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Full Length Research Paper

Analysis of organogenic competence of cotyledons of *Jatropha curcas* and their *in vitro* histological behavior

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Using cut pieces cotyledons from germinating zygotic embryos of *Jatropha curcas*, we monitored the series of anatomical events leading to the generation of shoot through organogenesis by histological analysis. 14 days old cotyledons that were pre-cultured in a half strength Murashige and Skoog (MS) media supplemented with 100 mg/L myoinositol and 10 mg/L thiamine HCl, were cultured in organogenic competence induction media (CIM) comprising of MS salts with 1.5 mg/L benzyl adenine (BA) and 0.05 mg/L indole-3-butyric acid (IBA) and incubated for the induction of organogenic competence. Following their sequential transfer to shoot induction media (containing MS + 1.5 mg/L BA, 0.05 mg/L IBA and 0.5 mg/L GA_3); shoot elongation media (containing MS + 0.3 mg/L BA) and rooting media (containing half strength MS + IBA at different concentrations), we selected individual explants and subjected them to histological analysis in order to study the morphological changes occurring during organogenesis. Our findings show that to induce organogenesis using cut pieces of cotyledons, these tissues must be harvested at least by the 14th day following germination. Optimal organogenic competence was attained after 21 days incubation period in the dark where most of the explants showed evidence of protruding shoots surrounded by calli with various morphological features. Up to 53.91% of the total explants cultured in this study produced well defined shoots with each explant producing an average number of 1.5 shoots bearing two to eight leaves. We recorded the highest percentage of root formation, which stood at 27.50% when the shoots were cultured in a rooting media containing 0.3 mg/L IBA. Histological analysis of the different events occurring during the process of organogenesis suggest that the protuberances arising from parenchymatous cells and perhaps bundle sheath forming meristematic centres acquiring organogenic competence have a multicellular origin, indicating that the regeneration process takes place through direct organogenesis.

Key words: *Jatropha curcas*, organogenesis, auxins, histological analysis.

INTRODUCTION

The growing concern over the diminishing supply of fossil fuel and increase in emissions of greenhouse gases have resulted in a corresponding increased interest in the use of cellulosic biomass to meet energy challenges through the exploration and utilization of non food sources of biofuels like *Jatropha curcas* (Carroll and Somerville, 2008; Lynd et al., 2008). A drought resistant shrub belonging to the Euphorbiaceae family, the plant is widely adapted to many regions of the world. It possesses enormous potentials as a source of biofuel and as a possible source of animal feed, if its residue of oil extraction is detoxified (Fairless, 2007). However, little is known about its physiology and biochemistry to allow for its manipulation using biotechnological tools capable of improving some of its desirable qualities. Typically, the goal of biotechnology based programs in *J. curcas* is to increase the levels of oils produced by the plant on one hand, and reduce the level of its toxic substances, on the other (Ye et al., 2009; Costa et al., 2010). An important starting point in achieving this is the development of regeneration protocols suitable for mass propagation of

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the plant, to be used in molecular and genetic studies. Although there are a number of reports on the regeneration of the plant (Kalimuthu et al., 2007; Deore and Johnson, 2008; Li et al., 2008; Khurana-Kaul et al., 2010; Varshney and Johnson, 2010), further studies are still required in order to standardize, and in some cases validate, the different regeneration systems reported so far. For example, only recently has a report on histological investigations of the origin and developmental stages of morphogenesis of the plant *in vitro* appeared (Varshney et al, 2011). Perhaps one of the most successful reports in the development of regeneration system on *J. curcas*, is that of Li et al. (2008), who used indole-3-butyric acid (IBA), benzyladenine (BA) and gibberallic acid (GA_3) to induce organogenesis in the plant. This regeneration protocol was used by the workers in subsequent *Agrabacterium* mediated transformation experiments. The reproducibility of this system as well as the transgenic approach can be extended to other provenances of the crop following their validation. In this work, we adopted the regeneration method of Li et al. (2008) to induce organogenesis using cut pieces of cotyledons of *J. curcas* while monitoring their different developmental stages leading to the formation of shoot and root *in vitro* using histological data generated from different stages of development which allowed us to demonstrate direct organogenesis.

MATERIALS AND METHODS

Plant materials

Mature seeds of *J. curcas* obtained from Instituto Fazenda Tamanduá, Santa Terezinha of Paraíba State in Brazil, were sterilized in 1% NaOCl and soaked in distilled water for about an hour in order to facilitate the removal of embryos. Following the removal of seed coat, the seeds were surface sterilized using 70% ethanol for 30 s, 1% NaOCl for 15 min and rinsing with sterile distilled water. The seeds were then germinated in half strength Murashige and Skoog (MS) media (Murashige and Skoog, 1962) containing 100 mg/L myoinositol, 10 mg/L thiamine-HCl and 3% sucrose solidified with 0.7% agar. Zygotic embryos arising from the germinating seeds were detached and transferred into a fresh media, with their radicle in direct contact with the media. The process of germination lasted for 14 days in a germination chamber maintained at 27°C and 16 h photoperiod provided by white fluorescent lamps (25.3 µmol m^{-2} s^{-1}). Cut pieces of cotyledons arising from the 14 days old plantlets were made to a size of 5 × 5 mm and used in the organogenesis experiment using similar method as described by Li et al. (2008). The explants were cultured in Petri dishes containing organogenic CIM which comprised of complete MS salts supplemented with 1.5 mg/L BA and 0.05 mg/L IBA with their abaxial sides in direct contact with the media. A total of 120 explants were kept in CIM over a period of 21 days in the dark, after which they were transferred to regeneration media.

