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ORIGINAL PAPER

# Establishment of long-term proliferating shoot cultures of elite *Jatropha curcas* L. by controlling endophytic bacterial contamination

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**Abstract** Leaf explants of the second or third node were collected from field-grown elite *Jatropha curcas* trees and incubated in Murashige and Skoog's (Physiol Plant 15:473–497, 1962) medium supplemented with growth regulators. Direct shoot organogenesis was induced when explants were incubated in a medium containing 0.5 mg $l^{-1}$ benzyladenine (BA) and 0.1 mg $l^{-1}$ indolebutyric acid (IBA). A maximum of seven shoot buds differentiated within 6 weeks of culture incubation. Indirect shoot organogenesis was obtained when explants were incubated in the medium supplemented with 0.5 mg $l^{-1}$ BA along with 1.0 mg $l^{-1}$ each of 2,4-dichlorophenoxyacetic acid (2,4-D) and indoleacetic acid (IAA). A pulse treatment of 0.5 mg $l^{-1}$ thidiazurone (TDZ) and 0.1 mg $l^{-1}$ IBA for 5 days was necessary for shoot organogenesis in green compact callus before subculture into 0.5 mg $l^{-1}$ BA and 0.1 mg $l^{-1}$ IBA containing medium. Leaf explants of *J. curcas,* collected from the field, contained endophytic bacterial contamination, which expressed itself after 2–3 subcultures. These bacteria were cultured and identified as *Enterobacter ludwigii*. After staining, these were found as gram-negative bacteria. Their sensitivity against different antibiotics has been tested by culturing them with different antibiotic stabs for 72 h. Finally, Augmentin® was found as the most effective and suitable antibiotic which not only controlled the bacteria within 2–3 subcultures but also supported the regeneration system and growth of the regenerated shoots and such cultures have been grown for a long-term of over 2 years without any contamination.

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**Keywords** Augmentin · Bacterial contamination · Direct shoot organogenesis · Indirect shoot organogenesis *Jatropha curcas* · Long-term culture

**Abbreviations**

| | |
|---|---|
| BA | 6-Benzyladenine |
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| Kn | Kinetin |
| MS | Murashige and Skoog's (1962) medium |
| NAA | α-Naphthaleneacetic acid |
| TDZ | Thidiazurone |

## Introduction

*Jatropha curcas* (Fam. Euphorbiaceae) is a biodiesel plant, which is perennial, fast growing, easy to propagate and can survive with minimum inputs (Bush and Leach 2007). It produces oil from the seeds, which can be combusted as fuel without being refined (Keith 2000; Li et al. 2007). It burns with clear smoke-free flame and has been tested successfully as green fuel for simple diesel engine (Forson et al. 2004). Although there are other sources available as biofuel crops, such as soybean, sunflower, corn and maize, but being a non-food crop *Jatropha* cultivation as biodiesel crop is most preferred, considering social, economic and humanitarian reasons (Chhetri et al., 2008; Fairless 2007; Runge and Senauer 2007). Millions of hectares of *Jatropha* plantation are being projected to be undertaken in the coming years worldwide. In view of *Jatropha*'s plant life being as much as 50 years, the quality of planting material

is bound to have long term tangible ramifications. A foolproof protocol for propagation of elite *Jatropha* plant through tissue culture is imperative for its quality planting material.

Field-grown material from elite mature trees is preferred for collecting explants due to its genetic stability and known characters. Rajore and Batra (2005) have propagated *J. curcas* by collecting shoot tip explants from two-year-old field-grown plant. Most of the reports refer to in vitro shoot organogenesis from different seedling explants (Sujatha and Mukta 1996; Wei et al. 2004), but there is no report on direct shoot organogenesis from field-grown *Jatropha* leaves. Recently, few reports on *Jatropha* propagation through somatic embryogenesis (Jha et al. 2007) and in vitro clonal propagation through organogenesis (Datta et al. 2007; Lin et al. 2002; Lu et al. 2003; Sujatha et al. 2005) have been published, but all these reports pertain to seedling explants. The present report is the first where leaf explants were collected from field-grown elite trees instead of shoot tip explants, because direct shoot organogenesis from mature leaves is a prerequisite for genetic transformation of a known elite tree. The possibility of developing a chimeric plant after transformation is minimal in case of leaf explants as compared to shoot tips. Gressel (2008) reviewed the importance of transgenics in order to increase the efficiency of biofuel crops. On the other hand, indirect shoot organogenesis, also reported in this communication, is more likely to produce somaclonal variation and could be used for selecting variants.

