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In vitro micropropagation of biofuel feedstock pongam [*Pongamia pinnata* (L.) Pierre] through cotyledonary node culture and assessment of clonal uniformity by RAPD markers

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ABSTRACT

Pongamia pinnata has recently been recognized as a viable source of seed-oil for production of biofuel to meet the demand of the burgeoning biofuel industry. Although this tree is propagated by seeds, germination and plant vigour decreases following storage of seeds for 3 months or more. Plants propagated through stem cuttings are not deep rooted, hence not a preferred method of cloning this valuable species. Hence, the aim of the present study was to develop an efficient and reproducible mass propagation protocol for raising clonal garden of P. pinnata through in vitro cotyledonary node culture and assessing its trueness to parent plant.

Key words*: cotyledonary nodes, genetic fidelity, micropropagation, Pongamia pinnata, thidiazuran.*

INTRODUCTION

Pongamia pinnata (L.) Pierre (Family: Fabaceae) commonly known as pongam is a fast growing, glabrous, deciduous and non-edible oil bearing tree. Historically, this plant has been used in India and neighbouring regions as a source of traditional medicines, animal fodder, green manure, timber, fish poison and fuel. More importantly, *P. pinnata* has recently been recognized as a viable source of seed-oil for production of biofuel to meet the demand of the burgeoning biofuel industry[1,2]. However, the present production of pongam seeds is grossly inadequate and planting of such oil bearing trees in a massive scale has the potential to not only provide a renewable energy resource but in addition will alleviate the competitive situation that exists with food crops as biofuels and associated arable land and water use. Hence, there is an urgency to increase the production of such tree crops as *P. pinnata* which can be cultivated on marginal land.

Although *P. pinnata* is propagated by seeds, germination and plant vigour decreases following storage of seeds for 3 months or more[2]. Plants propagated through stem cuttings are not deep rooted, hence not a preferred method of cloning this valuable species. On the other hand, propagation through tissue culture techniques offers the possibility for rapid multiplication of desired genotypes in a shorter span of time. Although tissue cultured plantlets are widely used for raising large commercial plantations of many important agri-horticultural plants, progress made in this direction in the case of oil bearing trees species is very much limited.

Tissue culture regenerated plants appear to be no longer behaving as mother plant, probably due to epigenetic changes. Clonal trueness is of major importance in micropropagation of tree species. Although a few reports on *in vitro* culture of various explants of *P. pinnata* such as axillary buds[3] cotyledon[4] and cotyledonary node [5] with varied responses, but none of the above reports explained whether the plantlets

produced was true-to-type. Molecular techniques are powerful and valuable tools used in analysis of genetic uniformity of micropropagated plants. Molecular markers have particularly been suggested to be useful for confirmation of genetic fidelity in micropropagated tree species, where life span is quite long and performance of micropropagated plants could only be ascertained after their long juvenile stage in field conditions[6]. While working on seedling explants, it is always desired to evaluate the clonal fidelity of the regenerants so as to ensure of getting clones of desired lines. Hence, the aim of the present study was to develop an efficient and reproducible mass propagation protocol for raising clonal garden of *P. pinnata* through *in vitro* cotyledonary node culture and assessing its trueness to parent plant.

MATERIALS AND METHODS

Explants source

Efforts were undertaken to screen high oil yielding strains of *P. pinnata* by collecting seeds from different parts of Odisha, India and analysed the oil content. Out of 20 germplasm screened, PPAK03 (collected from Dhenkanal, Odisha, India) strain showed higher oil yield up to 26 %. Mature and healthy pods were collected from selected germplasm (PPAK03) and dried under partial sunlight for 2 – 3 d. The seeds were separated from the pods and washed thoroughly in running tap water. Thereafter, they were surface sterilised with 0.1 % mercuric chloride ($HgCl_2$) solution for 7-8 min in laminar flow hood and rinsed three times in sterilised distilled water for 3 minutes each. These seeds were cultured in test tubes (Borosil, India) containing MS medium to obtain axenic seedlings for isolation of cotyledonary nodes.

Culture medium and growth regulators

All media used for the present study were based on[7]. The explants were cultured on MS medium containing 0.8 % (w/v) agar and 3 % (w/v) sucrose with different cytokinins BAP, KIN and TDZ in varied concentration of 0.25 - 2.0 mg l^{-1} were used for multiple shoot induction. For rooting half-strength MS medium supplemented with various concentrations of (0.5 - 2.0 mg l^{-1}) IAA, IBA and NAA were used. The medium pH was adjusted to 5.8 prior to autoclaving with 0.1N HCl or NaOH before adding 0.8 % (w/v) agar and the media were autoclaved at 121℃ for 15 min.

