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Efficient somatic embryogenesis of *Jatropha curcas* L. from petiole and leaf discs

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***Jatropha curcas* L. is peculiar treasured tree species for its uses and considerable economic potential as a biofuel plant. Propagation using seeds is limited by low viability and germination hence unable to provide high quality planting material for sustainable use. To achieve mass production of *J. curcas,* regeneration from leaf discs and petioles using different treatments of plant growth regulators (PGRs) including 6- benzyl aminopurine (BAP), Kinetin (KIN), Indole -3-acetic acid (IAA) and Thiadiazuron (TDZ) was explored. Maximum callus formation efficiency (85.00%) and the shoot proliferation per explants (8.25) was observed on MS medium supplemented with 1.5 mg/L BAP, 0.6 mg/L KIN, 0.3 mg/L IAA and 0.1 mg/L TDZ. Rooting was induced from elongated shoots cultured on half strength Murashige and Skoog (MS) medium fortified with different regimes of indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA). MS supplemented with 3.5 mg/L IBA and 3.5 mg/L NAA gave optimum root formation of 2.5 cm on JCO4, JN1, JE4 and JRV1 accessions. The rooted plants were established in forest soil, sand and manure mixed in the ratio of 2: 1:1 in the green house with 20% survival rate. This successful *in vitro* regeneration is vital for seedling system for mass production.**

Key words: Plant growth regulators, callus, shoot induction, root formation.

INTRODUCTION

The genus *Jatropha*, which is perceived to comprise approximately 170 known species, is distributed in the tropical and subtropical world. *Jatropha* has been considered as a plant for biofuel production mainly due to its high seed oil content of 40 to 50% and non-competing demand with edible oil supplies (Rajore et al., 2007). With the recent increase in fuel prices there is an increased demand in the use of *Jatropha* oil to alleviate energy crisis. Conversion of the oil to biodiesel is relatively simple by chemical (Berchmans and Hirata, 2008) or biological trans-esterification (Modi et al., 2007). Jatropha is also desired due to its drought hardiness, rapid growth, easy propagation, low cost of seeds, oil content and short maturity period with wide range of environmental adoption (Gubitz et al., 1999; Jones and Miller, 1991). The oil is also used in manufacturing candle, varnishes, soap and treatment of several diseases among others uses (Rajore et al., 2007). Although Jatropha has

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assumed paramount importance as a potential biodiesel crop in more than 50 countries, its production is met with limited success. This is due to unreliable seed production, low oil yields, vulnerability to pests and diseases and low economic returns (Heller, 1996; Kaushik et al., 2007).

Commercial propagation of Jatropha is typically through seed and vegetative cutting. Macro propagation of Jatropha through stem cuttings has been achieved however the established plants are not deep rooted and hence, they easily get uprooted when cultivated in lands with poor top soil (Heller, 1996; Openshaw, 2000). Despite their profuse vegetative growth, the number of seeds produced per plant is very low and the seeds show a low seed fecundity, which is reduced by 50% within 15 months. Plants propagated by cuttings show a lower longevity and possess a lower drought and disease resistance than those propagated by seeds (Sujatha et al., 2007). Traits like higher seed yield, oil content, synchronous maturity and early flowering can be introduced for production to be sustainable and viable. Application of plant biotechnological methods can improve the crop by producing disease free plants, large number of plantlets and conserve the germplasm (Rajore et al., 2007). Plant species with rich secondary metabolites like Jatropha have proved to be difficult for mass propagation through tissue culture (Roy, 1991). Development of an efficient *in vitro* regeneration system would be a remarkable progress for the Jatropha business and the field of alternative energy technology.

Although somatic embryogenesis and organogenesis using different explants have been reported in Jatropha (Deore et al., 2008; Varshney et al., 2010), low rates of *in vitro* multiplications were reported in these studies. Somatic embryogenesis is the gateway to efficient propagation and genetic transformation due to its single cell origin (Cai et al., 2011). Evaluation of tissue culture propagated plants of Jatropha revealed that they were at par with seed propagated plants in terms of yield and yield related traits (Sujatha and Mukta, 1996). There are no documented studies on micropropagation of Kenyan Jatropha genotypes. Moreover, no field transfers of *Jatropha* tissue culture plantlets have been successfully reported in previous studies. Rooting *in vitro* and acclimatization have also proven very difficult for *Jatropha* (Pankaj and Divay 2011). Therefore, the objective of this study was to develop an efficient *in vitro* regeneration system for micropropagation of Kenyan *Jatropha* genotypes.

