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Age and orientation of the cotyledonary leaf explants determine the efficiency of *de novo* plant regeneration and *Agrobacterium tumefaciens*-mediated transformation in *Jatropha curcas* L.

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Abstract

Effects of age and orientation of the explant on callus induction and *de novo* shoot regeneration from cotyledonary leaf segments of *Jatropha curcas* were studied. The callus induction and shoot regeneration capacity of cotyledonary leaf segments were found significantly related to the age of the explants and their orientation in culture medium. The youngest explant, derived from the cotyledonary leaf of germinated seed induced the highest regeneration response as compared to one- and two-week-old explants. A gradient response with age of the explant was observed in percentage of callus induction, shoot regeneration from callus and the number of shoots per regenerating callus. The explants cultured with their abaxial side in medium showed significantly higher regeneration response. The youngest explant was found to be most amenable to *Agrobacterium*-mediated transformation as compared to older explants. The fact that callus induced from the edges of the explant followed by *de novo* shoot induction, and strong transient *gus* expression observed in the edges of the explant are significant for routine *Agrobacterium*-mediated transformation and generation of stable transgenic plants in *J. curcas*.

Keywords: Abaxial; Age; Cotyledonary leaf; *Jatropha curcas*; Multiple shoots; Transformation

1. Introduction

Jatropha (*Jatropha curcas* L.), a hardy perennial shrub member of the Euphorbiaceae has attracted global attention in recent past as an important source of biodiesel (Annarao et al., 2008). The seeds of Jatropha contain 30–40% oil with a fatty acid pattern similar to that of edible oils (Gubitz et al., 1999). The seed oil can be used as a diesel engine fuel as it has characteristics close to those of fossil fuel, diesel. The non-edible oil of Jatropha seeds and its derivatives are also used for manufacturing a number of useful products, including candles, high quality soaps, cosmetics, biopesticide and fertilizer as well as for healing several skin disorders (Kochhar and Kochhar, 2005). Jatropha can be grown in marginal wastelands due to its ability to adapt to adverse agroclimatic conditions (Kaushik et al., 2007). It has an estimated annual production potential of 200,000 metric tonnes in India (Tiwari et al., 2007). However, conventional propagation of Jatropha is limited by poor seed germination, scanty and delayed rooting of seedlings and vegetative cuttings (Heller, 1996; Openshaw, 2000; Purkayastha et al., 2010). The low germination of whole seeds indicates that whole seed germination under controlled conditions considerably understates the potential germinability of Jatropha. This has significant implications on seed bank determinations and predictive seedling recruitment estimates, which rely on whole seed germination methods to assess seed viability. In this context, embryo or embryo derived explants culture offers a useful solution as well as being a promising method for initiating stock cultures for micropropagation of Jatropha as the explants derived from field grown plants are

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associated with high degree of microbial contaminants, and less responsive to *in vitro* culture manipulation (Purkayastha et al., 2010). Furthermore, it could offer a rapid means for conservation of elite germplasm. Although, the use of this process for clonal propagation, demanding seed tissues as starting material, requires evaluation of the heterogeneity in clones use of seeds from inbred elite Jatropha lines could circumvent the screening requirement.

