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Full Length Research Paper

An efficient plant regeneration protocol from petiole explants of physic nut (*Jatropha curcas* L.)

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Efficient and reproducible method for the *in vitro* regeneration of *Jatropha curcas* plants has been developed through direct shoot buds induction from petiole explants on Murashige and Skoog (MS) basal medium supplemented with different concentrations of thidiazuron (TDZ). The highest percentage of shoot buds induction (64.0%) was observed on MS medium supplemented with 0.52 mgL^{-1} TDZ with organic additives; adenine sulphate (50 mgL^{-1}) + glutamine (100 mgL^{-1}) + L-arginine (25 mgL^{-1}) + citric acid (0.0025%) + ascorbic acid (0.005%). A maximum of six shoots per explant were elongated when explants with induced shoot buds were transferred on MS medium containing 3.0 mgL^{-1} 6-benzyladenine (BA) and 1.0 mgL^{-1} indole-3-butyric acid (IBA). The regenerated shoots were rooted on half strength MS medium supplemented with 1.0 mgL^{-1} indole-3-acetic acid (IAA) and 0.2 mgL^{-1} IBA. The rooted plantlets were established in soil with more than 90% survival.

Key words: *Jatropha curcas*, thidiazuron, petiole, shoot buds, plantlets.

INTRODUCTION

The oil yielding plant *Jatropha curcas* (L.) or physic nut is a multipurpose and drought resistant large shrub or small tree. It is a native of tropical Central America which has now been domesticated in a widespread manner in Africa and Asia mainly due to its ability to grow in a number of climatic zones in tropical and subtropical regions of the world, particularly in marginal lands. *J. curcas* is easy to establish, grows relatively quickly and is hardy. *J. curcas* has immense economic potential and ecological and environmental significance. The uses of this crop ranges from traditional medicine for common human and animal ailments, protection against land erosion, as a boundary fence to newly found potential for fossil fuel replacement (Openshaw, 2000). In the recent years, energy conservation and its production has acquired significant importance in the wake of the world energy crisis. A number of options for the production of liquid fuel as an alternative source have been considered in many countries. The *J. curcas* oil has been identified as an efficient substitute to be used as fuel for diesel engines (Raina, 1985). The engine performance and fuel consumption with *J. curcas* oil has been compared favourably with normal diesel oil (Munch and Keifer, 1989). Hence, utilization of *J. curcas* oil as a new source of oil for diesel engine has tremendous scope in contributing to the growing needs of country for energy resources. Since, *J. curcas* does not compete with conventional crops for cultivation, the dilemma of food verses fuel does not arise. Seeds and cuttings are widely used for propagation of *J. curcas.*

Plant propagated through seeds lead to significant variations in seed yield and oil content (Jha et al., 2007). Furthermore, seeds of *J. curcas* have a limited viability and can only be stored for 15 months after which its viability is reduced by 50% (Kochhar et al., 2005).

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Abbreviations: BA, 6-Benzyladenine; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog's medium; **NAA,** α-naphthalene acetic acid; **TDZ,** thidiazuron.

Propagation through cuttings is subject to seasonal availability of cuttings and trees propagated by cuttings show a lower longevity and possess lower drought and disease resistance. Therefore, improvement programmes of *J. curcas* by modern methods of agro-biotechnology are of interest worldwide. To meet the large-scale demand and ensure easy supply of this elite material, there is a need to develop mass multiplication techniques.