Shoot induction and elongation

Having attained organogenic competence, the explants were transferred to shoot formation media (SM) which comprised of full strength MS salts supplemented with 1.5 mg/L BA, 0.05 mg/L IBA and 0.5 mg/L gibberalic acid (GA_3). The explants were then incubated in growth chamber for 30 days in a 16 h photoperiod. Those that produced clearly defined shoot apex and at least two primordial leaves were regarded as having formed shoots while those with protuberances were considered to be in their initial stage of shoot formation. Emerging shoots were cultured in elongation media (EM), which comprised of MS salts supplemented with 0.3 mg/L BA as reported by Li et al. (2008).

Rooting

Following elongation, the shoots were cultured in different rooting media (RM) having the following compositions: 1) ½ MS + 0.00 mg/L IBA; 2) ½ MS + 0.05 mg/L IBA; 3) ½ MS + 0.15 mg/L IBA and 4) ½ MS + 0.30 mg/L IBA, and were incubated in a growth chamber at 27°C and 16 h photoperiod. The explants were daily monitored for the emergence of roots. Each treatment consisted of eight replicates of five explants giving a total of 40 explants. Regression analysis was carried out in order to analyze the relationship between the concentrations of the auxins and number of explants that formed root.

Histological analysis

Anatomical characterization was conducted using cotyledons subjected to CIM at 0, 2, 4, 6, 12 and 20 days of incubation and for those in SM at 0, 4, 6, and 11 days of incubation. The explants were harvested and fixed using a modified Karnovsky solution (Karnovsky, 1965) containing 1% glutaraldehyde and 4% formaldehyde over a period of 24 to 48 h, after which they were washed five times in 0.02 M phosphate buffer at pH 7.2. The explants were then dehydrated using increasing concentrations of ethanol (10; 20; 30; 40; 50; 60; 70; 80; 90 and 100%) for each hour and then transferred into a pre-filtered solution comprising of absolute alcohol, and basic resin [(2-hydroxymethyl metacrylate and benzyl peroxide in a ratio of 1:1 (V/V)]. The explants were later embedded and used to form blocks during 2 to 8 days in a vacuum. The blocks were then cut to 5 µm size using rotating microtome (Leica – model 2065). The tissues were stained using 0.12% toluidine blue for 5 min followed by 0.05% basic fuscin for 1 min. Slides were mounted with synthetic resin of Entellan (Merck) and later analysed with optical microscope (Olympus – model CX40). Images obtained were captured by a coupled system of imaging (Moticam). From each treatment, four explants were used to prepare histological cuttings and this was repeated three times.

RESULTS AND DISCUSSION

Acquisition of organogenic competence

Within the first few days following incubation in CIM, the explants formed callus around their borders followed by the emergence of shoot-like structure within the central region, most of which actually formed shoot when placed onto SM media (Figures 1A and B). This observation differed from that of Li et al. (2008) where callus formation was observed from which shoots emerged thus implying the occurrence of indirect organogenesis. According to our observation, the calli arising from the edges of explants obtained in this work were typically non friable, whitish and/or hard. No green points were observed on the surface of any of these calli, which