MATERIALS AND METHODS

Plant material

Plant materials used in this study were collected between December 2008 to April 2009 from the Coastal, Eastern, Western, Central, Nairobi and Rift Valley provinces of Kenya. The plant materials collected consisted of mature seeds, cuttings and seedlings. They were stored in cool boxes and later transported to molecular laboratory in the Institute for Biotechnology Research (IBR), Jomo Kenyatta University of Agriculture and Technology (JKUAT). Seeds were dried at room temperature where some were germinated on sand in IBR greenhouse and others stored in papers bags in a cool well ventilated area in the laboratory. The seedlings and cuttings were transplanted in potting bags containing well mixed forest soil, sand and manure in the ratio of 2:1:1 and kept in the IBR green house. They were watered thrice a week using a spraying can. Seed that germinated were transplanted into potting bags containing soil, sand and manure in the ratio of 2: 1: 1 after two weeks. Well adapted plantlets were used as stock plants in subsequent experiments.

Jatropha accessions

Five accessions (JCO4, JE1, JN1, JNY1 and JRV1) were selected and used in subsequent tissue culture experiments.

Establishment of sterilization protocol and culture conditions

Murashige and Skoog (MS), basal medium (Murashige and Skoog, 1962) was used. All the media were supplemented with 3% (w/v) sucrose, plant growth regulators (PGRs) as per growth stage and the pH was adjusted to 5.8 using 0.1 N HCL or 0.1 N NaOH, 0.28% (w/v) Gelrite was added and the media dispensed in 200 ml culture jam jars (20 ml of medium per jar). The media, water, glassware and metallic equipment used were sterilized by autoclaving at 121°C at 1.06 kg cm-^{2}pressure for 20 min. The sterilized media was kept at room temperature for three days before culture. Chemicals used were of analytical grade and experiments were carried out under aseptic conditions in the laminar flow. Forceps and scalpels were sterilized in a Bead Sterilizer at 250°C before use. Cultures were maintained at 25± 2°C under a 16 h light and 8 h dark period in air conditioned growth chambers illuminated by 40 W provided by Philips white fluorescent tubes. The intensity of light was regulated between 2500 and 3000 µmol m^{-2} s^{-1}. Leaf discs and petiole explants were obtained from the 3rd and 4th leaves foliar of 3 to 4 months old mother plants raised in the greenhouse. They were placed in glass beakers and kept in running tap water for 30 min to remove physical impurities and latex. They were then transferred to new glass beakers containing 250 mL of water into which 100 µl/L Tween®20 and 2 ml/L of Dettol detergent had been added. The beaker was swirled gently at intervals for fifteen minutes followed by three times rinsing with distilled water to remove all the traces of detergent. Thereafter the explants were kept in 0.3% (w/v) Redomil® (fungicide) plus 100 µl/L Tween®20 for 1 h then washed thoroughly with double distilled water. Under a clean Lamina flow hood, the explants were subjected to 70% (v/v) ethanol for 30 s, rinsed with double distilled water thrice to remove all the ethanol and then subjected to 20% (v/v) sodium hypochlorite containing 100 µl/L Tween®20 at varying exposure times; 15, 20 and 25 min then rinsed three times with double distilled water. After sterilization, individual leaf disc explants were trimmed aseptically to 0.5 mm to 1 cm all round and petiole 1 to 2 cm before culturing them on full strength MS medium with the leaf abaxial in contact with initiation medium for leaf disc and petioles horizontally.

Effects of plant growth regulators on callus induction

Murashige and Skoog medium supplemented with different PGRs

at varying concentrations was used for callus induction on five *Jatropha* accessions; JNI, JNYI, JCO4, JE4 and JRVI. Combined PGRs applications at varying concentrations of 0.5 to 2.0 mg/L BAP, 0.4 to 0.7 mg/L Kin, 0.3 to 0.6 mg/L IAA and 0.1 mg/L TDZ and a control (MS media without PGRs) totaling to 25 treatments were set. The percentage proportion of callus induction on leaf discs and petioles were evaluated at an interval of 4 weeks after initiation.

Effects of plant growth regulators on somatic embryo development

MS medium combined with PGRs at varying concentrations as in callus induction was used to test the rate of somatic embryos formation from callus of JN1, JCO4, JE4 and JRVI accessions. Sub culturing after every 4 weeks was carried out. Somatic embryos formation per treatment was evaluated at an interval of 4 weeks for 2 months after sub culturing. The morphology, colour, texture and shape of the embryos were also determined using eye and microscopic observation.