Despite the best protein composition of Jatropha seed press cake, its use as feed for livestock carries potential concerns due to the presence of toxic substances, including a lectin (curcin) and phorbol esters (Makkar et al., 1998). Increased use of Jatropha seeds as a fuel source would risk increase in the production of the toxic by-product (meal), which could pile up as a potential waste (Becker and Makkar, 1998). Genetic engineering appears to be an effective approach to reduce the levels of these toxic substances in seeds, and increase resistance to biotic stresses, and furthermore, offers opportunities to modify seed oil for higher engine efficiency (Hossain and Davies, 2010). However, the development of an efficient regeneration system amenable to transformation is a prerequisite for genetic manipulation of this important biofuel plant. Plant regeneration in *J. curcas* has been accomplished through organogenesis from various explants, including mature leaf (Sujatha and Mukta, 1996; Sujatha et al., 2005; Soomro and Memon, 2007; Deore and Johnson, 2008), petiole (Sujatha and Mukta, 1996), hypocotyls (Sujatha and Mukta, 1996; Soomro and Memon, 2007), axillary node (Sujatha et al., 2005; Shrivastava and Banerjee, 2008) and via somatic embryogenesis from mature leaf explants (Jha et al., 2007). However, amenability of these regeneration systems to genetic transformation methods has not been evaluated. Seed-derived explants are, in general, known to be more responsive to rapid regeneration (Pradhan et al., 1998; Tiwari and Tuli, 2009) and *Agrobacterium*-mediated transformation (Patnaik et al., 2006; Paz, 2009). Our laboratory has recently developed a method for rapid and efficient plant regeneration from shoot apices, and generation of transgenic plants by direct DNA delivery to mature seed-derived shoot apices of Jatropha (Purkayastha et al., 2010). However, *Agrobacterium*-mediated transformation is preferred as it offers several advantages, such as the defined integration of transgenes, preferential integration into transcriptionally active regions of the chromosomes, and potentially single or low copy number with rearrangement being relatively rare (Birch, 1997; Hiei and Komari, 2006). Therefore, to obtain a large number of independent transgenic Jatropha plants for screening over expression or cosuppression of candidate genes, a high frequency regeneration protocol amenable to *Agrobacterium*-mediated transformation is required. Report on *Agrobacterium*-mediated transformation system in Jatropha using cotyledonary leaf explants (Li et al., 2007) has proven to be either inefficient or difficult to reproduce, a proposition attributed to the inappropriate age of the explant used. The age of the explant is a critical aspect in transformation experiment and therefore, appropriate biological condition of the explant is vital for optimal infection and T-DNA transfer by *Agrobacterium tumefaciens* (Purkayastha et al., 2010). Furthermore, the choice of explants of appropriate age, and the orientation of the explants in culture medium have been indicated to exert significant influence on the frequency of plant regeneration (Thomas, 2003; Bhatia et al., 2005). As a first step to achieving this goal, we have evaluated the effect of the age, and orientation of the cotyledonary leaf explants on plant regeneration, and *Agrobacterium*-mediated transformation.

2. Materials and methods

2.1. Plant material and explant preparation

Seeds of a local elite clone of *J. curcas* L. were collected from Rangia, Kamrup, Assam, India. The seeds were decoated and soaked in distilled water overnight at room temperature. The soaked seeds were treated with a 0.1% sodium hypochlorite solution containing few drops of Tween-20, for 10 min followed by washing under tap water for 20 min. The seeds were then surface sterilized with 70% alcohol for 5 min, and with 0.2% mercuric chloride for 10 min followed by four rinses in sterile double distilled water. After blot-dried on sterile filter paper, the endosperm was carefully dissected out to expose embryos with papery cotyledonary leaves. The papery cotyledonary leaves were separated out and cut into four segments (10 mm^2) with the edges removed and used as explants for callus induction, shoot regeneration and *Agrobacterium*-mediated transformation.

2.2. Induction of callus and shoot multiplication

The explants were placed with their abaxial surface firmly in contact with MS (Murashige and Skoog, 1962) medium supplemented with 6.66 μM of 6-benzylaminopurine (BAP) and 0.24 μM of indolebutyric acid (IBA) for 3 weeks in dark conditions for callus induction. The calli were transferred to MS medium supplemented with 6.66 μM BAP, 0.24 μM IBA and 1.44 μM gibberellic acid (GA_3) for induction of multiple shoots.

2.3. Influence of age and orientation of explants on shoot multiplication

To study the effect of the age of the explants on callus induction and shoot proliferation, and *Agrobacterium*-mediated transformation, cotyledonary leaves were obtained from freshly germinated seeds, and seedling developed from embryos, cultured for 1–2 weeks on MS medium. The leaves were cut into four segments (10 mm^2) with their edges removed to obtain the explants.