Using different explants, plant regeneration protocols have also been described in *J. curcas* (Sujatha et al., 2006; Wei et al., 2004; Jha et al., 2007; Rajore and Batra, 2007). Different lines of *J. curcas* showed differential behavior which indicates its genotype effect (Machado et al., 1997; Kumar, 2008; Kumar and Reddy, 2010). The petiole is a somatic tissue and plants raised from the petioles have been shown to be more resistant to genetic variation (Pierik, 1991). The suitability of thidiazuron (TDZ) or 6-Benzyladenine- indole-3-butyric acid (BA-IBA) medium for adventitious shoot regeneration from leaf petiole of *J. curcas* and its hybrids has previously been reported (Sujatha et al., 2006; Kumar and Reddy, 2010) but the effective concentrations favouring optimal shoot regeneration varied with genotype and agro-climatic conditions. The objective of this study was to develop a direct plant regeneration method without the requirement of an intervening callus, using a Murashige and Skoog (MS) medium supplemented with different concentrations of plant growth regulators (TDZ 0.0 to 0.70, BA 0.00 to 4.0, IBA 0.0 to 2.0, NAA 0.0 to 2.0 and IBA 0.0 to 2.0 mgL^{-1}) with and without organic additives (adenine sulphate, glutamine, arginine, citric acid and ascorbic acid).

MATERIALS AND METHODS

Source of explants

Young tender vegetative shoots of 5 to 10 cm in length were excised from the plants growing at the College of Agriculture, CCSHAU, Hisar (29˚08'N, 75˚42'E) Haryana, India.

Explant treatment

The shoots were washed with detergent (Teepol), followed by washing under running tap water and finally, rinsed with double distilled water. Petioles collected from leaves, which were close to apical buds, were surface sterilized with Streptocycline (0.1%, 8 min) and $HgCl_2$ (0.1%,15 min) followed by successive washing with autoclaved distilled water three to four times in a laminar flow.

Culture conditions and *in vitro* establishment of explants

The petioles were cultured on MS medium supplemented with various concentrations of TDZ with or without additives (adenine sulphate (50 mgL^{-1}) + glutamine (100 mgL^{-1}) + L-arginine (25 mgL^{-1}) + citric acid (0.0025%) + ascorbic acid (0.005%). The pH of the medium was adjusted to 5.8 using 1 N KOH or HCl, prior to the addition of agar. The culture medium was autoclaved at 1.05 $kgcm^{-2}$ pressure at 121°C for 20 min. The cultures were maintained at 25 ± 2°C under a 16 h photoperiod with light intensity of 35 to 40 µmol $m^{-2}s^{-1}$ (cool white fluorescent tubes). For each treatment, 25 explants were cultured and each experiment was repeated at least thrice. Cultures were observed every day and the data were recorded after 28 days of culture.

Induction of multiple shoots

Well-developed shoots (2 to 3 cm) were excised from the regenerating explants and inoculated onto multiplication medium in conical flasks (250 ml, Borosil, India) or magenta box. Full strength MS basal medium supplemented with varying concentrations of cytokinins BA (2.0 to 4.0 mgL^{-1}) and auxins, that is, α-naphthalene acetic acid (NAA) 0.0 to 2.0 mgL^{-1} or IBA 0.0 to 2.0 mgL^{-1} or IAA 0.0 to 2.0 mgL^{-1} was tested for multiplication of *in vitro* proliferated shoots. MS medium devoid of any plant growth regulators was used as control. The numbers of shoots developed were recorded after four and six weeks of culture. Sub culturing was carried out at periodic interval of two or three weeks.

Rooting of micro shoots

Green and healthy elongated shoots size (2 to 3 cm) with three to four leaves were excised and cultured on half-strength-MS medium supplemented with different concentrations and combinations of auxins, viz. IBA (0.4 to 2.0 mgL^{-1}) and IAA (0.1 to 2.0 mgL^{-1}) for rooting. For each treatment, 30 microshoots were cultured and each experiment was repeated at least thrice. Cultures were observed every day and percentage of root induction was recorded after 4 weeks.

Hardening and acclimatization

Shoots with well-developed roots (4 to 5 cm in length) were carefully taken out of the rooting medium and washed thoroughly in sterile distilled water to remove MS medium attached to the roots. The plants were transferred to polythene bags containing sterilised sand and soil (1:1) and wetted with 0.02% w/v carbendazim and covered with transparent plastic bags to maintain humidity. After 3 to 4 weeks, the established plants were transferred to a greenhouse at temperature 25 ± 3°C and relative humidity 70 to 80% for further growth. Uniform culture conditions were applied in all experiments.