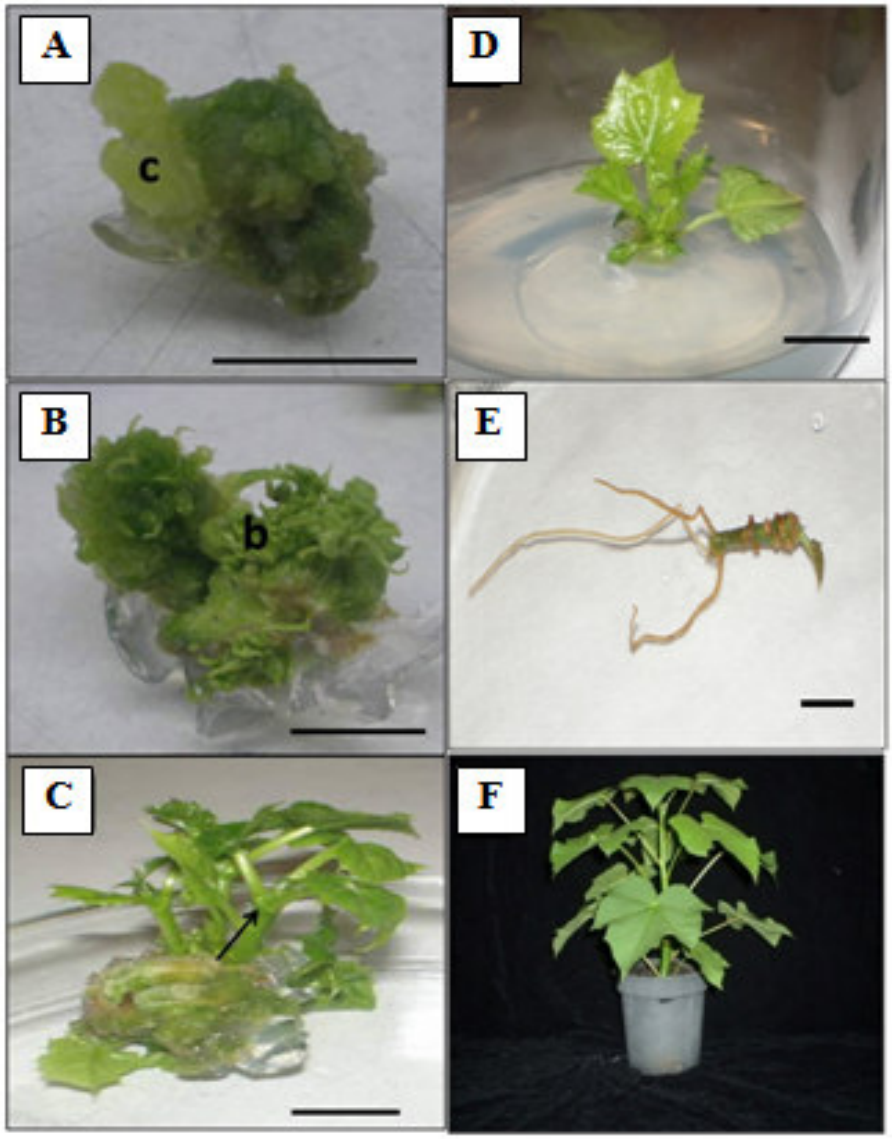

Figure 1. Regeneration of *J.curcas* through organogenesis. Cut pieces of cotyledons of *J. curcas* after 21 days in organogenic competence induction media (A); and 30 days in shoot induction media (B and C). Calli (c) and brotations (b) were formed at the edges of the explants. The arrow indicates the emerging point of the apical meristem, which resulted in shoot in the elongation media (D) and after 28 days, roots were formed (E) giving rise to a complete plant after 60 days (F). Line: 1 cm.

would have led to the possible conclusion that the calli were organogenic (Nabors et al., 1983). We hypothesized that these calli did not participate in the organogenic process and therefore did not give rise to the emerging shoot. This was confirmed by our histological studies described below. We attribute the formation the callus around the edges of these explants to the greater availability, in those regions of growth regulators present in the medium, due to injuries caused by cutting the cotyledons (Smith, 2000). Majority of the explants acquired organogenic competence after 21 days in CIM and upon transfer to SM, they developed shoots. Explants that were subjected to SM directly without passing through CIM did not generate shoot. This underscores the importance of the acquisition of organogenic competence induced by the combining IBA and BA within a concentration range (Sujatha and Mukta, 1996).

Shoot formation

The percentage of explants that gave rise to well defined shoots following transfer from CIM to SM was 53.91%

(Figure 1C). This represents regeneration efficiency higher than that reported by Li et al. (2008) under similar experimental conditions. Although we cannot deduce to what extent genotypic difference may have led to these discrepancies, similar result which placed the percentage at 50%, was reported by other authors (Sujatha and Mukta, 1996) where the same kind of explants were incubated in a media supplemented with a 1:2 concentration ratio of BA and IBA. In our case, up to 84.35% of the explants attained their initial stage of shoot formation five weeks after incubation although not all of them later developed into shoots. The remaining 15.65% of the explants did not show any morphological difference from their initial appearance when in CIM. Although the average number of well defined shoot regenerated per cut piece of cotyledon passing from CIM to SM observed in this work was about 1.5 shoots per explant, further exposure of explants bearing these shoots generated further shoots by the explants, which could be detached and cultured separately. Supplementation of the culture media with gibberallic acid and incubation in the light apparently stimulated further development of shoots. Similar morphological response was reported to have been observed in castor bean, where addition of GA_3 in combination with different concentrations of cytokinin led to rapid development of shoot (Kumari et al., 2008).

Shoot elongation

Five weeks after incubation in shoot induction media, the shoots were detached from each other and incubated in shoot elongation media (EM) for 15 days. Analysis of length of the shoots after incubation in EM media showed that increase in shoot length varied from 1 to 3 cm. This corroborates with the findings of Li et al. (2008). The shoots were robust with very short intermodal spaces (Figure 1D). Shoots having a length from 0.5 to 1 cm ended up forming callus at their basal points. The presence of callus might be attributed to the inherent effect of cytokinins in the stimulation of the production of genetic tumor when present in high concentration (Müller and Sheen, 2008). There was also increase in the number of leaves which varied from two to eight per shoot with longer shoots presenting higher number of leaves. The generally low shoot length recorded in the explants might be attributed to the cytokinin in the media which could have inhibited apical dominance but stimulated cell division and lateral growth (Müller and Sheen, 2008).

Rooting

Analysis of the percentage number of shoots that formed roots as well as the average number of root per shoot, when explants were cultured in rooting media containing different concentrations of IBA is presented in Table 1. The highest percentage (27.50%) of root formation was observed when the shoots were cultured in a rooting media containing 0.3 mg/L IBA. This was followed by those cultured in a media devoid of IBA. Intermediate IBA concentrations did not appear to generate high percentage of roots. After 28 days, the process of root formation stopped and by the 60th day, all shoots had started the process of senescence or died altogether. The percentage of explants that formed roots in this work was much lower than that reported by Li et al. (2008) who recorded 86% when shoots were cultivated in the same rooting media. The number of roots formed per explant varied from one to five. However, for most of the concentrations tested, the number of roots per explants was not more than two (Figure 1E). Taken as a whole, the percentage of shoots that formed roots and the different concentrations of IBA in the media into which they were subjected showed that the minimum percentage of 10% was recorded with 0.05 mg/L IBA. We observed higher number of shoots that formed roots at 0.0 and 0.3 mg/L IBA whereas intermediary concentrations generated lower roots. This suggests that the effect of IBA was positive when IBA level was higher than 0.05 mg/L. However, the value of correlation coefficient for the relationship between the percentage of shoots that actually formed root and increasing concentration of IBA was R = 0.871, suggesting a negative relationship between the application of increasing concentrations of IBA and rooting, at least up to certain level (Figure 2). The relationship observed between auxin level and rooting potential has been recorded for other plants where high levels of auxin may have inhibitory effect over the rooting process (Pedroti and Voltolini, 2001; Erig and Schuch, 2004; Henrique et al., 2006; Maia and Botelho, 2008). Following this, the regenerated plants were acclimatized and transferred to the green house (Figure 1F).