To investigate the effect of orientation of explants on callus induction and shoot proliferation, the cotyledonary leaf explants, and leaf explants (1–2 weeks old) were cultured with their abaxial or adaxial surface in firm contact with the medium.

2.4. Culture media and conditions

All culture media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar (Hi-media, Mumbai, India). The pH of the medium was adjusted to 5.8 prior to autoclaving at 15 psi and 121 °C for 20 min. All the cultures were maintained at 25±2 °C under a 16 h photoperiod with a photosynthetic photon flux

density of 35 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India).

After 3 weeks of incubation in the dark, the efficacy of each variant on callus induction, and after 4 weeks of incubation of calli in 16 h photoperiod, the shoot multiplication rate were determined by recording (1) the percentage of explants forming callus, (2) the frequency of shoot regeneration and (3) the average number of shoots per callus.

2.5. Shoot elongation, rooting and acclimatization

The individual shoots were separated from shoot clusters and transferred to MS medium supplemented with varied concentrations of GA_3 (0.5, 1.0, 1.5, and 2.0 μM) to allow for the elongation of shoots. Elongated shoots were cultured for rooting on half strength MS medium supplemented with varied concentrations (1.0, 2.5, and 5.0 μM) of IBA and α-naphthaleneacetic acid (NAA). Plantlets with well-developed roots were removed from the culture medium, washed gently under running tap water, and transferred to plastic pots containing soil, vermiculite, and vermicompost (1:1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (28 °C day, 20 °C night, 16 h day-length, and 70% relative humidity). After a week, the plastic covering was gradually removed and the plantlets were maintained in the greenhouse in plastic pots containing normal garden soil until they were transplanted to the nursery.

Experiments were set up in a completely randomized design and each treatment had three replicates of 20 explants each. All data are statistically analyzed by ANOVA followed by Newman–Keul's multiple range test for mean comparison.

2.6. Transformation procedure and histochemical GUS-assay

A. tumefaciens strain EHA105 harbouring a binary vector pCAMBIA2301 which contains *β*-glucuronidase (*gus*) with an intron in the coding region and neomycin phosphotransferase (*nptII*) genes, both driven by CaMV 35S promoter was used for transformation studies. Single colony of the bacterial strain was inoculated in 25 ml of liquid AB minimal medium (Chilton et al., 1974) containing 10 mg/l rifampicin and 50 mg/l kanamycin, and grown overnight at 28 °C until OD_{600} reached to 0.8. The cells were collected by centrifuging at 5000 rpm for 5 min and the pellet was resuspended in liquid cocultivation medium, LCM (MS medium containing 6.66 μM of BAP and 0.24 μM of IBA, pH adjusted to 5.7) supplemented with 100 μM acetosyringone. The cotyledonary leaf explants excised from seeds, and leaf explants (1–2 weeks old) were inoculated in bacterial suspension for 30 min with occasional shaking in dark. The explants were then blotted on sterile filter paper and cocultivated in petridishes lined with filter paper, moistened with LCM supplemented with 100 μM acetosyringone for 3 days at 25 °C. After 3 days of cocultivation, the explants were washed three to four times with sterile distilled water and blotted dry on sterile filter paper.

Histochemical GUS assays (Jefferson et al., 1987) were used to assess transient expression of the *gus* gene. Transient *gus* expression in leaf explants was scored after 3-day-cocultivation, and the number of explants showing transient *gus* expression at their edges were scored by immersing the tissue materials in GUS substrate solution for 24 h at 37 °C. Following incubation, tissues were bleached with 100% ethanol, and examined under microscope.

