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Research Article
Rapid *in vitro* Plant Regeneration From Nodal Explants And Assessment of Genetic Fidelity Using Inter Simple Sequence Repeats Markers in *Butea monosperma* (Lam.) Taub. var. lutea (Witt.)

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Abstract
Background and Objective: *Butea monosperma* (*B. monosperma*) (Lam.) Taub. var. lutea (Witt.), an important and threatened medicinal plant of Fabaceae. It is having potential role in certain biological activities and needs immediate conservation. The present study was focused on establishment of an efficient regeneration protocol and to assess genetic stability among *in vitro* regenerants. **Materials and Methods:** *In vitro* propagation was done on Murashige and Skoog (MS) basal medium supplemented with varying combination of growth regulators such as benzyl amino purine (BAP) and thidiazuron (TDZ) with a concentration ranged from 0.5-3.0 mg L^{-1} in combination with indole-3-acetic acid (IAA) (0.2 -0.5 mg L^{-1}) for shoot induction. For elongation of shoots, MS medium is supplemented with 0.1-1.0 mg L^{-1} of gibberellic acid (GA_3) and for rooting of elongated shoots indole-3-butyric acid (IBA) at 0.5-2.0 mg L^{-1} was used. For clonal fidelity analysis, ISSR-PCR method was used by extracting total genomic DNA from leaves of individual regenerated plantlet and its mother plant by cetyl triammonium bromide (CTAB) method. **Results:** Multiplication has been achieved through direct adventitious shoot formation from axillary buds of nodal explants at 2 mg L^{-1} BAP along with 0.2 mg L^{-1} IAA. Excised microshoots were elongated on 1.0 mg L^{-1} of GA_3, which enhanced shoot length to 80% within 2 weeks of culture (3.21$\pm$0.78). The fully elongated shoots were rooted with a frequency 89.65% on MS medium supplemented with IBA at 1.0 mg L^{-1}. Rooted microshoots were successfully acclimatized with a survival rate of 78%. **Conclusion:** The present investigation reports a high frequency regeneration system for its conservation and the reliability of current study was achieved by obtaining the true to type *in vitro* raised *B. monosperma* plants which showed monomorphic banding pattern with that of mother plant.

Key words: Nodal explants, *Butea monosperma*, ISSR, genetic fidelity, benzyl amino purine (BAP), thidiazuron

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Butea monosperma (Lam.) Taub. var. lutea (Witt.), is commonly known as White palash belonging to the family Fabaceae. It is a slow growing deciduous woody plant species with high enumerate medicinal properties[1,2]. During the past few years, the phytochemical analysis from this species have been isolated different secondary metabolites including flavonoids, chalcones and unsaturated fatty acids[3]. The extract from stem bark exhibited pharmacological properties including anti-diarrheal and anti-fungicidal activities[4].The isolated biological constituents from the seeds have been found to exhibit antimicrobial[5] and antiviral properties[6]. In view of its potential medicinal values, it is highly desirable to develop a rapid and efficient method for large scale multiplication of *B. monosperma* var. lutea. *In vitro* clonal propagation is a perquisite and a promising alternative for conservation of such forest tree species that is critically threatened. However its poor seed germination frequency has limited the conventional mode of propagation extensively and it is shown upto 65% response in seed viability[7]. To some extent a few protocols for standardization of *in vitro* regeneration was reported in same species through cotyledonary node explants[8,9].

The genetic integrity of micropropagated plantlets is another important aspect of this study. *In vitro* conditions are usually prone and susceptible to genetic changes due to culture stress[10,11]. PCR based molecular markers such as inter simple sequence repeats (ISSR) which are highly reproducible, polymorphic and easy to handle, have been successfully employed to assess the genetic stability of micropropagated plants in many plant species[12].

The objective of study was to study and describe an efficient micropropagation protocol of *B. monosperma* var. lutea from nodal segment explants using different hormonal concentrations and the genetic stability of *in vitro* raised plantlets using ISSR markers. This *in vitro* protocol can be used an attractive alternate for large scale production and conservation of such valuable medicinal plants.