*Jatropha curcas* impregnates endophytic bacterial contamination which expresses itself after 2–3 subcultures in the medium. Initially, these bacteria do not hinder formation and development of new shoots, but later they affect the growth of newly developed shoots. These shoots turn yellowish brown and become necrosed making it impossible to grow cultures beyond three subcultures, similar to the reports by Pirttilä et al. (2008) with Scots pine. In this respect, there are many reports where long term cultures were destroyed by endophytic bacteria, viz., watermelon (Thomas 2004; Thomas et al. 2006), grapes (Thomas 2006), papaya (Thomas et al. 2007) and banana (Thomas et al. 2008) which could be controlled by the use of antibiotics in the medium (Kulkarni et al. 2007; Reed et al. 1995).

In the present communication, besides developing a shoot organogenesis protocol through field-grown leaves, the intractable problem of endophytic bacterial contamination stands resolved by the use of appropriate antibiotics, and such cultures are being grown for a long-term of over 2 years without any contamination.

## Materials and methods

### Establishment of field-grown leaf explants under aseptic condition

Initially, four high yielding elite accessions of *Jatropha curcas,* aged 2 years, namely, JA-9, JA-18 and JA-135 and C2 from CSMCRI, Bhavnagar, India, growing in NBRI campus were used as experimental material. However, C2 accession from CSMCRI, Bhavnagar, being highly regenerative in culture, was selected for further experiments. Fully expanded leaves from field-grown plants of C2 accession were excised from 1st to 5th node and used for regeneration purposes (Fig. 1a). The leaves were initially washed with tap water and surface sterilized with 0.1% $HgCl_2$ followed by 3–4 washings with sterilized distilled water. All the thick veins and all the margins of the leaf lamina were removed and inoculated in the medium.

### Direct and indirect shoot organogenesis in field-grown leaf explants

For direct shoot organogenesis, the segments of leaf lamina were incubated in MS media supplemented with a combination of 0.25 to 1.0 mg $l^{-1}$ BA, kinetin (Kn) or TDZ

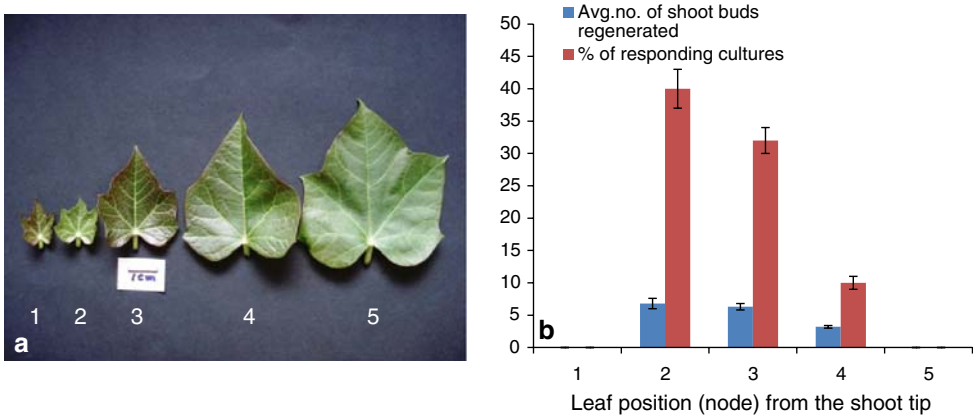


**Fig. 1** **a** Position of leaves from the shoot tips. **b** Shoot organogenesis and percent response in different leaves

**Table 1** Influence of growth regulators on direct shoot organogenesis from leaf lamina of *J. curcas* after 6 weeks of culture

| Treatment (Conc. mg $l^{-1}$) | | % of explants responding ± S.E. | Mean of regenerated shoot buds ± S.E.[a] | Associated callus | Health status of regenerated shoots |
|---|---|---|---|---|---|
| BA 0.25 | IBA 0.1 | 68.4 ± 1.08 | 4.6 ± 0.46 | – | Shoots were fresh, green and healthy |
| BA 0.5 | | 83.6 ± 0.46 | 6.8 ± 0.60 | – | |
| BA 1.0 | | 70.0 ± 1.52 | 5.8 ± 0.60 | + | |
| BA 0.25 | IBA 0.2 | 56.8 ± 0.96 | 4.2 ± 0.34 | + | |
| BA 0.5 | | 63.8 ± 1.22 | 6.2 ± 0.52 | + | |
| BA 1.0 | | 51.8 ± 1.15 | 3.8 ± 0.34 | ++ | |
| Kn 0.25 | IBA 0.1 | – | – | + | Shoots were yellowish green |
| Kn 0.5 | | – | – | + | |
| Kn 1.0 | | 13.4 ± 0.73 | 2.6 ± 0.22 | ++ | |
| Kn 0.25 | IBA 0.2 | – | – | + | |
| Kn 0.5 | | – | – | ++ | |
| Kn 1.0 | | 14.6 ± 0.88 | 1.2 ± 0.34 | ++ | |
| TDZ 0.25 | IBA 0.1 | 29.2 ± 1.31 | 3.6 ± 0.46 | + | Shoots were vitrified |
| TDZ 0.5 | | 42.0 ± 0.85 | 4.0 ± 0.63 | ++ | |
| TDZ 1.0 | | 35.4 ± 1.43 | 3.8 ± 0.52 | +++ | |
| TDZ 0.25 | IBA 0.2 | 39.6 ± 1.29 | 2.2 ± 0.34 | ++ | |
| TDZ 0.5 | | 46.2 ± 0.96 | 3.4 ± 0.46 | +++ | |
| TDZ 1.0 | | 38.0 ± 1.00 | 3.2 ± 0.52 | +++ | |

