In vitro clonal propagation of biodiesel plant (*Jatropha curcas* L.)

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In the present investigation, *in vitro* clonal propagation of seven-month-old *Jatropha curcas* L. was achieved employing nodal explants. Axillary shoot bud proliferation was best initiated on Murashige and Skoog's (MS) basal medium supplemented with 22.2 μM N^6-benzyladenine (BA) and 55.6 μM adenine sulphate, in which cultures produced 6.2 ± 0.56 shoots per nodal explant with 2.0 ± 0.18 cm average length after 4–6 weeks. The rate of shoot multiplication was significantly enhanced after transfer to MS basal medium supplemented with 2.3 μM 6-furfuryl amino purine (Kn), 0.5 μM indole-3-butyric acid (IBA) and 27.8 μM adenine sulphate for 4 weeks. Both shoot number (30.8 ± 5.48) and average shoot length (4.8 ± 0.43 cm) were found to increase significantly. About 52% of root induction occurred in MS basal medium supplemented with 1.0 μM IBA in 2–3 weeks. Further elongation of roots with average length of 8.7 ± 1.35 cm was obtained in unsupplemented MS basal medium for 2–3 weeks. The plantlets (12–16-week-old) were successfully acclimatized in soil with 87% survival frequency.

Keywords: Axillary shoot, clonal propagation, *Jatropha curcas*, nodal explants.

JATROPHA CURCAS L. (Euphorbiaceae) or physic nut is an all-purpose, zero-waste perennial plant. It is considered as a potential source of non-edible fuel-producing plant along with its different medicinal properties and grows well in the tropical and subtropical climate in India. The seeds contain 4–40% viscous oil known as 'curcas oil'. The oil is high in cetane value and can be used directly in diesel engines added to diesel fuel as an extender or transesterized to a biodiesel fuel. The oil is a clean fuel reducing greenhouse gas emissions, has greater lubricity and reduces engine wear. Pure *Jatropha* biodiesel is non-toxic in nature. This oil is a strong purgative, widely used as an antiseptic for cough, skin diseases and as a pain reliever in rheumatism. Refining crude *Jatropha* oil into bio-fuel products produces glycerine as by-product, which is in great demand as a raw material for cosmetic, medicine and food product industries.

India has growing energy and transport fuel demand, where *J. curcas* has the potential to become one of the world's key energy crops. However, inexpensive biodiesel can be produced from India's vast agribiotechnological resources offering a clean substitute for expensive fossil fuel imports, thus enabling the country to meet the objectives of economic growth, fuel security and cleaner air.

J. curcas is the most primitive species of the genus and forms artificial and natural hybrid complexes readily and possess a problem to the genetic fidelity[1]. Conventional agriculture uses seeds and cuttings for its propagation. But the seeds are heterozygous in nature and the cuttings are seasonal. Moreover, it is reported that vegetative cuttings are not deep-rooted and are easily uprooted as they do not form a taproot system[2]. Seed set has been reported to be low in vegetatively propagated plants[2].

Tissue culture studies were undertaken in different species of *Jatropha*. Morphogenesis from endosperm tissues has been reported in *J. panduraefolia*[3–5]. High frequency regeneration from various explants of *J. integerrima* has been reported[6]. Using different explants, plant regeneration protocols have also been described in *J. curcas*[2,7–9], but multiplication rate was low for field applications. Moreover, no lab-to-land transfer protocol of *J. curcas* using nodal meristems is available. Nodal meristems are an important source tissue of micropropagation and plants raised from these are comparatively more resistant to genetic variation[10]. Keeping in mind the economical importance of *J. curcas*, critical analysis of the earlier protocols necessitated formulating a well-documented, reproducible, *in vitro* micropropagation protocol.

In the present communication, we report an efficient regeneration system of micropropagation from nodal explants of a seven-month-old *J. curcas* plant and establishment of plants under field conditions.

J. curcas L. seeds collected from Ramakrishna Mission, Narendrapur, West Bengal were sown in the experimental garden of Presidency College, Kolkata. The seedlings were grown in sterile vermiculite at 25–30°C in light. All the explants were collected from this donor plant for the present investigation.

