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ORIGINAL ARTICLE

High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant

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Abstract A simple, high-frequency and reproducible protocol for induction of adventitious shoot buds and plant regeneration from leaf-disc cultures of *Jatropha curcas* L. has been developed. Adventitious shoot buds were induced from very young leaf explants of in vitro germinated seedlings as well as mature field-grown plants cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) (2.27 μM), 6-benzylaminopurine (BA) (2.22 μM) and indole-3-butyric acid (IBA) (0.49 μM). The presence of TDZ in the induction medium has greater influence on the induction of adventitious shoot buds, whereas BA in the absence of TDZ promoted callus induction rather than shoot buds. Induced shoot buds were multiplied and elongated into shoots following transfer to the MS medium supplemented with BA (4.44 μM), kinetin (Kn) (2.33 μM), indole-3-acetic acid (IAA) (1.43 μM), and gibberellic acid (GA_3) (0.72 μM). Well-developed shoots were rooted on MS medium supplemented with IBA (0.5 μM) after 30 days. Regenerated plants after 2 months of acclimatization were successfully transferred to the field without visible morphological variation. This protocol might find use in mass production of true-to-type plants and in production of transgenic plants through *Agrobacterium*/biolistic-mediated transformation.

Keywords *Jatropha curcas* · Direct shoot regeneration · Adventitious shoots · Thidiazuron · Biodiesel

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Abbreviations

BA	6-Benzylaminopurine
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	Kinetin
MS	Murashige and Skoog basal medium
TDZ	Thidiazuron

Introduction

Jatropha curcas L. is a hardy perennial shrub of Latin American origin that is widespread throughout the tropical regions of the world. *Jatropha* is a large genus comprising more than 170 species. The commonly occurring species in India are *J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. multifida*, *J. nana*, *J. panduraefolia*, *J. villosa,* and *J. podagrica*. Most of these species are ornamental, except for *J. curcas* and *J. glandulifera*, which are oil-yielding species (Swarup 2004). The seeds of *Jatropha* contain 30–40% oil with a fatty acid pattern similar to that of edible oils (Gubitz et al. 1999). Jatropha oil contains linolenic acid and oleic acid, which together account for up to 80% of the oil composition. Palmitic acid and stearic acid are other fatty acids present in this oil.

Of late, *J. curcas* has attracted particular attention as a tropical energy plant. The seed oil can be used as a diesel engine fuel, for it has characteristics close to those of fossil fuel, diesel. *J. curcas* seed yields approach 6–8 MT/ha with approximately 37% oil. Cultivation of *J. curcas* assumes utmost importance to meet the large-scale demand and ensure continuous supply of the elite material. Traditional propagation through stem cuttings is possible, but low seed

yield and easy uprooting of established plants on poor and marginal soils hamper the practical utility of this propagation method. Evaluation of tissue-culture-propagated plants of nontoxic *J. curcas* was comparable to seed-propagated plants (Sujatha and Dhingra 1993).

Intervention of biotechnological methods to introduce desirable traits in *Jatropha* species is the need of the hour. Tissue-culture protocols for endosperm cultures and the rapid propagation of selected genotypes of *Jatropha* have been reported by various researchers (Srivastava 1974; Srivastava and Johri 1974; Sujatha and Dhingra 1993; Sujatha and Reddy 2000). Plant-regeneration systems from various explants of *J. integerrima* have been reported by Sujatha and Dhingra (1993). Sujatha et al. (2005) developed a method for the differentiation of adventitious shoot buds interspersed with callus from vegetative explants of nontoxic *J. curcas*. Weida et al. (2003) reported callus-mediated shoot-bud induction from *J. curcas* on Murashige and Skoog's (MS) medium supplemented with 0.5 mg/l 6-benzylaminopurine (BA) and 1.0 mg/l indole-3-butyric acid (IBA). All the above studies reported either callus-mediated regeneration or direct-shoot morphogenesis with interspersed callus from hypocotyl, leaf, and petioles. Despite sufficient regeneration systems achieved from *Jatropha* leaf explants, the presence of intermediary callus or callus-mediated regeneration is least desired for the production of true-to-type plants.

In this study, we investigated in vitro adventitious shoot-bud induction competency of leaf discs from *J. curcas*. In the process, we established an efficient protocol for high-frequency direct regeneration of plantlets from leaf discs of *J. curcas*.