Histological analysis

Anatomical changes during competence induction

We followed the anatomical changes in the explants passing through the different regimes of regeneration starting from the organogenic competence induction media (CIM) through shoot development media (SM), elongation (EM) to rooting media (RM) using histological analysis". Anatomical section of the explants on CIM on day 0 revealed that the leaves of *J. curcas* is amphistomatic revealing uniseriate epidermis on both sides (Figure 3A). There were higher numbers of stomatics on the adaxial region than on the abaxial. The mesophyll cells were biphasic with palisade parenchymatous cells on the adaxial side and spongy parenchymatous cells on the abaxial. The central vein was characterized by the presence of vascular sheath

Table 1. Effect of different concentrations of IBA on root formation by the shoots of *J. curcas* L. Total explants were used per treatment: n = 40.

Concentration of IBA (mg/L)	Percentage of shoots that formed root	Number of roots formed per shoot
0.00	22.9	1.0
0.05	10.0	1.5
0.15	12.5	1.4
0.30	27.5	1.6

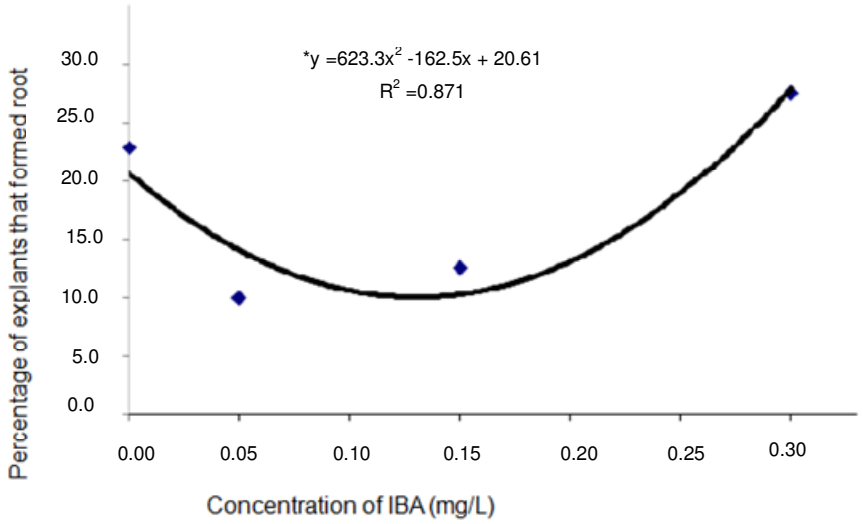

Figure 2. The relationship between the percentages of shoots that formed root to different concentration of IBA during the regeneration of *J. curcas* L.

with xylem and phloem surrounding a bundle sheath of parenchyma (Figure 3B).

The histological cuttings following incubation of the explants showed that the first few changes in these tissues began to occur only by the fourth day of incubation. The parenchymatous cells acquired more vacuoles surrounded by an amorphous intracellular matrix that gave intense blue color with toluidine (Figures 3C and D). The presence of this matrix might be crucial for the initial stage of regeneration because it is known to contain pectin like substance required for the synthesis of cell wall of newly emerging cells (Fernando, 2005).

By the sixth day, the parenchymatous cells presented intense cell division although the palisade cells masked their appearance. The direction of the cell division was both periclinal and anticlinal in the middle and marginal regions of the explant. It appears that the cells at the adaxial region of the explant were not involved in the regeneration process. However, cellular proliferation was much more intense in the abaxial region of the explant where meristem like tissues were seen whose cells presented reduced size, dense cytoplasm and clear nucleus (Figure 4). Indeed we could clearly distinguish the different stages of acquisition of organogenic competence starting from day six of incubation all the way to day 20 and beyond.

The 12th and the 20th day of incubation may be regarded as the ages at which there was the highest cell division (Figure 5). The cells at this stage were much higher in number with additional cells found around the border of the meristematic region (Figure 5D). However, these cells did not appear to be organogenic suggesting that they could have been the calli observed at the edges of the explants described earlier.

The formation of protuberance on the surface of the explant was also observed on the 12th day of incubation (Figures 5A and B). These structures resulted from the intense cell division of the parenchymatous cells earlier described. In these structures, parenchymatous and epidermal cells had high number of vacuoles while those in the vascular region had dense cytoplasm and were apparently actively dividing. The existence of this meristem like cells around the vascular region suggests that the regeneration process in *Jatropha* might have a multicellular origin. Indeed we can safely assume that new tissues are regenerated from meristematic cells arising from the “de-differentiation” of fundamental parenchyma like parenchymatous bundle sheath (Almeida et al., 2002).

