

MICROPROPAGATION OF *MUNTINGIA CALABURA* L. AND ASSESSMENT OF GENETIC FIDELITY OF *IN VITRO* RAISED PLANTS USING ISSR AND RAPD ANALYSIS

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Abstract: *Muntingia calabura* L. is a potent medicinal plant. Because of that several researchers reported phytochemical and pharmacological studies, but only minimal information is obtainable on tissue culture and genetic stability evaluation studies. Therefore, in the present investigation we attempted to establish a reliable direct and indirect regeneration study through leaf and node explants on MS medium containing cytokinins BAP and KN (0.5-3.0 mg l⁻¹) in combination with auxins IAA and NAA (0.5 mg l⁻¹). The breakage of bud and shoot initiation were noticed with the initiation of callus at lower concentrations of auxin alone at 0.5 mg l⁻¹. 2,4-D was found to be good in callus induction from leaf and nodal explants. A problem of browning callus was prevented by regular subculturing of callus cultures. Leaf explants exhibited maximum number of shoots (32±0.88a) with shoot length (7.6±0.17a) and nodal explants obtained optimal number of shoots (26±0.88a) with shoot length (9.6±0.30a) on MS medium supplemented with BAP (2 mg l⁻¹) and IAA (0.5 mg l⁻¹). Half strength MS medium fortified with IBA (2.0 mg l⁻¹) was effective and achieved 70% of rooting. These well-developed plantlets were shifted to pots containing soil and vermicompost in 1:1 ratio for acclimatization. The acclimatized plants were field transferred with survival rate of 85%. The ISSR and RAPD markers analysis revealed the genetic stability of *in vitro* regenerated plants with the mother plant.

Keywords: genetic fidelity, ISSR, *Muntingia calabura*, RAPD, regeneration.

Introduction

Muntingia calabura L. is a rapidly growing tree, domestic to the American continent, and is commonly cultivated in warm areas of the Asian region. The *Muntingia calabura* is also known locally as “Jamaican cherry”, belongs to the Family Muntingiaceae. *Muntingia calabura* is regularly grown as roadside trees [MORTON, 1987; YUSOF & al. 2011; ZAKARIA & al. 2006a,b, 2007a-f, 2008, 2010, 2011]. The different plant parts of *Muntingia calabura* possess several potential medicinal uses in herbal medicine for the treatment as antiseptic [ZAKARIA & al. 2006a], antipyretic [ZAKARIA & al. 2007f], antiulcer [IBRAHIM & al. 2012], antidiabetic [SRIDHAR & al. 2011], antihypertensive [SHIH & al. 2006], cardioprotective [NIVETHETHA & al. 2009], anti-inflammatory, antinociceptive [ZAKARIA & al. 2006a], antibacterial [YASUNAKA & al. 2005] and antiplatelet aggregation [CHEN & al. 2007], especially in leaves and roots. This plant is rich in flavonoids, flavones, and flavanones, rendering to its potent antitumor activities [KANEDA & al. 1991]. Despite of its traditional claims, different parts of the plant have been used to treat various ailments.

MICROPROPAGATION OF MUNTINGIA CALABURA L. AND ASSESSMENT OF GENETIC...

Muntingia calabura root extracts are screened for cytotoxic activities against A549 and HT-29 cells [KANEDA & al. 1991]. Plant parts like leaves, roots, and stems were yielded cytotoxic flavonoids: Chrysin 2, 4-Dihydroxychalcone, and galanin 3, 7-dimethyl ether. The boiled bark is used to reduce the swelling in the lower extremities [ZAKARIA & al. 2006a]. Whereas, the leaves decoction or steeped water is employed to decrease ulcers in the stomach, prostate gland swelling and to alleviate headache and cold [MORTON, 1987]. The fruits are often cooked and made into jams. The Flowers are used as tranquilizers and tonic in Colombia [PEREZ-ARBEALAEZ, 1975].