Materials and methods

Plant materials

Seeds from elite clones were collected from Reliance Life Sciences' agricultural farm at Kakinada, South of Andhra Pradesh, India. The decoated seeds were soaked in distilled water for 24–48 h at room temperature. After 48 h of soaking, decoated seeds were surface sterilized with 1% (w/v) Bavistin (BASF, India) for 30 min and subsequently rinsed three times with sterile distilled water. After treating with Bavistin, the seeds were treated with 0.1% (w/v) mercuric chloride for 6 min followed by sterile distilled water rinsing. The material was blotted dry on sterile filter paper and carefully dissected to expose embryos. Papery cotyledons including embryo axes were excised and placed onto MS basal salts (Murashige and Skoog 1962) distributed into 25 × 150-mm culture tubes. MS basal salts supplemented with 3% sucrose and solidified with 0.8% agar (Hi-Media, India) were used to grow excised embryos. The cultures were maintained at 26 ± 2°C under a 16-h photoperiod illuminated with cool fluorescent lamps at an intensity of 30 μmol m^{-2} s^{-1}.

Induction of adventitious shoot buds from leaf cultures

The 2-month-old in vitro germinated seedlings from excised embryos were used to isolate leaf discs. Fresh and young leaves were also obtained from 2-year-old mature trees. Very young leaves starting from one to four from nodal tips have been used for excising leaf discs. Leaf discs were prepared using a sterile cork borer approximately 3–5 mm in diameter and placed with the abaxial side in contact with the medium.

The culture medium for induction of adventitious shoot buds consisted of MS salts with 3% sucrose (w/v), and the explants were cultured in three groups of hormone combinations with different concentrations, which were termed as induction medium. The first group consisted of thidiazuron (TDZ) (2.27 and 4.55 μM) and 6-benzylaminopurine (BA) (2.22 and 4.44 μM) in combination with IBA (0.49 and 0.98 μM), the second group consisted of TDZ (2.27 and 4.55 μM) in combination with IBA (0.49 and 2.46 μM), and the third group consisted of BA (2.22 and 4.44 μM) in combination with IBA (0.49 and 2.46 μM). The cultures were incubated at 26 ± 2°C on a 16-h photoperiod of 30 μmol m^{-2} s^{-1} provided by white fluorescent tubes. Leaf-disc explants were incubated on induction medium for 4–6 weeks. For multiplication and elongation, the induced shoot buds were cultured on MS basal medium containing BA (0.44–8.88 μM), kinetin (Kn) (0.47–4.65 μM), indole-3-acetic acid (IAA) (0.29–5.71 μM), and gibberellic acid (GA_3) (0.14–1.30 μM) in combination.

Statistical analysis

Experiments were set up in completely randomized design (CRD) (single-factor CRD). For experiments on adventitious shoot-bud induction, each treatment consisted of 25 replicates and each replicate with eight leaf-disc explants. The frequency of callus formation and bud induction, expressed as percentage, was calculated as the proportion of the number of explants forming callus and adventitious buds. The percentage values were arcsin angular transformed and subjected to analysis of variance (*F* test). Significant differences in values were calculated where effects or interactions were statistically significant and were rated accordingly.

Results and discussion

Leaf discs placed on the induction medium enlarged and exhibited varied response. The leaf discs increased in size

and adventitious shoot-buds originated from cut ends exposed to the medium. Regeneration of adventitious buds was observed in leaf discs cultured on all three groups of culture media (Fig. 1a, b). However, the frequency of leaves showing adventitious shoot-bud induction varied with the presence of both the cytokinins. TDZ and BA in combination with IBA was best suited for maximum adventitious shoot-bud induction. TDZ at 2.27-μM concentration induced adventitious shoot buds in 53.5% leaf-disc explants whereas at 4.55 μM, the shoot-bud induction was only 37% (Table 1). The shoot-bud induction capacity was reduced in the absence of BA. TDZ and IBA in the absence of BA had less effect on shoot-bud induction. TDZ (2.27 μM) in combination with IBA (0.49 μM) had induced shoot buds in 24.5%, whereas TDZ (4.55 μM) in combination with IBA (2.46 μM) induced shoot buds in 32% of cultured leaf discs, respectively. Similarly, BA in the absence of TDZ promoted callus induction rather than shoot-bud induction. BA (2.22 μM) in combination with IBA (0.49 μM) induced callus in 58% and shoot buds in 12% of cultured leaf discs, whereas BA (4.44 μM) in combination with IBA (2.46 μM) induced callus in 62% and shoot buds in 20% of cultured leaf discs, respectively. Shoot-bud induction capacity was drastically reduced in the absence of TDZ. These results suggest that TDZ and BA together had a synergistic effect in adventitious