Nodal explants (2–3 cm in length) collected from the seven-month-old donor plant were kept for 3 h in a systemic fungicide, Bavistin (BASF India Ltd) prior to surface-sterilization. They were surface-sterilized in 0.1% $HgCl_2$ (w/v) for 20–25 min followed by repeated washing (five times) with sterile distilled water. After sterilization, the explants were trimmed (~1.0 cm) at the base and cultured with the cut surface in contact with the culture medium (Figure 1 *a*).

For axillary shoot bud proliferation, the nodal explants cultured on Murashige and Skoog (MS)[11] basal medium at full and half strength, supplemented with 3% (w/v) sucrose were studied. MS basal medium was supplemented with 55.6 μM adenine sulphate; different cytokinins, e.g. 2.3–37.2 μM 6-furfurylamino purine (Kn), 2.2–35.6 μM N^6-benzyladenine (BA), 2.3–36.4 μM (thidiazuron; TDZ) or 2.5–39.4 μM 2-iP (2-isopentenyladenine) individually for 4–6 weeks (Sigma, Aldrich, USA). All media were solidified with 0.75% (w/v) agar (Merck, India).

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All experiments were carried out in culture tubes (150 × 25 mm) containing 20 ml of culture medium. The pH of all media was adjusted to 5.7 ± 0.1 prior to autoclaving at 121°C at 1.1 kg sq. cm for 15 min. Cultures were incubated under 16 h/8 h light/dark cycles (artificial light, 80 μmol per sq. m per s) at 22 ± 2°C.

After 4–6 weeks of culture, the shoots cultured on MS basal medium supplemented with 3% (w/v) sucrose, 22.2–35.6 μM BA and 55.6 μM adenine sulphate were transferred to MS basal medium supplemented with 3% (w/v) sucrose, 27.8 μM adenine sulphate, 2.2–23.2 μM Kn and 2.2–22.2 μM BA, singly or in combination, as well as in combination with 0.5–5.0 μM IBA (indole butyric acid) for raising multiple shoots. The best response of (30.8 ± 5.48) shoots per node was obtained in medium containing 2.3 μM Kn, 27.8 μM adenine sulphate and 0.5 μM IBA.

For initiation of roots, the 8–10-week-old shoots (2.0–3.0 cm in length) were cultured on MS basal medium supplemented with 3% (w/v) sucrose and different concentrations of auxins were tested individually: 0.5–11.0 μM α-napthaleneacetic acid (NAA); 0.5–10.0 μM IBA; 0.5–11.4 μM IAA (indoleacetic acid) for 2–3 weeks. The shoots were also tested on unsupplemented full or half strength MS basal medium with 3% sucrose (w/v) for root initiation. The rooted (10–13-week-old) shoots were then transferred to MS basal medium supplemented with 3% sucrose (w/v) for 2–3 weeks for further elongation. Addition of activated charcoal (0.5, 1.0%) to MS basal medium supplemented with 3% sucrose (w/v) for root elongation was also tested.