Multiple shoot induction

The cultured cotyledonary node explants were induced multiple shoots after 12 days of culture. Multiple shoots were carefully sub-cultured onto MS medium containing the same
hormonal composition for further shoot proliferation and elongation. Once shoots attained 4.0 to 5.00 cm height they were clipped off from the explants and used for rooting purpose. The original explants were repeatedly sub-cultured onto shoot multiplication medium and subsequently onto shoot elongation medium after each harvest of the elongated shoots. The experiment was continued for two successive subcultures. The cultures were maintained at 25 ± 2℃ under 16h photoperiod with the light intensity of 60 $\mu Em^{-2}s^{-1}$ irradiance provided by fluorescent lamp at 25 ± 2 ^{o}C. Each treatment consisted of 14 explants, and all experiments were repeated at least twice.

Adventitious root induction and acclimatization

In vitro regenerated shoots were carefully excised and cultured on half-strength MS basal medium for rooting. After 5 weeks of cultures the rooted plantlets were carefully removed from the agar media, washed thoroughly under running tap water and transferred to root trainers (Nivedita Traders, India) containing vermiculite (TAMIN, India). The transplanted shootlets were shifted to automated green house where the temperature was maintained around 28 ± 2^{o}C with 85-90 % humidity for acclimatization. After 3 weeks of hardening the plantlets were transferred to poly bags containing garden soil + FYM (farm yard manure) and kept under a shade-net house for 2 weeks before planting in the field.

Statistical analysis

Experiments were setup in completely randomized design with 14 replications and repeated thrice. Data on percent response and the number of shoots and roots per explant were determined after a period of 6 wks following culture initiation. Data were subjected to analysis of variance (ANOVA). Duncan's new multiple range test[8] was used to separate the means to determine significant effects.

Histological observation

For anatomical investigations, cotyledonary nodes with or without shoots at different developmental stages were excised from seedlings after 1 wk of culture and were fixed in FAA (formaldehyde:ethanol:aceticacid:::90:5:5), dehydrated serially with ethanol and n-butanol and embedded in paraffin wax. Longitudinal sections (15 micron thick) were cut on a rotary microtome and were stained with safranin. For observation of microscopical features, Nikon microscope (Eclipse 80i, Japan) with photo micrograph attachment was used. For normal observations bright field was used.

Genomic DNA isolation

Leaves from 10 randomly selected micropropagated plants and mother plant were collected and used for DNA isolation. CTAB method[9] was employed for isolation of DNA from both the sources. Approximately 1.0 g of fresh leaves was ground to a powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a 25 ml sterile falcon tube containing 10 ml CTAB buffer. The buffer consisted of 2% (w/v) CTAB (Cetyltrimethyl ammonium bromide, Sigma), 1.4 mM NaCl, 20 mM EDTA, 100mM Tris HCl pH-9.5 and 0.2% (v/v) β-mercaptoethanol. The homogenate was incubated at 65ºC for 1 hour, extracted with an equal volume of chloroform, and centrifuged at 10,000 rpm for 20 min. DNA was precipitated from the aqueous phase by mixing with 1/10 volume 3 M sodium acetate and an equal volume of isopropanol. After centrifugation at 10,000 rpm for 10 min, the DNA pellet was washed with prechilled absolute ethanol. DNA was air dried and dissolved in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0). Quantification was made by running the dissolved DNA in 0.8% agarose gel along side uncut λ DNA of known concentration. The DNA was diluted to 25 ng μl^{-1} for RAPD analysis.