3. Results and discussions

3.1. Callus induction and shoot multiplication

Cotyledonary leaf explants (Fig. 1a) placed on the MS medium supplemented with 6.66 μM of BAP and 0.24 μM of IBA exhibited distinct morphological changes. The explants turned to green in color within a week of culture, enlarged, and swelled at their edges within the following two weeks of incubation showing the sign of dedifferentiation (Fig. 1b). The explants formed white friable callus after 3 weeks of culture. *De novo* shoot bud induction was observed on the calli (Fig. 1c and d) developed at the edges of explants, within two weeks of transfer of calli to MS medium containing 6.66 μM BAP, 0.24 μM IBA and 1.44 μM GA_3 at 16 h photoperiod regime. The cut edges of leaves provided a way for nutrients and growth regulators to be absorbed efficiently from the medium (Sarwar and Skirvin, 1997), and the synergistic effect of BAP and IBA was shown to trigger response for callus induction in Jatropha (Sujatha and Mukta, 1996; Weida et al., 2003; Li et al., 2007). Prior incubation in the dark was a very important process for dedifferentiation and redifferentiation of Jatropha. Prior incubation in the dark has been reported to increase shoot regeneration in various plant species, such as Zhanhua winter jujube (Gu and Zhang, 2005), campanula (Sriskandarajah and Serek, 2004), watermelon (Compton, 1999), quince (Baker and Bhatia, 1993), pear (Chevreau et al., 1989), apple (Fasolo et al., 1989), and blueberry (Billings et al., 1988). Incubation in the dark may delay degradation of endogenous and/or exogenous plant growth regulators (Rusli and Pierre, 2001). In addition, dark treatment may reduce the levels of cell wall thickness and cell wall deposits (cellulose and hemicellulose), facilitating translocation of plant growth regulators in plant cells (Herman and Hess, 1963). The calli developed cluster of multiple shoots (Fig. 1e) with an average of 12.56 shoots in 94% of the responded calli (Table 1) within 4 weeks of transfer of callus to shoot induction medium. Cotyledonary leaves have been most widely studied for *de novo* shoot formation in diverse plant species (Nikam and Shitole, 1997; Amutha et al., 2003; Vengadesan et al., 2003).

3.2. Influence of age and orientation of explants on callus induction and shoot multiplication

The factors that significantly influenced the efficiency of callus induction and prolific *de novo* plant regeneration in cotyledonary leaf segment explants of Jatropha were the age of the explant, and its orientation in culture. Although the explants of different age formed callus and subsequently induced clusters of multiple shoots, however, the percentage of explants that formed callus, the percentage of callus that induced multiple shoots and the average number of shoots per callus significantly