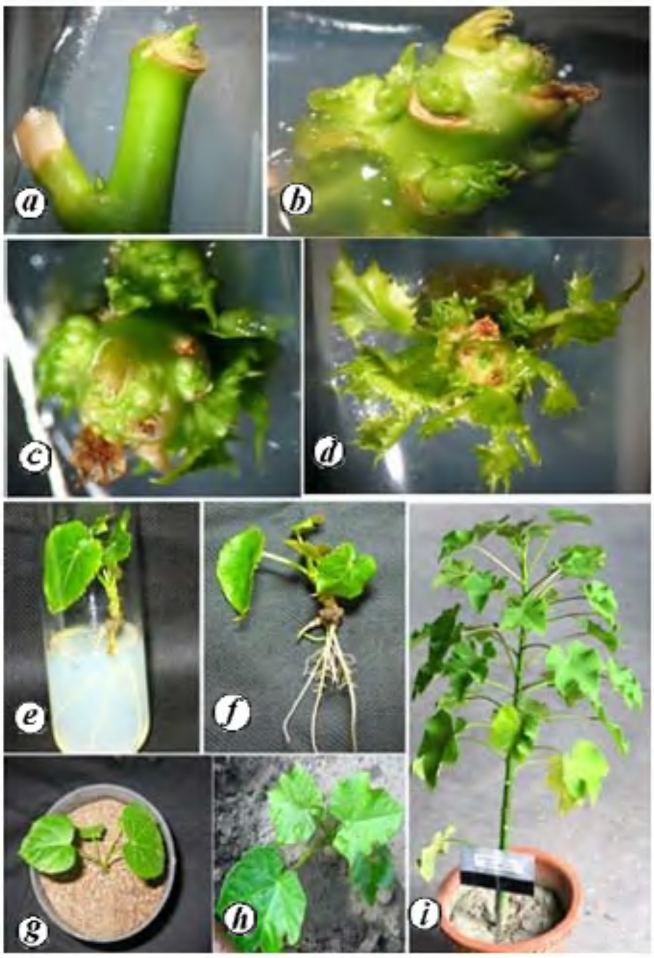

Figure 1. *In vitro* clonal propagation of biodiesel plant (*Jatropha curcas* L.). ***a***, Nodal explants from the donor plant. ***b***, Nodal explants after the first 2–3 weeks of culture. ***c***, Axillary bud breaking after 4–6 weeks of culture. ***d***, Profuse multiplication of shoots after 8–10 weeks of culture. ***e***, Initiation of roots in *in vitro* shoots. ***f***, *In vitro* shoots with slender and white taproot system. ***g***, Complete plantlet (12–16-week-old) grown under field conditions. ***h***, Plant after 10 weeks of hardening. ***i***, Ten-month-old *in vitro* grown plant.

The complete rooted plantlets (12–16-week-old) were hardened for three weeks in MS basal medium under diffuse light (16/8 h photoperiod) in culture room prior to field transfer. Then the plantlets were transferred to pots containing a mixture of soil and vermiculite in the ratio 1 : 1 and covered with polyethylene bags to maintain more than 80% relative humidity, with temperature ranging from 25°C to 30°C and kept in field conditions. About 87% of the plants survived after 3 weeks of hardening.

The experiments were set up in a completely randomized design. Data were analysed by analysis of variance (ANOVA) to detect significant differences between means[12]. Means differing significantly were compared using Duncan's Multiple Range Test (DMRT) at 5% probability level. Variability of data was expressed as mean ± standard error (SE).

Initiation of cultures from the nodal explants did not pose a major problem. During initiation, the explants did not show any leaching or browning of tissues. MS basal medium was the most effective for *in vitro* shoot multiplication from nodal explants. The nodal explants cultured on MS basal medium supplemented with 55.6 μM adenine sulphate and different cytokinins showed varied response (Table 1). Nodes cultured on half strength MS basal medium showed no visible signs of tissue differentiation. This was possibly due to a greater demand of nitrogen and potassium-containing compounds, which induce greater amount of new proteins[13]. These components are lower in half strength MS basal medium compared to full strength MS basal medium.

The best response (6.2 ± 0.56 shoots per nodal explant) was obtained in the presence of 55.6 μM adenine sulphate, 22.2 μM BA and was found to be significantly ($P \leq 0.05$ level) higher than shoots induced per nodal explant in other concentrations of cytokinins (Kn, 2-iP or TDZ) used in the present study (Table 1; Figure 1 *b*, *c*). However, at the same concentration of 22.2 μM BA, a lower initiation (1.5 ± 0.2 shoot buds per explant; 2.2 ± 0.6 shoots per nodal explant) of shoot bud proliferation was reported[2,8]. This differential response may be attributed to the specific age and physiological condition of the donor plant from which the nodal explants were excised. Nodes cultured on medium with different concentrations of Kn showed lower induction of axillary shoot-bud

Table 1. Effect of different types and concentrations of cytokinins on shoot multiplication of *Jatropha curcas*

