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RESEARCH ARTICLE

IMPACT OF TDZ (THIDIAZURON) PULSE TREATMENT IN SINGLE AND MULTIPLE SHOOT FORMATION IN CALLI OF *JATROPHA CURCAS* L.

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Abstract

Rather being the widely used green alternative to fossil sources of energy, *Jatropha* have so far been commercially utilized and has medicinal values too. As the remunerative benefits accruing it is now really important to micropropagate *Jatropha* in a large extent. Here in a new protocol being proposed to regenerate shoots from calli earlier in time through TDZ pulse treatment. Thidiazuron being a most active cytokinin like substance induces greater *in vitro* shoot proliferation. *Jatopha curcas* (CJC - 19) calli when given 3 – 5d TDZ (3mg/L) treatment were capable of giving rise to earlier shoots. Calli obtained from *ex vivo* leaves was used to study this effect. The distinct shoots were isolated and rooted in MS media containing IBA (0.3 mg/L). The rooted plants were finally transferred to soil in pots.

INTRODUCTION

Bio fuels are often regarded as an attractive 'green' alternative to fossil sources of energy due to their potential contribution to lowering carbon dioxide emissions [35]. There is growing interest in *Jatropha curcas* as biodiesel "miracle tree" to help alleviate the energy crisis and generate income in rural areas of developing countries. *Jatropha* is becoming a poster child among some proponents of renewable energy [28]. *Jatropha* biofuel contains more oxygen, with higher cetane value increasing the combustion quality, is clean, non-toxic, eco-friendly and economic due to its low production cost. The superior quality oil can be extracted from the seeds. The oil can be used as a mixed fuel for diesel/gasoline engines [5]. The oil is not edible due to the presence of toxic substance "Curcascine" [13]. It is conventionally used in making soaps, candles, paints, lubricants and medicinally as a purgative [1,10,16].Once the seeds have been pressed, the remaining cake can be used as feed in digesters and gasifiers to produce biogas for cooking and in engines, but not in animal feed due to anti-nutritional factor. Medicinally the oil is used as a purgative and emetic and against cutaneous disease [3]. Recent years, more and more investigations have been performed on the chemical and biological active constituents of *Jatropha curcas*, such as curcin [4, 11, 12, and 19], 12-Deoxy-10-hydroxyphorbol [6], esterase and lipase [17] have been isolated from seed and characterized in succession, yet the full potential of *Jatropha curcas* is far from being realized. Besides its medicinal value they have been used in agro forestry program. It grows throughout most of the tropics. It survives on poor stony soils and can be used to reclaim land [8]. Conventional propagation of *J. curcas* is beset with problems of poor seed viability, low germination, scanty and delayed rooting of seedlings and vegetative cuttings [15, 18]. To exploit it to the maximum extent, one method of plant reproduction and biological active component formation is the use of tissue culture. This has increased the importance of developing tissue culture methods to facilitate large scale production of true to type plants and for the improvement of species using genetic engineering techniques. There have been reasonable proposals with possible outcome of improving oil quality of *Jatropha* such

as, down regulation of JcFATB1 in transgenic *Jatropha* can be attempted to increase levels of oleic acid in seeds [36]. *ACCase* gene has cloned to improve oil content in seeds. After this many possible outcomes in transgenic approaches, the micropropagation should not delimit *Jatropha* being utilized. Micropropagation of *J. curcas* has been reported by various authors in axillary nodes [16], nodes [27, 32], axillary bud [19], in petiole explants [16, 33, 38], in cotyledon [40], hypocotyls [20], epicotyls [34] in leaves [25, 31], through embryogenesis [30]. However, the regeneration frequencies, especially adventitious bud inductive frequencies, were still low. Therefore, the objective of this work is to provide an alternative method which could lessen the time taken for the onset of regeneration. Here we go in for a simpler, additive free approach which gives earlier regeneration.

[II] MATERIALS AND METHODS

2.1. Plant material and source of explant

Leaf pieces were used as the primary explants. Young fully expanded leaves from second node were collected from 2 yr old mature *J. curcas L.* (cultivar CJC 19) tree growing in Tamil Nadu Agricultural University campus. The explants were washed under running water for 30 min, rinsed with Tween 20 followed by further washing under running water to remove traces of the detergent. These were then surface sterilized with 0.1% $HgCl_2$ for 5 min. They were then clearly rinsed with sterile distilled water consecutively for 3 to 5 times.

2.2. Media preparation and culture conditions

For callusing and subsequent regeneration MS (Murashige and Skoog) medium, [2] containing 100 mg/l Myo-inositol, 10 mg/l Thiamine-HCl, 3% (w/v) sucrose in combination with plant growth regulators was used and cultured in a growth chamber. All media were adjusted to pH 5.8 with 1 N KOH or 1 N HCl, solidified with 0.8% (w/v) agar, and autoclaved at 121°C for 20 min. The cultures were incubated at 25 ± 2°C in darkness or under 16 h photoperiod using cool, white fluorescent lights throughout the period of culture.