MATERIALS AND METHODS

Plant material: In the present study, experimental nodal explants (Juvenile) were collected from 10 year old *Butea monosperma* (Lam.) Taub. var. lutea (Witt.) located at Kothakonda village of Karimnagar district, Telangana. All the experiments were conducted during months of July to December at the Department of Biotechnology, Kakatiya University, Warangal. The nodal explants were excised into 0.8-1.0 cm in length and were thoroughly washed in running tap water to remove the adherent traces of dust and soil particles. The explants were dipped in 0.2% Bavistin for 30 min and washed with distilled water and then placed in 10% 20 for 5-10 min, followed by repeated washing with sterile distilled water for 3-4 times. The explants were then disinfected with 0.1% $HgCl_2$ for 10 min and rinsed again with autoclaved distilled water for 4-5 times to clear the chemical traces from the surface of nodal explants. Both the ends of nodal segments exposed to chemical sterilents were trimmed and inoculated in a vertically upright position on Murashige and Skoog[13] (MS) medium fortified with 3% sucrose and 0.8% agar. All the cultures were maintained at 25±2°C with 16/8 h light/dark period.

Multiple shoot proliferation and elongation: Different concentrations (0.5-3 mg L^{-1}) of N6-Benzylaminopurine (BAP) and thidiazuron (TDZ) alone and in combination with auxin like indole-3-acetic acid (IAA) (0.2-0.5 mg L^{-1}) was used for proliferation of shoots from excised nodal segments. The cultures were maintained under same conditions for 3 weeks for initiation of multiple shoots and sub cultured onto same regeneration medium for next 3-4 weeks for further proliferation. After 4 weeks of regeneration, the efficiency of hormonal concentration on shoot proliferation was determined by recording the frequency of primary shoot development, number of shoots per explant and shoot length. A single excised regenerated shoot was shifted to MS medium containing gibberellic acid (GA_3) at different concentration levels (0.2-0.5 mg L^{-1}) for shoot elongation. The cultures were allowed to maintain for elongation for 2 weeks and the effect of different concentrations of GA_3 on shoot elongation was noted in terms of shoot length (cm).

***In vitro* rooting and acclimatization:** The elongated shoots of 2-3 cm with fully expanded leaves were shifted to rooting medium supplemented with various auxins includes, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) at different concentration levels (0.5-2 mg L^{-1}). After 3 weeks of culture, mean number of roots/explant and their respective length in cm was recorded. Completely rooted *in vitro* regenerated plantlets were washed carefully to remove the traces of medium and agar and the individual plantlets were shifted to pots containing sterile soil and vermiculite (1:1). The pots were covered with polysheets in order to maintain the relative humidity. The plants were transferred to greenhouse and finally shifted to field.

Conditions for ISSR-PCR study: Genetic stability of plantlets cultured *in vitro*, with that of mother donar plant was assessed by Inter Simple Sequence Repeat (ISSR) pattern analysis. Total genomic DNA was extracted from leaves of individual regenerated plantlet and its mother plant by Cetyl Triammonium Bromide (CTAB) method[14]. PCR reaction was carried out in a DNA Thermal Cycler (BIORAD C1000™, USA) with final reaction mixture volume of 25 μL contained 10XPCR reaction buffer (500 mM Tris–HCl, 160 mM $(NH_4)SO_4$ pH 9.2), 17.5 mM $MgCl_2$, 1 U of Taq DNA polymerase, 200 μmol of each dATP, dTTP, dCTP and dGTP , 10 pmol of primer and approximately 50 ng of template DNA. PCR conditions used for amplification consist of an initial denaturation step at 94°C for 3 min, followed by 40 cycles of 1 min at 94°C for denaturation, 48°C for 45 sec of annealing and 72°C for 2.3 min of extension and finally a soak temperature of 10°C. The amplified DNA was separated by 1% agarose gel electrophoresis and the size of amplicon was estimated using 1 Kbp DNA ladder which is photographed and documented.