There is a need to proliferate (or) multiply wider uses by conventional and biotechnological approaches. The improvement of the tree by traditional breeding is a delayed process due to its long juvenile period and high heterozygosity [SINGH & al. 2002]. Micropropagation of tree species offers a renewable biomass, conserve the genetic traits and produce clonal saplings for afforestation [ROUT & al. 2008]. Several plant species, especially woody plants are recalcitrant to high frequency root and shoot regeneration due to their *in vitro* recalcitrance [MCCOWN, 2000]. *In vitro* regeneration studies are difficult in woody tree species due to the high presence of phenols and alkaloids, which leads to the problem of browning callus. At the same time as, many of the researchers have established protocols in *Gardenia latifolia* Aiton, *Elaeocarpus serratus* L., etc. The present investigation was aimed for the development of sustainable regeneration protocol for *Muntingia calabura* and the influence of various plant growth regulators (PGRs) in the micropropagation. The regenerated plants were analyzed for genetic homogeneity studies using ISSR and RAPD markers. Moreover, the *Muntingia calabura* is growing at pollution releasing areas such as roadside and also heavy metal-containing areas. In future *in vitro* regenerated acclimatized plantlets can be used for isolating and screening of secondary metabolites from various plant parts and also these acclimatized plantlets roots are treated with bioinoculates (PGPR, *Azotobacter*, PSM and *Mycorrhiza*) for evaluating their survival rate in abiotic stress conditions.

Material and methods

Explants source and surface sterilization. The fruits of *Muntingia calabura* were squeezed and mixed in soil, later they were rubbed in sandy soil to separate the seeds. The seeds were used for the establishment of healthy plants in the polyhouse and field of Department of Biotechnology, Kakatiya University, Warangal, Telangana State, India. Young leaves, nodes, internodes, stem and other explants of *Muntingia calabura* were collected from the polyhouse or field during the month of January to December. The shoots were excised into 1.5 cm nodal segments and leaves were cut into 1 cm rectangular bits. Then the explants were washed rigorously under running tap water for 15-20 minutes for removing dust particles on the surface of explants. Then followed by surface sterilization by 1% Bavistin (a fungicide) solution for about 5 minutes and cleaned 3-4 times with sterilized double-distilled water. The explants are now transferred into laminar airflow for further sterilization. The explants (leaf and node segments) are now sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for about 2-3 minutes and then rinsed again with sterilized distilled water to remove any traces of $HgCl_2$.

Culture medium and conditions. MS medium [MURASHIGE & SKOOG, 1962] was prepared by adding 30 g/l sucrose, 100 mg/l Myo-inositol and with varying concentrations of cytokinins BAP or kinetin KN (0.5-3 mg l⁻¹) and auxins IAA or NAA (0.5 mg l⁻¹). The pH of

the medium is set to 5.6 ± 0.1 with 1N HCl or 1N NaOH before the addition of agar (0.8%). Now the culture media was sterilized in an autoclave with 1.1 kg cm^{-2} pressure at $121 \text{ }^\circ\text{C}$ for 15 min. The surface sterilized explants were inoculated onto the sterilized culture medium (one explant per culture tube). All inoculated cultures were transferred into a controlled condition chamber for the incubation at $25 \pm 2 \text{ }^\circ\text{C}$ temperature with a relative humidity of $65 \pm 5\%$ under a 2000-2500 lux for 16h Photoperiod. Data was collected and analyzed after a period of 45 days.

Callus induction and shoot proliferation. Initially after two weeks, brown and light green color callus was initiated from cut margins of leaf and node explants cultured on MS medium containing 0.5 mg l^{-1} 2,4-D. After the third week, the brown and thick green callus expunged was subcultured onto medium with 0.5 mg l^{-1} IAA or NAA with various concentrations of KN (0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg l^{-1}) or with BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg l^{-1}) to identify the utmost appropriate combination of PGR's for the multiplication of regenerated shoots from leaf and node segment of *Muntingia calabura*. The data on percentage of regeneration, number of regenerated shoots per explants and their shoot length (cm) was recorded after fourth week of culture.

Rooting of in vitro regenerated shoots. After achieving healthy and elongated *in vitro* regenerated shoots upto a height of 1.0 to 1.5 cm, they are excised and cultured onto 0.8% agar-gelled with half strength MS medium fortified with various concentrations of IBA 0.5, 1.0, 1.5, 2.0, 2.5 mg l^{-1} for induction of *in vitro* rooting from regenerated shoots. The percentage of rooting, number of roots and their length (cm) was recorded after fourth and fifth week of culture.