In addition, Figure 5B shows that due to the

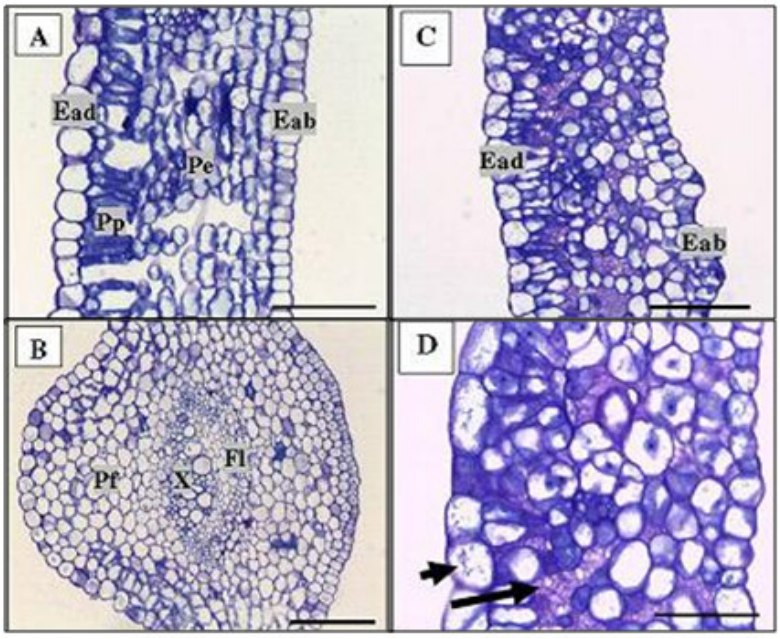

Figure 3. Transverse sections of cut pieces of cotyledons of *J. curcas* cultured in organogenesis media on day 1 (A and B) and on day 4 (C and D). A represents the marginal region of the explants (Ead, epidermis adaxial; Eab, epidermis abaxial; Pp, palisade parenchyma; Pe, spongy parenchyma); B represents the medial region (Pf, fundamental parenchyma; X, xylem; Fl, Phloem), C. represents the marginal region (Ead, adaxial epidermis; Eab, abaxial epidermis) and D represents details of the marginal region. Scale: A = 500 µm; B and C = 250 µm; D = 150 µm.

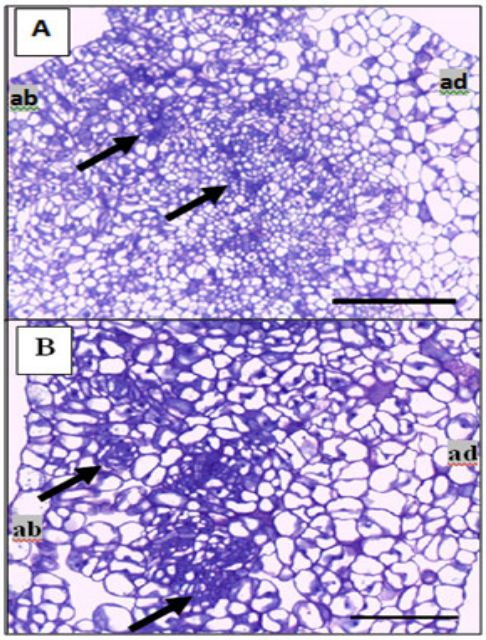

Figure 4. Transverse sections of cut pieces of cotyledons of *J. curcas* cultured in organogenesis media after 6 days. A, Middle region; B, marginal region (ab, face abaxial region of the leaf; ad, adaxial region of the leaf). Arrows indicate cell division). Scale: A = 500 µm e B = 250 µm.

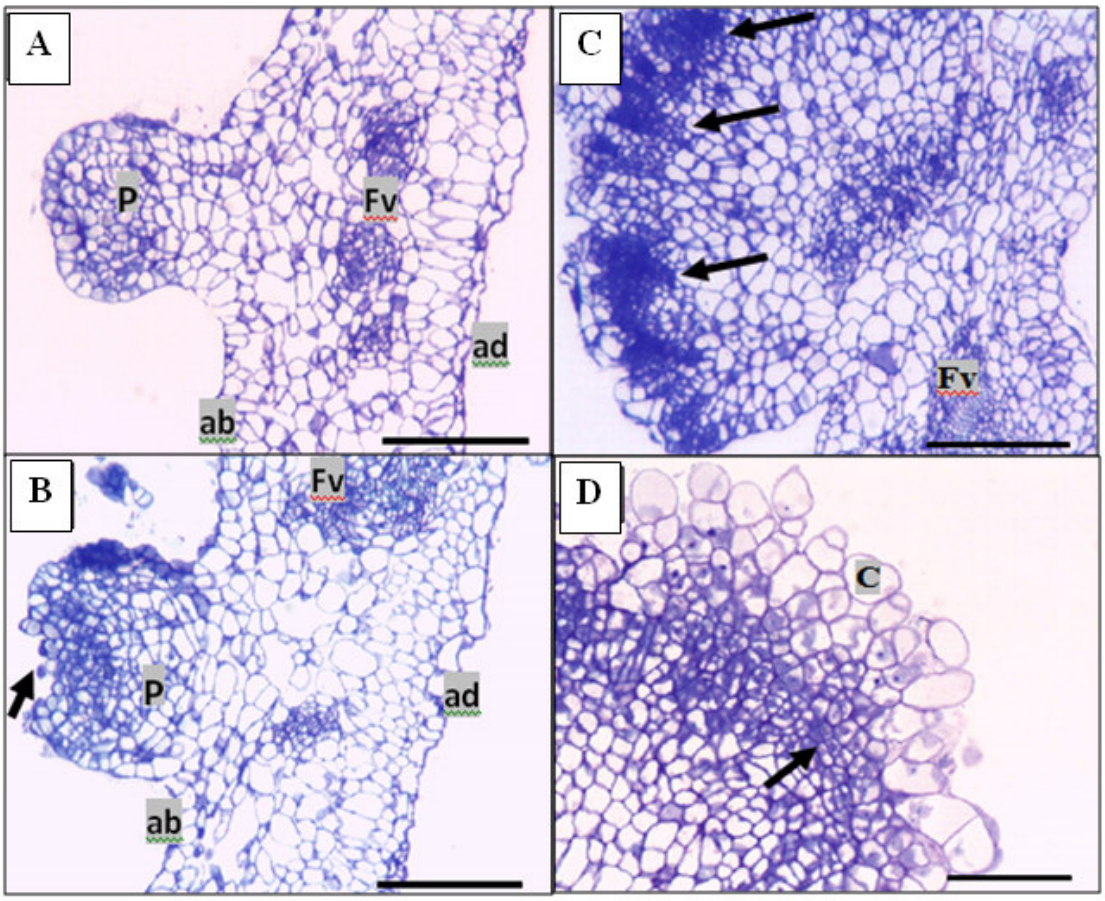

Figure 5. Transverse sections of cut pieces of cotyledons of *J. curcas* cultured in organogenesis media after 12 days (A and B) and 20 days (C and D). Protuberance formed at the abaxial region of the explants which later ruptured around the epidermis region of the explants. D. Represents the border region where c indicates calli formed (ab, abaxial region of the leaf; ad, adaxial region of the leaf; Fv, vascular region; P, protuberance). Arrows indicate rupture of the epidermis (B), the formation of meritematoids (C) and cell division (D). Scale: A, B and C = 500 µm; D = 250 µm.