RAPD analysis

RAPD analysis was performed following method described by[10]. A total of 20 RAPD primers initially screened, out of which 10 random decamer Operon primers (Operon Tech, Alameda, USA) from A, C and N series (OPA01, OPA02, OPA04, OPA08, OPA12, OPA18, OPA19, OPA20, OPC04, OPC08) (Table 1) were selected taking 10 randomly selected tissue culture raised plants and a mother/source plant. RAPD primers were selected on the basis of the clarity of banding patterns. Each amplification reaction mixture of 25 µl volume contained 2.5 µl of 10X assay buffer (10 mM Tris-Cl, pH 8.3, 500 mM KCl, 50 mM $MgCl_2$ and 0.1% gelatin), 200µM of each dNTP (dATP, dCTP, dGTP, dTTP) (Biotools, Spain), 15 ng of primer (Bangalore Genei Pvt. Ltd, India), 0.5 unit of Taq DNA polymerase (Banglore Genei Pvt. Ltd, India) and 25 ng of template DNA. The amplification reaction was carried out in a programmed for 44 cycles as follows: 1st cycle for 5 min at 94ºC, 1 min at 37ºC, 2 min at 72ºC; followed by 43 cycles each of 1 min at 92ºC, 1 min at 37ºC, 2 min at 72ºC followed by one final extension cycle of 7 min at 72ºC for complete polymerization. Amplified products were separated by electrophoresis on 1.5% agarose gel in TAE (1×) buffer stained with ethidium bromide for 20 mins and photographs were taken using Gel documentation system (Bio-Rad Corporation, USA). RAPD analysis using each primer was repeated at least twice to establish reproducibility of banding pattern of different DNA samples of *P. pinnata*.

RESULTS

In vitro shoot induction

About 80% seeds were germinated after 8-10 days of incubation in MS medium devoid of any growth regulators. Cotyledonary nodes (1–1.5 cm) from 2 wk old seedlings (Fig. 1a) were cultured on MS medium supplemented with different growth regulators. The shoot forming capacity of cotyledonary nodes was greatly influenced by the type of growth regulator and its concentration in the medium present. There was complete failure of explants on MS basal medium devoid of growth regulators (Table 2).

Cotyledonary nodes cultured on MS medium supplemented with growth regulators started swelling after 3–4 days of inoculation followed by formation of either callus or shoots depending on the media composition. Wherever higher concentration of cytokinin used, there was callus formation at the basal portion and direct shoot regeneration occurred at the apical ends without callus formation. Of the various cytokinin tested, TDZ was more effective than BAP and KIN. Maximum (100 %) regeneration response

was obtained in the medium composed of MS + 1.0 mg l^{-1} TDZ with an average shoot number of 12.79 per cotyledonary node culture (Fig. 1b). Shoot inducing capacity of TDZ was declined above 1.0 mg l^{-1} concentration. The next highest response was achieved on the explants cultured in the medium MS + 1.0 mg l^{-1} BAP where an average of 85.71 % cultures responded with a mean shoot numbers of 8.07. Explants maintained in KIN augmented medium showed comparatively poor response. A maximum of 42.85 % explants responded at 1.0 mg l^{-1} concentration with a mean shoot numbers of 2.43. Though percent response and number of shoot produced per explants was least in KIN augmented medium, shoots exhibited healthy growth.

Shoots induced in the culture medium was transferred to half-strength MS basal medium with the same concentration of cytokinins for further shoot elongation. On transfer to the fresh medium with reduced MS salt concentration, shootlets developed in BAP and KIN augmented medium showed shoot elongation after 2 wk of transfer. However, shoot bud induced in presence of TDZ did not elongate and suppressed the growth of the shoot buds. Repeated transfers at 3 days interval for 4 times resulted in better shoot elongation. Visible shoot development was observed after 2 wk of culture period. In 5 wk time 90 percent of shoots elongated and attained reasonable length (< 3.0 cm), suitable for root induction (Fig. 1c). In order to obtain large-scale multiplication, shoot culture was established by repeatedly sub-culturing the mother cotyledonary nodes on fresh shoot regeneration medium after one harvest of *in vitro* regenerated shoots. On re-culture, they produced 6-8 shoots in 6 wk culture. Each original cotyledonary explant was sub-cultured for one more time but shoot regeneration potential was reduced (up to 60 %).

Histological observation

Transverse sections from the cotyledonary nodal region after three wks of inoculation revealed origin of shoot apical meristems from the peripheral cortical regions. The cortical cells during shoot bud formation become meristematic and start dividing actively resulting in swelling up of nodal part and reorientation of the cortex and vascular cylinder. The meristematic regions in the cortex showed small densely staining cells (Fig. 1d). The superficial cells in these regions at later stages of development gradually become organized into layers and finally exhibit a tunica-corpus organization which underwent differentiation to produce multiple shoots that had a distinct vascular connection with the parent tissue.