Hardening and acclimatization. The *in vitro* regenerated plantlets were carefully removed from the culture vessels and washed thoroughly with sterile distilled water to get rid of adhered agar and then transplanted into plastic cups filled with sterile garden soil and vermicompost (1:1) and maintained inside a plant growth chamber at $25 \pm 1 \text{ }^\circ\text{C}$ under 16hr daylight provided by white fluorescent lamps. After acclimatization, these plantlets were transferred under greenhouse conditions and covered with polythene sheets for maintaining moisture and humidity for further acclimatization. After sixth and seventh weeks these acclimatized plants were hardened by transplanting into large pots filled with garden soil and manure. The percentage of survival was recorded after 3 weeks of the field transfer and found to be 85% same to the mother plant.

Genomic DNA Isolation and genetic homogeneity analysis. The genomic DNA was isolated from the mother plant and regenerated plantlets by using cetyltrimethyl ammonium bromide (CTAB) method [DOYLE & DOYLE, 1990]. The DNA concentration was checked by 0.8% agarose gel electrophoresis. Assessment of genetic fidelity of *Muntingia calabura* plants was performed through Inter-Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) analysis. Each set of 10 ISSR and RAPD primers was selected for the genetic stability study. The PCR reaction mixtures and conditions are followed based on the protocol described by PENDLI & al. (2019). The PCR bands were calculated using the 100 bp DNA ladder (Himedia). The experiments were repeated thrice to verify the reproducibility of PCR bands.

Data analysis and observations. The cultures were regularly subcultured onto fresh medium with an interval of 4-5 weeks. The cultures were observed and recorded after every six days of inoculation. All the experimental work was conducted thrice with 60 explants for each combination. The significant differences for various parameters recorded were calculated by mean and standard error variance (ANOVA) using SPSS software.

Results and discussions

The sterilization treatments of explants with 0.1% (w/v) mercuric chloride (HgCl_2) yielded 95% of contamination-free leaf and nodes of *Muntingia calabura*. The responses of both the explants inoculated shown the capacity of shoot regeneration differently to the two cytokinins (BAP and KN) and combination with (IAA and NAA) with various concentrations of PGR's. The 15-day callus subcultured on MS modified medium showed efficient shoot induction. The age of explants showed to play a key role in morphogenesis [DISTABANJONG & GENEVE, 1997].

Callus induction from leaf and node explants. *Muntingia calabura* is a woody tree species and contain a wide variety of secondary metabolites like alkaloids or phenols, the browning of the medium may be due to the production of alkaloids or phenols in the medium. The mixture of antioxidant was found to reduce the phenolic exudation and browning of callus in *Ceratonia siliqua*, *Pterocarpus santalinus* [ROMANO & al. 2002; PRAKASH & al. 2006]. Callus was induced from the excised portions of midrib and lateral veins of leaf lamina and basal part of nodal regions of the node. The initiation of callus was achieved even at lower concentrations of auxin alone with 0.5 mg l^{-1} 2,4-D. 2,4-D found to be good in inducing callus from leaf and nodal explants (Figure 1a and Figure 2a). 2,4-D plays a major role in callus induction in an ample range [RANI & GROVER, 1999]. An identical report on high percentage of callus induction was obtained from stem and leaf explants on MS medium with IAA (0.5 mg l^{-1}) and 2,4-D (1.0 mg l^{-1}) in *Citrullus colocynthis* [SHASTHREE & al. 2012] and *Momordica cymbalaria* [CHAITANYA & al. 2020]. A problem of browning callus in the node segment (Figure 2a) and blackish light green color callus in leaf (Figure 1a) explant was observed during the initiation of callus from *Muntingia calabura*. *Acacia auriculiformis*, *Strelitzia reginae*, and *Anemone coronaria* have also been reported that this problem can be prevented by adding up of activated charcoal (AC) to the medium which discolors and stimulates the shoot growth [YADAV & al. 2016]. Whereas the present work promoted by frequent subculturing of explants for every 10-20 days to reduce the browning of callus and the growth of callus kept increased.