RESULTS

Multiple shoot formation and proliferation: Multiple shoot induction and proliferation from nodal segment explants has been initiated (Fig.1a-c) within 4 weeks of culture on BAP+IAA containing media. BAP at 2 mg L^{-1} along with 0.2 mg L^{-1} IAA showed the highest shoot regeneration frequency (89%) and number of regenerated shoots are 9.15±0.68 per explant. A decrease in frequency of axillary shoot proliferation and the number of shoots per explant was found with increasing concentration of BAP further to 3 mg L^{-1} with 8.65±0.66 number of shoots/explant (Table 1).

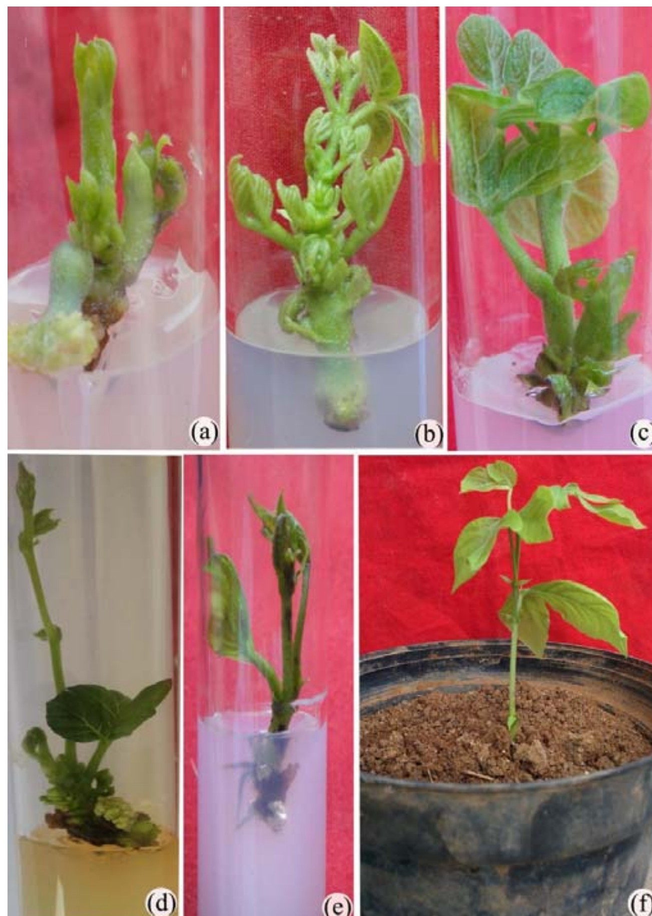

Fig. 1(a-f): *In vitro* regeneration from nodal segments and plantlet establishment of Butea *monosperma* (Lam.) Taub. Var. lutea (a) Bud breaking from the nodal region of explants, (b,c) Multiple shoot induction and proliferation from nodal explants of *B. monosperma* (Lam.) Taub. Var. lutea within 4 weeks of culture on MS fortified with 2 mg L^{-1} BAP and 0.2 mg L^{-1} IAA, (d) Elongation of shoots from primary culture of *B.monosperma* within 2 weeks of culture on MS and 1.0 mg L^{-1} GA_3, (e) A rooted shoot of *B. monosperma* on MS medium supplemented with 1 mg L^{-1} IBA and (f) Acclimatized plant in soil

Table 1: Effect of various hormonal concentrations of BAP and TDZ alone and in combination with IAA on shoot proliferation from nodal explants of *Butea monosperma* (Lam.) Taub. var. lutea (Witt.)