Fig. 1 Adventitious shoot-bud induction and plant regeneration from leaf-disc cultures of *Jatropha curcas*. **a**, **b** Adventitious shoot buds induced from leaf-disc cultures on Murashige and Skoog's (MS) medium with 2.27 μM thidiazuron (TDZ), 2.22 μM 6-benzylaminopurine (BA) and 0.49 μM indole-3-butyric acid (IBA) (*bar* 5 mm). **c** Profuse shoot-bud clumps on multiplication and elongation medium with MS medium supplemented with 4.44 μM BA, 2.33 μM kinetin (Kn), 1.43 μM indole-3-acetic acid (IAA) and 0.72 μM gibberellic acid (GA_3) (*bar* 5 mm). **d** In vitro root induction (*bar* 1.0 mm). **e** Four-month-old acclimatized plant (*bar* 100 mm). **f** Field-transferred plant (*bar* 100 mm)

shoot-bud induction in *J. curcas*. The induced shoot buds were later transferred to the multiplication medium for further growth and multiplication.

To optimize type and concentration of plant-growth regulators to achieve the highest multiplication rate, well-developed shoots were transferred to the MS medium containing BA, Kn, IAA, and GA_3 in combination. A wide range of concentrations of BA and Kn, used singly or in combination, did not show a considerable increase in number of shoots (results not shown). However, BA, Kn, IAA, and GA_3, together recorded the highest number of shoots (11.5 shoots) after 12 weeks of culture (Table 2). The morphogenic competence for shoot regeneration was highest with 4.44 μM BA, 2.33 μM Kn in combination with 1.43 μM IAA and 0.72 μM GA_3 (Fig. 1c). Higher concentrations of BA (4.44–8.88 μM), Kn (2.33–4.65 μM), IAA (1.43–5.71 μM), and GA_3 (0.72–2.89 μM) recorded the maximum number of shoot clumps (10.6–11.5) after 12 weeks in culture with subculturing at 4 weeks interval. Proliferation and elongation of shoot buds could be achieved due to higher cytokinin/auxin ratio along with addition of GA_3 in the media.

Shoots of 2–3 cm were separated individually and transferred to the root-induction medium containing full-strength MS basal medium supplemented with IBA (0.5 μM).

After 30 days of culture on root-induction medium, 80% rooting was observed without any intermediary callus growth (Fig. 1d). The well-developed plantlets were washed with sterile water to remove traces of agar from the roots and dipped in 0.1% (w/v) of a broad-spectrum fungicide solution (Bavistin, BASF, India) for 10 min and transferred to the potting mixture containing 1:1 ratio of cocopeat:garden soil in 3 inch diameter pots. Primary hardening took place in 3–4 weeks under high-humidity conditions created by covering pots with polythene bags. The well acclimatized plants were further transferred to the field with more than 80% survival rate (Fig. 1e, f).

Plant regeneration through the leaf disc is a highly practiced method in tissue cultures (Landi and Mezzetti

Table 1 Effect of various plant-growth regulators on direct induction of adventitious shoot buds from leaf discs of *Jatropha curcas*

Plant-growth regulator concentration (μM)			Response of leaf-disc explants (%)		
TDZ	BA	IBA	Callus formation	Bud induction	Nonresponsive
2.27	2.22	0.49	40^{e}	53.5^{a} (47.040)	6.5 (2.160)
4.55	4.44	0.98	$50^{d,e}$	$37^{b,c}$ (37.320)	13 (3.376)
2.27	–	0.49	$52.5^{c,d}$	24.5^{d} (29.328)	23 (4.767)
4.55	–	2.46	$50^{d,e}$	$32^{b,c}$ (34.368)	18 (4.244)
–	2.22	0.49	$58^{a,b,c}$	12^{f} (21.072)	30 (5.435)
–	4.44	2.46	62^{a}	20^{e} (26.220)	18 (4.100)

Data scored after 6 weeks of culture inoculation, Means in each column followed by same letters are not significantly different