Indirect regeneration studies from leaf explants. Regeneration of high number of shoots of *Muntingia calabura* with proper growth was achieved by testing varying combination of cytokinins (BAP and KN) with auxins (IAA and NAA). In leaf explants, 2 to 4 micro shoots were initiated from second week's brown or coffee colored callus (Figure 1b). After third-week brown colored callus slowly changed to green colored callus and few shoots formation was occurred simultaneously (Figure 1c). The leaf explants exhibited a highest shoot regeneration of 60% when cultured on multiplication medium with BAP (2.0 mg l^{-1}) or KN (2.0 mg l^{-1}) in combination with 0.5 mg l^{-1} IAA or 0.5 mg l^{-1} NAA. The explants inoculated onto the medium containing a low level of auxin and cytokinin have shown their response by early enlargement of nodes with axillary bud break within 25 days (Figure 1d). The proliferated shoot buds are shifted to a medium where the concentration of cytokinin is kept increased and the auxin concentration was maintained constant (Figure 1e). The number of shoots developed from leaf explants ranged from 13 ± 0.3 to 32 ± 0.8 on medium fortified with 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} IAA (Figure 1f). The highest shoot length was found to be 7.6 ± 0.1 on BAP (2 mg l^{-1}) and IAA (0.5 mg l^{-1}) MS medium (Figure 1g and 1h). The synergistic effect of BAP with IAA on shoot elongation has also reported in *Acacia auriculiformis* [YADAV & al. 2016]. An average of 4 ± 0.6 to 30 ± 0.6 (Table 1)

number of shoots were obtained on medium containing 2.5 mg l⁻¹ KN with 0.5 mg l⁻¹ IAA and 1±0.5k to 24±0.1e (Table 1) with 2.0 mg l⁻¹ KN and 0.5 mg l⁻¹ NAA in leaf explants. Excellent high frequency regeneration of *Populus deltoides*, a hardwood tree was also reported in combination of KN and IAA [YADAV& al. 2009]. Whereas, in *Gardenia latifolia* BA (4.0 mg l⁻¹) and IAA (0.5 mg l⁻¹) had the best results for shoot regeneration from leaf [REDDY & SARITHA, 2013]. The hostile effect of cytokinin percentage in shoot induction decreases as the concentration of cytokinin increases. This effect has also been reported in *Pterocarpus marsupium* [ANIS & al. 2005]. The BAP is more efficient and more energetic cytokinin in many tree species like *Syzygium alternifolium* [SHA VALLI KHAN & al. 1999] and *Pterocarpus marsupium* [CHAND & SINGH, 2004]. Decisively, the combination of auxins and cytokinins in the medium can improve organogenesis and regeneration of the cultured explants.



Figure 1. Effect of PGRs on indirect plant regeneration from leaf explants: a – Initiation of callus on 2,4-D at 0.5 mg l⁻¹; b – Initiation of shoots in combination with 0.5 mg l⁻¹ BAP + IAA 0.5 mg l⁻¹; c – Proliferation of micro-shoots at 0.5 mg l⁻¹ IAA + BAP 1.0 mg l⁻¹; d – Elongation of shoots at 0.5 mg l⁻¹ IAA + 1.0 mg l⁻¹ BAP; e – Formation of multiple shoot clusters on 0.5 mg l⁻¹ + IAA + 1.5 mg l⁻¹ BAP; f – High frequency of multiple shoots formed on 2 mg l⁻¹; g & h – Elongation of shoot with roots at alone IBA 2.0 mg l⁻¹; i – Partially hardened plant in an earthen pot containing 1:1 vermiculite inside the greenhouse; j – Green house grown plants transferred into field.

MICROPROPAGATION OF MUNTINGIA CALABURA L. AND ASSESSMENT OF GENETIC...

Table 1. Effect of different concentrations of PGR's on multiple shoot induction using leaf and node explants of *Muntingia calabura* L. – a pharmaceutically and medicinally important woody tree.