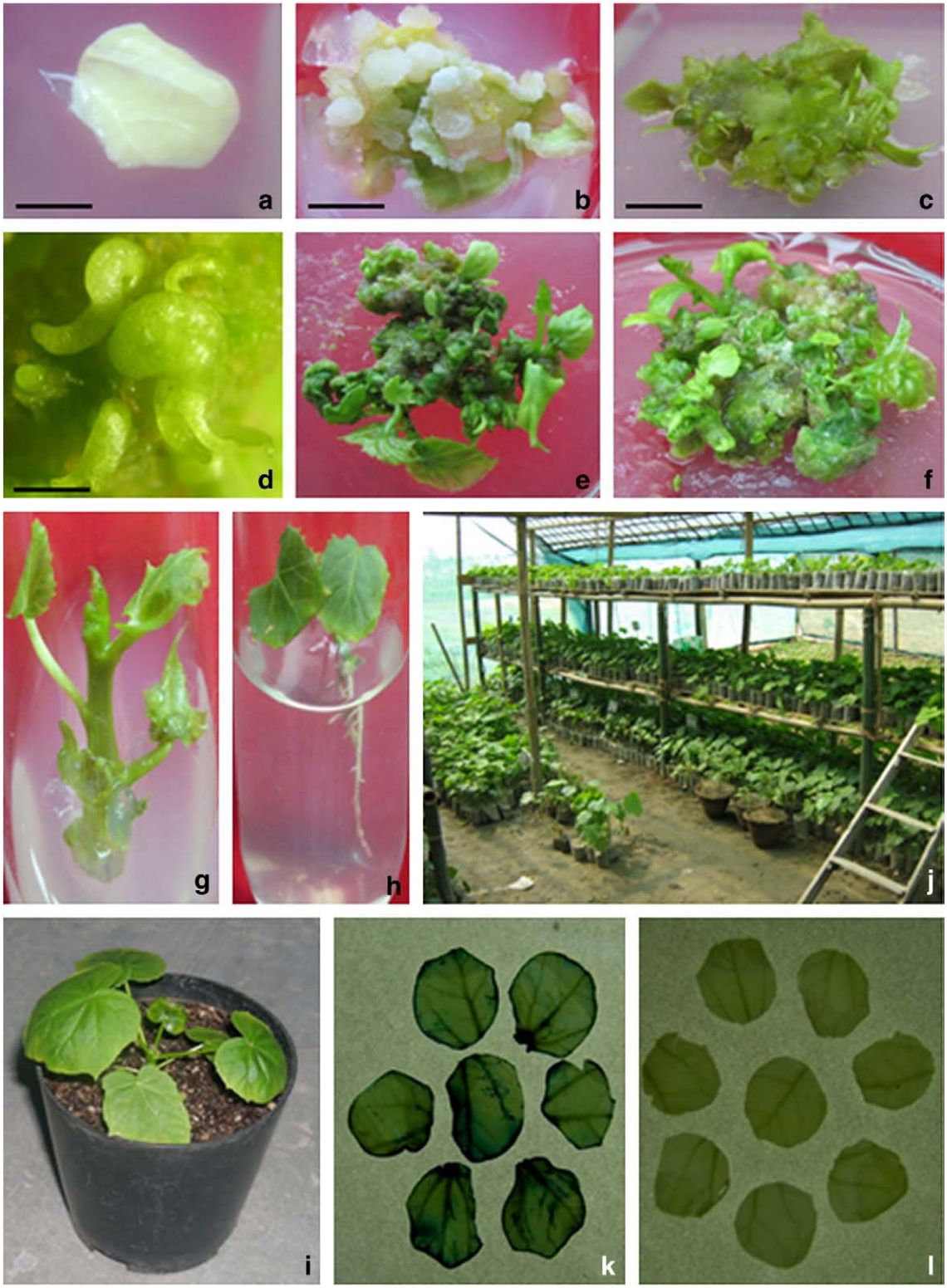

Fig. 1. (a–l). *De novo* shoot bud induction and plant regeneration from cotyledonary leaf explant of *Jatropha curcas* L. (a) Cotyledonary leaf segment explant of freshly germinated seed (bar: 0.7 cm). (b) White, friable callus induced from cotyledonary leaf segment on MS medium with BAP (6.66 μM) and 0.24 μM indole-3-butyric acid (IBA) after 3 weeks of dark incubation (bar: 1.4 cm). (c) *De novo* shoot bud induction from cotyledonary leaf segment derived callus on MS medium supplemented with BAP (6.6 μM), IBA (0.24 μM) and GA_3 (1.44 μM) after 2 weeks of culture at 16 h photoperiod regime (bar: 1.8 cm). (d) Microscopic view of leaf edge showing shoot bud induction (bar: 0.15 mm). (e, f) Profuse shoot bud clump formation from cotyledonary leaf segment derived callus. (g) Shoot elongation on MS medium supplemented with GA_3 (1.0 μM) after 2 weeks of culture. (h) Rooting of *in vitro* regenerated shoot on half strength MS medium supplemented with NAA (5.00 μM) within 17 days of culture. (i) An acclimatized plant. (j) Plants established in nursery. (k, l) GUS expression at the cut edges of cotyledonary leaf segment immediately after cocultivation with *Agrobacterium tumefaciens* EHA105pCAMBIA2301 (k) and untransformed control leaf explant (l).