Hormones (mg L^{-1})	Regeneration of shoots (%)	Number of Shoots/Explant (Mean±S.E)	Mean shoot length (cm)
BAP			
0.5	63.95±0.57	6.15±0.15	2.25±0.19
1.0	75.75±0.56	8.25±0.22	2.05±0.15
2.0	73.25±0.90	7.85±0.18	1.75±0.16
3.0	69.25±0.75	7.15±0.22	1.45±0.15
BAP+IAA			
0.5+0.2	76.25±0.84	7.45±0.21	1.55±0.18
1.0+0.2	83.25±0.75	8.95±0.18	2.85±0.18
2.0+0.2	89.25±0.90	9.15±0.68	3.15±0.30
3.0+0.2	72.25±0.78	8.65±0.66	1.35±0.18
TDZ			
0.5	65.25±0.60	4.25±0.26	1.15±0.11
1.0	74.25±0.75	6.45±0.32	1.95±0.27
2.0	71.25±0.98	5.45±0.25	1.25±0.16
3.0	68.25±0.90	4.55±0.15	1.05±0.15
TDZ+IAA			
0.5+0.2	57.75±0.78	3.25±0.16	0.85±0.11
1.0+0.2	66.95±0.88	5.25±0.26	0.65±0.15
2.0+0.2	59.75±0.78	3.95±0.25	1.10±0.13
3.0+0.2	46.75±0.90	3.45±0.21	0.95±0.23

Data was recorded after 4 weeks of culture and values are given as Mean±Standard error (SE) represents the No. of Shoots/Explant of 20 replicates per each treatment and repeated thrice

Table 2: Effect of GA_3 on elongation of proliferated shoots regenerated from nodal segments of *Butea monosperma* (Lam.) Taub. var. lutea (Witt.)

GA_3 (mg L^{-1})	Response (%)	Shoot length (cm)
0.1	35	1.28±0.26
0.5	68	2.12±0.56
1.0	80	3.21±0.78

Data were collected after 2 weeks of culture and each treatment consisted of 20 replicates and repeated thrice. Mean±Standard error (SE)

The frequency of shoot proliferation in medium supplemented with TDZ at 1 mg L^{-1} alone was relatively low with 74% and there were fewer shoots/explant (6.45±0.32). The combination of TDZ with IAA has affected the frequency rate to 66% with 5.25±0.26 mean number of shoots. Thus, the hormonal combination of BAP when added with IAA in the medium was the most effective for multiple shoot regeneration indicating the cytokinin specificity of nodal explants of *B. monosperma* (Lam.) Taub. var. lutea.

Elongation and rooting: In the present study, BAP along with IAA at various concentrations induced both multiple shoot buds and their proliferation but failed to elongate even after transfer onto the same medium or hormone free MS medium. GA_3 at 0.1–1.0 mg L^{-1} was used in order to stimulate shoot elongation. Incorporation of 1.0 mg L^{-1} of GA_3 enhanced the shoot elongation in 80% of shoot cultures within 2 weeks at 3.21±0.78 in shoot length (Fig.1d), whereas the complete absence of shoot elongation in medium devoid of GA_3 (Table 2). IBA was found most effective among the different auxins tested, in inducing roots without an intervening callus phase. The maximum frequency of root formation (89%), number of roots (3.86±0.23) and root length (2.1 cm) were achieved within 2 weeks when shoots were cultured on medium containing 1 mg L^{-1} IBA (Table 3, Fig. 1e). In contrast, rooting of shoots occurred at low frequency on IAA and NAA supplemented media.

Well developed plantlets with four to five fully expanded leaves were successfully acclimatized in the greenhouse, in pots containing soil, vermiculite (1:1) within 2 weeks (Fig. 1f) that which showed normal growth and similar morphological characteristics. About 78% of survival rate was exhibited in micropropagated plants.

Genetic stability assessment: Among different ISSR primers tested for determining genetic level differences in plants raised under *in vitro* conditions and their donor mother, resulted in showing similar banding pattern with different primers in both the lines, where the size of bands ranges from 100 bp to 1500 bp, which indicates the absence of polymorphism (Table 4). Visual observation of plants did not showed any morphological variations. These results confirm the retaining of genetic fidelity in *Butea monosperma* Var. lutea that was obtained from nodal proliferation under *in vitro* conditions (Fig. 2a-c).