Statistical analyses

Experiment was done in triplicate and each treatment consisted of ten replicates. The data was analyzed using ANOVA of completely randomized design (CRD). The significance level was determined as at P = 0.05. Mean values of treatments were compared with Newman-Keul's multiple range.

RESULTS AND DISCUSSION

Induction of shoot buds

The effect of different concentrations of TDZ either alone or in combination with organic additives was assessed on the induction of shoots from petiole explants of *J. curcas* (Table 1). The percent induction of shoot buds increased with increasing concentrations of TDZ up to 0.52 mgL^{-1}

Table 1. Induction of shoot buds from petiole explants of *J.* curcas under different concentrations of TDZ with and without additives.

TDZ concentration (mgL^{-1})	Percentage induction of shoot bud	
	(-) Additive	(+) Additive
0.00	0.00	0.00
0.17	12.0 ± 0.12^{d}	14.62 ± 0.14^{d}
0.35	17.5 ± 0.21^{c}	20.3 ± 0.18^{c}
0.52	59.0 ± 0.10^{a}	64.0 ± 0.20^{a}
0.70	54.2 ± 0.32^{b}	61.0 ± 0.15^{b}

Mean followed by the same letter are not significantly different according to Newman-Keul's multiple range test (p=0.05). Culture medium: MS basal medium; TDZ, thidiazuron. '-', without additive (TDZ alone); '+', with additive.

Table 2. Effect of plant growth regulator combinations on elongation of shoot buds into shoots in *J. curcas.*

Plant growth regulators (mgL^{-1}) in MS medium			Mean number of shoot/explant		
BA + NAA	BA + IAA	BA + IBA	BA + NAA	BA + IAA	BA + IBA
0.00	0.00	0.00	0.00	0.00	0.00
2.0 + 0.0	2.0 + 0.0	2.0 + 0.0	0.0	0.0	0.0
2.5 + 0.5	2.5 + 0.5	2.5 + 0.5	0.6 ± 0.13^{c}	0.8 ± 0.33^{c}	3.0 ± 0.22^{c}
3.0 + 1.0	3.0 + 1.0	3.0 + 1.0	1.0 ± 0.22^{b}	1.2 ± 0.27^{b}	6.0 ± 0.25^{a}
3.5 + 1.5	3.5 + 1.5	3.5 + 1.5	1.4 ± 0.20^{a}	2.0 ± 0.12^{a}	5.4 ± 0.11^{b}
4.0 + 2.0	4.0 + 2.0	4.0 + 2.0	1.0 ± 0.33^{b}	1.5 ± 0.23^{b}	2.0 ± 0.19^{c}

Mean followed by the same letter are not significantly different according to Newman-Keul's multiple range test (P=0.05). BA, 6-Benzyladenine; IBA, indole-3-butyric acid, IAA, Indole-3- Acetic acid and NAA, -napthalene acetic acid.

and thereafter decreased irrespective of the organic additives. However, the response was higher in the presence of organic additives. The highest induction of shoot buds (64.0) was observed on the medium containing 0.52 mgL^{-1} TDZ and organic additives (adenine sulphate 50 mgL^{-1} + glutamine 100 mgL^{-1} + L-arginine 25 mgL^{-1} + citric acid 0.0025% + ascorbic acid 0.005%). TDZ is a potent cytokinin for tissue culture of woody plants including *J. curcas* (Huetteman and Preece, 1993; Deore and Johnson, 2008). In the present investigation, it was observed that low concentrations of TDZ induced relatively fewer shoot buds, but these developed rapidly into shoots in subsequent culture. In contrast, media containing high concentration of TDZ had more visible primordia but, only a few were able to develop into shoots. Similar results were obtained in *Miscanthus sinensis* (Nielsen et al. 1993). In *Capsicum annuum*, 2.20 mgL^{-1} TDZ also induced multiple shoots which failed to elongate (Hyde and Phillips, 1996). However, further proliferation of shoot buds was inhibited because of compact shoot bud induction at this concentration. It is evident from the data (Table 1) that as the concentration of TDZ increased from 0.52 to 0.70 mgL^{-1}, percent induction of shoot buds decreased both in the presence or absence of additives. The inhibitory effects of high concentrations of TDZ on shoot induction have been reported previously (Preece and Imel, 1991; Feyissa et al., 2005; Raghu et al., 2006; Kumar and Reddy, 2010) and our results are in agreement with these findings.