PGR'S and Concentration				Regeneration		No. of shoots		Shoot length(cm)	
BAP	KN	IAA	NAA	Leaf	Node	Leaf	Node	Leaf	Node
0.5		0.5		67	61	13±0.3h	16±0.5cde	1.5±0.2h	1.7±0.1g
1		0.5		70	68	23±0.5e	16±0.8cde	2.8±0.1fg	3.7±0.1d
1.5		0.5		77	71	27±0.1c	20±0.4b	6.4±0.3b	9.3±0.4a
2		0.5		93	88	32±0.8a	26±0.8a	7.6±0.1a	9.6±0.3a
2.5		0.5		84	80	24±0.7de	18±0.6b	5.5±0.4c	6.9±0.3b
3		0.5		67	76	18±0.8f	10±0.1gh	3.0±0.3ef	3.6±0.2de
	0.5	0.5		46	40	4±0.6j	2±0.2jkl	0.5±0.2i	0.1±0.1i
	1	0.5		55	48	10±0.4i	8±0.3hi	1.4±0.3h	1±0.2hi
	1.5	0.5		55	50	23±0.5e	14±0.8f	5.1±0.4d	3.2±0.4def
	2	0.5		62	58	25±0.5cde	17±0.5cd	5.5±0.3cd	5.4±0.2c
	2.5	0.5		70	66	30±0.6b	25±0.6a	7.5±0.5a	6.3±0.3b
	3	0.5		68	58	25±0.8cde	16±0.4cdef	7.5±0.2a	3.5±0.2def
0.5			0.5	54	58	4±0.5j	1±0.3l	1.6±0.2h	0.5±0.2hi
1			0.5	67	60	10±0.5i	3±0.1jk	3.6±0.2e	1.5±0.2g
1.5			0.5	77	69	15±0.3gh	9±0.5ghi	3.6±0.2e	2.6±0.1f
2			0.5	80	75	26±0.2d	17±0.8cd	6.4±0.4b	3.6±0.2ef
2.5			0.5	78	64	16±0.2g	11±0.5b	2.4±0.3f	3.2±0.4def
3			0.5	60	50	10±0.4i	7±0.33i	1.5±0.2h	0.9±0.05i
	0.5		0.5	57	46	1±0.5k	0.6±0.33l	0.5±0.2i	0.2±0.2i
	1		0.5	66	60	5±0.5j	2±0.2jkl	2.0±0.2f	0.5±0.2hi
	1.5		0.5	70	55	19±0.6f	10±0.6gh	2.7±0.1ef	0.5±0.2hi
	2		0.5	75	64	24±0.1de	15±0.5ef	5.1±0.4c	2.7±0.1ef
	2.5		0.5	66	52	16±0.2g	9±0.3ghi	4.7±0.1d	2.7±0.1ef
	3		0.5	56	45	8±0.33i	3±0.5j	1.8±0.1h	1.3±0.4g

Direct regeneration studies from node explants. Development of new axillary shoots on medium with varying concentrations of BAP or KN in addition to IAA (0.5 mg l⁻¹) and NAA (0.5 mg l⁻¹) has revealed that BAP is efficient over KN in all the combinations. The combination of BAP 2 mg l⁻¹ + IAA 0.5 mg l⁻¹ is found to be efficient for shoot bud elongation in node than in leaf (Figure 2c). Similarly, the regeneration of the node had shown less response compared to leaf with BAP 2 mg l⁻¹ in combination with NAA 0.5 mg l⁻¹ and IAA 0.5 mg l⁻¹. Node explants showed maximum number of shoots from 16±0.5cde to 26±0.8a on BAP 2 mg l⁻¹ + IAA 0.5 mg l⁻¹ and from 1±0.3i to 17±0.8cd on BAP 2 mg l⁻¹ + NAA 0.5 mg l⁻¹ (Figure 2d and Table 1) and the highest shoot length of 9.6±0.3a was also developed on BAP 2 mg l⁻¹ + IAA 0.5 mg l⁻¹. In nodal explants a range of 2±0.2jkl to 25±0.6a (Figure 2e and Table 1) mean number of shoots was noticed on medium with KN 2.5 mg l⁻¹ and IAA 0.5 mg l⁻¹ whereas 0.6±0.33i to 15±0.5ef number of shoots were developed with KN 2.0 mg l⁻¹ + NAA 0.5 mg l⁻¹ g/l after 6 weeks. In *Artemisia nilagirica*, a high regeneration frequency occurred when nodal explants were cultured on MS medium with 2.5 mg l⁻¹ BAP and 7.5 mg l⁻¹ 2-iP [SHINDE & al. 2016]. In *Elaeocarpus* family, the species *Elaeocarpus sphaericus* and *Elaeocarpus robustus* an efficient micropropagation method using BA+KN+casein hydrolysate was reported from node explants. The best

results for shoot induction was reported in *Elaeocarpus sphaericus* [SAKLANI & al. 2015, LAKSHMI & al. 2010] on MS+2.2 μ M BA+2.2 μ M KN, whereas with equal concentrations of BA + KN + casein hydrolysate in *Elaeocarpus robustus* [ROY & al. 1998]. ARSHAD & KUMAR (2006) have also developed a protocol using axillary buds of *Elaeocarpus tuberculatus* on MS medium containing equal concentrations of only cytokinins in the combination of BA and KN.