Effects of plant growth regulators on shoot proliferation

MS medium combined with PGRs at varying concentrations as in callus induction was used in shoot formation, multiplication and elongation of JN1, JCO4, JE4 and JRVI. This was done for 2 months to evaluate the regeneration rate. Induction of micro shoots and their length was evaluated at an interval of 4 weeks after sub culturing.

Effects of plant growth regulators on root induction

Half strength MS containing 3% (w/v) sucrose, 0.28% (w/v) gelrite fortified with IBA and IAA combined at varying concentrations were used. 1.5 to 4.0 mg/L IBA, 1.5 to 4.0 mg/L IAA, and a control (MS media without PGRs) totaling 25 treatments were set and used in root initiation of JN1, JCO4, JE4 and JRVI. This was done for 2 months to evaluate the regeneration rate. Induction of roots and their length was evaluated at an interval of 4 weeks after sub culturing.

Acclimatization

Plants from the tissue culture were then placed in the green house in pots containing forest soil, sand and manure at the ratio of 2:1:1. They were watered thrice a week using a spraying can.

Experimental design, data collection and analysis

Experiments on sterilization, callus induction, shooting and rooting were set up in a complete random design in 3 replicates. The frequency of callus formation, expressed as percentage, was calculated as the proportion of number of explants forming callus. Observation on somatic embryo germination, shoot proliferation and root induction rate were recorded. Data was collected in MS Excel spreadsheets and analyzed using Statistical Analysis System (SAS) 9.1 and GenStat 12th Edition, statistical softwares. Results were subjected to analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using Tukey's test at 5% probability.

RESULTS

Callus induction

Explants enlargement and swelling was observed after three days of culture. After 6 days mass of undifferentiated cells originated from petiole ends exposed to the medium whereas on leaf discs calli were formed all over the surface on all four groups of culture media used (Figure 1a and b). The callus colour varied from green, whitish green, yellowish green, brownish green, white and brown. The texture was both compact and friable. When the callus induction rates of the different accessions of Jatropha were compared using Turkeys test, significant differences were observed. Explants cultured in absence PGRs senesced without producing callus. The medium containing 1.5 mg/L BAP, 0.6 mg/L KIN, 0.5 mg/L IAA and 0.1 mg/L TDZ (C) induced callus significantly faster and in high rates compared to that induced by lower or higher levels. Induction rates of 18% JNY1, 65% JE4, 66% JCOE, 67% JRV1 and 85% JN1 were recorded (Table 1).

Embryo development and germination

After 8 weeks of culture on different concentrations, JNY1 callus produced few whitish somatic embryos which soon became necrotic. Necrosis began on the periphery and eventually the whole plant. Callus from other accessions remained green, whitish green, light green, white and brown in different concentrations and retained high embryogenic potential. During embryo development friable green and compact green callus were observed to undergo globular stage, heart stage, torpedo stage and maturity stage (Figure 1c, d and e). White and brown callus did not generate any embryos but died after subsequent culturing.

Shoot multiplication and elongation media

After transfer of embryos to shoot inducing medium (SIM), an average number of shoots were formed on each explant which varied significantly. The generated plants showed true Jatropha morphology (Figure 1f, g, h and i). Shoot length of 1.25 to 4.75 cm was achieved (Table 2). Concentration C was the best across all the accessions.

Root induction

Root induction and development required approximately 4 weeks with significant treatment effects on the rate of rooting (Figure 1j). Root development was induced in the