Culture incubation: 45 days

[a] Mean of five replicate cultures

'+' sign denotes increasing amount of callus

along with 0.1 or 0.2 mg $l^{-1}$ IBA, as shown in Table 1. Indirect organogenesis involved three phases, (1) for callus induction, 1 or 2 mg $l^{-1}$ 2,4-D was used in combination with 0.5 mg $l^{-1}$ BA or Kn and 1.0 mg $l^{-1}$ IAA or α-naphthaleneacetic acid (NAA, Fig. 3a, b); (2) for shoot organogenesis from the callus, a pulse treatment of 0.25–2.0 mg $l^{-1}$ TDZ and 0.1 mg $l^{-1}$ IBA was given for 2, 5, 10 or 15 days and (3) after the pulse treatment, the callus was subcultured in different concentrations of BA (0, 0.1, 0.25 or 0.5 mg $l^{-1}$) along with 0.1 mg $l^{-1}$ of IBA.

The pH of all the media was adjusted to 5.8 prior to the addition of agar and autoclaved at 121°C for 20 min. All the media were solidified with 7 g $l^{-1}$ agar. Cultures were incubated at 25 ± 2°C under 16/8 h light/dark period. Light at 60 µmol photon $m^{-2}$ $s^{-1}$ was supplied by fluorescent tube lights fitted on culture racks.

## Endophytic bacterial contamination

After 2–3 subcultures of 4 weeks each, all the regenerating cultures showed bacterial contamination. The experiments were revised 3–4 times with all the accessions collected from NBRI campus. The fresh explants of C2 accession remained infection-free up to 2–3 subcultures beyond which a similar type of bacterial contamination was observed visually. This bacterial contamination was latent in the explants, despite all the precautions taken during manual subculture.

## Tissue homogenization and microscopic examination

The in vitro growing contaminated regenerating cultures from C2 accession, as well as the clean cultures (procured after use of antibiotics mentioned later) were used directly for homogenate preparation following the procedure as mentioned by Thomas et al. (2008). Shoots and leaf tissues (2 g each) were homogenized aseptically in 0.1× nutrient broth (100 mg $ml^{-1}$) using a sterilized mortar and acid washed sand. It was centrifuged at 1,000 rpm for 5 min at 4°C. The sand particles were allowed to settle down at 4°C for 20–30 min. The tissue homogenate of the samples was serially stained through sterile cheesecloth followed by Whatman filter No.1 to remove tissue debris and organelles. A thin smear of the filtrate was taken in the glass slide. A total of 10 µl of gram crystal violet (0.5% concentration) and 10 µl of gram safranin (0.5% concentration) were added over the smear. Cover slip was carefully kept without allowing any air bubble. The samples were observed under inverted microscope (Nikon Eclipse TE 300 Nikon Cameras and accessories, photovision, 223, Okla Industrial Estate) at 450X, and the images were captured as described elsewhere (Thomas et al. 2008).

### Bacteriological media, culture and identification of bacteria

Bacterial isolate was taken from in vitro growing contaminated regenerating cultures and streaked on YEB minimal medium (Lichtenstein and Draper 1986) without any antibiotic. The plates were grown at 28°C in the dark. After 24 h the plates have been sent to Microbial Type Culture Collection and Gene Bank, Chandigarh, for identification of the bacteria.

### Sensitivity test of the bacteria using different antibiotics

The bacterial smear was grown on YEB medium with stabs of different antibiotics available in the local market, so as to test bacterial sensitivity towards various antibiotics. These bacterial cultures were grown for 72 h of incubation at 28°C. Thereafter the ring diameter around the antibiotic stab was measured. Antibiotics producing more than 1.1 cm of ring were selected as highly effective (++), while those with less than 1.0 cm as moderately effective (+), whereas without ring as resistant (R). The data is being presented in Table 2.