Media formulations: MS basal medium + 3% sucrose (w/v) + 55.6 μM adenine sulphate +	Concentration of cytokinin used (μM)	Average number of shoots (~> 0.5 cm) developed per nodal explant	Average length of shoots developed (cm ± SE)
Kinetin	2.3	2.2 ± 0.17^{b}	0.8 ± 0.02^{a}
	4.6	2.0 ± 0.10^{b}	0.8 ± 0.04^{a}
	11.6	2.4 ± 0.12^{b}	0.8 ± 0.06^{a}
	23.2	3.5 ± 0.35^{c}	0.9 ± 0.07^{a}
	37.2	1.0 ± 0.01^{a}	0.8 ± 0.02^{a}
BA	2.2	1.0 ± 0.01^{a}	0.8 ± 0.02^{a}
	4.4	2.0 ± 0.13^{b}	0.8 ± 0.05^{a}
	11.1	2.7 ± 0.23^{bc}	0.9 ± 0.03^{a}
	22.2	6.2 ± 0.56^{f}	2.0 ± 0.18^{b}
	35.6	5.3 ± 0.40^{e}	1.4 ± 0.12^{ab}
2-iP	2.5	1.0 ± 0.03^{a}	1.0 ± 0.03^{a}
	4.9	1.2 ± 0.05^{a}	0.9 ± 0.02^{a}
	12.3	1.0 ± 0.02^{a}	0.8 ± 0.02^{a}
	24.6	1.0 ± 0.01^{a}	0.9 ± 0.05^{a}
	39.4	1.0 ± 0.03^{a}	0.8 ± 0.04^{a}
TDZ	2.3	3.0 ± 0.02^{c}	0.8 ± 0.03^{a}
	4.5	1.2 ± 0.04^{a}	0.9 ± 0.06^{a}
	11.4	1.0 ± 0.05^{a}	0.9 ± 0.02^{a}
	22.7	1.0 ± 0.02^{a}	0.8 ± 0.03^{a}
	36.4	1.0 ± 0.01^{a}	0.9 ± 0.02^{a}

Data were recorded after 4–6 weeks of culture. Each treatment was repeated thrice and each replicate consisted of 3–5 nodal explants. Means having different letters as superscripts are significantly different from each other ($P \leq 0.05$) according to DMRT.

proliferation. Though TDZ is known to induce cytokinin-like effects in a number of plant species, particularly woody[14] as well as herbaceous crop species[15], the present study showed negligible effects of TDZ on induction of shoot multiplication as also reported in *J. curcas* earlier[2]. *Jatropha* belongs to the family Euphorbiaceae and our observation confirms earlier reports[16,17] that among the cytokinins BA plays an important role in initiation of shoot-bud proliferation in many members of the Euphorbiaceae family.

The 4–6-week-old-nodal cultures on MS basal medium supplemented with 55.6 μM adenine sulphate and 22.2–35.6 μM BA on transfer to MS basal medium supplemented with 27.8 μM adenine sulphate with cytokinin singly or in combination, as well as in combination with IBA showed varied response (Table 2). In the present study, transfer of cultures to MS basal medium with lower concentration of adenine sulphate (27.8 μM) and with same (22.2 μM) or reduced (2.2–11.1 μM) concentration of BA, led to induction of callus at the base and failed to induce further shoot proliferation (Table 2). Results also indicated that shoots cultured on medium with combinations of BA and Kn did not proliferate further. On the other hand, transferring the cultures from a set having higher concentration of 22.2 μM BA and 55.6 μM adenine sulphate to a set with lower concentration of 2.3 μM Kn, 27.8 μM adenine sulphate and 0.5 μM IBA, led to a significantly higher nodal shoot multiplication (30.8 ± 5.48 shoots per nodal explant) within the next 4 weeks, which was not recorded previously (Table 2; Figure 1 *d*). This was possibly due to a combined effect of different growth regulators along with other additives. Lowering of growth regulators in micropropagation studies to achieve higher rate of multiplication has been reported in *Holarrhena antidysenterica*[18]. However, it has been observed in *Jatropha* that it requires higher concentration of only one type of cytokinin (BA) for induction phase and favours lower concentration of another type of cytokinin (Kn) along with other additives for escalation and proliferation of shoot cultures. A recent report[2] indicated similar effect in *J. curcas* with BA and TDZ, but the authors obtained lesser number of shoots (2.0 ± 0.8 to 12.3 ± 1.7) with comparatively higher amount of cytokinin.