2.3. Tissue culture and maintenance of cultured explants

Leaves were cut into 0.5 cm^2 segments by avoiding main midrib and are placed with abaxial side in contact with the medium. Thirty explants were inoculated for each experiment and three replicas of the same were made and kept under observation. The explants gave rise to small protrusions of tissue, which gradually organized into calli mass in a week. After 2 wk, the cellular clumps consisting of small green very friable callus grew at the cut ends of the explant. The calli were then maintained under the same condition by transferring to fresh medium every 2 wk once.

The well developed calli were transferred to MS medium fortified with 0.3mg/L TDZ for 3, 5 and 11d before being transferred to medium for shoot regeneration. The regenerants were maintained in same fresh medium with frequent subculture.

Healthy and sturdy shoots were separated and transferred onto MS medium fortified with 0.3 mg/l IBA for rooting. Roots were found to develop directly at the base of the shoots with no intervening callus. The percentage of rooting was recorded after 3 wk. Rooted shoots were carefully taken out of the medium and washed thoroughly in sterilized distilled water to remove the medium found adhering to roots. The plants were transferred to sterilized potting mixture (sand and silt at 1:1) for hardening.

[III] RESULTS

The role of cytokinin and auxins in different stages of somatic embryogenesis is well known. But the most important is finding out the triggering combination and concentrations of plant growth regulators besides other factors that vary from cell to cell even within a particular type of tissue of a plant species. Thidiazuron (TDZ) is among the most active cytokinin like substances and it induces greater *in vitro* shoot proliferation than many other cytokinins in many plant species. It is very soluble in DMSO and has slight solubility in water [7]. TDZ a substituted phenyl urea, is known to regulate varied morphogenic responses, such as breaking bud dormancy in apple [9] regeneration and multiple shoot formation in soy bean [26] and somatic embryogenesis in peanut and African violet respectively [14, 21]. It is more potent than BA in *in vitro* regeneration of shoots in dicotyledonary species [22]. Addition of TDZ and silver nitrate to shoot initiation medium on cotton increased the number of multiple shoots formed on the proximal end of hypocotyl explants [24]. Addition of TDZ has given more number of shootlet formations both in stem nodes and cotyledonary nodes in two different varieties of tomato [39].

Both the petiole (data not shown) and leaf explants (Table) shown higher percentage of callusing in MS+ BAP (1.5 mg/l) + IBA (0.5 mg/l). Increase in IBA concentration did not favor much callusing. The callus initiation was very evident in the first week, the complete proliferation was over within a period of 4 weeks. At this particular developmental stage, the calli were transferred to MS basal medium containing TDZ of concentration 0.3 mg/l for 3d before transferring them to regeneration medium; MS with BAP 1.5 mg/l, IBA 0.1 mg/l and GA_3 0.25 mg/l or BAP 1.5 mg/l, IBA 0.5 mg/l and GA_3 1.5 mg/l. To know that such a pulse treatment has its impact on regeneration efficiency, calli at same stage were transferred to regeneration medium without any TDZ treatment. From this study

it was very apparent that those calli which were given TDZ treatment were early to show regeneration in a week, whereas the calli without TDZ treatment took more than 3wks to give rise to the first shoot. TDZ treatment to calli in MS+BAP 1.5 mg/l, IBA 0.5 mg/l and GA_3 1.5 mg/l gave rise to multiple shoots with more than 3 distinctly separable shootlets within 2 weeks. Moreover extended TDZ treatment to calli resulted in necrosis, from sixth day onwards. When the treatment was being prolonged for 11d, the calli died in the same medium. Therefore 3 to 5 day TDZ pulse treatment could be helpful in advancing the regeneration initiation from the completely developed calli.

TDZ Treatment	1.5 mg/L BAP + 0.1 mg/L IBA + 0.25 mg/L GA_3		1.5 mg/L BAP + 0.5 mg/L IBA + 1.5 mg/L GA_3	
	Range of shoot initiation	Days took for initiation	Range of shoot initiation	Days took for initiation
3 days	63.3 ± 0.5	9	73.3 ± 0.5	11
Without	60 ± 1.0	22	66.6 ± 0.5	26

Table1: Range of callus induction percentage and standard deviation for the same, using various hormonal concentrations,

Hormones	Concentration (mg/L)	Range of callusing (%)
BAP+IBA	0.5+1.0	84.4±1.1
BAP+IBA	1.0+0.5	85.5±1.5
BAP+IBA	1.5+0.5	87.7±1.1
BAP+IBA	1.0+2.0	77.7±1.5
BAP+IBA	1.5+3.0	76.6±1.7

Table 2: Range of callus initiation percentage and standard deviation for the same in two different hormonal concentrations with and without TDZ treatment.