In vitro root induction and acclimatization

In vitro shoots obtained from primary and secondary cultures were excised from mother explants and transferred to root induction medium (Table 3). Of all the auxin used for root induction, IBA responded better than IAA and IBA. Almost all concentrations of IBA responded positively by producing healthy root system except the highest concentration (2.0 mg l^{-1}). Root inducing capacity was maximum in the concentration of 1.5 mg l^{-1} IBA where 90 percent culture responded with an average root number of 2.5 (Table 3 & Fig. 1e). Also time taken for root formation was less (14-16 days) than that of the other auxins. There was very poor response in the NAA augmented medium where most of the cultures showed callusing at the base. Plantlets with well-developed roots were carefully removed from the culture tubes and after thorough washing they were placed in root trainers filled with vermiculite soaked in ¼ strength MS basal salts and kept for 4 wks in a growth chamber. Later they were established in the field (Fig. 1f).

The main objective of this study was to obtain the plantlets of true-to-type. Hence, in order to confirm whether the plantlets produced through cotyledonary node cultures of *P. pinnata* are similar to mother plant, RAPD analysis was carried out on 2 months old *in vitro* regenerated plants and compared with mother plant. Out of 20 primers initially screened 10 produced clear amplification products in the clones. Number of bands produced by a single primer ranged from 2 to 7. The 10 selected RAPD markers utilized in the present study gave rise to a total of 44 scorable bands ranging from 350 to 1000 bp with an average of 4.4 bands per RAPD primer. A total of 50 plantlets analyzed gave rise to 2200 bands (number of plantlets analyzed X number bands with all 10 primers) giving rise to monomorphic pattern. The number of monomorphic bands was highest in the case of OPC08 (Fig 2).

Table 1. Effect of MS basal medium + growth regulators on induction of adventitious shoot buds on cotyledonary node segments of *Pongamia pinnata*[1, 2 *]

MS + Plant Growth Regulators (mg l^{-1})	Days to bud break	% response	shoots/explant
BAP			
0.25	18-20	50.0	2.64^{H}
0.5	16-18	64.28	6.79^{E}
1.0	14-16	85.71	8.07^{C}
2.0	16-18	57.14	3.36^{G}
KIN			
0.25	-	0	0^{K}
0.5	20-22	28.57	0.71^{J}
1.0	18-20	42.85	2.43^{H}
2.0	20-22	28.57	1.36^{I}
TDZ			
0.25	18-20	71.42	5.93^{F}
0.5	18-20	71.42	8.5^{B}
1.0	14-16	100.0	12.79^{A}
2.0	14-16	78.57	7.43^{D}

[1] Data pooled from three independent experiments each with 14 replicates per treatment.
[2] Data presented after 6 weeks of culture.
[*] Mean value within column followed by the same letter are not significantly different ($p \leq 0.05$; Duncan's Multiple Range Test)

Table 2. Effect of MS basal medium + plant growth regulators (auxin) on induction of roots of *Pongamia pinnata*[1, 2 *]

