogen effectively contributes to cellular metabolism and to the regulation of the osmotic potential of the culture medium. The response obtained supports the hypothesis that most of the plants prioritize the absorption of NH_4^+ over NO_3^-. However, the inverse situation is also true, and this situation can be observed in Figure 1a. From this result, it can be inferred that the level of inorganic nitrogen in the *in vitro* culture substrate influenced the metabolic processes in the culture that effect tissue growth and differentiation. The influence of nitrogen concentrations on plant cell and organ culture systems designed to produce proliferation of organs and metabolites was reported for various plant species, such as *Withania somnifera* (Nagella and Murthy, 2011), *Bacopa monnieri* (Naik et al., 2011) and *Panax ginseng* (Yu et al., 2001). This observation demonstrated that the ammonium/ nitrate ratio controls the pH of the growth media, stimulates morphogenesis and is therefore important for inducing callus formation in many species. However, all of the aforementioned effects of the culture media differ from one

species to another and from one compound to another (Aoki et al., 1997). Microscopy allows clear observations to be made of morphogenetically important states, such as the conversion of disorganized structures into the elongated arrangements that characterize a prominently meristematic stem cell. The dedifferentiation phase was resuming instantly the red-differentiation phase marked by induction of shoot with subsequent development. This observation validates the results from this study that certified that only those areal parts that were successfully produced during regeneration and that can originate a complete plant were important. Wrongly interpreting the formation of leaf primordia and/or roots as being shoots affects the success of *in vitro* cultures. It is known that cells are autonomous and have the potential to regenerate complete plants if they receive adequate treatments that would support the results obtained in this study. Based on these results, a "step by step" procedure can be developed for the induction of adventitious shoots that would be capable of continuing on with subsequent development. To obtain shoots from the superficial portion of the explants, the explant can be cultured in a dark room for nine weeks and can then be transferred to a lit growth chamber that would allow for the continued development of the regenerated explant with the subsequent formation of vegetative primordia as presented in Figures 4a-h. The development of shoots during the initial stages and of shoot elongation after 2 months occurs in half-strength MS medium in the presence of 150 % NH_4NO_3, 50% KNO_3, vitamin B5 and the growth regulators BAP and IAA (Fig. 4c). The images presented in Figures (4a-h) illustrate the concept of organogenesis *in vitro*, which is defined as a process in which plant cells and tissues are induced to make changes that produce a unipolar structure called a vegetative primordium. The vascular system of this structure is connected to the original tissue. Various authors have observed vegetative primordia from various types of explants during the formation of callus tissue in *J. curcas* (Deore and Johnson, 2008; Singh et al., 2010; Kumar et al., 2011). After 2 months, the explants were again subcultured and transferred to culture medium for another two months to potentialize the multiplication phase (Fig. 4g). During this phase, the explant cells gained higher competency and the ability to respond to hormones, consequently increasing the induction of organs. During this phase, the authors visualized multiple formations and the growth of vegetative organs under the influence of the balanced nutrition investigated in this study. Finally, shoots approximately 3 cm in length were separated and cultured in MS medium in the absence of growth regulators (Fig. 4h). These shoots displayed defined structures and the ability to respond to rooting stimulants during the final phase of the process of plant formation. In this manner, the process of manipulating tissue cultures of *J. curcas* can be exploited to maximize multiplication through *in vitro* organogenesis. In addition, these results demonstrated a gain in the number of shoots/explants that had significant advantages because this gain is an important growth parameter if the objective is to have a high multiplication rate for large-scale production of the plant. The present work achieved satisfactory results demonstrating that the methodology applied can be used for the multiplication of *J. curcas*. The techniques support the production system for the species to be cultivated and achieve economic relevance. In addition, the research facilitates investigations into the genetic improvement and genetic transformation of the species. The information generated *in vitro* during this study can be used by producers, breeders, geneticists and the pharmaceutical industries as an additional tool for producing economic benefits.

Materials and Methods

Sources of explants

Seeds of the matrix plant variety Oracília were collected from the germplasm bank of *J. curcas* located in the town of Janaúba, Minas Gerais, Brazil. After the seed coats were removed, the seeds were disinfected with a solution containing 70% v/v alcohol and 1% v/v sodium hypochlorite. They were then washed three times using distilled water. The embryos were extracted from the seeds and placed in MS medium (Murashige and Skoog, 1962) to produce the plantlets. The plantlets obtained from the culture were used to extract the following explants: leaf cotyledonary obtained from plantlets originated from *in vitro* (CIV) germination after ten days of culturing, and leaf cotyledonary (CGH) and mature leaves (MGH) (3^{rd} leaf from apex) of plantlets after 30 days of acclimatization in the greenhouse. The leaf segment from the central part of the leaf explants was used for callus induction and regeneration. Three separate experiments were conducted to select the source of the explants, to investigate the effects of coconut water and flask sealing and to investigate the effects of ammonium/nitrate ratios on shoot induction.