### Effects of antibiotics on control of contamination, shoot regeneration and growth

Antibiotics, either highly effective (++), or moderately effective (+) for bacterial contamination (Table 3), were used at different concentrations and combinations in the regeneration medium after filter sterilization. For initial optimization of antibiotics, contaminated leaf explants having just differentiated shoots, almost uniform in appearance, were subcultured in antibiotic containing medium. Later, all the contaminated cultures at different stages of regeneration were used in the optimized concentration of antibiotic, so as to check the removal of bacterial contamination completely. Augmentin®, a combination of amoxycillin and clavulanic acid (GlaxoSmithKline Pharmaceuticals Limited, Mumbai, India) and Novamox® (Cipla) containing only amoxycillin (generic name) antibiotic were used. Gatiquin® (Cipla) for gatifloxacin, Lomadey® (Dr. Reddy's Lab) for lomefloxacin, Pelox® (Wockhardt) for pefloxacin, Genticin® (Nicholus Piramal) for gentamycin and Ciplox® (Cipla) for ciprofloxacin, were used in the regeneration medium. Lower concentrations of these antibiotics were also used in combination. Data with regard to the effect of various antibiotics on control of bacterial contamination, number of cycles to control bacterial contamination along with the growth and regeneration of proliferating shoots was recorded (Table 3).

### Statistical analysis

Morphological changes were recorded by visual observations. Results were subjected to analysis of variance and significance test. The values of data are mean ± standard error of five replicate cultures. Each experiment was repeated twice.

## Results and discussion

### Establishment of field-grown leaf explants under aseptic condition

Among four accessions, JA-9 and JA-135 could not be established in culture due to heavy bacterial contamination. However, C2 accession from CSMCRI, Bhavnagar, being more regenerative than JA-18 accession (data not shown), was selected for all the further experiments on shoot organogenesis. The position of leaf on the plant was evaluated in terms of number of shoots regenerated, percentage of responding cultures and condition of the explants in culture. The results are presented in Fig. 1b. It was found that the segments from the youngest leaf turned brown due to leaching of phenols and did not respond in culture. However, segments from the second and third leaves, measuring approx. 2.0–2.5 cm in width and 3.0–3.5 cm in length (Fig. 1a), responded well in culture by regenerating maximum number of shoots (6.8 and 6.3, respectively) within 4–6 weeks of incubation. Conversely, the fourth leaf responded very late i.e. only few shoot buds (3.2) were visible after 8 weeks; these too, later turned

**Table 2** Antibiogramme of different antibiotics showing sensitivity of *Enterobacter ludwigii* contamination in *J. curcas*

| Sl. no. | Name of antibiotic | Sensitivity response | Diameter of the ring (cm) |
|---|---|---|---|
| 1 | Augmentin | Highly sensitive (++) | 1.2 |
| 2 | Gatifloxacin | Highly sensitive (++) | 1.4 |
| 3 | Lomefloxacin | Highly sensitive (++) | 2.0 |
| 4 | Pefloxacin | Highly sensitive (++) | 1.5 |
| 5 | Ciprofloxacin | Moderately sensitive (+) | 1.1 |
| 6 | Gentamycin | Moderately sensitive (+) | 0.9 |
| 7 | Levofloxacin | Moderately sensitive (+) | 1.0 |

**Table 3** Effect of commonly used plant antibiotics on contaminated regenerating cultures of *J. curcas*

| Name of antibiotics (Conc. mg $l^{-1}$) | Removal of bacteria | Growth of the proliferating shoots | | |
|---|---|---|---|---|
| | | Associated callus | Mean number of regenerated shoots/explant[a] | Health status of the shoots |
| Novamox® | | | | |
| 250 | +++** | ++ | 0 | Yellowish green shoots |
| 500 | +++* | ++ | 0 | |
| Augmentin® | | | | |
| 100 | +++*** | +++ | 4.2 ± 0.52 | Green, healthy & growing shoots |
| 200 | +++** | +++ | 7.2 ± 0.77 | |
| 300 | +++* | ++ | 4.6 ± 0.46 | |
| 400 | +++* | ++ | 3.8 ± 0.34 | |
| Pelox® | | | | |
| 250 | +++** | – | 0 | Shoot necrosis |
| 500 | +++* | – | 0 | |
| Gatiquin® | | | | |
| 250 | +++** | – | 2.7 ± 0.32 | Shoot necrosis |
| 500 | +++* | – | 0 | |
| Lomadey® | | | | |
| 250 | +++** | – | 0 | Shoot necrosis |
| 500 | +++* | – | 0 | |
| Genticin® | | | | |
| 250 | +++** | +++ | 0 | Shoots remained green and healthy |
| 500 | +++* | +++ | 0 | |
| Ciplox® | | | | |
| 250 | +++** | + | 1.4 ± 0.46 | Shoots became yellowish green |
| 500 | +++* | – | 0 | |

Culture incubation: 30 days

'+' sign denotes positive response to some extent; '++' sign denotes positive response to moderate extent; '+++' sign denotes complete positive response

'–' sign denotes no response

'*' sign denotes number of cycles

[a] Mean of five replicate cultures

yellow and ultimately died. The segments of the fifth leaf did not respond in culture may be due to its maturity. Therefore, the second and third leaves were selected for shoot bud differentiation.