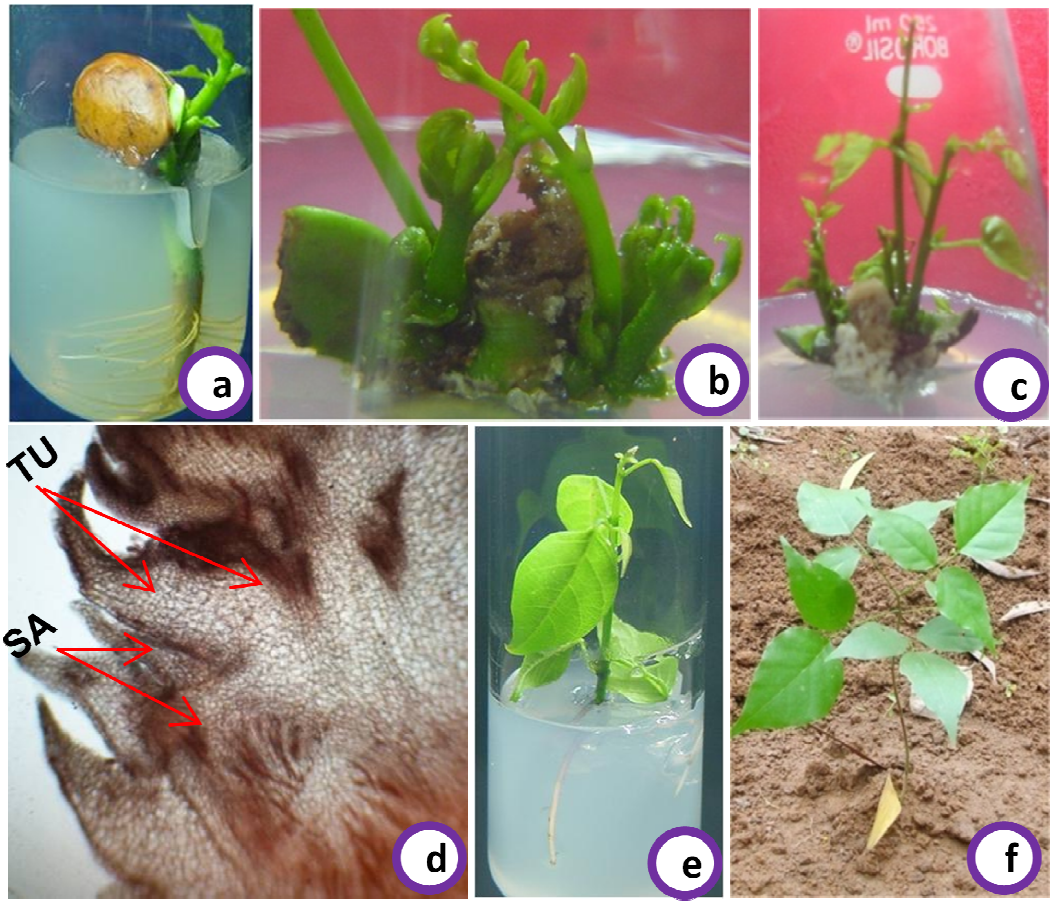
MS + Plant Growth Regulators (mg l^{-1})			Days taken to root initiation	% response	roots/shoot
IBA	IAA	NAA			
0.5	-	-	18-20	56.6	1.2^{C}
1.0	-	-	16-18	73.3	1.6^{B}
1.5	-	-	14-16	90.0	2.5^{A}
2.0	-	-	14-16	43.3	0.9^{D}
-	0.5	-	20-22	26.6	0.5^{E}
-	1.0	-	20-22	40.0	0.9^{D}
-	1.5	-	18-20	53.3	1.0^{D}
-	2.0	-	18-20	53.3	0.6^{E}
-	-	0.5	-	0	0^{F}
-	-	1.0	-	0	0^{F}
-	-	1.5	16-18	20	0.45^{E}
-	-	2.0	16-18	33.3	0.52^{E}

[1] Data pooled from three independent experiments each with 14 replicates per treatment.
[2] Data presented after 6 weeks of culture.
[*] Mean value within column followed by the same letter are not significantly different ($p \leq 0.05$; Duncan's Multiple Range Test)

Table 3. RAPD banding pattern of micropropagated and field-grown mother plants of *P. pinnata*

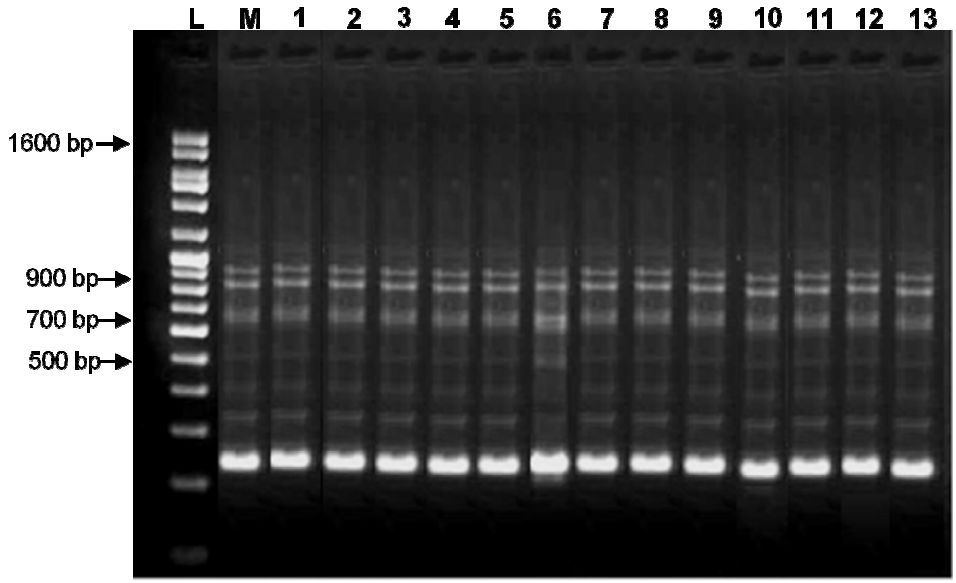
Primers	Sequences	Total bands	Range of amplification (bp)
OPA01	CAGGCCCTTC	5	600-1000
OPA02	TGCCGAGCTG	5	400-900
OPA04	AATCGGGCTG	4	1000-1400
OPA08	GTGACGTAGG	2	700 - 850
OPA12	TCGGCGATAG	3	600 - 900
OPA18	AGGTGACCGT	4	700 -1200
OPA19	CAAACGTCGG	5	600 - 1100
OPA20	GTTGCGATCC	3	600 - 900
OPC04	CCGCATCTAC	6	550 – 1100
OPC08	TGGACCGGTG	7	350 - 900
	Total	44	