Table 1
Effect of age of explant on callus formation and multiple shoot induction from cotyledonary leaf explants of *J. curcas* L.

Age of the cotyledonary leaf explant (days)	Response of the cotyledonary leaf explant		
	Callus formation (%)	Average no of shoots	Regeneration frequency (%)
0	87.50[a]±3.46	1256[a]±0.38	87.50[a]±3.11
7	75.83[ab]±3.46	4.35[b]±0.32	65.83[b]±3.27
14	70.83[bc]±3.46	2.66[c]±0.35	57.50[bc]±3.24

The values are the means (±SE) of six replicates with 20 explants each. Means within a column followed by different letters are statistically significant at $P<0.05$ by Newman–Keul's multiple range test.

declined with the increase in age of the explants (Table 1). The best regeneration response was observed in explants, prepared from cotyledonary leafs of freshly germinated seeds with 87.5% of the explants forming callus, out of which 94% of the callus regenerated forming an average of 12.56 shoots, a response which was significantly higher than one- and two-week-old explants (Table 1). Famiani et al. (1994) proposed that explants from young leaves show more regeneration potential than older leaves as the younger leaves, still developing, have less differentiated and more metabolically active cells, and therefore, under suitable hormonal and nutritional conditions show improved plant regeneration. Furthermore, explants of different age may have different levels of endogenous hormones and, therefore, the age of explants would have a critical impact on the regeneration efficiency, similar results have been reported in other plants, including *Platanus occidentalis* (Sun et al., 2009), *Morus alba* (Thomas, 2003), *Cajanus cajan* (Dayal et al., 2003), *Rosa hybrida* (Ibrahim and Debergh, 2001), *Aerides maculosum* (Murthy and Pyati, 2001), *Cercis canadensis* (Distabanjong and Geneve, 1997), *Malus* (Famiani et al., 1994), *Cydonia oblonga* (Baker and Bhatia, 1993), *Aegle marmelos* (Islam et al., 1993), *Lachenalia* (Niederwieser and Van Staden, 1990) and *Prunus* (Mante et al., 1989). In contrast with the above results, a higher regeneration response was observed from explants of older leaves as compared to the younger explants in apple (Antonelli and Druart, 1990).

Success of regeneration from leaf explants depends not only on the age of the explant chosen, but also the way explants are placed on the culture media (Duzyaman et al., 1994; Bhatia et al., 2005). Explants can be inoculated on the culture media in abaxial (lower surface facing down) or adaxial (upper surface facing down) orientation for the cotyledons and leaves (George, 1993). The best results were obtained when the leaf abaxial side was touching the culture medium as in this position the regeneration obtained was nearly two-fold higher than the leaf explants cultured with their adaxial side in contact with the medium (Table 2). Similar observations have been reported with the leaf abaxial side touching the culture medium (Dolcet-Sanjuan et al., 1990; Fiola et al., 1990; Leblay et al., 1990; Stamp et al., 1990; Welander and Maheswaran, 1992; Duzyaman et al., 1994; Bartish and Korkhovoi, 1997). Shoots produced from the adaxial orientation protruded into the culture medium and then turned upwards, trying to emerge from the medium. This resulted in a reduced number of shoots being produced per explant. The contrasting response of the explants in its two different orientations is attributed to the total surface area of the explants that come in contact with the medium.

Table 2
Effect of orientation of explant in the culture medium on callus formation and multiple shoot induction from cotyledonary leaf explants of *J. curcas* L.

Orientation of the cotyledonary leaf disc attached to the medium	Response of the leaf disc		
	Percentage of callus formation	Regeneration frequency (%)	Average no of multiple shoots
Abaxial	87.84[a]±4.32	87.62[a]±4.06	12.54[a]±0.38
Adaxial	54.23[b]±3.63	56.34[b]±4.45	07.35[b]±0.42

The values are the means (±SE) of four replicates with 20 explants each. Means within a column followed by different letters are statistically significant at $P<0.05$ by Newman–Keul's multiple range test.