## Shoot organogenesis in field-grown leaf explants

### *Direct shoot organogenesis*

Shoot buds were visible to the naked eye within 4–6 weeks of incubation. Shoot buds differentiated directly from leaf lamina (Fig. 2a), from the petiolar end and also from the cut surfaces (Fig. 2b, c). This morphological ability which increases from the tip towards the base of the leaf had also been reported in Asiatic hybrid lily (Misra and Kochhar 2008). It was explained by the fact that leaves reach maturity first at the distal part and subsequently basipetally towards the proximal part. A nutritional medium having a combination of 0.1 mg $l^{-1}$ IBA and 0.5 mg $l^{-1}$ BA was optimum where a maximum of seven shoot buds could be regenerated from one leaf segment within 6 weeks of incubation (Table 1). These results were similar to those of Datta et al. (2007) and Wei et al. (2004), where BA and IBA (at low concentration) were found beneficial for direct shoot organogenesis in *J. curcas*. The concentrations and combinations of these growth hormones were very crucial to avoid callus formation. In a preliminary experiment, IAA enhanced callus formation in *Jatropha* as against IBA, which was reported beneficial for shoot organogenesis similar to the reports by Datta et al. (2007), Jha et al. (2007), Sujatha et al. (2005) and Wei et al. (2004). In the presence of 1.0 mg $l^{-1}$ Kn, only 1–2 shoots could be induced, that too were yellowish green in color. However, Jha et al. (2007) reported best embryo induction in the presence of Kn and IBA which was further stimulated in the presence of adenine sulphate (AdS). Addition of TDZ instead of BA, induced shoot buds at low concentration of 0.25 or 0.5 mg $l^{-1}$, but shoots formed were vitrified. A large callus was developed along with the shoots, due to which further growth of the regenerated shoots was hindered (Table 1).

The analysis of variance showed highly significant differences ($P > 0.01$) between the concentrations of BA for both the parameters, i.e., percentage of explants responding and the number of shoots differentiated. However, IBA and its interaction with BA revealed significant differences

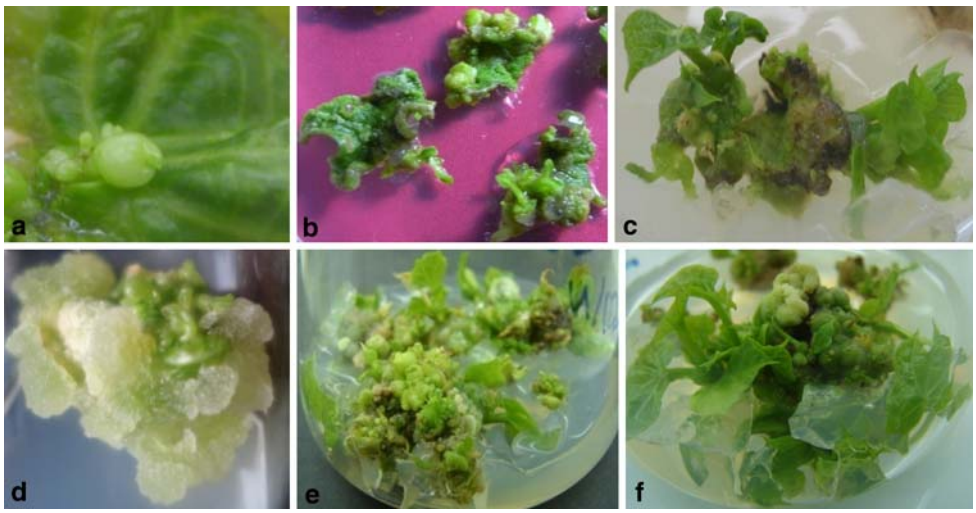


**Fig. 2** Clean cultures of *Jatropha curcas*. **a–c** Direct shoot organogenesis from leaf lamina, **a** from basal portion of leaf having veins, **b** from margins/cut surfaces of segments of leaf lamina, **c** from adaxial and abaxial surfaces of segments of leaf lamina. **d–f** Indirect shoot organogenesis in leaf callus, **d** shoot organogenesis from leaf callus, **e** different stages of developing shoot buds, **f** growing shoots

($P > 0.01$) only for percentage of explants responding, whereas, it remained non significant for the number of shoots differentiated. The treatments of TDZ and IBA showed highly significant differences ($P > 0.01$) for percentage of explants responding and significant differences ($P > 0.05$) for the number of shoots differentiated. Although, their interaction was significant ($P > 0.05$) for percentage of explants (data not shown) only.