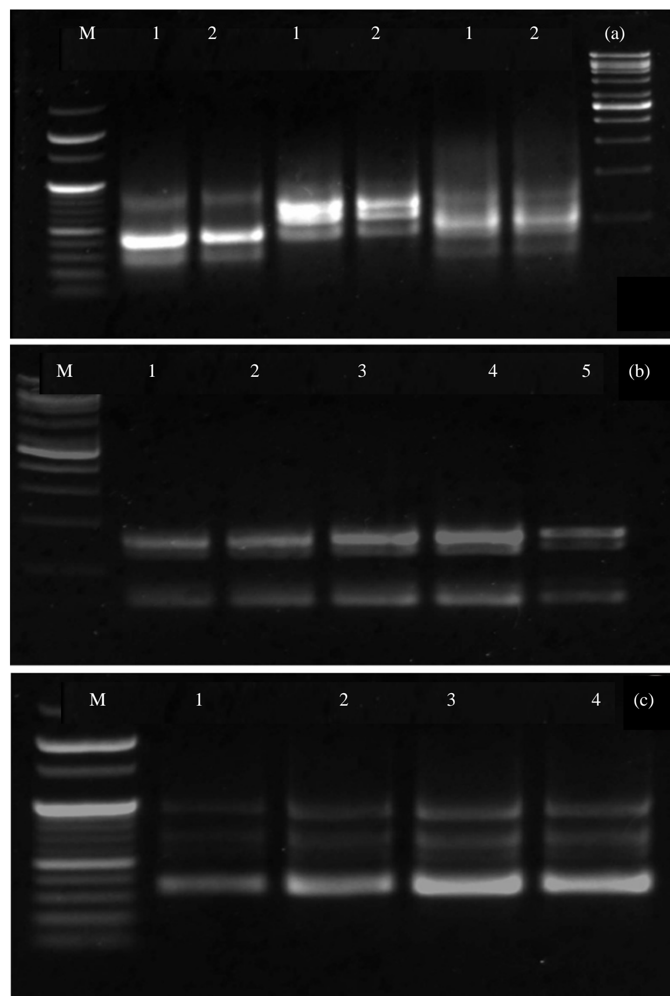

Fig. 2(a-c): ISSR amplification pattern obtained with different Primers, (a) Banding pattern with primers P1, P2 and P3 Lane M represents molecular marker, Lane 1: DNA from mother plant and Lane 2: DNA from micropropagated plants, (b) Banding pattern with primer P4, Lane M represents molecular marker, Lane 1: DNA from mother plant and Lane 2-5: DNA from micropropagated plants, (c) Banding pattern with primer, P5, Lane M represents molecular marker, Lane 1: DNA from mother plant and Lane 2-4: DNA from micropropagated plants

Table 3: Effect of various auxins on rooting efficiency of *in vitro* regenerated shoots from nodal explants of *Butea monosperma* (Lam.) Taub. var. lutea (Witt.)

Auxins (mg L^{-1})				Number of roots/shoots	
IAA	IBA	NAA	Response (%)	(Mean±S.E)	Length of roots (cm)
0.5	-	-	64.95±0.66	1.55±0.23	0.63±0.08
1.0	-	-	68.75±0.84	1.96±0.21	0.77±0.03
2.0	-	-	71.31±0.82	2.11±0.22	0.95±0.05
-	0.5	-	73.05±0.84	2.65±0.21	1.13±0.06
-	1.0	-	89.65±0.28	3.86±0.23	2.11±0.09
-	2.0	-	75.55±0.96	3.18±0.34	1.41±0.09
-	-	0.5	64.85±0.94	1.76±0.13	0.46±0.07
-	-	1.0	59.45±0.70	1.65±0.20	0.43±0.06
-	-	2.0	55.85±0.62	1.25±0.31	0.42±0.02

Data were collected after 3 weeks of culture and values are given as Mean±SE, Each treatment consisted of 20 replicates and repeated thrice, IAA = Indole-3-acetic acid, IBA = Indole-3-butyric acid, NAA = α-naphthalene acetic acid

Table 4: List of ISSR primers used in assessing genetic stability of *in vitro* regenerated plants of *Butea monosperma* (Lam.) Taub. var. lutea (Witt.)