3.3. Elongation of shoots

In the present study, shoot clumps produced on caulogenesis medium, either took prolonged duration for elongation of a few shoot buds or majority of the shoots buds turned necrotic and later died on transferring to phytohormone-free basal medium, a response which is quite prevalent in plant regeneration from callus (Ibrahim and Debergh, 2001). It appears that the hormone levels present in the medium used for shoot induction have a carry-over effect in the shoots. This situation caused prolific multiplication of shoot initials from the calli, and this process prevented individual shoot elongation. It was necessary to develop a suitable medium for faster elongation of shoot buds. Incorporation of 1.0 μM GA_3 to MS media significantly enhanced the shoot elongation within one to two weeks of culture (Table 3; Fig. 1g), as compared to medium devoid of GA_3. The promotive effect of GA_3 on elongation of stunted shoots has been reported in several other plant species (Demeke and Hughes, 1990; Jordan and Oyanedel, 1992; Purohit and Singhvi, 1998; Sugla et al., 2007; Purkayastha et al., 2008). GA_3 is considered to stimulate shoot elongation by inhibiting the action of auxins in meristematic regions (Taiz and Zeiger, 1998).

3.4. Rooting and transplantation

Rhizogenesis is the final step in the formation of complete plantlet in *in vitro* culture. Root formation occurred in 40% of the shoots within 20–22 days of culture on half strength MS medium.

Table 3
Effect of gibberellic acid (GA3) on elongation of shoots from cotyledonary leaf derived callus of *J. curcas* L. on MS medium after two weeks of culture.

GA3 (μM)	Mean shoot length (cm)	Fold increase in shoot length
0	1.37[a]±0.07	0
0.5	2.90[b]±0.03	1.11[b]±0.03
1.0	5.12[c]±0.03	2.73[a]±0.03
1.5	3.22[d]±0.03	1.35[b]±0.03
2.0	3.35[e]±0.03	1.44[b]±0.03

The values are the means (±SE) of three replicates with 20 cultures each. Means within a column followed by different letters are statistically significant at $P<0.05$ by Newman–Keul's multiple range test.

No roots could be induced on full strength MS basal medium, wherein the shoot bases turned brown, indicating necrosis effect. Roots were not induced on medium containing IBA, and the shoots formed varying degree of callus at shoot base (Table 4). However, NAA containing medium induced roots without any callus induction at the shoot base (Table 4). Highest percentage of root induction occurred in 75% of the shoots in the medium containing 5.0 μM NAA within 17–20 days of culture (Fig. 1h). The contrasting difference between the present observation and our previous report (Purkayastha et al., 2010), wherein all the shoot apex-derived shoots induced roots in half strength medium could be attributed to the origin of shoots. Plantlets with well-established roots were successfully acclimatized (Fig. 1i) in pots containing soil, vermiculite and vermicompost (1:1:1) and eventually established in a nursery (Fig. 1j) with survival frequency of 97%. The established plants were apparently uniform and did not show any observable variation.