Figure 2. Effect of PGRs on direct regeneration from node explants: a – initiation of callus from mature nodal explants on MS medium with 0.5 mg l⁻¹ 2,4-D; b – proliferation of shoots from nodal brown calli on MS medium with 1.0 mg l⁻¹ BAP + 0.5 mg l⁻¹ IAA; c – elongation of multiple shoots with 1.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ IAA; d – elongation of multiple shoots with 2.0 mg l⁻¹ BAP + 0.5 mg l⁻¹ IAA; e – formation of roots from the lower part of the *in vitro* shoots on MS medium containing 2.0 mg l⁻¹ IBA; f – *in vitro* regenerated plantlets transferred to plastic cup possessing soil and vermicompost (1:1) and the plantlets covered with polythene sheet for acclimatization.

Rooting for *in vitro* shoots. Individual shoots were excised and inoculated onto half-strength MS medium containing IBA. IBA at a concentration of 2.0 mg l⁻¹ induced rooting with a root length of 2±0.33b with maximum 25±0.57a (Figure 2e-r and Table 2) number of roots. But the maximum root length of 3±0.3a was observed at IBA 2.5 mg l⁻¹. At lower concentrations of IBA, the number of roots grown was less comparatively at 2.0 mg l⁻¹. Interestingly, as the concentration of IBA is increased above 2 mg l⁻¹ basal callusing was observed all through the surface of shoots and there was also a decrease in root length. IBA has been identified to induce the best rooting response in woody tree species. Similar reports were observed in *Gardenia latifolia* with IBA at 4.0 mg l⁻¹ of concentration on half-strength MS medium [REDDY & SARITHA, 2013].

Table 2. Effect of different concentrations of IBA on rooting of *in vitro* regenerated shoots of *Muntingia calabura* after 6 weeks ½ strength MS medium.

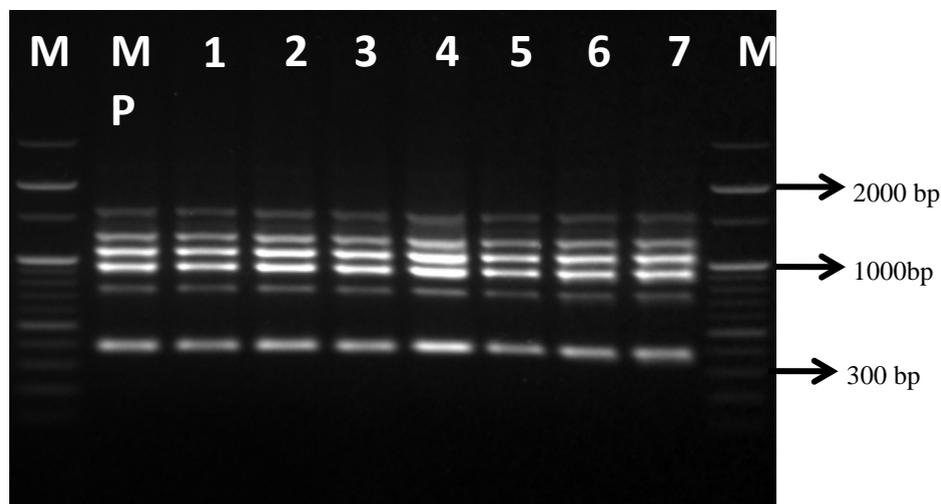
Auxin IBA	% of rooting	Root number	Root length (cm)
0.5	20	5±0.33e	0.2±0.3d
1.0	39	12±0.57d	0.5±0.3cd
1.5	55	18±0.33c	1±0c
2.0	60	25±0.57a	2±0.2b
2.5	51	21±0.57b	3±0.3a

Hardening and acclimatization. *In vitro* grown complete plantlets of *Muntingia calabura* are carefully taken out and washed gently to remove the adhered media. Polythene cups containing garden soil and vermicompost in 1:1 ratio was found to be best for hardening (Figure 2f). Hardened plantlets were maintained initially in greenhouse conditions by irrigating with MS salt solution (half-strength) for four weeks (Figure 1i). The poly cups with hardened plantlets were covered with polythene sheets and proper watering was done to maintain humidity (50-70%) and moisture. Later, plantlets were planted and acclimatized in field conditions with high survivability of 85% was observed (Figure 1j).