development of these protuberances, the epidermal layer appeared to rupture and may therefore not have participated in the actual regeneration process. However, protuberances at the age of 20 days after culture developed meristemoids (Figure 5C). Similar result was obtained using *Saintpaulis ionanthana* × *confuse* (Lo et al., 1997); *Cucumis melo* (Gaba et al., 1999) and *Passiflora edullis* (Fernando, 2005). Typically, meristemoids occur side by side with the region next to the protuberances, forming an intense layer of meristematic cells.

Anatomical changes during shoot formation

During the last days of the explants in the CIM (having spent 21 days), which coincided with the first day of the same in SM, the central meristematic cells remained actively dividing leading to the emergence of the protuberances (Figure 6) which resulted in the formation of shoots. We conclude that organogenesis in *J. curcas*, at least for the genotype used in this work, takes place through direct organogenesis and not by indirect organogenesis as suggested by Li et al. (2008). This is because the calli arising from the treatment of the explant did not participate in anyway, in the regeneration of shoots. Furthermore, parenchymatous cells and perhaps bundle sheath are responsible for the emergence of protuberances that gave rise to meristematic centers known as meristemoids, which later elongated to form shoots. Our observations also differ from that of Varshney et al. (2011) who reported that meristemoids were found to originate from the peripheral region of organogenic callus and the same time observed meristemoids structures from periphery and deeper layers of cells from cotyledons.

Explants that were previously cultured in CIM were transferred to SM where they remained for approximately

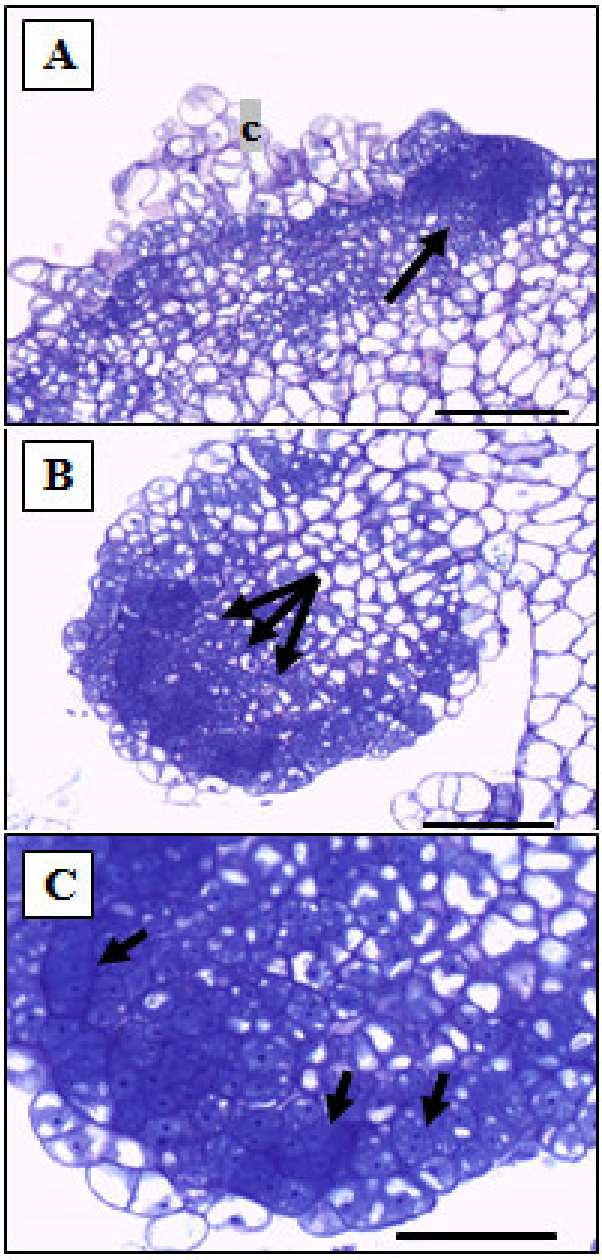

Figure 6. Transverse sections of cut pieces of cotyledons of *J. curcas* cultured in organogenesis media after 21 days and on 0 day at induction media. A represents the border region where c indicates callus and the arrow points at meristematic centre. B indicates the protuberance at the inferior region of the explants with its arrow pointing at meristmoids and C shows the details of the protuberances in which arrows point at intense cell division. Scale in A and B = 250 µm and in C = 150 µm.

four weeks. We followed the series of anatomical changes resulting from this treatment over a period of 11 days. The most significant anatomical event started to take place by the 4th day of incubation, where the number of competent cells increased considerably and from which several protuberances corresponding to initial shoots described earlier, emerged (Figure 7A).

