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DIRECT PLANTLET REGENERATION FROM SHOOT TIP EXPLANTS THROUGH CLONAL PROPAGATION OF PHYSIC NUT (*JATROPHA CURCAS*.L)

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Abstract:
In the present investigation, *in vitro* clonal propagation of seven-month-old *Jatropha curcas* L. was achieved employing shoot tip explants. Axillary node were cultured on MS medium fortified with Thidiazuron (TDZ) (1.0-3.0 mg/L), IAA (0.5 mg/L)+ TDZ (1.0-3.0 mg/L) for multiple shoot induction. Multiple shoots proliferation was best observed at IAA (0.5 mg/L) + (1.5 mg/L) TDZ from the axillary node explants within four weeks of culture. Shoot number per explants ranged between 2 and 6 Individual shoots were aseptically excised and sub cultured in the same media for shoot elongation. The elongated shoots were transferred to NAA (1.0mg/L–3.0mg/L) for root induction. Rooting was observed within two weeks of culture. Rooted plantlets were successfully hardened under culture conditions and subsequently established in the field conditions. The recorded survival rate of the plants was 86%. Plants looked healthy with no visually detectable phenotypic variations. The plantlets (12–16- week-old) were successfully acclimatized in soil with 87% survival frequency

Keywords: Axillary Shoot, clonal propagation, *Jatropha curcas*, shoot tipexplants.

Introduction:

Jatropha curcas L., a soft wood perennial plant belongs to family Euphorbiaceae (Nasir, 1986), commonly known as Physic nut. *Jatropha curcas* is one of the most valuable crude drugs of primitive times and is still widely used in modern medicine. It has natural distribution covering the Neo tropic from Mexico to Brazil including the Caribbean Island (Grim, 1996). It is now distributed throughout the entire tropics of Africa and Asia as well (Grim, 1996). In recent years this plant has received extensive attention of many scientists in view of its great economic importance, medicinal significant and for its seed oil as commercial source of fuel (Datta and Pandy, 1993). The superior quality oil can be extracted from the seeds. The oil can be used as a mixed fuel for diesel/gasoline engines (Yoshifumi, 1982). The oil is not edible due to the presence of toxic substance" Curcascine" (Gandhi *et al.,* 1995). It is conventionally used in making soaps, candles, paints, lubricants and medicinally as a purgative (Dastur, 1951; Sujatha and Mukta, 1996). It is also recommended as a drought resistant plant suitable for erosion control and is not palatable to grazing animals due to the toxicity (Munch and Kiefer1989).

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 agri biotechnological 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 (Prabakaran *et.al.,* 1991). 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 (Sujatha *et.al.* 2006). Seed set has been reported to be low in vegetatively propagated plants (Sujatha *et.al.* 2006).

Tissue culture studies were undertaken in different species of *Jatropha*. Morphogenesis from endosperm tissues has been reported in *J. panduraefolia* (Srivastava1971, Johri and Srivastava 1973 and Srivastava and Johri1974) High frequency regeneration from various explants of *J. integerrima* has been reported

(Sujatha and Dhingra 1993). Using different explants, plant regeneration protocols have also been described in *J.* curcas (Sujatha and Makkar 2006, Qin, *et.al.,* 2004 Sujatha and Mukta 1996) 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 (Pierik, 1991). 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 shoot tip explants of a seven-month-old *J. curcas* plant and establishment of plants under field conditions.

Methodology:

J. curcas L. seeds collected from Mahadevapur Reserve Forest Mahadevapur Karimnagar District Telangana state were sown in the experimental garden of S.R.R. Govt Arts & Science College Karimnagar. 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. Shoot tip explants (2–3 cm in length) collected from the Two-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*).

Selection of explants: - Shoot tips with one or two leaf primordia, of 60-d old *in vitro* raised seedlings were selected as explants for direct shoot multiplication. The shoot tips, segments of 5–8 mm in length were excised aseptically.

Culture media and culture conditions

MS media containing 3.0% sucrose and supplemented with various concentrations cytokinin such as BAP/Kn/TDZ (1.0 – 5.0 mg/L) and in combination of (0.5mg/L) IAA were used. The initial pH of the culture media was adjusted to 5.8 before addition of 0.8% (w/v) agar- agar. The medium was dispensed into culture tubes (25 + 150 mm) each containing 15 ml of the culture medium capable with non-absorbent cotton and was autoclaved at 121^0 C for 15 minutes. In each cultures tube one shoot tip explants was implanted. The cultures were maintained under 16h light provided with white fluorescent tubes (40 µ mol m-2s-2) at 25 ± 2^0C.