Glutamine and glutamic acid are directly involved in the assimilation of NH_4^+. A direct supply of these amino acids should therefore enhance the utilization of both nitrate and ammonium nitrogen and its conversion into amino acids (George, 1993). Glutamine proved most effective to control leaf fall in multiple shoots (Sanjaya et al., 2005). Adenine sulphate shows synergistic effects with cytokinins, it stimulates cell growth and gently enhances shoot formation (Raha and Roy, 2001). L-arginine increased number of shoots per explants (Sotiropoulos et al., 2005). The effect of citric acid has been reported to increase shoot length in some plants (Sanjaya et al., 2005).

Elongation of shoots buds into shoots

The induced shoot buds were transferred to the medium containing either BA alone, or BA supplemented with different auxins (NAA, IAA and IBA) for elongation. BA alone failed to elongate the shoot buds. However, BA in the presence of auxins elongated the shoots which varied with the type of auxin. BA (3.0 mgL^{-1}) in combination of IBA (1.0 mgL^{-1}) induced the elongation of the maximum number of shoots (six shoots per explant) (Table 2). Increasing or decreasing this concentration reduced the number of shoots per explants.

Prabhakaran and Sujatha (1999) reported that BA in

Table 3. Effect of different concentrations and combinations of auxins in half-strength-MS medium on the percentage of root induction in regenerated elongated shoots from petiole explants.

Media combination	Root induction (%)
½ MS	8.11 ± 0.22^{e}
½ MS + IAA (0.1 mg/L) + IBA (1.0 mg/L)	46.4 ± 0.32^{c}
½ MS + IAA (0.2 mg/ L) + IBA (2.0 mg /L)	59.0 ± 0.12^{b}
½ MS + IAA (1.0 mg/ L) + IBA (0.2 mg/L)	67.6 ± 0.29^{a}
½ MS + IAA (2.0 mg/ L) + IBA (0.4 mg/L)	22.2 ± 0.20^{d}

BA, 6-Benzyladenine; IBA, indole-3-butyric acid and IAA, Indole-3- acetic acid.