Fig1. Micropropagation of *P. pinnata* through cotyledonary node culture



(a) 2 week old axenic seedling; (b) Multiple shoot bud development from cotyledonary node after 3 week of culture in MS + 1.0 mg/l TDZ; (c) shoot elongation on half-strength MS medium; (d) anatomical detail showing shoot development; (e) root induction with ½ MS + 1.5 mg/l IBA; (f) an field established plant (3 month old); *TU- Tunica*; *SA –Shoot Apex*

Fig 2. RAPD banding pattern with primer OPC08 in both micropropagated and field grown mother plants of *P. pinnata*



The lane L represents the molecular marker used (100 bp ladder), lane M, mother plant; lanes 1 – 13, micropropagated plants

DISCUSSION

The ability of *P. pinnata* cotyledonary node explants to form new shoots and root varied with composition and concentration of phytohormone present in the medium. Although the explants remained green and fresh in the absence of cytokinin in the medium, failure to produce shoots may suggest the vital role of phytohormones in inducing bud break in cotyledonary nodes. Similar observations have been revealed by many authors in a number of tree species[11, 12, 13]. The response of excised cotyledonary node segments of *P. pinnata* to produce shoots clearly indicates that the cotyledonary node is suitable explants materials for the micropropagation of this valuable biofuel species. This is primarily due to the fact that cotyledonary nodes play an important role in production of shoots from seedling explants as they supply endogenous growth regulators to the cultures[14, 15]. Micropropagation through cotyledonary node segments has been successfully used in the micropropagation of several tree species such as *Azadirachta indica*[14], *Gmelina arborea*[13], *Commiphora whitii*[15] also in *Pongamia pinnata*[4, 5].

Parallel reports on cotyledonary node culture of *P. pinnata* authenticate need of a cytokinin for effective shoot induction[4, 5, 3]. Sugla et al.[3] reported to have obtained an average shoot numbers of 7.0 from the cotyledonary nodes implanted in MS medium supplemented with 7.5 µM BAP. Similarly, Srivastava and Kant[5] obtained a mean shoot number of 3.0 with the aid of BAP and NAA. Comparatively higher shoot numbers were obtained in this present study. We could obtain shoot numbers ranging from 7.0 to 14.0 numbers from the primary culture using TDZ supplement in the MS medium. As reported by the earlier workers, in the present study, BAP supplement too gave good response but inferior to TDZ treatment. Our present set of results are in close conformity with the earlier report on meristematic bud[11] and cotyledon segment[4] cultures of *P. pinnata* where TDZ treatment resulted in best response than the other cytokinins such as BAP, KN, and Z.

Though shoot bud induction frequency was high in TDZ augmented medium, shoot elongation was not possible in presence of TDZ. Mehta et al.[16] are of the opinion that TDZ induce proliferation of the existing meristem, but suppress differentiation of the buds to shoot. Supporting to the above statement Sujata et al.[4], observed suppressed shoot bud development in presence of TDZ while working on cotyledon explants of *P. Pinnata* in the initial stage. Further, they proposed that removal of TDZ by repeated culture passage helps to overcome such problem. This was true in the present finding that removal of TDZ by repeated sub-culture to half-strength proved beneficial.