***Statistical analysis*:** Number of shoots and number of leaves were recorded. Data were analysed by Duncan's New Multiple Range Test.

Results:-

Data on multiple shoot induction from shoot tip explants cultured on MS medium fortified with different concentrations of BAP/Kn/TDZ alone is presented in (Table-1). The important part of the present study was the preparation of contamination free explants. This was achieved by using *in vitro* germinated seedlings as an explant source. Sterilization of seeds required 0.1% (w/v) $HgCl_2$ 5 min treatment for maximum germination (95%) and minimum contamination (Narashimhulu and Reddy, 1983). A similar observation was also reported in *Vigna aconitifolia*, confirming the view that the pretreatment of seeds with specific surface sterilizing agents would predetermine the regenerating behavior of explant tissues (Godbole, *et al.,* 1984). The use of direct and large sized explants had higher survival and growth rates than the smaller ones (Hu Wang, 1983).

After 4–6 weeks of culture, the shoots cultured on MS basal medium supplemented with 3% (w/v) sucrose, (1.0–3.0mg/L) BAP/Kn/TDZ were transferred to MS basal medium supplemented with 3% (w/v) sucrose, (1.0–3.0mg/L) BAP/Kn/TDZ singly or in combination, as well as in combination with (0.5mg/L) IAA for raising multiple shoots. The best response of (6.0 ± 0.32) (Fig-a, b, c and d) shoots per shoot tip was obtained in medium containing (0.5mg/L) IAA (1.5mg/L) TDZ. 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 (Table 1).

***In vitro* Rooting and Acclimatization:-**

The shoots were also tested on un supplemented 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 (Table 2).

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 (Table 3).

C

D

E

F

Figure 1 : Direct plantlet regeneration from shoot tip explants through Clonal propagation of Physic nut (*Jatropha curcas*.L) (a) Formation of multiple shoots on MS+BAP (2.5) mg/L from shoot tip, (b) Proliferation of multiple shoots on MS+Kn (1.0mg/L) from shoot tip with rooting, (c) Multiple shoot induction on MS+ TDZ (2.0mg/L) from cotyledonary node, (d) Multiple shoot induction from shoot tip on MS+ IAA (0.5mg/L) + TDZ 1.5mg/L (e) Rooting of individual shoots, (f) Hardening of plantlets

Table 1: Effect of different concentration of BAP/Kn/TDZ on multiple shoot induction from shoot tip explants of *J. curcas*.

Hormone concentration (mg/L)	% of cultures response	Mean number of shoots /explants ±(S.E.)*
BAP		
0.5	60	1.0 ± 0.01
1.0	70	2.0 ± 0.13
1.5	80	2.7 ± 0.42
2.0	90	4.5 ± 0.05
2.5	85	3.6 ± 0.46
3.0	60	1.0 ± 0.35
Kn		
0.5	62	2.3 ± 0.17
1.0	73	2.4 ± 0.12
1.5	85	3.5 ± 0.32
2.0	92	2.6 ± 0.01
2.5	88	1.2 ± 0.35
3.0	65	1.0 ± 0.34
TDZ		
0.5	65	2.0 ± 0.45
1.0	75	2.5 ± 0.32
1.5	86	3.8 ± 0.34
2.0	95	1.8 ± 0.45
2.5	80	1.2 ± 0.45
3.0	68	1.0 ± 0.35

***SE Standard Error 1**

Table 2: Direct multiple shoots Proliferation from Shoot tip explants of *J. curcas*. on MS medium IAA (0.5mg/L) supplemented with various concentrations of BAP, Kn and TDZ.

Hormone Concentration (mg/L)	% of cultures response	Mean number of shoots / explants ±(S.E.)*
IAA+BAP		
0.5+0.5	63	1.3 ± 0.05
0.5+1.0	65	2.3 ± 0.32
0.5+1.5	70	2.9 ± 0.36
0.5+2.0	85	4.9 ± 0.05
0.5+2.5	70	3.7 ± 0.03
0.5+3.0	50	2.0 ± 0.35
IAA+Kn		
0.5+0.5	65	2.2 ± 0.35
0.5+1.0	75	2.4 ± 0.05
0.5+1.5	86	3.8 ± 0.04
0.5+2.0	90	3.0 ± 0.05
0.5+2.5	75	2.3 ± 0.32
0.5+3.0	56	1.3 ± 0.05
IAA+TDZ		
0.5+0.5	65	2.2 ± 0.43
0.5+1.0	78	2.8 ± 0.34
0.5+1.5	88	4.0 ± 0.32
0.5+2.0	93	2.0 ± 0.34
0.5+2.5	78	2.2 ± 0.34
0.5+3.0	60	1.8 ± 0.34

***SE Standard Error**

Table 3: Rooting ability of regenerated shoots from Shoot tip explants culture of *J. curcas*. Cultured on MS medium supplemented with NAA and IBA.