## Indirect shoot organogenesis

### *Callus induction from leaf segments*

The entire leaf segments were callused within 2–3 subcultures of 25 days cycle. Among different combinations used, the optimum treatment for callus induction in leaf segments was 0.5 mg $l^{-1}$ BA with 1.0 mg $l^{-1}$ each of 2,4-D and IAA (Fig. 3). At a low concentration of 1.0 mg $l^{-1}$ 2,4-D, callus was more green and compact as compared to higher concentration of 2.0 mg $l^{-1}$, where it was more yellowish as reported by Tao et al. (2002).

### Shoot organogenesis in callus

Both types of calli (green and compact; yellow and friable) were employed for shoot organogenesis. TDZ played a major and crucial role for shoot bud formation. A pulse treatment of 0.5 mg $l^{-1}$ TDZ and 0.1 mg $l^{-1}$ IBA was optimum to induce shoot buds in green compact callus raised in the presence of BA. Whereas, the yellow callus developed in the presence of Kn could neither develop shoot buds nor somatic embryos, as reported by Jha et al. (2007). Yellow friable callus is an attractive tissue for somatic embryogenesis (Brisibe et al. 2000), while green compact callus is good for shoot organogenesis (Tao et al. 2002). Similar results were obtained in the present communication. The maximum shoot organogenesis could be obtained in 5 days pulse treatment of 0.5 mg $l^{-1}$ TDZ, beyond which a large callus was developed. It was seen that any other callus, like, shoot tip callus, could also be made to differentiate using TDZ for pulse treatment.

Callus after pulse treatment of TDZ was subcultured in 0.5 mg $l^{-1}$ BA and 0.1 mg $l^{-1}$ IBA, as used for direct

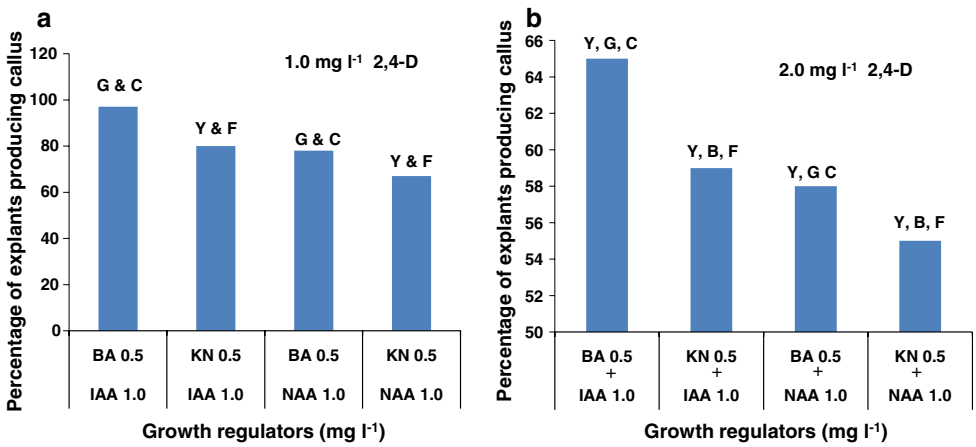


**Fig. 3** Callus induction in leaf segments of *Jatropha curcas* Y-*yellow*; G-*green*; B-*brown*; C-*compact*; F-*friable callus*, **a** 1.0 mg $l^{-1}$ 2,4-D, **b** 2.0 mg $l^{-1}$ 2,4-D

shoot organogenesis. Approximately, 7–10 shoot buds started appearing from 1 $cm^2$ of callus explants within 10 days of incubation (Fig. 2d, e). The process of shoot bud formation was asynchronous, where new buds continued to form, while the older ones grew into shoots (Fig. 2f). These results were similar to those of Sujatha et al. (2005), who showed the presence of TDZ initially for some period in primary cultures and then subculture in BA and IBA containing media was necessary for shoot bud regeneration.

## Endophytic bacterial contamination

Endophytic bacterial contamination expressed itself after 2–3 subcultures of *J. curcas* in the medium. The regenerated shoots along with the associated callus slowly turned yellow and brown (Fig. 4a–c). Although some part of the tissue continued to form new shoot buds, within 2 weeks all the tissues necrosed (Fig. 4d). It became impossible to continue cultures any longer. This problem is very much similar to the report by Thomas et al. (2008), who found that apparently clean stocks of banana harbored viable but-non-culturable bacteria initially which, after repeated subculture, was expressed and became culturable. Only then it was possible to control these endophytic bacteria.

## Identification of organism and microscopic examination

The bacterial colonies isolated and cultured from C2 accession were sent to MTCC, Chandigarh, India, and identified as *Enterobacter ludwigii*. After staining, these were found as gram-negative bacteria. The efforts to localize the endophytes in the intercellular spaces through stereomicroscopy in liquid film were unsuccessful on account of the inability to discriminate the bacterial cells from host tissue constituents. However, inverted microscopy on tissue homogenate of both the samples like, leaf samples and shoot tips filtered serially, to remove the tissue debris and cell organelles, from in vitro- infection-free explants in liquid film showed a host of cell organelles only (Fig. 5a). Conversely, in vitro-infected samples showed a host of cell organelles along with motile bacterial cells in the background (Fig. 5b). These bacteria occurred singly as short rods with entire margin, flat elevation and smooth surface with 0.5–1.0 $\mu$ in size. The wriggling bacterial cells could be viewed under bright field (450×) in liquid film with the use of crystal violet stains.