Genetic fidelity analysis. The ISSR and RAPD markers were employed to identify the genetic fidelity of the mother plant and the regenerated plants of *Muntingia calabura*. Earlier reports suggested that using of more than one type of molecular marker in genetic homogeneity studies of *in vitro* regenerated plants is always useful for reliability [ROHELA & al. 2019]. In the current investigation, ISSR (10) and RAPD (10) primers (Table 3 & Table 4) were used for the screening of genetic homogeneity of *in vitro* regenerated plants with the parent plant. In out of 10 ISSR primers, seven primers were produced reproducible and clear bands, the PCR banding pattern of MC4 primer shown in Figure 3. In out of 10 RAPD-primers, six primers were amplified with scorable and clear reproducible bands, the PCR banding profile of OPA6 shown in Figure 4. ISSR and RAPD analysis confirmed that there is no polymorphic nature in regenerated and mother plants; both plants exhibited similar monomorphic banding patterns. Therefore, the results prove that the mother plant and regenerated plant contains the same gene pool (Figure 3, 4). A similar kind of genetic fidelity evaluation using ISSR and RAPD markers was successfully achieved in different plant systems like *Rauwolfia tetraphylla* [ROHELA & al. 2019], *Citrullus colocynthis* [RAMAKRISHNA & al. 2012], *Solanum trilobatum* [PENDLI & al. 2019], and *Flemingia macrophylla* [SIRIKONDA & al. 2020].

Table 3. List of ISSR and RAPD primers used in genetic fidelity analysis of *Muntingia calabura* L. – a pharmaceutically and medicinally important woody tree.

ISSR			RAPD		
S.No.	Primer code	Primer sequence (5'-3')	S.No.	Primer code	Primer sequence (5'-3')
1	MC1	TCTCTCTCTCTCTCC	1	OPA1	TGGGCGTCAA
2	MC2	TCTCTCTCTCTCTCG	2	OPA2	CAGGCCCTTC
3	MC3	AGAGAGAGAGAGAGAGC	3	OPA3	GGCATGACCT
4	MC4	AGAGAGAGAGAGAGAGG	4	OPA4	GGGTAACGCC
5	MC5	GAGAGAGAGAGAGAGAC	5	OPA5	CCAGCAGCTT
6	MC6	GAGAGAGAGAGAGAGAT	6	OPA6	AGTCAGCCAC
7	MC7	GAGAGAGAGAGAGAGAYT	7	OPA7	TGCCGAGCTG
8	MC8	AGAGAGAGAGAGAGAGYT	8	OPA8	AATCGGGCTG
9	MC9	GAGAGAGAGAGAGAGAYG	9	OPA9	GACTGCACAC
10	MC10	AGAGAGAGAGAGAGAGTC	10	OPA10	AGGGGTCTTG

**Figure 3.** PCR banding profile of ISSR based genetic fidelity analysis of *Muntingia calabura* amplified with MC4 primer. Lane M: 100 bp DNA ladder (Himedia, India). Lane MP: PCR banding pattern of mother plant. Lane 1–7: PCR banding pattern of regenerated plants.