By the 6th day in this media, several meristematic cells could be seen forming promeristems from which shoot meristem later emerged (Figures 7B and C). Indeed, the

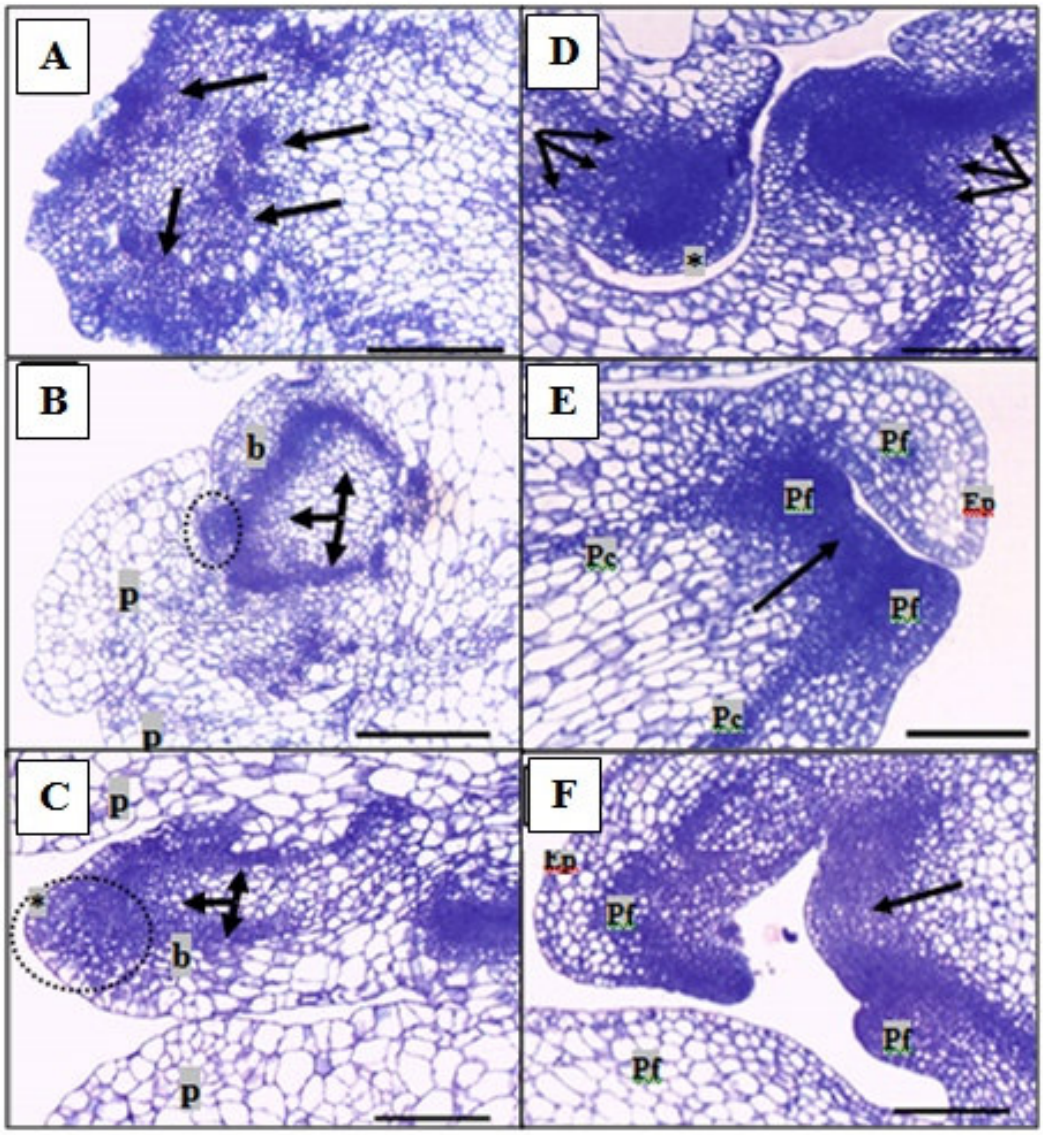

Figure 7. Transverse sections of cut pieces of cotyledons of *J. curcas* cultured in shoot formation media after 4 days (A), 6 days (B and C) and 11 days (D, E and F). A: Expanding protuberances formed at the abaxial region with arrows identifying competent cells. B and C: Expansion of protuberances (p), where early brotations (b) can be seen. The arrows in B and C show cellular organization with the * and broken circle showing the formation of protoderme and promeristems respectively. D, E and F, initial shoot meristem was formed (arrow indicate cellular organization and * shows formation of protoderm) in D. Regenerated apical meristem is seen in E and F (Pf, primordial leaf; Pc, procambium; arrow shows apical meristem and Ep,show epidermis cells). Scale in A and B = 500 µm and in C, D, E and F = 250 µm.

presence of these structures was a precondition for the eventual emergence of complete shoot from the explants. The promeristems became evident with the appearance of *tunica-corpus* structure present at the distal region of the apical meristem and formed by one or two layers of cells dividing in both periclinal and anticlinal direction (Figures 7B and C). Some of the protuberances appeared to contain large vacuoles and were therefore not considered as part of the emerging shoots. Figure 7C shows the presence of a protodermal layer of cells which originated from the meristematic tissues below them. This was particularly evident by day 6. Indeed by this time, all organogenic cells were committed to forming specific tissues and that the minimum time required attaining this was indeed 6 days.

In subsequent days, similar structures were observed. However, it was only by day 11 that an unmistakable apical meristem could be discerned clearly (Figures 7D,

E and F). We observed different developmental stages of the emergence of apical meristem within the same cut, suggesting that the process takes place in a random manner. However, the stage observed at day 11 represents the crucial stage for the emergence of apical meristem. An advanced stage in this development can be seen in Figures 7E and F where apical meristem and primordial leaves were apparent. The epidermal layer of this new shoot can be seen with its characteristic cubic nature similar to those observed in zygotic embryos. Below these structures were meristematic cells responsible for the formation of different organs.