Growth Hormones (mg/L)		Percentage of response	Average no of roots (S.E)*
NAA	**IBA**		
00	00	23	1.0 ± 0.12
0.5	-	60	2.3 ± 0.37
1.0	-	70	3.2 ± 0.38
2.0	-	73	5.6 ± 0.38
-	0.5	54	No roots
-	1.0	73	No roots
-	2.0	70	No roots

*** Mean ± Standard Error**

Discussion:-

We were successful in shoots regenerating plants from shoot tip explants cultures on MS medium fortified with different concentrations of cytokinines i.e. BAP Kn and TDZ individually maximum number of shoots were induced at (2.0mg/L) BAP in comparison to (1.5 mg/L Kn) and (1.5mg/L) TDZ as a role of growth regulators. However the shoot bud proliferation was found to be more on (2.0 mg/L) BAP compared to (1.5 mg/L) Kn might have triggered the action of BAP in proper way for inducing more number of plant let regeneration among all hormonal combinations and concentrations used Influence of explanting season on culture establishment was also noted. The best response (6.0 ± 0.32 shoots per shoot tip explant) was obtained in the presence of (0.5mg/L) IAA and in combination of (1.5mg/L) TDZ was found to be significantly higher than shoots induced per nodal explant in other concentrations of cytokinins (Kn, BAP) used in the present study (Table 2; Figure 1 *b*, *c*). However, at the same concentration of (1.0mg/L) BAP, a lower initiation (1.0 ± 0.01) shoots buds per explant; this differential response may be attributed to the specific age and physiological condition of the donor plant from which the shoot tip explants were excised. Shoot tips cultured on medium with different concentrations of Kn and BAP showed lower induction of axillary shoot-bud proliferation. Though TDZ is known to induce cytokinin like effects in a number of plant species, particularly woody (Barrueto *et.al.,* 1999) as well as herbaceous crop species (Huetteman and Preece 1993), the present study showed negligible effects of BAP/Kn/TDZ on induction of shoot multiplication as also reported in *J. curcas* earlier (Sujatha and Makkar 2002) *Jatropha*

belongs to the family Euphorbiaceae and our observation confirms earlier reports (Ripley and Preece 1986) (Tiedman and Hawker 1962) that among the cytokinins BAP plays an important role in initiation of shoot-bud proliferation in many members of the Euphorbiaceae family.

The 4–6-week-old-Shoot tip cultures on MS basal medium supplemented with (0.5mg/L) IAA and in combination of (1.5mg/L) TDZ on transfer to MS basal medium supplemented with (0.5mg/L) IAA and in combination of (1.5mg/L) TDZ as well as in combination with (0.5mg/L) IAA and in combination of (1.5mg/L) BAP/Kn showed varied response (Table 2). In the present study, transfer of cultures to MS basal medium with lower concentration of TDZ (1.5mg/L) and with same (2.0mg/L) or reduced (1.0–3.0) concentration of TDZ, 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 BAP and Kn did not proliferate further. On the other hand, transferring the cultures from a set having higher concentration of (3.0 mg/L) TDZ and (1.5mg/L) BAP to a set with lower concentration of (1.0mg/L) Kn, (0.5 mg/L) IAA and (1.5mg/L) BAP/Kn to a significantly higher shoot shoot multiplication (4.5 ± 0.5) and (3.5 ± 0.32) shoots per shoot explants) 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* (Kumar *et.al.* 2005). However, it has been observed in *Jatropha* that it requires higher concentration of only one type of cytokinin (TDZ) for induction phase and favours lower concentration of another type of cytokinin (BAP/Kn) along with other additives for escalation and proliferation of shoot cultures. A recent report (Sujatha *et.al.* 2006) indicated similar effect in *J. curcas* with BAP, Kn 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 mg/L) NAA roots were induced in 52% of the shoots within three weeks (Table 3; Figure 1 *e*). Other concentrations of (0.5–2.0 mg/L) 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 charcoal8 for another 2–3 weeks. It is to be mentioned in this regard that rooting was obtained in MS basal medium supplemented with (1.0 mg/L) NAA, and in MS basal medium supplemented with higher amount of (2.0mg/L) NAA. 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. Plants were then transferred to potted soil for further growth. The plants ranged from (56.0 ± 0.12 to 82.0 ± 0.21 cm) in height after 10 months.

The present study describes a well-documented and reliable clonal propagation protocol of *J. curcas* from shoot 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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