Fig 1: time chart depicting complete morphogenesis of *jatropha* plantlet

	Stages	Time period in week															
		1	2	3	4		5	6	7	8	9	10	11	12	13	14	15	16
Callus	Initiation					4 d In TDZ												
	Proliferation																	
Regeneration	Shoot bud																	
	Shoots																	
Elongation	Elongation																	
Rooting	Initiation																	
	Acclimatization																	

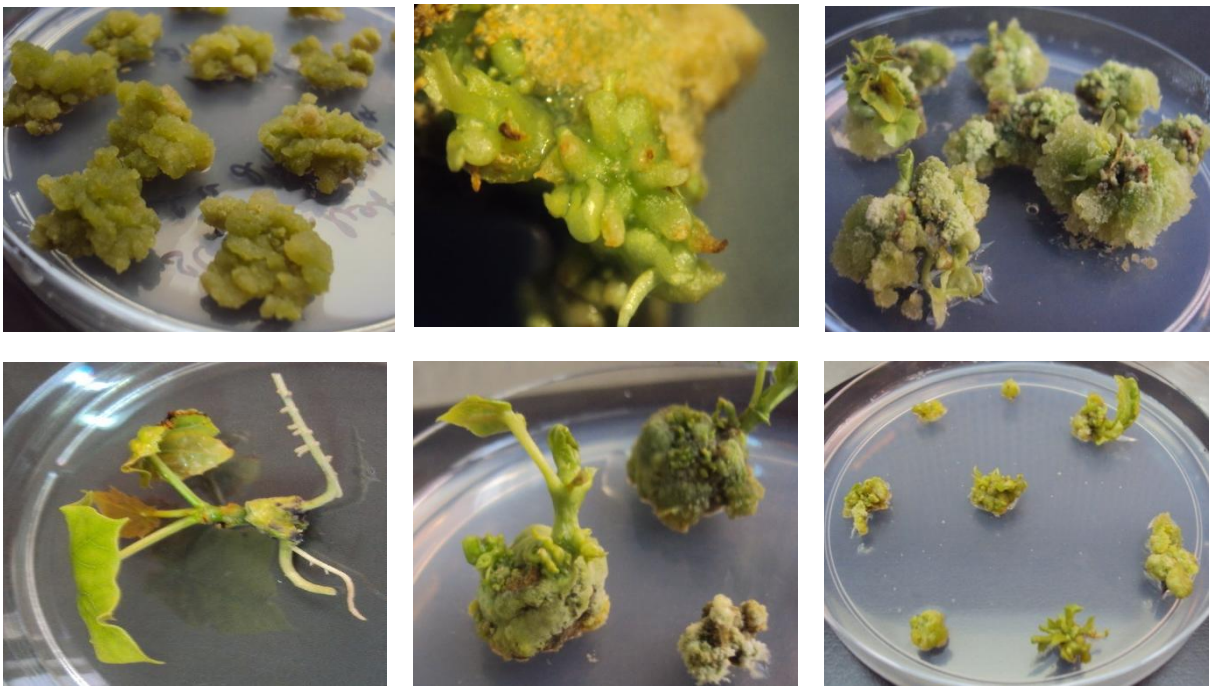

Fig 2: From left top to bottom clockwise – completely proliferated callus, embryos developing at the surface of the calli, embryos transformed to shoots, multiple shoots from single calli, sturdy individual shoots, and roots developed without intervening calli.

[IV] DISCUSSION

Except for the main mid rib the whole leaf lamina was used to study callusing. The leaf edges with midrib showed earlier callusing. The whole leaf bit was found to enlarge at first and the cut ends show the primary response. Later whole explants show callusing. By constant subculture at every 2 weeks in a fresh medium, the calli were maintained live and healthy. The calli were transferred as a whole during subculture without cutting or separating it. Both initiation (2wk) and maturation (3wk) of calli were accomplished in same hormonal combination. Highly organized, solid, greenish globular structures differentiated on the surface of callus by the fifth week of culture. At this stage the calli were given a pulse treatment of 3-5 d in MS+3 mg/L TDZ +3% Sucrose medium. After pulse treatment the calli were maintained in BAP 1.5 mg/l, IBA 0.1 mg/l and GA_3 0.25 mg/l or BAP 1.5 mg/l, IBA 0.5 mg/l and GA_3 1.5 mg/l for shoot development. Later the distinct shoots were isolated and rooted in IBA containing medium. Weida *et* al, 2003 reported callus mediated shoot-bud induction from *J. curcas* on MS medium supplemented with 0.5 mg/l BAP and 1.0 mg/l IBA. Meiru li in 2007 reported similar BAP, IBA, GA_3 combination for regeneration. The number of shoots developed from each calli is determined by the GA_3 in medium, which ranges from 2 to 5 individual, hard, distinct shoots. But simply by increasing the concentration of GA_3 in the medium the number of shoots from a single calli could possibly not be increased. Because, the triggering combination and concentrations of plant growth regulators is a delimiting factor. Even in case of TDZ treatment prolonged maintenance of calli in TDZ medium for more than 5d was found to be hazardous. Thus developed shoots were rooted in IBA (3mg/L) and hardened. By this *Jatropha* micropropagation could be made possible within 16 wks, with high frequency shoot formation also. Successful reports in *Jjatropha* micropropagation have probably used additives in high concentration. Timir *et al*, 2007 used adinene sulphate for Somatic embryo maturation. Khurana *et al*, 2010 used $CuSO_4$ at various levels of shoot formation. Sarika and Meenakshi, 2008 used citric acid along with adenine sulphate and few amino acids as nitrogen source for shoot induction and multiplication. Whereas this protocol is free from any additives or any extra C/N sources.

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