No roots could be induced in either MS or half strength MS basal medium, but profuse callusing at the base of the shoots was noted in the presence of NAA and IAA. However, when 2.0–3.0 cm elongated shoots were placed on MS medium with lower concentration of 0.5–1.0 μM IBA, roots were induced in 52% of the shoots within three weeks (Table 3; Figure 1 *e*). Other concentrations of 2.5–10.0 μM IBA did not induce any roots. A distinct taproot system developed with slender and white secondary roots; this was considered important for hardening and field transfer (Figure 1 *f*). Significant increase in root length occurred on transfer to only MS basal medium without addition of charcoal[8] for another 2–3 weeks. It is to be mentioned in this regard that rooting was obtained in MS basal medium supplemented[2] with 5.4 μM NAA, and in MS basal medium supplemented[8] with higher amount of 15.0 μM IBA.

Table 2. Effect of growth regulators on shoot multiplication from nodal explants of *J. curcas*

Growth regulators (μM)*				
BA	IBA	Kinetin	Number of shoots per explant	Length of shoots
2.2	0	0	1.0 ± 0.01[a]	0.8 ± 0.02[a]
4.4	0	0	2.0 ± 0.13[b]	0.8 ± 0.05[a]
11.1	0	0	3.0 ± 0.25[a]	1.0 ± 0.05[a]
22.2	0	0	6.5 ± 0.60[c]	2.2 ± 0.19[b]
2.2	0	2.3	1.0 ± 0.02[a]	0.9 ± 0.06[a]
4.4	0	4.6	1.2 ± 0.05[a]	0.8 ± 0.05[a]
11.1	0	11.6	1.0 ± 0.03[a]	0.9 ± 0.08[a]
22.2	0	23.2	1.0 ± 0.01[a]	0.8 ± 0.04[a]
2.2	0.5	0	1.2 ± 0.08[a]	1.0 ± 0.05[a]
4.4	1.0	0	1.5 ± 0.04[a]	0.8 ± 0.06[a]
11.1	2.5	0	1.3 ± 0.03[a]	0.9 ± 0.03[a]
22.2	5.0	0	1.0 ± 0.02	0.9 ± 0.01[a]
0	0.5	2.3	30.8 ± 5.48[g]	4.8 ± 0.43[d]
0	1.0	4.6	14.6 ± 2.93[f]	3.9 ± 0.48[c]
0	2.5	11.6	11.2 ± 2.73[e]	3.6 ± 0.32[c]
0	5.0	23.2	9.8 ± 1.86[d]	3.0 ± 0.29[c]
0	0	2.3	2.4 ± 0.25[a]	0.9 ± 0.04[a]
0	0	4.6	2.2 ± 0.22[a]	0.9 ± 0.06[a]
0	0	11.6	2.6 ± 0.28[a]	0.9 ± 0.05[a]
0	0	23.2	3.7 ± 0.36[b]	1.0 ± 0.08[a]

*MS basal medium + 3% sucrose + adenine sulphate (27.8 μM) along with different plant growth regulators. Data were recorded after 3–4 weeks of culture. Each treatment was repeated thrice and each replicate consisted of 3–5 nodal explants. Means having different letters as superscripts are significantly different from each other ($P \leq 0.05$) according to DMRT.

Table 3. Effect of IBA on initiation of roots in excised shoots of *J. curcas*

IBA (μM)*	Percentage of root induction	Number of roots per shoot	Length of roots (cm ± SE)
0	0	0	0
0.5	15	1.0 ± 0.06[a]	1.2 ± 0.09[a]
1.0	52	5.6 ± 0.42[b]	8.7 ± 1.35[b]
2.5	0	0	0
5.0	0	0	0
10.0	0	0	0

*MS basal medium + 3% sucrose.
Data were recorded after 2–3 weeks of culture. Each treatment was repeated thrice and each replicate consisted of 3–5 shoots. Means having different letters as superscripts are significantly different from each other ($P \leq 0.05$) according to DMRT.