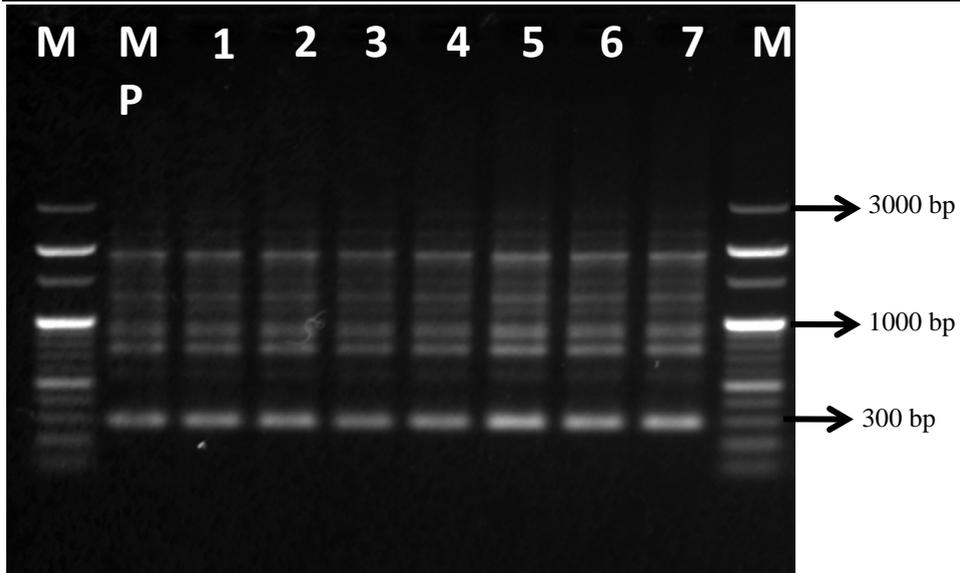


Figure 4. PCR banding profile of RAPD based genetic fidelity analysis of *Muntingia calabura* amplified with OPA6 primer. Lane M: 100 bp DNA ladder (Himedia, India). Lane MP: PCR banding pattern of mother plant. Lane 1–7: PCR banding pattern of regenerated plants.

Conclusions

The present investigation appears to be the first report on regeneration studies of medicinally important woody tree *Muntingia calabura* and assessment of genetic fidelity demonstrates the true-to-type nature of regenerated plants comparatively to the mother plant. Our research findings established the protocol of regeneration studies in the woody tree using PGRs at lower concentrations with eradicating the problem of browning callus without using any anti-browning agents through regular subculturing of cultures. The highest number of shoots was observed to be $32 \pm 0.8a$ in leaf explants on MS medium along with BAP 2.0 mg l^{-1} + IAA 0.5 mg l^{-1} and $26 \pm 0.8a$ in node explants on BAP 2.0 mg l^{-1} + IAA 0.5 mg l^{-1} . The highest rooting frequency is $25 \pm 0.57a$ was obtained on IBA 2.0 mg l^{-1} . The regeneration in leaf and node explants cultured on to MS medium with BAP or KN in combination with IAA and NAA exposed that BAP is more efficient to KN in all tried combinations. BAP has given the maximum efficiency of shoots comparative to KN, with the addition of IAA produced a high organogenic response of the explants. Further, *in vitro* regenerated acclimatized plantlets are using for isolating and screening of secondary metabolites from various plant parts. For the above reasons, the biotechnological analysis studies are required in *Muntingia calabura*.

Notes on contributors

Suvarchala VANKUDOTH is PhD student and she has conducted the total experimental work. Ramakrishna DASARI is a research scientist and expert in plant tissue culture and Biotechnology. Pavani CHIRUMAMILLA is a PhD student and she has conducted the total experimental work. Chaitanya GOPU is also a PhD student and she has designed the figures and tables. Phanikanth JOGAM is a PhD completed student and he has conducted genetic fidelity study. Srinivas KOTA is a PhD student and he has edited the whole manuscript.

Shasthree TADURI is a senior grade professor and plant biotechnologist. His work focuses on developing regeneration protocols for the medicinal and endangered plants. His research group is actively involving in isolation, screenings, and enhancement of bioactive compounds using biotic and abiotic elicitors. Shasthree TADURI availed Dr. CV Raman Fellowship in Mississippi State University, USA and Associated fellow of Telangana Academy of Sciences. He also attended and presented his research findings in national and international conferences.

Acknowledgements

The first author gratefully acknowledges the National fellowship for Higher Education (NFST-2015-17-ST-TEL-2837) for providing financial assistance.

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MICROPROPAGATION OF MUNTINGIA CALABURA L. AND ASSESSMENT OF GENETIC...

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How to cite this article:

VANKUDOTH S., DASARI R., CHIRUMAMILLA R., GOPU C., JOGAM P., KOTA S. & TADURI S. 2020. Micropropagation of *Muntingia calabura* L. and assessment of genetic fidelity of *in vitro* raised plants using ISSR and RAPD analysis. *J. Plant Develop*. **27**: 33-45. <https://doi.org/10.33628/jpd.2020.27.1.33>