Altogether, it requires a minimum of 32 days for the formation of an average shoot through organogenesis from parenchymatous cells and perhaps bundle sheath which might have acquired meristematic character. At this stage, the cells have reached the third phase of organogenic process characterized by differentiation (Christianson and Warnick, 1983). The differences between the results obtained in this work and that reported by Li et al. (2008) can perhaps can be related to genetic variability due to the use of different genotypes. Moreover, even the explant (cotyledon leaf segments) may present genetic variability due to its origin from seed, because *J. curcas* is a pollinating species. The relationship between the concentrations of auxin and cytokinin also affect the process.

In conclusion, we have shown for the first time, the development of shoots from explants of *J. curcas* through direct organogenesis as opposed to indirect organogenesis and this was supported by the histological analyses which represent important strategy for understanding the morphogenesis of *in vitro* regeneration of *J. curcas*. Our findings will form the basis for the development of different propagation protocols as well as genetic transformation strategies of *J. curcas*.

REFERENCES

Almeida WAB, Mourão Filho FAA, Mendes BMJ, Rodriguez APM (2002). *In vitro* organogenesis optimization and plantlet regeneration in *Citrus sinensis* and *C. limonia*. Scientia Agricola, 59: 35-40.

Carroll A, Somerville C (2008). Cellulosic Biofuels. Annu. Rev. Plant Biol., 60: 165-182.

Christianso N, ML; Warnick, DA (1983). Competence and determination in the process of *in vitro* shoot organogenesis. Dev. Biol., 95: 288-293.

Costa GGL, Cardoso KC, Del Bem LEV, Lima AC, Cunha MAS, Campos-Leite L, Vicentini R, Papes F, Moreira Rc, Yunes Já, Campos FAP, Da Silva MJ (2010). Transcriptome analysis of the oil-rich seed of the bioenergy crop *Jatropha curcas* L. BMC Genomics, 11, artn 462.

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 DOI 10.1007/s11816-008-0042-y.

Erig AC, Schuch MW (2006). Ação da 6-benzilaminopurina e da qualidade da luz na multiplicação *in vitro* de macieira (*Malus domestica* Borkh) cvs. Galaxy e Mastergala. R. Bras. Agrociência, 12:.151-155.

Fairless D (2007). Biofuel: the little shrub that could–maybe. Nature, 449: 652–655.

Fernando JA (2005). Estudos anatômicos e ultra-estruturais da organogênese *in vitro* de *Passiflora edulis* Sims f. *flavicarpa* Deg. 106 f. Tese (PhD Thesis) -Universidade Estadual de Campinas, Campinas.

Gaba V, Schlarman E, Elman C, Sagee O, Watad AA, Gray DJ (1999). *In vitro* studies on the anatomy and morphology of bud regeneration in melon cotyledons. *In Vitro* Cell Dev. Biol. Plant, 35: 1-7.

Henrique A, Campinhos EN, Ono EO, Pinho SZ (2006) Effect of plant growth regolators in rooting of *Pinus* cuttings. Brazilian Archives Biol. Technol., 49: 189-196.

Kalimuthu K, Paulsamy S, Senthilkumar R, Sathya M (2007) *In vitro* propagation of the biodiesel plant *Jatropha curcas* L. Plant Tissue Cult. Biotech., 17:137–147

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol., 27: 137-138.

Khurana-Kaul V, Kachhwaha S, Kothari SL (2010) Direct shoot regeneration from leaf explants of *Jatropha curcas* in response to thidiazuron and high copper contents in the medium. Biol. Plant, 54: 369–372.

Kumari KG, Ganesan M, Jayabalan N (2008). Somatic organogenesis and plant regeneration in *Ricinus communis*. Biologia Plantarum, 52: 17-25.

Li M, Li H, Jiang H, Pan X, Guojiang W (2008). Establishment of an *Agrobacterium*-mediated cotyledon disc transformation method for *Jatropha curcas*. Plant Cell Tiss. Organ. Cult., 92: 173-181.

Lo KH, Giles KL, Sawhney VK (1997) Histological changes associated with acquisition of competence for shoot regeneration in leaf discs of *Saintpaulia ionantha* x *confuse* hybrid (African violet) cultured *in vitro*. Plant Cell Reports, 16: 421-425.

Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, Mcmillan JD, Sheehan J, Wyman CE (2008). How biotech can transform biofuels. Nature Biotechnol., 26: 169-172.

Maia AJ, Botelho RV (2008). Reguladores vegetais no enraizamento de estacas lenhosas de amoreira-preta *cv Xavante*. Semina: Ciências Agrárias, 19: 323-330.

Müller B, Sheen J (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis Nature, 453: 1094-1097, doi:10.1038/nature06943.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Nabors MW, Heyser JW, Dykes TA, Demott KJ (1983). Long-duration, high frequency plant regeneration from cereal tissue cultures. Planta, 157: 385-391.

Pedrotti EL, Voltolini JA (2001). Enraizamento *ex vitro* e aclimatação do porta-enxerto de macieira. Revista Brasileira de Fruticultura, 24: 234-239.

Smith RH (2000). Regeneration and morphogenesis. Plant Tissue Culture. Techniques and experiments. 2 ed. California: Academic press, pp. 107-121.

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

Varshney A, Johnson TS (2010). Efficient plant regeneration from immature embryo cultures of *Jatropha curcas*, a biodiesel plant. Plant Biotechnol. Rep., 4: 139-148.

Varshney A, Sangapillai R, Patil MS, Johnson TS (2011) Histological evidence of morphogenesis from various explants of *Jatropha curcas* L. Tree DOI 10.1007/s00468-011-0546-x.

Ye J, Qu J, Thi H, Bui N, Chua NM (2009). Rapid analysis of *Jatropha curcas* gene functions by virus-induced gene silencing. Plant Biotechnol. J., 7: 964-976.