Culture conditions

The abaxial side of the explants was placed in the MS culture medium. The MS medium was prepared by adding 50% salt, 3% sucrose and vitamin B5 (Gamborg et al., 1968). The medium was then solidified with 0.6% agar and adjusted to pH 5.8 before being autoclaved at 121 ºC for 20 min. The culture was maintained in a dark growth room at a temperature of 25±2ºC. After nine weeks of incubation, the rates of callus induction and shoot proliferation were determined based on the coloring of the callus, the presence of regenerative callus, the number of shoots/explant, the average length of the shoots, the percentage of explants with shoots and the number of leaf and root primordia/flask. A scale of 0 to 2 was used as the criterion for evaluating the percentages for each variable, where (0) corresponded to an absence of response (0%), (1) corresponded to 50% and (2) corresponded to a 100% response for the variables. The same scale was also used to quantify the coloring of the callus, which measures the level of regenerative development, where (0) referred to dark beige, (1) was beige and (2) was light beige. Dark beige represented a deep black coloring of the callus but was not oxidized. All of the data were subjected to ANOVA and the Scott-Knott mean comparison test using polynomial regression. All statistical analysis was done using SISVAR® (Ferreira, 2011).

Explant source affecting shoot induction

Leaf segments obtained from plantlets *in vitro* after 10 days of culturing, leaf cotyledonary and mature leaves (3^{rd} leaf from the apex) of plants in the greenhouse after 30 days of acclimatization were placed in MS culture medium supplemented with different combinations of growth regulators: (thidiazuron) - TDZ, (6-benzylaminopurine) – BAP, (indole-3-butyric acid) - IBA and (indole-3-acetic acid) - IAA in µM: (1) 2.3 TDZ + 2.2 BAP + 0.5 IBA (Deore and Johnson, 2008), (2) 4.5 TDZ, (3) 4.4 BAP, (4) 4.5 TDZ + 2.5 IBA, (5) 9.0 TDZ + 5.0 IBA, (6) 4.4 BAP + 2.5 IBA, (7) 8.8 BAP + 5.0 IBA, (8) 4.5 TDZ + 2.8 IAA, (9) 9.0 TDZ + 5.7 IAA, (10) 4.4 BAP + 2.8 IAA and (11) 8.8 BAP + 5.7 IAA. The experiment was designed as an 11 x 3 factorial with a

completely randomized design using five replications with five tubes in each replication.

Coconut water and flask sealing effects on shoot induction

Leaf cotyledonary taken from plantlets *in vitro* after 10 days of culture were placed in a 300 mL glass flask containing 40 mL/flask of MS culture medium with 13.3 µM of BAP and 17 µM IAA. The culture medium treatments were also supplemented with 0, 50, 100, 150 and 200 $mL.L^{-1}$ of coconut water derived from fruits and sealed with transparent plastic film (PF), polypropylene lids with filter membranes permeable to gases (PLF) and polypropylene lids without filters (PL). The experiment was designed as a 5x3 factorial using a completely randomized design with eight replications. Each replication consisted of four explants.

Ammonium/nitrate ratio effects on shoot induction

Leaf cotyledonary derived from plantlets pre-established *in vitro* after 10 days of culture were placed in tubes (25 x 150 mm) containing 15 mL of MS culture medium with half the salt and without ammonium nitrate salt (NH_4NO_3) and potassium nitrate (KNO_3). The other components of the treatments included interaction with concentrations of 0, 50, 100, 150, and 200% NH_4NO_3 and KNO_3 in MS medium with 4.4 µM BAP and 2.8 µM IAA. A 5x5 factorial completely randomized design with four replications was used. Each replication contained three tubes.

Micromorphological aspects of callus

To achieve a high resolution of the external morphology of the callus regenerated, an analysis was performed using a scanning electron microscope. To confirm if the regenerated callus contained meristematic cells with regenerative competence, *in vitro* culture samples were collected and dehydrated by adding ethanol until reaching the critical point. Consequently, the samples were coated with metal and observed with a Zeiss DSM 940 scanning electron microscope from the Microscopy Laboratory of the Department of Plant Pathology, according to the established protocol (Alves, 2004).

Conclusion

The absence of light and the specific culture conditions are the parameters that influence the process of induction and the morphogenetic response. To obtain these responses, the present study recommends the utilization of explants of leaf cotyledonary *in vitro* because they produced greater regeneration compared with mature explants. The use of the growth regulator BAP produced highly significant regenerative responses in combination with MS medium without coconut water, 50% KNO_3 and 100% NH_4NO_3 and cultures maintained in flasks sealed with plastic film or lids with polypropylene filters. The characteristics of shoot formations on the surfaces of explants of *J. curcas* can be used as descriptors of cellular dedifferentiation and the subsequent initiation of the meristematic characteristics that confirm the regeneration of explants through organogenesis.