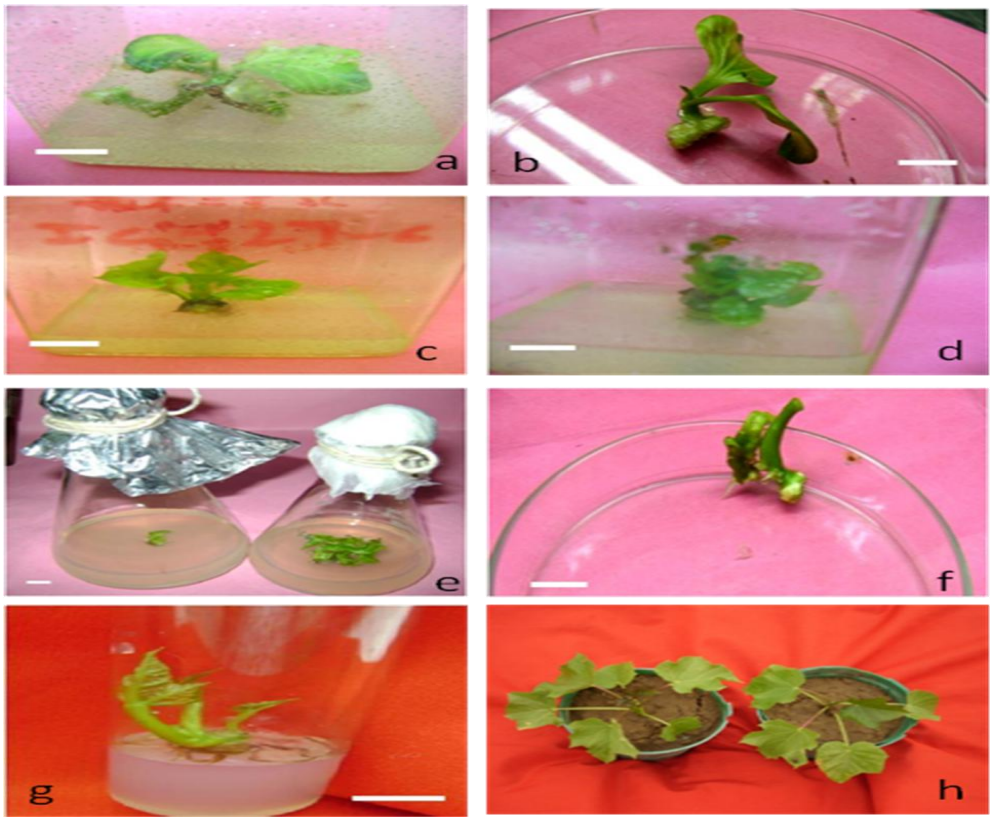

Figure 1. Direct regeneration from petiole explants of *J. curcas.* a, b = Induction of shoot buds from petiole explants (bar 1 cm); c, d = elongation of regenerated plantlets (bar 1 cm); e = multiple regenerated plantlets (bar 1 cm); f, g = induction of rooting from regenerated plantlets (bar 1 cm); h = plants transfer to pots.

combination with IBA in MS medium elongated the shoots developed from leaf discs of *J. curcas* and its hybrids. There are some reasons for preferred use of BA as cytokinin. Slow degradation of BA is one of the reasons and it can be autoclaved without loosing its activity. BA is considered as one of the most useful cytokinin for achieving the multiplication and micropro-pagation of the plants (Stfaan et al., 1994) .The combination of cytokinin with auxins are very fruitful for axillary apical bud sprouting in certain species (Dhar and Joshi, 2005; Baker et al., 1999). This has also been confirmed by our results.

Rooting of shoots and transplantation

Two to three centimeter shoots were transferred to MS medium supplemented with IBA and IAA for rooting. Of the two auxins tested, IAA along with IBA was found to be the most effective. Maximum percent (67.6%) of root induction was observed on ½ MS + IAA (1.0 mgL^{-1}) + IBA (0.2 mgL^{-1}) followed by 59.0% in ½ MS + IAA 0.2 mgL^{-1} + IBA 2.0 mgL^{-1} (Table 3). Minimum number of percent induction was observed in ½ MS media without hormonal supplementations.

Auxins are potent hormones for rooting (Vuylasteker et al., 1998; Nandagopal and Kumari, 2007). The role of auxin in root development is well established and reviewed (Scott, 1972). Interaction of a number of different exogenous and endogenous factors influenced the ability of plant tissue to form roots.

Rooted shoots were carefully taken out of the medium and washed thoroughly in sterile distilled water to remove MS medium attached to the roots. The rooted shoots were transferred to polythene bags containing sterilized sand and soil (1:1) and wetted with 0.02% w/v carbendazim and covered with transparent plastic bags to maintain humidity. After 3 to 4 weeks, the established plants were transferred to a greenhouse at temperature 25 ± 2°C and relative humidity 70 to 80% for further growth. After 6 to 8 weeks, more than 18 plants (90%) survived. No morphological abnormalities were observed in regenerated plants (Figure 1).

To conclude, this communication describes an additional successful protocol for regeneration of plants from petiole explants with the use of additives which has not been reported in the literature so far for *J. curcas*.

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