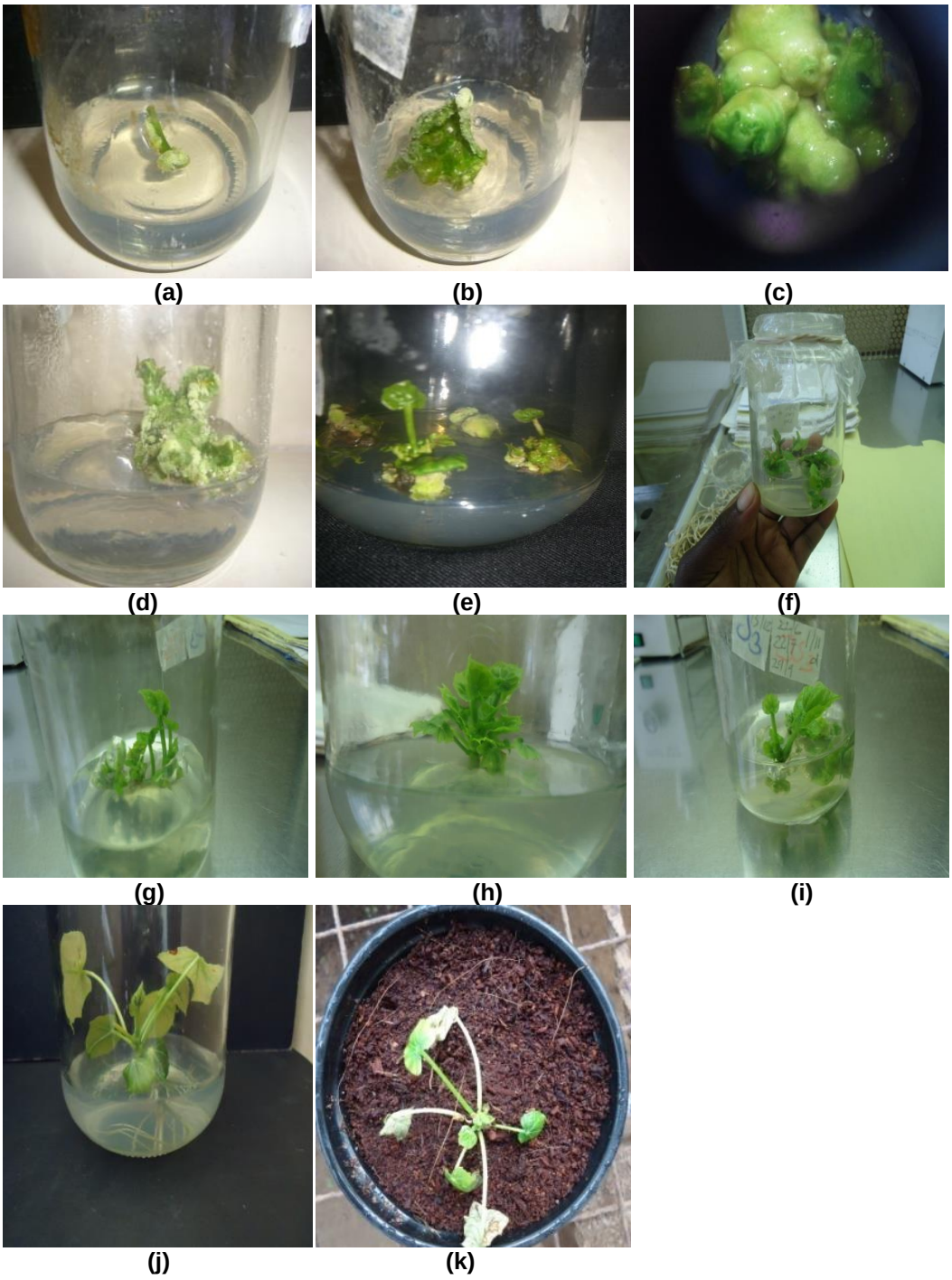

Figure 1. (a.) Callus induction on petiole; (b) callus induction on leaf discs; (c) embryo development, (d) embryo maturation, (e) embryo germination, (f) shoot formation, (g) shoot development, (h) shoot multiplication, (i) shoot elongation, (j) rooting (k) plantlet undergoing acclimatization.

shoot cultured on ½ MS supplemented with IBA and NAA for all accessions. No rooting occurred in the absence of auxins. Generally, these treatments resulted in the formation of average number of roots regardless of the accessions type with concentrations 3.0 mg/L IBA and 3.5 mg/ L NAA being the most effective for root induction. The lowest root number was 1.81 while the highest was only 20% survived.

Table 1. Effect of different concentration and combination of BAP, Kinetin, IAA and TDZ upon callus induction from leaf disc (n=60).