## Sensitivity test using antibiotics in the bacteriological medium to control endophytic bacteria

The results of antibiogramme are presented in Table 2. A range of 22 antibiotics tested against *E. ludwigii*, 4 were found highly effective, 3 moderately effective, while amikacin, ampicillin, azithromycin, cefazolin, cefexim, cefoperazone, cefotaxim, cefpodoxim, ceftazidime, ceftriaxone, moxifloxacin, nitrofurantoin, norfloxacin, ofloxacin and septran were found ineffective.

## Effects of antibiotics on control of contamination, shoot regeneration and growth

Antibiotics found highly or moderately effective against bacterial colonies were used in the regeneration media at

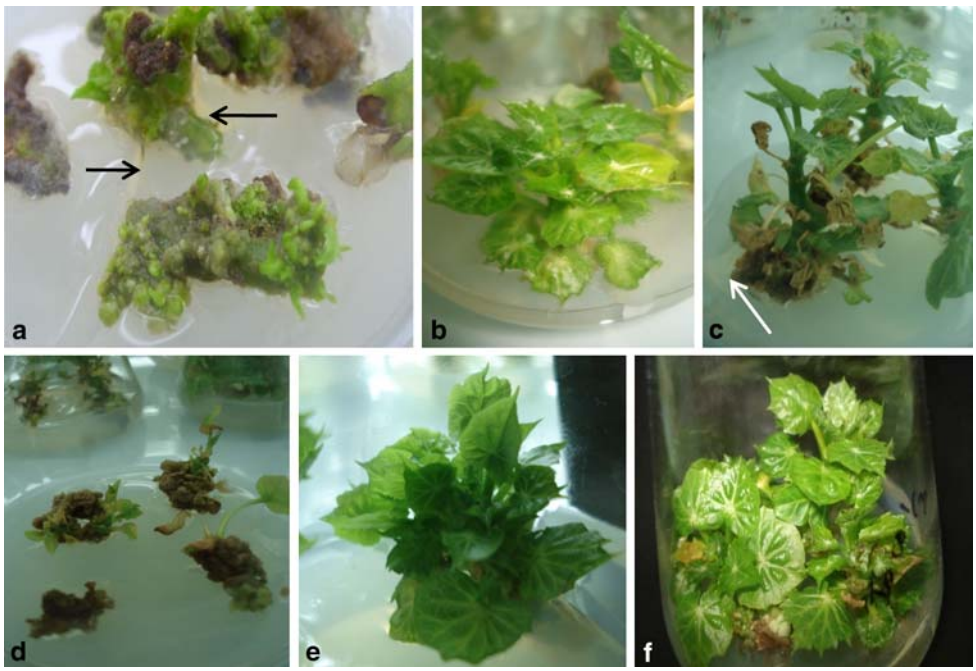


**Fig. 4** **a–d** Cultures with endophytic bacterial contamination. **a** Regenerating shoots despite the bacterial contamination, **b** Yellowing of leaves, **c** Browning of leaves, **d** Necrosis of cultures, **e** Growing clean shoots with 400 mg $l^{-1}$ augmentin and, **f** 45-days-old proliferating clean shoots with 50 mg $l^{-1}$ augmentin in the medium