Acknowledgements

The authors would like to acknowledge the financial support of the Foundation that supports Research in Minas Gerais State (FAPEMIG) and the Coordination for the Improvement of Personnel in Higher Education (CAPES) for providing a post-graduate fellowship.

References

Alves E (2004) Introdução à microscopia eletrônica. Lavras: UFLA/FAEPE: 88

Andrade SRM (2003) Transformação de Plantas. Planaltina DF:Embrapa Cerrados (Embrapa Cerrados. Documentos 102), 8p

Aoki T, Matsumoto H, Asako Y, Matsunaga Y, Shimomura K (1997) Variation of alkaloid productivity among several clones of hairy roots and regenerated plants of *Atropa belladonna* transformed with *Agrobacterium rhizogenes* 15834. Plant Cell Rep 16:282–286

da Camara Machado A, Frick NS, Kremen R, Katinger H, da Camara Machado ML (1997) Biotechnological approaches to the improvement of *Jatropha curcas*. In: Proceedings of the international symposium on *Jatropha*, Nicaragua

Deore AC, Johnson TS (2008) High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. Plant Biotechnol Rep 2: 7-11

Ferreira D (2011) SISVAR: A computer statistical analysis system. Ciência Agrotecnologia 35: 1039-1042

Gamborg O, Miller R, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158

Gray WM (2004) Hormonal regulation of plant growth and development. PLoS Biol 2:1270–1273

Ibrahim R, Debergh PC (2001) Factors controlling high efficiency adventitious bud formation and plant regeneration from in vitro leaf explants of roses (*Rosa hybrida* L.). Sci Hortic 88: 41–57

Khemkladngoen N, Cartagena J, Shibagaki N, Fukui K (2011) Adventitious shoot regeneration from juvenile cotyledons of a biodiesel producing Plant, *Jatropha curcas* L. J Biosci Bioeng 111: 67-70

Kumar N, Anand VKG, Reddy MP (2011) In vitro regeneration from petiole explants of non-toxic *Jatropha curcas*. Ind Crop Prod 33: 146-151

Kumar N, Reddy MP (2010) Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant. Ann Appl Biol 156:367–375

Kumar N, Vijay Anand KG, Reddy MP (2010) Shoot regeneration from cotyledonary leaf explants of *Jatropha curcas*: a biodiesel plant. Acta Physiol Plant 32:917–924

Li Zhong-Guang, Gong M, Yang Shi-Zhong, Long Wei-Biao (2012) Efficient callus induction and indirect plant regeneration from various tissues of *Jatropha curcas*. Afr J Biotechnol 11: 7843-7849

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15: 473- 479

Nagella P, Murthy HN (2011) Effects of macroelements and nitrogen source on biomass accumulation and withanolide-A production from cell suspension cultures of *Withania somnifera* (L.) Dunal. Plant Cell Tiss Organ Cult 104:119–124

Naik PM, Manohar SH, Murthy HN (2011) Effects of macro elements and nitrogen source on biomass accumulation and bacoside A production from adventitious shoot cultures of *Bacopa monnieri* (L.). Acta Physiol Plant 33:1553-1557

Rajore S, Batra A (2005) Efficient plant regeneration via shoot tip explant in *Jatropha curcas*. J Plant Biochem Biot 14: 73-75

Sharma S, Pamidimarri DVNS, Anand KGV, Reddya MP (2011) Assessment of genetic stability in micropropagules of *Jatropha curcas* genotypes by RAPD and AFLP analysis. Ind Crop Prod 34: 1003-1009

Shrivastava S, Banerjee M (2008) In vitro clonal propagation of physic nut (*Jatropha curcas* L .): Influence of additives. Int J Integr Biol. 3: 73-79

Silva GN, Faroni LRA, Sousa AH, Freitas RS (2012) Bioactivity of *Jatropha curcas* L. to insect pests of stored products. J Stored Prod Res 48:111-113

Singh A, Reddy MP, Chikara J, Singh S (2010) A simple regeneration protocol from stem explants of *Jatropha curcas*—A biodiesel plant. Ind Crop Prod 31: 209-213

Soomro R, Memon RA (2007) Establishment of callus and suspension culture in *Jatropha curcas*. Pak J Bot 39: 2431-2441

Staubmann R, Ncube I, Gubitz GM, Steiner W, Read JS (1999) Esterase and lipase activity in *Jatropha curcas* L. Seeds J Biotechnol 75: 117-126

Sujatha M, Mukta N (1996) Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas*. Plant Cell Tiss Org Cult 44:135-141

Wei Q, Lu W, Liao Y, Pan S, Tang L, Chen F (2004) Plant regeneration from epicotyl explant of *Jatropha curcas*. J Plant Physiol Mol Biol 30:475-478

Yu KW, Gao WY, Hahn EJ, Paek KY (2001) Effects of macro elements and nitrogen source on adventitious root growth and ginsenoside production in ginseng (*Panax ginseng* C. A. Meyer). J Plant Biol 44:179–184