Accessions	Plant growth regulators (mgL^{-l})			
	*A	*B	*C	*D
JNY1	8.75±1.25^{d}	15.00±2.04^{d}	17.50±2.50^{c}	11.25±1.25^{b}
JCO4	46.25±2.39^{b}	55.00±2.04^{c}	66.25±2.39^{b}	52.25±2.39^{a}
JE4	15.00±2.04^{c}	50.00±2.04^{c}	65.00±2.04^{b}	50.00±2.04^{a}
JN1	8.75±1.25^{d}	75.00±2.04^{a}	85.00±2.04^{a}	17.50±2.50^{b}
JRV1	57.50±3.23^{a}	65.00±2.04^{b}	67.50±3.23^{b}	50.00±2.04^{a}

Means (± SE) followed by different alphabets in each column were significantly different at P ≤0.05 using Tukey's test. *A = (0.5 mg/L BAP, 0.4 mg/L Kin, 0.3 mg/L IAA and 0.1 mg/L TDZ); *B = 1.0 mg/L BAP, 0.5 mg/L Kin, 0.4 mg/L IAA and 0.1 mg/L TDZ; *C = 1.5 mg/L BAP, 0.6 mg/L Kin, 0.3 mg/L IAA and 0.1 mg/L TDZ. *D = 2.0 mg/L BAP, 0.7 mg/L Kin, 0.6 mg/L IAA and 0.1mg/L TDZ.

Table 2. Effect of different concentration and combination of BAP, Kinetin, IAA and TDZ on shoots elongation (cm), (n=48).

Accessions	Plant growth regulators (mgL^{-l})			
	A	B	C	D
JCO4	1.25^{b}	2.75^{c}	4.75^{c}	3.00^{b}
JE4	2.00^{b}	2.50^{c}	4.50^{c}	5.00^{a}
JN1	1.25^{b}	4.25^{b}	8.25^{a}	6.00^{a}
JRV1	5.00^{a}	6.25^{a}	6.50^{b}	5.50^{a}
$L.S.D_{0.05}$	0.832	1.01	1.371	1.177

Means (± SE) followed by same alphabets in each column were not significantly different at P ≤0.05 using Tukey's test.

DISCUSSION

Somatic embryogenesis enables production of large number of plants throughout the year and is also a powerful tool for genetic improvement of all plant species as a result of its single cell origin as reported by Bhansali et al. (1990). In this study a reproducible regeneration system of Jatropha through somatic embryogenesis was attempted. The PGRs concentration, source of explants and genotype significantly influenced the regeneration response this is in consistent with observation made by Kumar et al. (2010). Young leaf discs and petiole explants from the 3rd and 4th leaves of the five Jatropha accession; JNI, JNY1, JCO4, JRV1 and JE4 cultured on different media formed callus. Embryogenesis induction proficiency was accession and explant specific whereby petioles of accession JN1 had 85% callus induction while JRV1, JCO4, JE4 and JNY1 had 67.5, 66.25, 65.0% and 17.5% respectively (Table 3). This concurs with study done on cotton (Nick et al., 1986), using both petioles and leaf discs. The developmental stage of explants was crucial in regeneration whereby young explants portrayed high cell differentiation. This finding is in agreement with the observation made by Ahuja (1993) who reported that Juvenile explants were more responsive to tissue culture than old ones. In this study sterilization using mercuric chloride was replaced by Sodium hypochlorite, due its harmful environmental effects as described by Ahmad et al. (2011). Induction of callus was the critical stage in this study where the type and quality of callus influenced subsequent plant regenerations. Plant growth regulators, especially cytokinins and auxins alone or in combination play a very important role in callus induction process and its proliferation according to Thomas and Maseena (2006). Similarly, in this study it was observed that combination of BAP, KIN, IAA and TDZ is essential for high frequency induction of callus and multiple shoots. However, finding out the triggering combination of these plant growth regulators was the most important as reported by Timir et al. (2007). Although BAP has been reported to be more effective than other cytokinins in micropropagation of various members of the Euphorbiaceae (Tideman and Hawker, 1982; Ripley and Preece, 1986), TDZ was found to play an important role in callus induction in this study. When BAP, KIN and IAA were used on nodal explants by Kalimuthu et al. (2007) they induced somatic embryogenesis without TDZ. The differential response of cytokinins and auxin in this case, is attributed to differences in uptake, levels of endogenous growth regulators and recognition by cells as described by Pedrose and Pais (1995) and Souter and Lindsey (2000).

Low concentration of TDZ was maintained throughout the regeneration process and especially during shoot

Table 3. Effects of auxins at different concentrations and combination on rooting in excised shoots of various *J. curcas* Accessions.