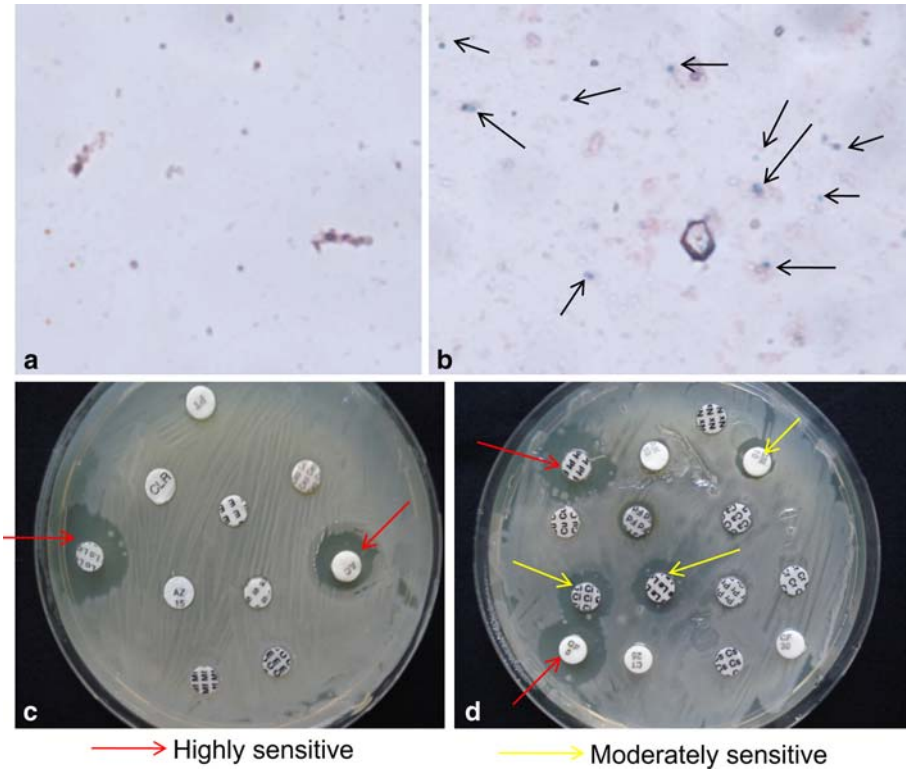


**Fig. 5** Inverted microscopy of homogenate prepared from leaf samples of *J. curcas* stained with crystal violet. **a** Bacterial contamination is not visible in cultures grown with antibiotic, **b** *Arrow heads* showing *blue* coloured bacterial contamination in cultures without antibiotic, **c**, **d** Sensitivity of bacteria towards different antibiotics

different concentrations. The results are presented in Table 3. It was found that all the antibiotics used were able to control the bacterial contamination completely in the first cycle itself at higher concentration of 500 mg $l^{-1}$, and within two cycles when used at lower concentration of 250 mg $l^{-1}$. However, all the effective antibiotics could control bacterial contamination but did not support plant growth and regeneration of shoots. Regeneration of only few shoots was achieved in the presence of lower concentrations of Gatiquin® and Ciplox® but the regenerated shoots turned yellowish and necrosed in due course of time. Only Augmentin® was effective at all the four concentrations used and did not adversely affect regeneration of shoots even at a higher concentration of 400 mg $l^{-1}$ for one cycle. Use of high concentration during subsequent cycles inhibited regeneration of shoots. The concentrations of Augmentin® were found inversely proportional to the number of cycles taken to control bacterial contamination. Initially, it was used at higher concentration of 400 mg $l^{-1}$, but cultures remained fresh, green and healthy. Later, depending on the intensity of bacterial contamination its concentration was decreased from 300, 200 to 100 mg $l^{-1}$ in subsequent cycles/subcultures. Even 100 mg $l^{-1}$ was sufficient to control bacterial contamination without any inhibitory effect on plant growth system (Fig. 4e). Further, for maintenance of long-term proliferating cultures, only 50 mg $l^{-1}$ Augmentin® was required (Fig. 4f). It was observed that augmentin was universally effective for controlling bacterial contamination in other accessions also, which generalizes its use in *J.curcas*. Amoxycillin is a moderate-spectrum ß-lactam antibiotic used to treat bacterial infections caused by susceptible microorganisms. It is usually the drug of choice within the class because it is better absorbed in plant systems also, than other beta-lactam antibiotics. It is susceptible to degradation by ß-lactamase-producing bacteria, and when given with clavulanic acid (named as Augmentin®), decrease its susceptibility. Amoxycillin is the most potent aminopenicillin and acts by inhibiting the synthesis of bacterial cell walls.

Antibiotics in combination did not respond well (data not presented). When Augmentin® was used with other antibiotics, although the bacterial contamination was controlled completely, the explants could not regenerate shoot buds. This showed the inhibitory action of other antibiotics towards cell differentiation in *J. curcas*. The use of Augmentin® was found beneficial in *Agrobacterium*-mediated transformation systems. After cocultivation Augmentin® was used in the medium to suppress the bacterial growth, but at the same time it supported growth of plant system with regard to cell division (plant growth) and cell differentiation (shoot organogenesis) as reported in many plant systems (Ieamkhang and Chatchawankanphanich 2005). Other antibiotics like ampicillin and antifungal agents like benomyl had also been used to control contamination in *Annonaceae* (Santana et al. 2003). The proliferating shoots of *J.curcas* remained fresh, green and regenerative in this

optimized medium up to 45 days without any subculture (Fig. 4f). After excision of isolated grown-up shoots, the remaining regenerative mass of shoots could be used as a repository of shoots for a longer period.

In *Jatropha, m*ost of the plant parts regenerate shoots very early but due to latent bacterial contamination, it was very difficult to establish the field-grown leaves under aseptic condition. The problem of endophytic bacterial contamination has been resolved in this report, where aseptic cultures remained green and regenerative with the addition of growth hormones as well as antibiotics in the medium up to 45 days of incubation without any subculture. Long-term proliferating cultures could be grown over 2 years without any contamination.

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