The figures in the parenthesis are arcsine angular transformation values (for bud induction) and square root transformation values (for nonresponsive)

TDZ thidiazuron, *BA* 6-benzylaminopurine, *IBA* indole-3-butyric acid

Table 2 Effect of plant-growth regulators on adventitious shoot formation and number of shoot-clump formation from leaf-disc cultures of *Jatropha curcas*

Plant-growth regulator concentration (μM)				Number of adventitious shoots per leaf disc	Number of shoot clumps/leaf disc		
BA	Kn	IAA	GA_3		After 4 weeks	After 8 weeks	After 12 weeks
8.88	4.65	5.71	2.89	1.30^{b}	2.55^{b}	$5.00^{a,b}$	10.60^{a}
6.66	2.33	2.86	1.45	1.60^{a}	3.20^{a}	5.60^{a}	11.20^{a}
4.44	2.33	1.43	0.72	1.50^{a}	$2.75^{a,b}$	5.45^{a}	11.50^{a}
2.22	2.33	0.69	0.35	$1.35^{a,b}$	2.50^{b}	4.70^{b}	9.20^{b}
1.11	1.16	0.29	0.14	1.20^{b}	2.00^{c}	4.00^{c}	$9.00^{b,c}$
0.44	0.47	0.29	0.14	1.15^{b}	2.00^{c}	3.80^{c}	8.20^{c}

Means in each column followed by same letters are not significantly different

Data for adventitious shoot-bud formation was scored after 4 weeks of culture

BA 6-benzylaminopurine, *Kn* kinetin, *IAA* indole-3-acetic acid, *GA_3* gibberellic acid

2006) and gene-transfer techniques (Tsugawa et al. 2004). Krikorian (1982) observed that juvenile plant tissues and organs, especially seedling parts, are highly responsive compared with mature, differentiated tissues. In case of *J. curcas*, very young leaf explants from 2-month-old in vitro germinated seedlings as well as mature trees exhibited great propensity for direct regeneration without the intervening callus growth. Plant-growth regulators such as TDZ and BA alone or in combination with auxins such as IBA (at 0.50–2.46 μM) have been tested for their ability to induce adventitious shoot buds. It was observed that these plant-growth regulators exhibited greater variation in shoot-bud induction. In our investigation, TDZ and BA in combination with IBA had a more pronounced effect on shoot-bud induction (Fig. 1a). Weida et al. (2003) reported callus-mediated regeneration of plantlets from hypocotyls, petioles, and leaf explants of *J. curcas* on medium supplemented with BA and IBA. Sujatha and Mukta (1996) found that among various *Jatropha* species, *J. integerrima* was most responsive and that regeneration occurred through organogenesis in seedlings and mature explants including leaves, petioles, and pedicels. However, they observed intermediary callus growth in leaf explants.

TDZ is able to induce diverse morphogenic responses ranging from tissue proliferation to adventitious shoots and somatic embryo formation. The ability of TDZ to induce high-shoot regeneration efficiency in woody plant tissues has been reported (Huetteman and Preece 1993; Meng et al. 2004). In strawberry-leaf tissues, TDZ in combination with IBA induced high-frequency shoot induction (Landi and Mezzetti 2006). Our results further support the role of TDZ on high-frequency shoot-bud induction from *J. curcas* leaf discs. TDZ is shown to play an important role in cultures with cytokinin-like activity. Apart from cytokinin-like activity, TDZ has been suggested to be a modulator of the endogenous auxin levels. There is experimental evidence that TDZ stimulates de novo synthesis of auxins by increasing the levels of IAA and its precursor, tryptophan (Murthy et al. 1995). Increases in endogenous auxin, cytokinin, and ethylene have been seen in response to TDZ treatment (Murthy et al. 1995). As a consequence, TDZ has been shown to be useful for rapid plant regeneration in several recalcitrant species through organogenesis (Malik and Saxena 1992). Similarly, in our studies, it was observed that TDZ is essential for the high-frequency induction of multiple adventitious shoot buds from leaf-disc cultures.

The availability of highly efficient regeneration protocol using leaf discs without the intervening callus phase is highly desirable for *Agrobacterium tumefaciens*-mediated genetic transformation. Therefore, the high-frequency and efficient plant regeneration protocol reported in the present investigation could be useful for mass production of true-to-type plants and the production of transgenic plants through *Agrobacterium*/biolistic-mediated transformation.

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