Accessions	Plant growth regulators (mgL^{-1})			
	1.5 IBA +1.5 NAA	2.5IBA +2.5 NAA	3.0IBA + 3.5NAA	4.0IBA + 4.0NAA
JCO4	1.813[a]	2.36[a]	3.36[a]	2.36[a]
JE4	1.813[a]	2.29[a]	3.50[a]	2.38[a]
JN1	1.938[a]	2.54[a]	3.50[a]	2.56[a]
JRV1	2.06[a]	2.24[a]	3.50[a]	2.36[a]
$L.S.D_{0.05}$	0.658	0.4178	0.794	0.4531

Means (± SE) followed by same alphabets in each column were not significantly different at $P \leq 0.05$ using Tukeys test.

elongation as high concentration have inhibitory effects (Preece and Imel 1991; Huetteman and Preece, 1993; Feyissa et al., 2005). Combination of 1.5 mg/L BAP with 0.6 mg/ L KIN, 0.5 mg/ L IAA and 0.1 mg/ L TDZ promoted the highest callus induction of 85% within 8 weeks on all accessions in comparison to lower or higher concentrations. The rate of somatic embryo induction varied from one accession to another as reported by Shibli et al. (2001). Within the JN1 accessions the highest rate of 85% was observed and lowest rate of 18% on JNY1 was recorded. JCO4, JRV1 and JE4 accessions recorded 66, 67.5 and 65% respectively. According to Ghimire et al. (2010) leaf explants produced more callus than petiole explants. This agrees with the findings of this study where by callus formation on leaf explants was significantly higher than petiole explants. Majority of the calli differentiated to organized embryos while other areas remained white, brown and translucent. Subsequent transfer induced embryogenic callus which later formed shoots on SIM at varied levels within 10 to 12 weeks of culturing. During embryo development heart, globular and torpedo shaped embryos were observed which originated from both compact and friable whitish green callus. The embryos germinated into plantlets in all accessions except in JNY1 where cultures senesced without producing shoots. This coincides with earlier reported findings where *in vitro* regeneration of *J. curcas* was observed to be genotype dependent (Kumar, 2008; Kumar and Reddy, 2010; Kumar et al., 2010a).

Of the four concentrations tested concentration C gave a higher shoot formation. The accessions also responded differently on medium C with accession JNI having the highest rate of shoot formation of 8.25, while accession JCO4 had the lowest shoot formation score of 4.50. The critical step of inducing roots on the elongated plantlets was obtained on the transfer of the 16 weeks old plants on rooting medium of ½ MS supplemented with 3.0 mg/L IBA and 3.5 mg/L NAA for 4 weeks. This resulted in a significant difference in the mean of root number (3.50 JN1 accessions, 3.36 JCO4 accessions, 3.50 JE4 accessions and 3.50 JRV1 accessions) P ≤0.05. This is in agreement to a study done in India using the same PGRs (Shrivastava et al., 2008). The acclimatization of the rooted shoots was accomplished successfully after transfer to pots containing forest soil, sand and manure in the ratio of 2: 1:1 under greenhouse conditions.

In all developmental stages the use of MS in the absence of plant growth regulators led to senesces of the explant used. This establishes well the role of cytokinins and auxins in different stages of somatic embryogenesis as reported by Fujimura and Komamime (1980), Loschiavo et al. (1989) and Litz and Gray (1995). The most important research finding in his study was discovering the triggering combination and concentration of plant growth regulators besides other factors like type of tissue of a plant. It also confirms earlier studies which have reported that presence of auxins throughout the regeneration promotes the completion of embryogenesis stages. In this study the type of explants used in regeneration was an important factor (Shen et al., 2008). Combination of 1.5 mg/L BAP with 0.6 mg/ L KIN, 0.5 mg/ L IAA and 0.1 mg/ L TDZ is a breakthrough in high callus and shoot formation. The plant survival in the greenhouse indicates a successful establishment of an efficient *in vitro* regeneration protocol for *J. curcas.* This research finding could be useful in producing true to type plants of *Jatropha* in mass and genetic transformation for desirable traits.

Conclusion

A rapid and efficient *in vitro* regeneration system for *Jatropha* from both leaf discs and petiole explants was developed. The study has revealed that interactions among BAP, KIN, IAA and TDZ PGRs at varying concentrations, explant nature and genotype significantly influenced the regeneration process. This protocol expands the base for the creation of new *Jatropha* cultivars with increased yield, pest and disease resistance and oil for biofuel industry through genetic transformation.

Conflict of Interests

The authors have not declared any conflict of interests.

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