The shoots (12–16-week-old) were removed from the medium, thoroughly washed with water, dipped for 1 h in 0.1% (w/v) bavistin (systemic fungicide), and transplanted to plastic pots containing a mixture of (1 : 1) soil and vermiculite (Figure 1 *f*). The plantlets were irrigated with tap water as and when required. Polythene covers were completely withdrawn after 3–5 weeks of hardening (Figure 1 *g*). Plants were then transferred to potted soil for further growth (Figure 1 *h*). The plants ranged from 86.0 ± 0.12 to 102.0 ± 0.21 cm in height after 10 months (Figure 1 *i*).

The present study describes a well-documented and reliable micropropagation protocol of *J. curcas* from nodal meristems with much higher rate of multiplication. This protocol can be used as a basic tool to commercialize cultivation of the biodiesel plant.

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A new species of the Indian caecilian genus *Gegeneophis* Peters (Amphibia: Gymnophiona: Caeciliidae) from the surroundings of Mahadayi Wildlife Sanctuary, Western Ghats

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A new species of Indian caecilian, *Gegeneophis mhadeiensis* (Amphibia: Gymnophiona: Caeciliidae) is described based on three specimens collected from the Western Ghats, northern Karnataka, India. The species is distinguished from all other *Gegeneophis* by a combination of the number of primary and secondary annuli. One of the paratypes is an albino.

Keywords: Caecilians, *Gegeneophis mhadeiensis*, Mahadayi Wildlife Sanctuary.

THE Indian caecilian (Gymnophiona) genus *Gegeneophis* contains nine nominate species[1], with six of these having been described since 1999 in a wave of new systematic work[2]. We recently collected three specimens resembling each other which fit the generic diagnosis given by Ravichandran *et al.*[3] for *Gegeneophis*, but which differ from all known species. Here we describe this form as a new species.

Gegeneophis mhadeiensis sp. nov. Figures 1–3. Holotype: Bombay Natural History Society, Mumbai, India (BNHS/4643). A mature male, collected from the surroundings of Rameshwar temple, 15°39′ N lat., 74°08′E long. (Chorla Village, Khanapur Taluk, Belgaum District, Karnataka), July 2006. The locality is situated at 728 msl, adjacent to the Mahadayi Wildlife Sanctuary in the Western Ghats region.

Paratypes: Zoological Survey of India, Calicut (ZSI/WGFRS/V/A/640), an immature female, collected from Chavatyaar, Chavato vaddo, Chorla village at 732 msl. This locality is about 2 km from the type locality and the other collection details are as for the holotype. BNHS/4643, an albino, mature male collected from Chavatyaar; other collection details are as for the holotype.

Diagnosis: A species of *Gegeneophis* that fits the generic diagnosis of Ravichandran *et al.*[3] differing from all other species in the genus except *G. carnosus*, *G. krishni* and *G. nadkarnii* in having more than 100 primary annuli (117–122), but differing from *G. carnosus* (secondary grooves 7) and *G. krishni* (secondary grooves 13–15) in having far more (29–31) primary annuli with secondary annular grooves, and from *G. nadkarnii* (secondary grooves 86) in having lower number of secondary annular grooves (29–31).

Description of holotype: Some morphometric and meristic data are given in Table 1. The specimen is in good condition generally, except for minor artifacts associated with preservation; notably mid-ventral longitudinal groove of 134 mm length extends between the third nuchal groove and the vent. There is a 20 mm long mid-ventral incision into the body cavity beginning 41 mm in front of the vent. There are several small scratches on the skin on both the dorsal and ventral surfaces made during the search for scales.

The body in life is sub-cylindrical and slightly dorsoventrally compressed (Figure 1), though almost uniform in its width throughout (Table 1). In dorsal view, the head tapers strongly from the level of the occiput to the tentacular apertures. Anteriorly, the head tapers and terminates in a bluntly rounded but narrow snout tip. The posterior part of the head is slightly narrower than the nuchal region. In lateral view, the top of the head is straight and without any strong bulges. The margin of the upper lip is slightly arched. The distance between the jaw angle and the top of the head is less than the distance between the jaw angle and the ventral surface of the lower jaw. In ventral view, the anterior margin of the lower jaw is more broadly rounded than the anterior margin of the snout.

The small sub-circular nostrils are close to the front of the snout tip, and are visible dorsally and laterally but not

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