3.5. Influence of age of explants on Agrobacterium-mediated transformation

Agrobacterium-mediated transformation is the preferred method for its simplicity, cost effectiveness, little rearrangement of transgenes, ability to transfer relatively longer DNA segments (Hamilton et al., 1996) and preferential integration of foreign genes into transcriptionally active regions (Ingelbrecht et al., 1991) ensuring thereby proper expression of transgenes in transgenic plants (Hernandez et al., 1999). However, *Agrobacterium*-mediated transformation has often proved unsuccessful due to the accumulation of secondary metabolites in explants used, and particularly complex mixtures such as the oils (Sugimura et al., 2005) or bacteriostatic polyphenols (Kumar et al., 2004). Therefore, selecting leaves at a developmental stage that possesses low amounts of oil, without compromising their prolific regeneration ability of the explants is crucial for establishing successful *Agrobacterium* infection and transgenic plant generation. In order to select leaf materials for their greater amenability to *Agrobacterium*-mediated genetic transformation, the transient *gus* expression analysis was performed on explants from cotyledonary leaves of different age, following three days of cocultivation with *A. tumefaciens*. The cotyledonary leaf disc explants excised from one- and two-week-old *in vitro* raised seedlings were compared with cotyledonary leaf disc explants excised from freshly germinated seeds for their transformation efficiency. Strong *gus* expression was observed at their edges (Fig. 1k), and the explants demonstrated ability for *de novo* shoot formation. The endogenous *gus* activity (color) was not detected in non-transformed (control) explants (Fig. 1l). The *gus* activity at the cut ends indicated the susceptibility of explants to *Agrobacterium*-mediated transformation. However, a significant difference in percentage of explants showing *gus* expression was observed among the explants of different ages (Fig. 2). The number of *gus* expressing explants was significantly higher in the case of cotyledonary leaf explants, excised from the freshly germinated seeds, as compared to the explants excised from one- and two-week-old leaves (Fig. 2).

In conclusion, the age of explants, and its orientation in culture medium were found as the most critical factors influencing induction of callus and *de novo* shoot regeneration from cotyledonary leaf explants in *J. curcas*. The data presented in this report clearly suggested that the cotyledonary leaf segment explants prepared from seeds, cultured with their abaxial surface placed in the medium showed significantly better regeneration response as compared to older explants, and the explants cultured with their adaxial surface in culture medium. Furthermore, cotyledonary leaf segment explants, prepared from freshly germinated seeds were found to be most amenable to *Agrobacterium*-mediated transformation as compared to older explants. The method established here could be employed for routine transformation by *Agrobacterium*-mediated method for transformation of cotyledonary leaf explants and *de novo* generation of transgenic plants in *J. curcas*.

Table 4
Effect of different concentrations of auxins, IBA and NAA added to half strength MS medium on rooting of shoots raised from cotyledonary leaf derived callus of *Jatropha curcas* L.

Auxin (μM)	Percentage of rooting	Degree of callus formation at shoot base	No of days required for rooting
–	40[a]±3.71	–	20–22
IBA			
1.0	–	+	–
2.5	–	++	–
5.0	–	++++	–
NAA			
1.0	–	–	–
2.5	55[b]±4.26	–	19–22
5.0	75[c]±4.39	–	17–20

The values are the means (±SE) of three replicates with 20 cultures each. Means within a column followed by different letters are statistically significant at $P<0.05$ by Newman–Keul's multiple range test.

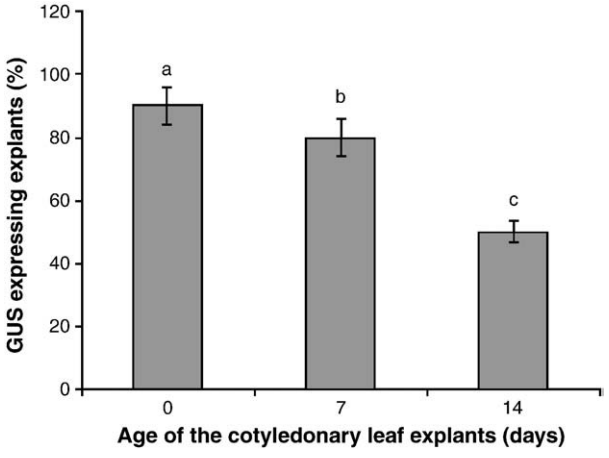

Fig. 2. Effect of age of the explant on efficiency of T-DNA transfer in *Agrobacterium*-mediated transformation of cotyledonary leaf explants of *J. curcas* L. Different letters denote significant differences at $P<0.05$ between treatments. Bars represent standard errors.

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