ISSR marker no.	Primer sequence (5'-3')	Size range (bp)
P1	(AG)8G	200-700
P2	(GA)8YT	500-700
P3	ACTGCT(AG)7	200-500
P4	(GA)8YG	100-500
P5	(TC)8C	400-900
P6	(TC)8RG	No amplification
P7	GCC(GA)7	No amplification

Y = T or C: R= A or G

DISCUSSION

The number of plants produced through conventional method of propagation which accounts low seed germination rate and low viability offers limited advantages over *in vitro* approach. On the other hand, formation of multiple shoots from *in vitro* culturing of nodal segments having pre-existing meristems has proved to be an effective method for rapid mass multiplication with maintaining clonal fidelity[15,16]. In this experiment, the effect of different plant growth regulators (BAP, TDZ) was evaluated on morphogenetic response from nodal segments of *B. monosperma* (Lam.) Taub. Var. lutea. The potent ability of these hormones in multiple shoot formation form different explants were reported earlier for several medicinal and aromatic plant species[17-19]. Of the two cytokinins tested, BAP in combination with IAA was more effective than others in inducing and development of multiple shoots using nodal segments with highest shoot proliferation frequency. This combination has been demonstrated efficiently and was found to be effective in achieving regeneration with a variety of explants in different plant systems[20]. While in this case, TDZ was the less effective one with lower frequency of shoot induction and however similar studies were reported with different plant explants[21,22].

The effect of BAP has often been reported to stimulate multiple shoot proliferation while inhibiting shoot elongation[23,24]. Hence, it was necessary to develop a suitable media for elongation of proliferated shoots. The enhanced shoot elongation activity of GA_3 is upto 80% in *Butea monosperma* Var. lutea and its vital role is also reported earlier in some medicinally important plants, such as *Canavalia virosa*[25] and *Eclipta alba*[26]. The superior effects of IBA on roots development of *in vitro* raised shoots of *Butea monosperma* var. lutea has proved better when compared to IAA and NAA. Similar results were in accordance with *Garcinia mangostana* L[27]. The shoots produced continuously during successive subculture on MS medium supplemented with optimized concentrations to multiplicate in large number required for field transfer. The equivalent number of shoots further regenerated depicting the high frequency regeneration potential of *B. monosperma* var. lutea using nodal explants.

In the present study, PCR based ISSR markers system has been chosen as a molecular tool to assess the clonal fidelity among *in vitro* regenerates. The results obtained here showed similar identical tendencies with the absence of polymorphic variations. However, these are in good agreement and were supported by earlier studies on Strawberry, *Momordica charantia* L., *Citrullus colocynthis* (L.) Schard[28-30]. where ISSR showed relatively high value of genetic similarity determination. This study provides the first report on rapid *in vitro* regeneration protocol from nodal segments of *Butea monosperma* and genetic assessment of regenerants through ISSR markers. This is which may open up a new area of research in the field of micropropagation and genetic transformation of recalcitrant tree species.

CONCLUSION

The present study demonstrated a simple, efficient and direct method for high frequency shoot regeneration from nodal explants of *Butea monosperma* (Lam.) Taub. Var. lutea. Such a high and rapid regeneration frequency starting with the initiation of tissue culture and ending with the transplanting of regenerants to soil within 8–9 weeks, would be useful for mass propagation and multiplication of this valuable medicinal plant. And further this protocol can be exploited for genetic manipulation experiments. Moreover, employing ISSR marker system for studying genetic stability in regenerants could be fascinated in identifying clonal variations in brief.

SIGNIFICANCE STATEMENT

This study ensured the development of rapid regeneration protocol and assessment of genetic homogeneity among *in vitro* raised plantlets with that of mother plant. Different growth regulators found to have significant effect on regeneration ability of explants. Overall the results obtained are highly significant to carry out further studies.

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