Success of *in vitro* culture relies on the percent of plantlets survival in field condition which in turn depends on the efficient *in vitro* root induction on micro shoots. IBA was more effective than IAA and NAA for root induction and also increased the number of roots formed by shoots. Similar observations have been made for other tree species [17,18,19,20] opined that the slow movement and slow degradation of IBA felicitated its localization near the site of application hence better root formation with IBA treatment. Increasing the concentration of IBA was found to stimulate the callusing and decrease the number of roots formed. Several authors have reported promotion of callus growth in tree species by high concentrations of IBA. High auxin concentrations are also known to interfere with rooting and inhibit full root development [21,22].

In the present work, histological evaluation on 2 wk old cotyledonary node culture clearly indicated that the shoot bud developments are directly from the meristematic tissue of the cultured node as the differentiated buds had a distinct vascular connection with the parent tissue. Structural analysis is an important first step in the study of the organisation and changes in the plant body and it is an extremely useful in the study of plant morphogenesis[23,24]. Histological evaluation to understand early tissue development has been attempted in tissue culture of various explants by different workers[25].

Although, all the tissue cultured plants are expected to be genetically identical yet the possibility of some genetic variation emerging during the *in vitro* process due to various factors such as the duration, auxin and cytokinin concentration and their ratio (hormonal balance), other nutritional conditions and *in vitro* stress, are all known to induce somoclonal variation[26, 27]. They mostly occur as a result of the stress causing alteration in DNA methylation patterns, DNA damage and mutation, alteration of cell's ability to repair damaged and mutated DNA[28], chromosomal rearrangements and point mutations[29]. We observed that banding pattern of micropropagated plants along with their donor plant were found to be monomorphic for all the RAPD. Thus genetic stability of micropropagated plants of *P. pinnata* which could be established through clonal fidelity using RAPD technique. RAPD marker technique has been used as a powerful tool to evaluate the variation in micropropagated plants. The clonal fidelity of micropropagated plants has also been determined by RAPD technique in a number of species such as *Pinus thungergii*[30], *Drosera*[31], *Citrus jambhiri* [32] and *Celastrus paniculatus*[33].

CONCLUSION

The present study offers a feasible protocol for mass clonal propagation of the elite lines of *P. pinnata* through cotyledonary node culture. While histological studies confirmed the development of shoot buds were of meristematic origin, RAPD analysis revealed the genetic purity of the micropropagated plants. In summary, the results could be of significance for large scale commercial cultivation of this potential biofuel feedstock tree species.

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REFERENCES

1. Vivek & Gupta AK, Biodiesel production from *Pongamia* oil. *J Sc Ind Res* **63:** 39-47 (2004)
2. Scott PT, Pregelj L, Chen N, Hadler S, Djordjevic MA & Gresshoff PM, *Pongamia pinnata*: An untapped resource for the biofuels industry of the future. *Bioenergy Res* **1:** 2–11 (2008)
3. Sugla T, Puryastha J, Singh SK, Solleti SK & Sahoo L, Micropropagation of *Pongamia pinnata* through enhanced axillary branching. *In Vitro Cell Dev Biol-Plant* **43:** 409-414 (2007)
4. Sujatha K, Panda BM & Hazra S, De novo organogenesis and plant regeneration in *Pongamia pinnata*, oil producing tree legume. *Trees* **22:** 711-716 (2008)
5. Shrivastava V & Kant T, Micropropagation of *Pongamia pinnata* (L.). Pierre- a native Indian biodiesel tree from cotyledonary node. *Int J Biotechnol Biochem* **6(4):** 555-560 (2010)

6. Gheorghe RN, Visoiu E, Popescu CF & Pamfil D, Assessment of genetic stability and fidelity of some micropropagated *Vitis vinifera* L. "Feteasca neagra" clones by ampelometric and RAPD markers. *Bulletin UASVM Hort* **66(1):** 51-57 (2009)
7. Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15:** 473 – 497 (1962)
8. Gomez KA, & Gomez AA, Statistical procedures for agricultural research (2nd edn). *John Wiley & Sons, New York* (1984)
9. Saghai MA, Soliman KM, Jorgensen RA & Allard AW, Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proc Nat Acad Sci USA* **81:** 8014-8018 (1984)
10. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA & Tingey SV, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl Acid Res* **18:** 6531-6535 (1990)
11. Sujatha K,& Hazra S, Micropropagation of mature *Pongamia pinnata* Pierre. *In vitro Cell Dev Biol-Plant* **43:** 608–613 (2007)
12. Nayak P, Behera PR & Thirunavoukkarau M, High frequency plantlet regeneration from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *In Vitro Cell Dev Biol–Plant* **43:** 231-236 (2007)
13. Behera PR, Nayak P, Thirunavoukkarasu M & Sahoo SB, Plant regeneration of *Gmelina arborea* Roxb. From cotyledonary node explants. *Indian J Plant Physiol* **13(3)**: 258-265 (2008)
14. Reddy AR, Bavaji M & Rao JVS, Micropropagation of *Azadirachta indica* A. Juss. via cotyledonary nodes. *Indian J Biotechnol* **5:** 309-311 (2006)
15. Kant T, Prajapati S & Parmar AK, Efficient micropropagation from cotyledonary node cultures of *Commiphora wightii* (Arn.) Bhandari, an endangered medicinally important desert plant. *J Plant Develop* **17:** 37-48 (2010)
16. Mehta UJ, Barreto SM & Hazra S, Effect of thidiazuron in germinating tamarind seedlings. *In Vitro Cell Dev Biol – Plant* **40:** 279–283 (2004)
17. Nandagopal S & Kumari BDR, Effectiveness of auxin induced *in vitro* root culture in chicory. *J Cent Eur Agr* **8(1):** 73-80 (2007)
18. Perez C & Postigo P, Micropropagation of *Betula celtiberica*. *Ann Bot* **64:** 67–69 (1989)
19. Thirunavoukkarasu M, Parida S, Rath SP & Behera A, Micropropogation of *Enterolobium cyclocarpum* (Jacq.) Griseb. *J Sustain Forest* **23 (4):** 1-12 (2007)
20. Nikell GL, Kirk-Othmer *encyclopedia of Chemical Technology Wiley, New York* **18:** (1982)
21. Lane WD, Regeneration of apple plants from shoot meristem tips. *Plant Sci Lett* **13:** 281–285 (1978)
22. Wetmore R & Wardlaw CW, Experimental morphogenesis in vascular plants. *Ann Rev Plant Physiol* **2:** 269-292 (1951)
23. Robinson JP, Britto SJ & Senthilkumar S, Comparative anatomical studies on *Emilia zeylanica* C. B. Clarke with *in vitro* regenerated plants. Middle-East *J Sci Res* **4 (3):** 140-143 (2009)
24. Ogita S, Yeung EC & Sasamoto H, Histological analysis in shoot organogenesis from hypocotyl explants of *Kandelia candel* (Rhizophoraceae*). J Plant Res* **117:** 457–464 (2004)
25. Negi RS, Sharma KC & Sharma M, Micropropagation and anatomical comparision of *in vivo* and *in vitro* developed shoot and root in *Cassia auriculata* L. - A medicinally important plant. *Indian J Fundam Appl Life Sci* **1(1):** 21-29 (2011)
26. Devarumath RM, Nandy S, Rani V, Marimuthu S, Muraleedharan N & Raina S, RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp (Assam-India type). *Plant Cell Rep.* **21:** 166-173 (2002)
27. Shu QY, Liu GS, Qi DM, Chu CC, Liu J & Li MJ, An effective method for axillary bud culture and RAPD analysis of cloned plants in tetraploid black locust. *Plant Cell Rep* **22:** 175-180 (2003)

28. Leroy X, Leon K & Branchard M, Plant genomic instability detected by microsatellite-primers. *Electron J Biotechnol* **3:** 1–9 (2000)
29. Kaeppler SM & Phillips RL, DNA methylation and tissue culture-induced variation in plants. *In Vitro Cell Dev Biol-Plant* **29:** 125–130 (1993)
30. Goto S, Thakur RC & Ishii K, Determination of genetic stability in long-term micropropagated shoots of *Pinus thunbergie* Parl. Using RAPD marker. *Plant Cell Rep* **18:** 193-197 (1998)
31. Kawiak A, & Lojkowska E, Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* plantlets. *In Vitro Cell Dev Biol– Plant* **40:** 592-595 (2004)
32. Savita, Bhagat A, Pati PK, Virk GS & Nagpa A, An efficient micropropagation protocol for *Citrus jambhiri* Lush. and assessment of clonal fidelity employing anatomical studies and RAPD markers. *In Vitro Cell Dev Biol-Plant* **48:** 512–520 (2012)
33. Senapati SK, Aparajita S & Rout GR, Micropropagation and assessment of genetic stability in *Celastrus paniculatus*: *An endangered medicinal plant. Biologia* **68(4):** 627-632 (2013)